Identification and Expression Analysis of the bHLH Transcription Factor Family in Rhododendron delavayi Postprint

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Abstract

Water deficit is a key factor limiting the horticultural application of *Rhododendron delavayi*, and bHLH transcription factors play important regulatory roles in plant growth, development, and stress response processes. This study utilized the genome files and transcriptional expression data of *Rhododendron delavayi* as materials, employing bioinformatics methods to identify members of the *Rhododendron delavayi* bHLH transcription factor (RdbHLH) family, and analyzed characteristics including gene structure, conserved motifs, phylogeny, protein physicochemical properties, cis-acting elements, protein interaction networks, and expression patterns. The results showed that: (1) A total of 116 RdbHLH genes were identified, with significant differences in amino acid numbers and molecular weights among different proteins; overall, they are weakly acidic hydrophilic proteins that primarily function in the nucleus. (2) The RdbHLH family was divided into 17 subfamilies, with conserved gene motif structures within each subfamily but substantial differences between subfamilies; the vast majority of RdbHLH proteins contain both Motif 1 and Motif 2, and the promoter regions contain numerous cis-acting elements related to plant growth and development, hormone response, light response, and stress response. (3) *Rhododendron delavayi* responds to drought stress primarily by activating signal transduction pathways and the osmotic adjustment and flavonoid synthesis systems to alleviate stress damage; drought stress affected the expression of 36 RdbHLH genes and strongly induced the expression of 12 RdbHLH genes, among which RdbHLH49 and RdbHLH95 may play important regulatory roles in plant drought resistance. These findings provide a theoretical basis for further investigation of the biological functions of RdbHLH genes and also offer targeted gene resources for breeding superior horticultural varieties of *Rhododendron delavayi*.

Full Text

Preamble

Identification and Expression Analysis of the bHLH Transcription Factor Family in *Rhododendron delavayi*

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Abstract: Water deficiency is a critical limiting factor for the horticultural application of *Rhododendron delavayi*. bHLH transcription factors play crucial regulatory roles in plant growth, development, and stress responses. This study identified members of the *R. delavayi* bHLH transcription factor (RdbHLH) family using genomic and transcriptomic data, analyzing their gene structure, conserved motifs, phylogeny, protein physicochemical properties, cis-acting elements, protein interaction networks, and expression patterns through bioinformatics approaches. The results revealed: (1) A total of 116 RdbHLH genes were identified, with considerable variation in amino acid number and molecular weight among different proteins. These proteins are generally weakly acidic and hydrophilic, functioning primarily in the nucleus. (2) The RdbHLH family was divided into 17 subfamilies with conserved motif structures within each subfamily but substantial differences between subfamilies. The vast majority of RdbHLH proteins contained both Motif 1 and Motif 2, and their promoter regions harbored numerous cis-acting elements related to plant growth and development, hormone response, light response, and stress response. (3) *R. delavayi* responds to drought stress primarily by activating signal transduction pathways and modulating osmotic adjustment and flavonoid biosynthesis systems to alleviate stress damage. Drought stress affected the expression of 36 RdbHLH genes, with 12 showing strong induction. Notably, RdbHLH49 and RdbHLH95 may play important regulatory roles in drought resistance. These findings provide a theoretical foundation for further investigation of RdbHLH gene functions and offer target gene resources for breeding superior horticultural varieties of *R. delavayi*.

Keywords: *Rhododendron delavayi*, bHLH, bioinformatics, drought stress, expression patterns

