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Genome-Wide Identification and Characterization of bHLH Gene Family in *Hevea brasiliensis*

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Abstract: The basic helix-loop-helix (bHLH) transcription factors play crucial roles in plant growth, development, and stress responses. However, their identification and insights into the understanding of their role in rubber trees remain largely uncovered. In this study, the bHLH gene family was explored and characterized in rubber trees using systematic bioinformatics approaches. In total, 180 bHLH genes were identified in the rubber tree genome, distributed unevenly across 18 chromosomes, and phylogenetic analysis classified these genes into 23 distinct subfamilies. Promoter regions revealed a high density of cis-elements responsive to light and hormones. Enrichment analysis indicated involvement in numerous biological processes, including growth, development, hormone responses, abiotic stress resistance, and secondary metabolite biosynthesis. Protein interaction network analysis identified extensive interactions between *HbbHLH* genes and other functional genes, forming key clusters related to iron homeostasis, plant growth, and stomatal development. Expression profiling of *HbbHLH* genes have demonstrated varied responses to endogenous and environmental changes. RT-qPCR of eleven *HbbHLH* genes in different tissues and under ethylene, jasmonic acid, and cold treatments revealed tissue-specific expression patterns and significant responses to these stimuli, highlighting the roles of these genes in hormone and cold stress responses. These findings establish a framework for exploring the molecular functions of bHLH transcription factors in rubber trees.

Keywords: bHLH transcription factor; rubber tree; cis-element; expression analysis; transcriptome; abiotic stress; RT-qPCR; gene structure; subcellular localization

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1. Introduction

The rubber tree (*Hevea brasiliensis* Müll. Arg) is the primary source of natural rubber, which is an indispensable raw material for industrial production. More than 90% of natural rubber in the world is produced from rubber trees [1]. The regulation of rubber biosynthesis and related physiological processes is governed by complex genetic networks in which transcription factors play a crucial role. Rubber trees grow in tropical regions and are often subjected to various stresses, such as cold temperatures, pests, and diseases. Improving stress tolerance and understanding the regulatory networks involved in rubber biosynthesis are areas of focus for researchers. Among these, the basic helix-loop-helix (bHLH) transcription factor family has recently gained attention for its involvement in various biological processes in plants, including growth, development, and responses to environmental stress.

The basic helix-loop-helix (bHLH) family is the second-largest family of eukaryotic transcription factors, following the MYB family, and is known for its basic helix-loop-helix

(HLH) domain, which consists of approximately 60 amino acids, including a basic region and an HLH region. The basic region, located at the N-terminus, is rich in basic residues and serves as a DNA binding site. The HLH region, at the C-terminus, acts as a dimerization domain, enabling the formation of protein dimers through specific interactions, and is primarily composed of hydrophobic amino acids forming two amphipathic α-helices separated by a loop segment with a variable sequence and length [2].

bHLH transcription factors are found in both animals and plants. In animals, they are typically classified into six groups (A to F) based on phylogenetic relationships and DNA-binding patterns [3,4]. However, there are differences in the classification of plants and animals because of the relative independence of bHLH transcription factor genealogy [5]. To date, no definitive classification standard for bHLH transcription factors has been established in plants. According to bHLH protein sequence homology, conservation of specific amino acids at key positions, and the presence of other conserved functional domains outside the bHLH domain, researchers have classified bHLH transcription factors of plants into 15–35 subfamilies (Figure 1). For instance, in *Arabidopsis* (*Arabidopsis thaliana* (L.) Heynh.), 162 bHLH proteins have been characterized and grouped into 12 major groups and 25 subgroups [6,7]. Lorenzo Carretero-Pauletd, by including lower plants such as algae and mosses in the analysis, has classified the bHLH transcription factors into 32 subgroups from a broader evolutionary perspective [8]. Rice (*Oryza sativa* L.) has 167 bHLH family members, which are classified into 22 subfamilies [9]. Tomato (*Solanum lycopersicum* L.) possesses 152 bHLH genes, organized into 26 subfamilies, whereas [10] cucumber (*Cucumis sativus* L.) has 142 bHLH genes distributed among 32 subfamilies [11]. In potato (*Solanum tuberosum* L.), 124 bHLH members have been recognized and divided into 15 subfamilies [12].