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Introduction

Plants have evolved a series of defense strategies to adapt to adverse environmental conditions during their growth and development. Transcriptional regulation forms the basis of plant growth, development, and environmental adaptation, with transcription factors serving as key proteins that regulate the spatiotemporal expression of downstream target genes (Wu et al., 2022). In stress signaling networks, transcription factors bind to cis-acting elements to repress or activate gene expression, thereby participating in the regulation of plant growth, development, and stress responses (Strader et al., 2022). The bHLH transcription factor family represents one of the most important and largest transcription factor families in plants, being widely present in nearly all eukaryotes and regulating diverse biological processes at the transcriptional level (Zhang et al., 2020). bHLH proteins are named for their highly conserved basic region and helix-loop-helix (HLH) domains (Zhang et al., 2018; Pires & Dolan, 2010). The domain spans 50–60 amino acids, with the basic region located at the N-terminus (10–20 amino acids) functioning as a DNA-binding domain that recognizes and specifically binds to E-box sequences (5'-CANNTG-3') in target gene promoters (Feller et al., 2011). The HLH region at the C-terminus (40–50 amino acids) consists of two α-helices containing conserved hydrophobic residues separated by a variable-length loop. Interactions between these α-helices enable homodimerization or heterodimerization, allowing binding to different regions of target gene promoters to regulate transcription (Lang & Liu, 2020).

Plant bHLH families are generally classified into 15–25 subfamilies, with some special species containing up to 32 (Bano et al., 2021). With advances in sequencing technology, bHLH transcription factor families have been identified and analyzed in numerous model plants and crops, including *Arabidopsis thaliana* (Hao et al., 2021), peanut (*Arachis hypogaea*) (Li et al., 2021), cucumber (*Cucumis sativus*) (Li et al., 2020), and tea (*Camellia sinensis*) (Liu et al., 2021). Existing studies demonstrate that bHLH transcription factors participate in regulating photomorphogenesis (Akmakjian et al., 2021), flower and fruit development, anthocyanin accumulation (Zhao et al., 2019), and stress response (Sun et al., 2019).

Rhododendron delavayi is a perennial alpine woody ornamental in the subgenus *Hymenanthes* of the genus *Rhododendron*, family Ericaceae. It possesses elegant tree morphology, large and colorful flowers, and a long flowering period, offering high ornamental, medicinal, and horticultural value (Sun et al., 2021). However, *R. delavayi* has strict habitat requirements and requires greenhouse conditions for successful cultivation. Water deficiency is the key limiting factor constraining its landscape application (Cai et al., 2019; Wang et al., 2020). Previous studies have shown that bHLH transcription factors play important roles in stress-related regulatory networks and signaling pathways (Li et al., 2019), yet systematic analysis of the bHLH transcription factor family in Ericaceae plants remains scarce.

Based on the *R. delavayi* genome and transcriptomic data, this study employed bioinformatics methods to systematically identify and analyze RdbHLH family members, addressing: (1) basic information on RdbHLH family members and protein physicochemical properties; (2) characteristics of phylogenetic classification, gene structure, conserved motifs, and cis-acting elements; and (3) features and functional predictions of protein interaction networks, transcriptional expression patterns, and three-dimensional structures. The aim was to provide a theoretical basis for further investigation of RdbHLH transcription factor functions in drought stress resistance.

Materials and Methods

1.1 Materials

Genomic sequences, CDS sequences, protein sequences, GFF annotation files, and transcriptomic data under different drought treatments for *R. delavayi* were obtained from the Rhododendron Plant Genome Database (http://bioinfor.kib.ac.cn/RPGD/index.html). *Arabidopsis thaliana* AtbHLH data were retrieved from the TAIR database (https://www.arabidopsis.org/).

1.2 Identification, Physicochemical Properties, and Localization Prediction of RdbHLH Family Members

The hidden Markov model file for bHLH genes (PF00010) was downloaded from the Pfam database (http://pfam.xfam.org/). An initial domain search was performed using HMMER software, and the conserved domain sequences from the top one-third of genes were used to construct a *R. delavayi*-specific hidden Markov model for a second domain search. Candidate genes were screened with an E-value cutoff of 0.001. To verify whether candidate genes contained bHLH domains, corresponding protein sequences were submitted to Pfam and SMART (http://smart.embl.de/) databases for confirmation (Zhang et al., 2022). Protein physicochemical properties of RdbHLH proteins, including amino acid number, molecular weight, isoelectric point, instability index, aliphatic index, and hydrophilicity, were analyzed using ProtParam (https://web.expasy.org/protparam/). Subcellular localization was predicted using PSORT (https://www.genscript.com/wolfpsort.html).

1.3 Multiple Sequence Alignment, Phylogeny, and Classification of RdbHLH Proteins

Full-length amino acid sequences of all RdbHLH and selected AtbHLH proteins were aligned using ClustalW in MEGA11 software. A phylogenetic tree was constructed using the Neighbor-Joining (NJ) method with 1,000 bootstrap replicates to assess nodal reliability. The tree was visualized using ChiPlot (https://www.chiplot.online/), and RdbHLH family members were classified into subgroups and subfamilies according to the AtbHLH classification system (Heim et al., 2003).

1.4 Gene Structure, Conserved Domains, and Motif Analysis of the RdbHLH Family

MEME (http://meme-suite.org/) was used to analyze RdbHLH protein motifs, searching for 15 motifs with lengths ranging from 6 to 100 amino acids. Exon and intron positions were extracted from the GFF annotation file. Protein sequences were uploaded to NCBI to search for conserved domains, and hit data files were downloaded. TBtools was used to visualize gene structures alongside the phylogenetic tree. Conserved domain sequences from the second domain search were extracted, aligned using ClustalW, and analyzed using WebLogo (https://weblogo.berkeley.edu/logo.cgi) and GeneDoc software.

1.5 Analysis of Cis-Acting Elements in RdbHLH Gene Promoters

Promoter DNA sequences (2,000 bp upstream of the start codon) were extracted and submitted to Plant CARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) to identify cis-acting elements. The types, quantities, and functions of these elements were statistically analyzed.

1.6 Expression Analysis of RdbHLH Family Genes Under Different Drought Treatments

Gene expression levels were calculated from transcriptomic data and normalized to FPKM (Fragments Per Kilobase of transcript per Million mapped reads) values, yielding relative expression levels under four treatments: normal irrigation (CK), irrigation cessation for 5 days (D5), irrigation cessation for 9 days (D9), and irrigation cessation for 10 days followed by 6 hours of re-watering (REC). Differential expression analysis was performed using the limma R package, with significance defined as $|\log_2(\text{Fold Change})| > 1$ and $P(\text{padj}) < 0.05$ compared to the control. Expression data were standardized using TBtools, with FPKM values transformed as $\log_2(\text{FPKM value} + 1)$ to represent relative expression levels (Zhang et al., 2020). Heatmaps of differentially expressed RdbHLH genes under various drought treatments were generated.

1.7 Three-Dimensional Structure Prediction of RdbHLH Proteins

Based on transcriptional expression analysis, protein sequences of drought-responsive RdbHLH proteins were subjected to PSI-BLAST homology searches against the PDB database (https://saves.mbi.ucla.edu/) to identify matching 3D structure templates. Swiss-model (https://swissmodel.expasy.org/) was then used for homology modeling to construct RdbHLH protein 3D structures, which were evaluated using the SAVES server (https://saves.mbi.ucla.edu/).

1.8 Protein Interaction Network Analysis of RdbHLH Transcription Factors

OrthoVenn2 (https://orthovenn2.bioinfotoolkits.net/home) was used to perform orthologous comparisons between all *R. delavayi* protein sequences and *Arabidopsis* sequences, identifying homologous pairs between RdbHLH and *Arabidopsis* genes. The STRING database (https://string-db.org/) was then used to analyze interaction networks among *Arabidopsis* genes in these homologous pairs, mapping the interactions onto RdbHLH genes (interaction score ≥ 0.65).

Results

2.1 Identification, Physicochemical Properties, and Localization of RdbHLH Transcription Factor Family Members

This study identified 116 RdbHLH transcription factor family members, renamed RdbHLH1–RdbHLH116. Detailed information is provided in Table 1 . Compared with previous studies, the RdbHLH family contains fewer genes: 95 in peach (*Prunus persica*) (Zhang et al., 2018), 100 in Japanese apricot (*Prunus mume*) (Wu et al., 2022), 142 in cucumber (Li et al., 2020), 164 in tartary buckwheat (*Fagopyrum tataricum*) (Sun et al., 2020), 190 in potato (*Solanum tuberosum*) (Wang et al., 2018), 208 in maize (*Zea mays*) (Zhang et al., 2018), and 230 in Chinese cabbage (*Brassica rapa*) (Song et al., 2014), indicating substantial variation in bHLH family size across species.