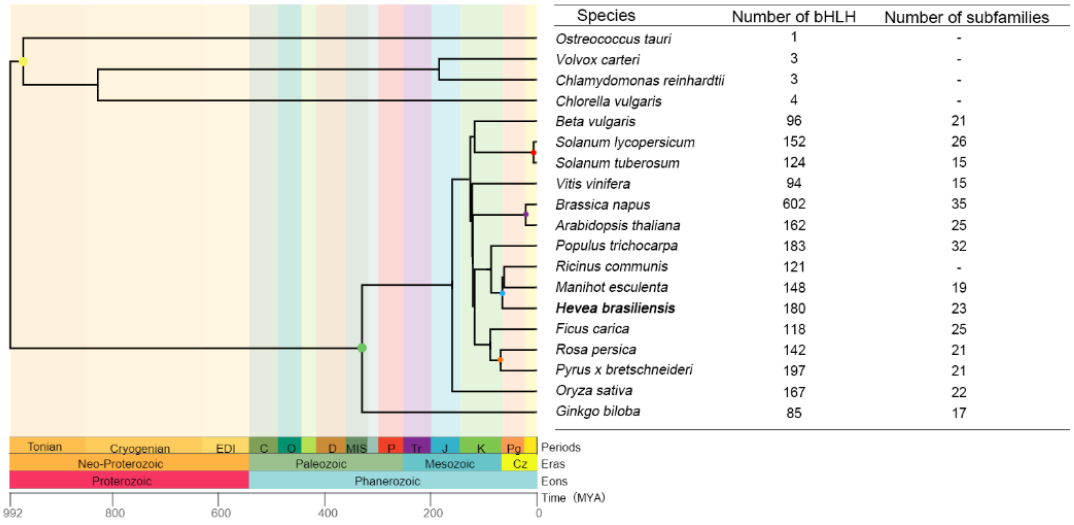

Figure 1. Phylogenetic tree of diverse species showing the number of bHLH families. The phylogenetic tree reflects the evolutionary relationships and divergence times of various plant species as determined using the TimeTree database (http://www.timetree.org, accessed on 28 March 2024). Different colored nodes represent various classifications, including *Chlorophyta* (yellow), *Spermatophyta* (green), *Solanaceae* (red), *Brassicaceae* (purple), *Euphorbiaceae* (blue), and *Rosaceae* (orange), among others. A linear scale of time in MYA (millions of years ago) and a geological timescale are shown at the bottom of the tree.

The ubiquitous presence of bHLH transcription factors further underscores their profound genetic significance and functional specialization throughout the plant kingdom. Recent studies have shown that the influence of the bHLH superfamily extends across a broad range of plant growth and developmental processes. For instance, bHLH transcription factors are instrumental in regulating a multitude of biological processes, such as seed

germination [13,14], flowering time control [15–17], responses to iron deficiency [18,19], mechanisms for cadmium tolerance [20], and enhancement of cold tolerance [21]. Furthermore, they play a pivotal role in mediating responses to light signals [22–24] and phytohormones, notably, jasmonic acid (JA) [25,26] and abscisic acid (ABA) [27–29], which are vital for environmental stress adaptation and physiological regulation. Collectively, these roles emphasize their critical importance in the intricate networks governing plant stress responses and development.

Prior to this study, researchers analyzed the functions of various bHLH factors in *H. brasiliensis*. For instance, Yamaguchi et al. subjected four *HbbHLH* genes to RNA-seq and in vitro binding assays, discovering that *HbMYC2* might function as a receptor in JA signaling, contributing to latex cell differentiation. In contrast, the other two *HbbHLH* genes likely promote rubber biosynthesis [30]. Chen et al. found that one bHLH transcription factor, *ICE*-like transcription factor *HbICE2*, is involved in the cold stress tolerance mechanism regulated by jasmonic acid in rubber trees [31]. Guo et al. found that *HbMYC2b* significantly increased the activity of the *HbSRPP* promoter, suggesting that *HbMYC2b* may be a positive regulator of *HbSRPP* in rubber trees [32]. These findings indicate that certain members of the bHLH family in rubber trees are involved in latex biosynthesis and the environmental stress response. However, our understanding of the overall function and characteristics of the bHLH family genes in rubber trees remains limited. In this study, we aimed to identify and characterize the complete bHLH family members of the rubber tree and investigate their transcriptional changes during different treatments, thereby providing clues for further functional characterization of genes.

2. Materials and Methods

2.1. Plant Materials and Treatments

The materials used in this study were planted at the Experimental Station of the Chinese Academy of Tropical Agricultural Sciences, Danzhou, Hainan, China (19°28′ N, 109°29′ E). For ethylene and methyl jasmonate treatments, 7-year-old virgin trees were selected and latex was harvested using a half-spiral tapping method. Latex samples were first collected without any treatment as 0 h samples. In the control group, 0.03 M methylcellulose was applied 2 cm above and below the tapping line, while in the treatment groups, 1% ethylene (prepared with methylcellulose) and 1% methyl jasmonate were applied similarly. Latex was collected at 4 h, 8 h, 12 h, 24 h, and 48 h after treatment, with each tree tapped only once. A total of 216 trees were used, with 12 trees per treatment at each time point. Samples were frozen in liquid nitrogen and three trees were combined into one sample, forming four biological replicates per treatment and time point.

Six-year-old virgin trees were selected for the cold treatment experiment. Branches and leaf samples were collected without prior treatment. Afterwards, the branches were exposed to cold treatment at 4 °C, and leaves were collected at 2 h, 8 h, and 24 h post-treatment. The samples were frozen in liquid nitrogen, and three biological replicates were prepared. For tissue-specific expression analysis, 10-year-old virgin trees were used and five tissue samples (bark, leaves, male flowers, female flowers, and latex) were collected, with three biological replicates for each sample type.

2.2. Identification of Hevea bHLH Genes

The genome sequences of H. brasiliensis originated from a preliminary assembly (unpublished) performed by our research group, and the Hidden Markov Model (HMM) profile of bHLH (PF00011) was downloaded from the Pfam protein database (http://pfam-legacy.xfam.org/, accessed on 25 March 2024) [33]. Putative *HbbHLH* sequences were identified using the profile HMM, generated, and calibrated using the HMMER v. 3.4 [34] software with default parameters. Then, the candidates were checked for the conserved bHLH domain using the Batch CD-search tool within the Conserved Domain Database (CDD) (https://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml, accessed on 25 March 2024) [35], Pfam (http://pfam-legacy.xfam.org/, accessed on 25 March 2024) [33],

and SMART (https://smart.embl.de/, accessed on 25 March 2024) [36]. The remaining sequences were preserved as the final collection of the putative *HbbHLH* sequences. The amino acid count, molecular weight, isoelectric point, instability index, aliphatic index, and grand average of hydropathicity (GRAVY) of the proteins encoded by the *HbbHLH* genes were determined using TBtools v. 2.119 [37]. Subcellular localization predictions were made using the WoLF PSORT [38] web server (https://wolfpsort.hgc.jp/, accessed on 25 March 2024). All domain sequences of *HbbHLH* genes were aligned with ClustalW v. 2.1 [39], and the alignment results were submitted to the online website WEBlogo (https://weblogo.berkeley.edu/logo.cgi, accessed on 25 March 2024) [40] to create a sequence logo.