Analysis of protein physicochemical properties and subcellular localization (Table 1) revealed that RdbHLH protein lengths ranged from 92 to 1,040 amino acids, with 84.48% comprising 200–600 amino acids. Molecular weights varied from 10,552.83 to 113,904.83 Da, with 81.03% between 20,000–60,000 Da. Isoelectric points ranged from 4.87 to 9.93 (mean = 6.69), with 65.52% of RdbHLH proteins being weakly acidic ($pI < 7$). Instability indices exceeded 40 for 111 proteins (95.69%). Aliphatic indices ranged from 47.7 to 102.5, with over 90% of proteins scoring above 60. All proteins had negative average hydrophobicity indices (mean = -0.54). Except for three genes localized to the cytoplasm, three to mitochondria, and one to the extracellular region, the remaining RdbHLH genes (93.97%) were nuclear-localized.

2.2 Phylogenetic Analysis and Classification of RdbHLH

The AtbHLH gene family is well-characterized in the model plant *Arabidopsis thaliana*. This study selected 131 AtbHLH genes as a reference for constructing a phylogenetic tree with the RdbHLH family. RdbHLH subfamily members were classified based on tree topology and the AtbHLH subfamily classification system.

As shown in Figure 1 [Figure 1: see original paper] and Table 2 , the phylogenetic tree was divided into 30 subgroups and 21 subfamilies. The 116

RdbHLH members were distributed across 17 of these subfamilies: Ia, Ib1, Ib2, II, IIIa+b+c+d+e+f, IVa, IVb2+c, IVd, Va+b, VIIa+b, VIIIa, VIIIb+c, IX, Xa, XI, XII, and XIII. RdbHLH111 and RdbHLH84 could not be assigned to any AtbHLH group and were thus classified into subfamily XIII. Subfamily IIIa+b+c+d+e+f contained the most members (20), while subfamilies IVb1, Xb, Xc, and VI contained no RdbHLH members. In plants, members of the same subfamily with closer evolutionary distances tend to share similar structures, pathways, and biological processes (Zhang et al., 2018). Therefore, RdbHLH functions could be inferred from phylogenetic relationships and AtbHLH functions. For example, members of subfamilies XI, X, and VIIIc may regulate root growth and development, while most members of subfamilies XII, Va+b, X, IIIa+b+c+d+e+f, IVd, Ia, and IX may participate in regulating plant growth (cell elongation, flower development, organ morphogenesis) and abiotic stress responses (drought, cold, salt) (Salih et al., 2021).

2.3 Gene Structure, Conserved Domains, and Motif Analysis of RdbHLH

Gene structure analysis (Figure 2 [Figure 2: see original paper]c) revealed substantial variation among RdbHLH subfamilies, with gene lengths ranging from 467 bp (RdbHLH82) to 11,869 bp (RdbHLH33). Intron and exon numbers also varied considerably: intron counts ranged from 0 to 13, with 12 genes (10.34%) lacking introns (distributed in subfamilies III-1, VIII-1, and X), 15 genes (11.21%) containing one intron (distributed in subfamilies I-1, I-3, III-1, Vb, and VIII-1), and four genes (RdbHLH28, RdbHLH96, RdbHLH109, and RdbHLH110) having $ $10 introns (distributed in subfamilies III-2, IV-2, and XII). The majority of RdbHLH genes (84, 72.4%) contained 2–8 introns. Exon numbers ranged from 1 to 14, with 12 genes (10.34%) having a single exon (distributed in subfamilies III-1, VIII-1, and X) and 98 genes (84.48%) containing 2–8 exons.