2.3. Sequence Conservation and Phylogenetic Analysis

The bHLH amino acid sequences of *A. thaliana* were obtained from The *Arabidopsis* Information Resource (TAIR, https://www.Arabidopsis.org/, accessed on 26 March 2024). The *AtbHLH* and *HbbHLH* sequences were aligned using ClustalW v. 2.1 [39] with default parameters, and the resulting alignments were subsequently used to construct an unrooted neighbor-joining phylogenetic tree using MEGA7 [41], employing the following parameters: 1000 bootstrap replicates, Poisson model, and pairwise deletion of gaps. Thereafter, the phylogenetic tree was visually rendered using evolview (https://www.evolgenius.info/evolview/#/treeview, accessed on 27 March 2024) [42].

2.4. Gene Structures, Conserved Motifs, Promoters, and Chromosomal Location Analysis

The structures of the *HbbHLH* genes were analyzed using TBtools v. 2.119 [37]. Conserved motif analysis was conducted with the MEME Suite [43] online program (https://meme-suite.org/meme/tools/meme, accessed on 30 March 2024), setting the maximum number of motifs to 20. Additionally, the 1500 bp upstream DNA sequences of the *HbbHLH* genes were submitted to the PlantCARE database (https://bioinformatics.psb.ugent.be/webtools/plantcare/html/, accessed on 27 March 2024) [44] for the prediction of *cis*-acting regulatory elements, with the results visualized using TBtools v. 2.119 [37]. *HbbHLH* genes were mapped to chromosomes using the MapChart v. 2.32 software [45].

2.5. Gene Duplication Patterns Analysis

The entire protein sequence of the *H. brasiliensis* genome served as queries in a self-alignment process executed with Blastall v. 2.16.0, employing an E-value threshold of 1×10^{-10}. The results were analyzed using MCScanX [46] to produce segmental duplications and tandem duplications across the whole genome. Segmental duplications belonging to the bHLH family were extracted to draw a collinearity map using CIRCOS v. 0.69.6 [47].

2.6. Gene Ontology Enrichment Analysis

To investigate the functional enrichment of the 180 *HbbHLH* genes, Gene Ontology (GO) analysis was performed based on gene function annotations obtained from previous studies within the research group. The R package "clusterProfiler v. 4.14.0" [48] was employed for this analysis, with significance thresholds set at $Q < 0.05$ and $P < 0.05$.

2.7. Interaction Network of Hevea Homologues in Arabidopsis

Homologous genes of *H. brasiliensis* were identified in *A. thaliana* using OrthoVenn3 (https://orthovenn3.bioinfotoolkits.net/, accessed on 30 August 2024) [49]. The *HbbHLH* homologous gene sets were further analyzed in STRING (https://cn.string-db.org/, accessed on 30 August 2024) with a minimum required interaction score of 0.4, limiting the results to no more than 50 interactors. Genes with homologues in *Hevea* were filtered from the interaction results and visualized using the Cytoscape v. 3.10.2 [50] software.

2.8. Expression Patterns Analysis

The expression levels of the *HbbHLH* genes were quantified using the average of normalized expression data (measured in FPKM—Fragments Per Kilobase of transcript

per Million mapped reads) obtained from HeveaDB (http://hevea.catas.cn, accessed on 22 May 2024) [51]. An expression heatmap was generated using the R package "pheatmap v. 1.0.12". Expression pattern clustering was performed using Mfuzz [52].

Total RNA was extracted using an RNA Prep Pure Plant Plus Kit (TIANGEN, Beijing, China). The PrimeScript™ RT Reagent Kit with gDNA Eraser (Takara, Dalian, China) was used for first-strand cDNA synthesis. RT-qPCR was conducted using the 2 × Q3 SYBR qPCR Master Mix (TOLOBIO, Shanghai, China) with primers designed using Primer3 (Table S5), and the rubber tree *YLS8* gene (Gene ID: 110644482) was used as an internal reference gene. The relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method. Each analysis was repeated in at least three independent experiments. One-way ANOVA and Tukey's multiple comparisons tests were performed using GraphPad Prism v. 9.0.0 software.

3. Results

3.1. Genome-Wide Identification of Hevea bHLH

Using Hidden Markov Model search results and screening through the SMART and CDD databases, 180 rubber tree bHLH family genes were identified and named *HbbHLH1-HbbHLH180* based on their chromosomal locations. These genes display distinct features (Table S1); all *HbbHLH* genes have negative grand average of hydropathicity values, indicating that they are hydrophilic. Subcellular localization analysis revealed that the vast majority (91%) of *HbbHLH* genes were predicted to localize to the nucleus, highlighting their potential roles in transcriptional regulation, while the remaining genes were localized to other organelles such as mitochondria, chloroplasts, and the cytoplasm.

3.2. Conserved Domain and Phylogenetic Analysis of Hevea bHLH

The analysis of conserved motifs within the HbbHLH family revealed a notable level of conservation, with 23 amino acid residues exhibiting over 50% conservation and 10 residues showing more than 75% conservation across all proteins (Figure 2). The majority of the conserved residues were consistent with previous research, particularly in regions associated with DNA-binding and dimerization. For instance, in the basic region of animal bHLH proteins, all bHLH proteins binding to the E-box (5′-CANNTG-3′) have glutamic acid at position 9 [53]. In rubber trees, the critical E9 residue is highly conserved and is found in 86% of the analyzed proteins; 62% of rubber tree bHLH proteins possess the characteristic configuration H5-E9-R13 of Group B animal bHLH proteins [6], which has been shown to bind to the variant of the E-box (5′-CACGTG-3′) [54]. Highly conserved hydrophobic residues in helices 1 and 2 are essential for dimerization [55]. In rubber trees, leucine (L) residues are present at position 23 in each bHLH protein, suggesting that this residue may play a significant role in dimerization.

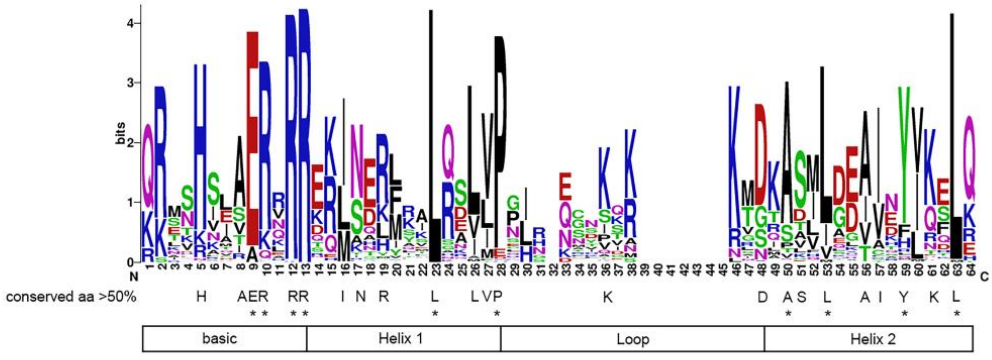

Figure 2. The bHLH domain is highly conserved across all the *HbbHLH* proteins. The overall height of the stack indicates the sequence conservation at that position. Capital letters indicate amino acids with more than 50% conservation, whereas asterisks indicate amino acids with more than 75% conservation across the 180 *HbbHLH* domains.

To classify the bHLH gene family of the rubber tree and identify its evolutionary relationship with the *Arabidopsis* bHLH family, a phylogenetic tree was constructed using 180 rubber tree bHLH protein sequences and 121 *Arabidopsis* bHLH protein sequences (Figure 3). According to the classification criteria of the *AtbHLH* family by Heim et al. [6], the 180 rubber tree bHLH family members were divided into 23 subfamilies. Subfamily XII was the largest, containing 26 *HbbHLH* proteins, while the smallest subfamilies, VIIIa and IIIe, contained only two *HbbHLH* proteins. No rubber tree bHLH proteins were classified into the families IVd and VI.