Multiple sequence alignment and conserved domain analysis (Figure 2b and Figure 3 [Figure 3: see original paper]) confirmed that all RdbHLH genes contained the typical bHLH domain, with 36 genes possessing the bHLH_{SF} superfamily domain. Domain composition was highly similar within the same subfamily.

A total of 15 motifs (Motif 1–Motif 15) were identified (Figure 2a and Figure 3), with each gene containing 1–8 motifs ranging from 21 to 100 amino acids in length (Table 3). Except for RdbHLH8, RdbHLH68, RdbHLH71, RdbHLH82, RdbHLH100, RdbHLH102, and RdbHLH108 (which lacked the basic region) and RdbHLH99 (which lacked the HLH region), the remaining 108 RdbHLH proteins (93.10%) contained both Motif 1 and Motif 2. These two motifs were closely positioned in most RdbHLH proteins except RdbHLH42, RdbHLH57, and RdbHLH88. Motif 1 comprised a basic region and one helix, while Motif 2 contained a loop region and another helix, together forming the bHLH domain (Figure 3 and Figure 4). The RdbHLH conserved domain contained 28 relatively conserved amino acids (sequence identity > 50%) and nine highly conserved residues (sequence identity > 90%). Generally, RdbHLH proteins on adjacent phylogenetic branches exhibited similar motif structures, with subfamilies I-2, II, IV-1, Vb, VII, VIII-1, IX-1, XI, and XII showing highly conserved motif patterns.

2.4 Analysis of Cis-Acting Elements in RdbHLH Gene Promoters

Upstream promoter sequences serve as transcription factor binding sites and function as important cis-regulatory elements in plant biological processes (Schmitz et al., 2022). To further investigate RdbHLH functions, cis-elements in promoter regions were identified and analyzed. The results (Figure 5 [Figure 5: see original paper] and Table 4) revealed 2,731 cis-acting elements of 32 types across 116 RdbHLH genes, which could be classified into four categories (Schmitz et al., 2022): (1) Plant growth and development-related elements such as meristem and protein metabolism regulation elements (AT-rich element, CAT-box, O2-site), accounting for 4.58% of total elements; (2) Hormone response elements including abscisic acid, auxin, gibberellin, methyl jasmonate, and salicylic acid (ABRE, GARE-motif, P-box, TATC-box, TCA-element, TGA-element, etc.), comprising 33.54%; (3) Light-responsive elements including ACE, AE-box, GA-motif, G-box, GT1-motif, and MRE, representing 43.39%; and (4) Stress-responsive elements for low temperature, drought, wounding, and anaerobic conditions (ARE, ATC-motif, LTR, TC-rich repeats, WUN-motif, etc.), accounting for 18.49%. Notably, 47 RdbHLH genes (40.52%) contained drought-responsive cis-acting elements.

2.6 Expression Analysis of RdbHLH Family Genes Under Different Drought Treatments

Differential expression patterns of RdbHLH genes under various drought treatments are shown in Figure 6 [Figure 6: see original paper]. Under drought stress, 36 RdbHLH genes exhibited significant expression changes. RdbHLH40, RdbHLH21, and RdbHLH48 showed high expression in the CK group but low expression under drought and re-watering conditions, suggesting these genes regulate normal plant growth and development, with drought stress inhibiting their expression by limiting normal physiological processes. RdbHLH28, RdbHLH104, and RdbHLH103 showed dramatically increased expression under D5 treatment, indicating high sensitivity to drought stress and important regulatory roles during moderate drought resistance. RdbHLH56, RdbHLH7, RdbHLH91, and RdbHLH65 exhibited high expression under CK and D5 but decreased expression under D9, suggesting severe drought stress suppressed these genes. RdbHLH23, RdbHLH100, RdbHLH52, RdbHLH82, RdbHLH98, and RdbHLH81 showed sharply elevated expression under D9, indicating induction by severe drought stress. Nine genes including RdbHLH51, RdbHLH16, RdbHLH50, RdbHLH27, and RdbHLH69 displayed high expression under REC treatment, suggesting involvement in post-stress recovery mechanisms. RdbHLH49, RdbHLH95, and RdbHLH64 showed high expression under both D5 and D9 treatments, indicating participation throughout the drought response. Among these, RdbHLH49 and RdbHLH52 exhibited the strongest expression responses, suggesting pivotal regulatory functions in drought resistance.