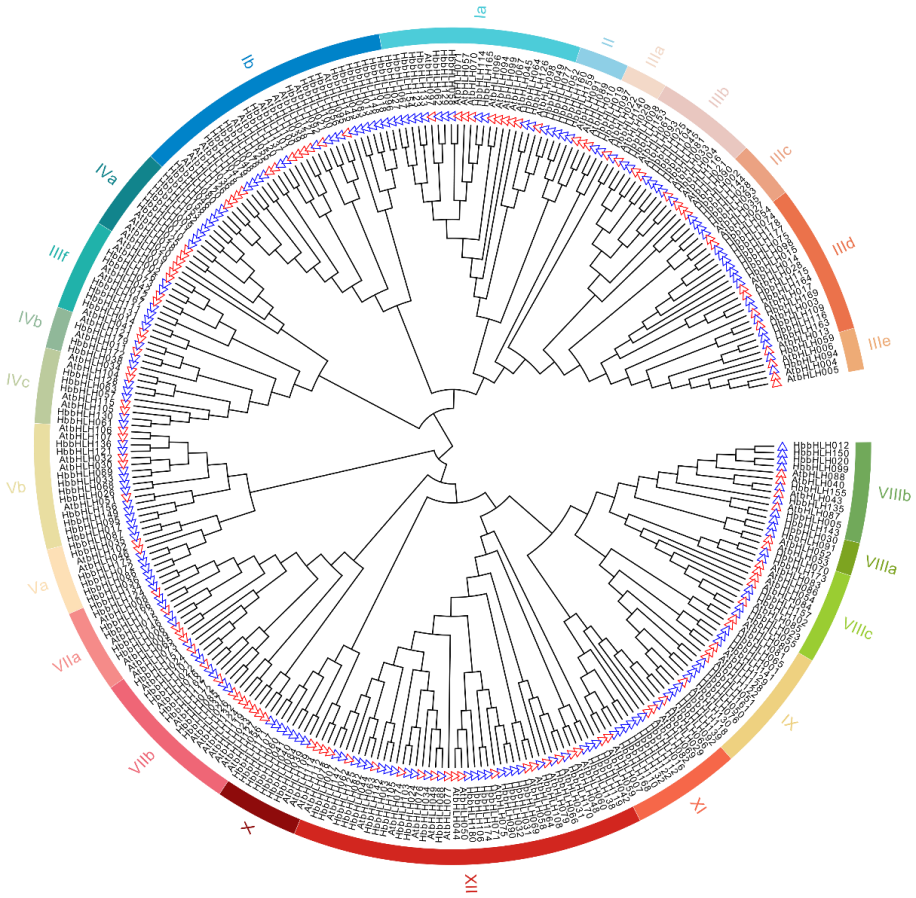

Figure 3. Phylogenetic analysis of bHLH gene families. The phylogenetic tree was generated using MEGA 7.0 with 1000 bootstrap replicates. Different colors indicate different subgroups. Red triangles represent *AtbHLH* proteins and blue triangles represent *HbbHLH* proteins.