2.7 Three-Dimensional Structure Prediction of RdbHLH Proteins

Based on transcriptional expression analysis, 3D structures of 12 strongly drought-responsive RdbHLH proteins (RdbHLH28, RdbHLH104, RdbHLH103, RdbHLH23, RdbHLH100, RdbHLH52, RdbHLH82, RdbHLH98, RdbHLH81, RdbHLH49, RdbHLH95, and RdbHLH64) were predicted (Figure 7 [Figure 7: see original paper]). All RdbHLH proteins exhibited α-helices, β-sheets, β-turns, and random coil conformations. The overall 3D structures showed certain similarities, with all members possessing the conserved bHLH structure.

2.8 Protein Interaction Network Analysis of RdbHLH Transcription Factors

The protein interaction network (Figure 8 [Figure 8: see original paper]) revealed that RdbHLH33 (homologous to triose phosphate isomerase, AtTIM) strongly interacts with key glycolytic enzymes glyceraldehyde-3-phosphate dehydrogenase (AtGAPA-2, AtGAPB, AtGAPC2, and AtGAPCP-2) (Simkin et al., 2023; Suzuki et al., 2021). Since *Arabidopsis* AtGAPCp plays a critical role in drought response and ABA signaling (Zhang et al., 2019), we hypothesize that RdbHLH33 induces expression of GAPA-2, GAPB, GAPC2, and GAPCP-2 to facilitate signal transduction, coordinately regulating the photosynthetic reductive pentose phosphate pathway and glycolysis to maintain cellular ATP levels and enhance resistance to high temperature and drought.

RdbHLH46 (homologous to AtbHLH29, FER-like regulator of iron uptake, FRU), RdbHLH50 (homologous to AtbHLH105, IAA-leucine resistant 3, ILR3), RdbHLH47 (homologous to AtbHLH104), and RdbHLH52 (homologous to AtbHLH39) co-regulate gene expression to increase iron absorption, distribution, and utilization, maintaining Fe homeostasis and providing photoprotection during iron deficiency to reduce reactive oxygen species damage (Akmakjian et al., 2021; Jakoby et al., 2004). AtbHLH13 (Huang et al., 2018), MYC2 (Zhu et al., 2023), JAZ1 (Feng et al., 2020), and TIFY7 (Singh & Mukhopadhyay, 2021) all participate in jasmonic acid-induced stress responses. RdbHLH56 (homologous to AtbHLH13) and RdbHLH7 (homologous to AtMYC2) showed strong interactions with AtJAZ1 and AtTIFY7. Additionally, jasmonic acid-responsive transcription factors AtRERJ1 (homologous to RdbHLH23) (Kiribuchi et al., 2004) and AtbHLH92 (homologous to RdbHLH48) (Shen et al., 2021) also strongly interact with AtJAZ1, suggesting that RdbHLH56, RdbHLH7, RdbHLH23, and RdbHLH48 play key regulatory roles in jasmonic acid signaling and osmotic/salt stress responses.

Transcription factors SPCH and MUTE regulate stomatal development, promoting epidermal cell conversion to stomata (Yang et al., 2022; Zuch et al., 2023). Regulator of G-protein signaling E1 (RGE1) modulates embryonic growth and development (Zuo et al., 2021), while FAMA promotes guard cell differentiation (Ohashi-Ito & Bergmann, 2006). Inducer of CBF Expression 1 (ICE1) is a transcriptional activator regulating cold responses and mediates epidermal stomatal differentiation by controlling SPCH, MUTE, and FAMA (Feng et al., 2013). We therefore hypothesize that RdbHLH19 (homologous to AtICE1) interacts with RdbHLH74 (homologous to AtMUTE), RdbHLH42 (homologous to AtSPCH), and RdbHLH67 (homologous to AtRGE1) to regulate cold stress responses, stomatal development, and embryogenesis (Liang & Yang, 2015).