3.3. Gene Structure, Regulatory Elements, Chromosomal Location, and Synteny Analysis of Hevea bHLH

The gene structure of the *Hevea* bHLH family exhibited substantial variation, with intron numbers ranging from 0 to 12 across the 180 identified genes (Figure S1). Notably, 20 genes are intronless, primarily distributed across subfamilies IIId, IIIe, and VIIIb, suggesting potential functional specialization or evolutionary pressure favoring rapid transcriptional responses [56]. Members of the same subfamily have similar structures. For example, all members of the IIId family, except *HbbHLH109*, were intronless (Figure 4a). Families Ia, Ib, IIIa, IIIb, IVc, IVb, Vb, VIIIa, and X contain 1–4 introns, while subfamilies such as IIIf, Va, VIIa, VIIb, and XII exhibit more complex structures with 5 to 10 introns (Figure S1). Conserved motif analysis using the MEME tool revealed that all *HbbHLH* genes share a core motif (motif 3), most of genes contain 3–5 conserved motifs, while members of families IIId, IIIe, and IIIf contain more conserved motifs, ranging from 7 to 11. The most conserved motifs are distributed at the C-terminus, such as those in families I, IIIb, IVa,

VIIb, VIIa, XII, XI, IX, VIIIa, VIIIb, and VIIIc. Families IIId, IIIe, and IIIf had a segment of conserved motifs at the N-terminus (Figure S1).