Arabidopsis TIFY transcription factor AtTIFY7 (Liu & Chen, 2019), bHLH transcription factors Transparent Testa 8 (AtTT8) (Shin et al., 2013) and Glabra 3 (AtGL3) (Zhang et al., 2016) all regulate anthocyanin biosynthesis. Based on interaction patterns, we predict that RdbHLH37 (homologous to AtTT8), RdbHLH53 (AtGL3), and TIFY7 co-regulate anthocyanin and other flavonoid compounds. Cryptochromes 2 (CRY2), phytochrome A (PHYA), phytochrome-interacting factors 3/7 (PIF3/7), and cryptochrome-interacting bHLH1 (CIB1) regulate photoperiodic growth and flowering time (Liu et al., 2013; Zuo et al., 2011). Strong interactions among RdbHLH105 (homologous to AtPIF3), AtPHYA, AtCRY2, RdbHLH21 (homologous to AtPIF7), and RdbHLH65 (homologous to AtCIB1) suggest that RdbHLH105, RdbHLH21, and RdbHLH65 participate in regulating photomorphogenesis, metabolic biosynthesis, and signal transduction. DYT1 (Dysfunctional Tapetum1) is crucial for anther development and tapetum differentiation in *Arabidopsis* (Li et al., 2017). AtbHLH10 (Huang et al., 2020), AtbHLH30 (Reisfeld et al., 2022), and AtbHLH41 (Reisfeld et al., 2022) are involved in tapetum and pollen development, suggesting that RdbHLH13 (homologous to AtbHLH10), RdbHLH2 (homologous to At-DYT1), RdbHLH8 (homologous to AtbHLH41), RdbHLH89 (homologous to AtbHLH30), and RdbHLH39 (homologous to AtbHLH144) co-regulate flower development.

Discussion and Conclusion

This study identified 116 RdbHLH genes, fewer than in Chinese cabbage, maize, and potato. This discrepancy may reflect differences in evolution, genome duplication, genome size, and habitat among species. The RdbHLH family may not have undergone large-scale gene duplication events, which drove bHLH family expansion in Chinese cabbage, maize, and potato (Carretero-Paulet et al., 2010; Xu et al., 2015). The negative average hydrophobicity indices indicate that RdbHLH transcription factors are hydrophilic proteins. The wide isoelectric point range (4.87–9.93) suggests these proteins can function in diverse pH environments. Most RdbHLH proteins had instability indices > 40, indicating overall poor stability. Nuclear localization of most RdbHLH proteins (93.97%) suggests primary nuclear function.

The RdbHLH family was classified into 17 subfamilies, consistent with the general 15–25 subfamily range reported for plant bHLH families (Pires & Dolan, 2010). Subfamily III contained the most members, while no RdbHLH members were assigned to subfamily VI, possibly due to loss during *R. delavayi* evolution. Genes within the same subfamily showed similar structures, conserved domains, and motif compositions, while substantial differences between subfamilies likely drive functional divergence, enabling regulation of complex biological processes (Wani et al., 2021).

RdbHLH gene promoter cis-regulatory elements fell into four categories: plant growth and development, hormone response, light response, and stress response, indicating multifaceted regulatory roles. Light-responsive elements were most abundant and widely distributed, suggesting light signals play important roles in RdbHLH transcriptional regulation (Nawaz et al., 2014). The presence of drought-responsive cis-elements in 47 RdbHLH genes indicates extensive involvement in drought response. Differential expression analysis revealed distinct expression patterns under drought stress, suggesting functional divergence and different roles in drought response (Zhang et al., 2022). Severe drought induced high expression of RdbHLH49, RdbHLH95, RdbHLH64, RdbHLH100, RdbHLH52, and RdbHLH82, with RdbHLH49 and RdbHLH52 showing the strongest responses. Reduced expression after re-watering suggests these genes may alleviate drought damage and play regulatory roles in drought resistance.

RdbHLH46 (homologous to AtFRU), RdbHLH50 (AtILR3), RdbHLH47 (AtbHLH104), and RdbHLH52 (AtbHLH39) regulate iron uptake, distribution, and homeostasis while providing photoprotection during iron deficiency. RdbHLH37 (AtTT8), RdbHLH53 (AtGL3), and AtTIFY7 co-regulate anthocyanin and flavonoid biosynthesis, consistent with Kazemitabar et al.'s findings in sesame (*Sesamum indicum*) where FRU, ILR3, and TT8 regulate stress responses, iron homeostasis, and anthocyanin synthesis (Kazemitabar et al., 2020). RdbHLH19 (AtICE1) interacts with RdbHLH74 (AtMUTE), RdbHLH42 (AtSPCH), and RdbHLH67 (AtRGE1) to regulate cold stress, stomatal development, and embryogenesis, consistent with *Arabidopsis* studies showing ICE1 forms heterodimers with SPCH, MUTE, and FAMA to positively regulate stomatal development (Pillitteri & Torii, 2007; Liu et al., 2009). RdbHLH105 (AtPIF3), RdbHLH21 (AtPIF7), RdbHLH65 (AtCIB1), PHYA, and CRY2 co-regulate photomorphogenesis, metabolic biosynthesis, and signal transduction, supporting Liu et al.'s finding that CRY2 activates CIB1 to promote FT mRNA expression in response to blue light (Liu et al., 2018). RdbHLH13 (AtbHLH10), RdbHLH2 (AtDYT1), RdbHLH8 (AtbHLH41), RdbHLH89 (AtbHLH30), and RdbHLH39 (AtbHLH144) co-regulate flower development, consistent with Cheng et al.'s report that AtbHLH10 forms a protein complex with DYT1 to regulate anther differentiation (Cheng et al., 2023). Key drought-responsive proteins identified in differential expression analysis were also predicted to have significant interactions, suggesting that *R. delavayi* drought response primarily involves activating stress signaling pathways, osmotic adjustment systems, and flavonoid synthesis to scavenge reactive oxygen species and alleviate stress damage.

The phylogenetic relationships and motif structures corresponded with functional predictions. For instance, *Arabidopsis* Ia subfamily members AtbHLH45, AtbHLH97, and AtbHLH98 are associated with stomatal development control (Zhou et al., 2020), and RdbHLH74 and RdbHLH42 from the same subfamily showed similar functions in the interaction network. AtbHLH37, AtbHLH40, AtbHLH43, and AtbHLH88 in subfamily VIIIb regulate flower and fruit development (Carretero-Paulet et al., 2010), and RdbHLH72, RdbHLH106, RdbHLH107, RdbHLH11, and RdbHLH85 from subfamily VIIIb showed comparable functions. AtbHLH38, AtbHLH39, AtbHLH100, and AtbHLH101 participate in iron deficiency responses (Wang et al., 2013), and RdbHLH102, RdbHLH68, and RdbHLH52 from subfamily Ib1 were predicted to share this function. Furthermore, RdbHLH56, RdbHLH7, RdbHLH23, and RdbHLH48 predicted to function in jasmonic acid signaling contained methyl jasmonate-related cis-elements (G-box, CGTCA, and TGACG motifs) in their promoters, while those predicted to participate in light response contained light-responsive cis-elements (G-box, Box 4, GT1-motif), demonstrating consistency between gene structure and function.

In summary, this study identified 116 RdbHLH genes, performed systematic characterization and evolutionary classification, and analyzed expression patterns under drought stress using transcriptomic data. Two candidate genes, RdbHLH49 and RdbHLH52, were identified as potentially important for drought resistance in *R. delavayi*, though their specific functions require further experimental validation. These results provide a theoretical basis for investigating RdbHLH gene functions and offer target gene resources for breeding superior horticultural varieties of *R. delavayi*.

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