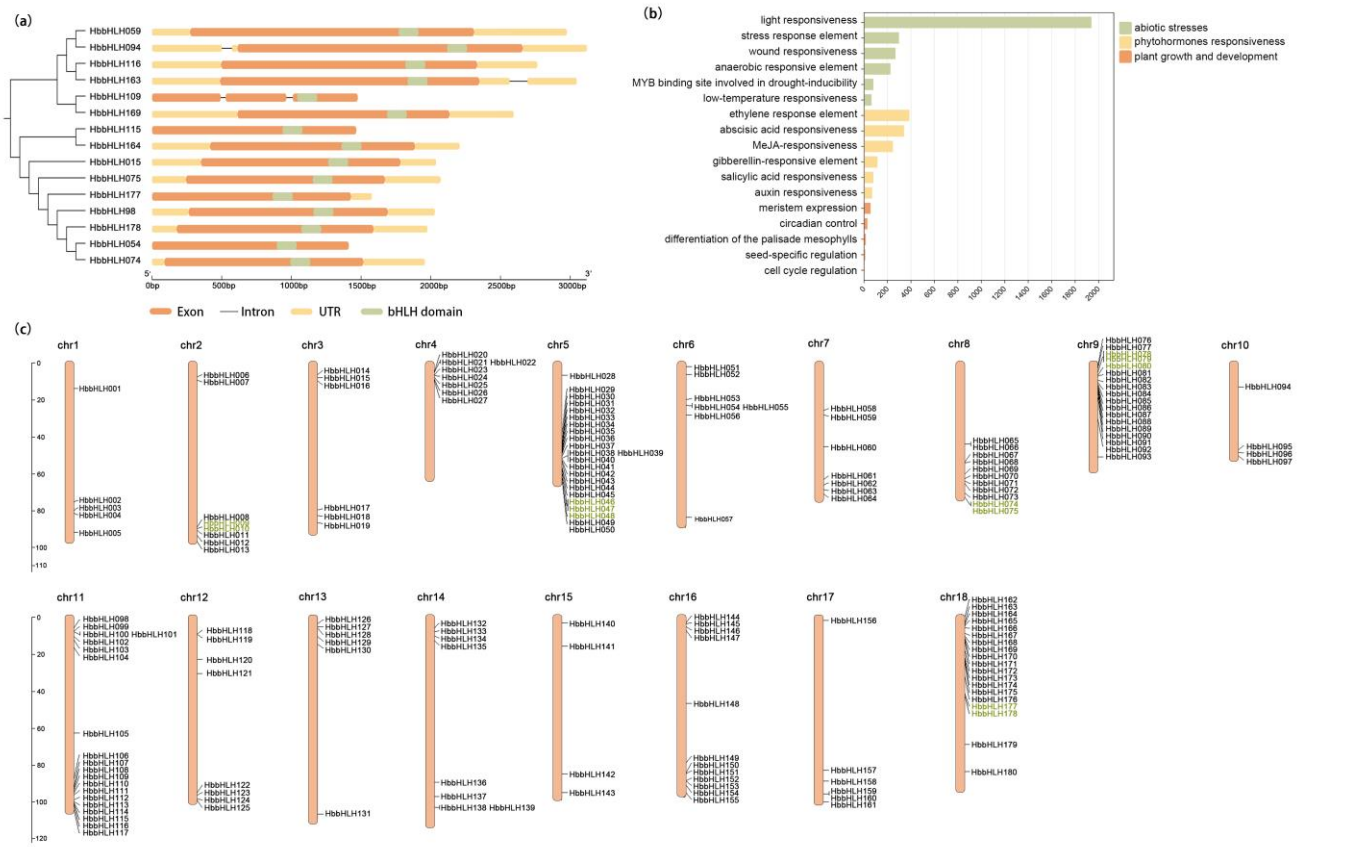

Figure 4. Gene structure, *cis*-regulatory elements, and chromosomal localization of *HbbHLH* genes. (**a**) Gene structure and domain positions of *HbbHLH* IIId and IIIe subfamilies. (**b**) Statistics of the three categories of *cis*-regulatory elements in *HbbHLH* genes. (**c**) Chromosomal localization of *HbbHLH* genes, with tandemly duplicated genes marked in green.

An analysis of *cis*-regulatory elements within the 1500 bp promoter regions of the *HbbHLH* genes uncovered a rich diversity of regulatory motifs (Figure S2), reflecting the complex regulatory networks governing their expression. The upstream *cis*-regulatory elements of *HbbHLH* genes were divided into three main categories. The first category is related to abiotic stress, such as light-responsive elements (Box 4, G-box, GT1-motif), antioxidant response elements (ARE), low-temperature response elements (LTR), drought-induced elements (MBS), and wound response elements (WUN-motif, W box). The second category is related to phytohormone responsiveness, such as ethylene-responsive elements (ERE), abscisic acid-responsive elements (ABRE), methyl jasmonate-responsive elements (TGACG-motif), salicylic acid-responsive elements (TCA-element, SARE), gibberellin-responsive elements (P-box, GARE-motif), and auxin-responsive elements (TGA-element, AuxRR-core). The third category is related to plant growth and development, such as circadian rhythm regulatory elements (circadian), cell cycle regulatory elements (re2f-1), seed-specific regulatory elements (RY-element), endosperm-specific regulatory elements (AACA_motif), and meristem expression-related elements (CAT-box).

Among the 4187 *cis*-regulatory elements found, the first category had the highest frequency, with 2858 elements, accounting for 68% of the total. Light-responsive elements were particularly abundant, accounting for 46% of the total elements. The second category contained 1225 elements, mainly ethylene, abscisic acid, and methyl jasmonate-responsive elements. The third category of regulatory elements was less frequent with only 104 el-

ements (Figure 4b). This suggests that *HbbHLH* genes may be primarily involved in the responses to various plant hormones and abiotic stresses.

Chromosomal localization results showed that the 180 *HbbHLH* genes were unevenly distributed across 18 chromosomes of the rubber tree (Figure 4c). Most of the *HbbHLH* genes were concentrated in the terminal regions of the chromosomes; chromosomes 10 and 15 had the fewest, with only 4 genes, while chromosome 5 had the most, containing 23 *HbbHLH* genes. Among the 180 *HbbHLH* genes, 12 tandemly duplicated genes formed five gene clusters, which are marked in green in Figure 4c.

Synteny analysis indicated that segmental duplications were the predominant factor driving the expansion of the bHLH gene family in the rubber tree. Among the 180 identified *HbbHLH* genes, 136 (approximately 75%) were segmentally duplicated, underscoring the critical role of genome-wide duplication events in shaping the diversity of this transcription factor family (Figure 5). These results suggest that segmental duplications significantly contributed to the retention and diversification of *HbbHLH* genes, allowing for functional divergence and adaptation in response to the unique selective pressures experienced by rubber trees.

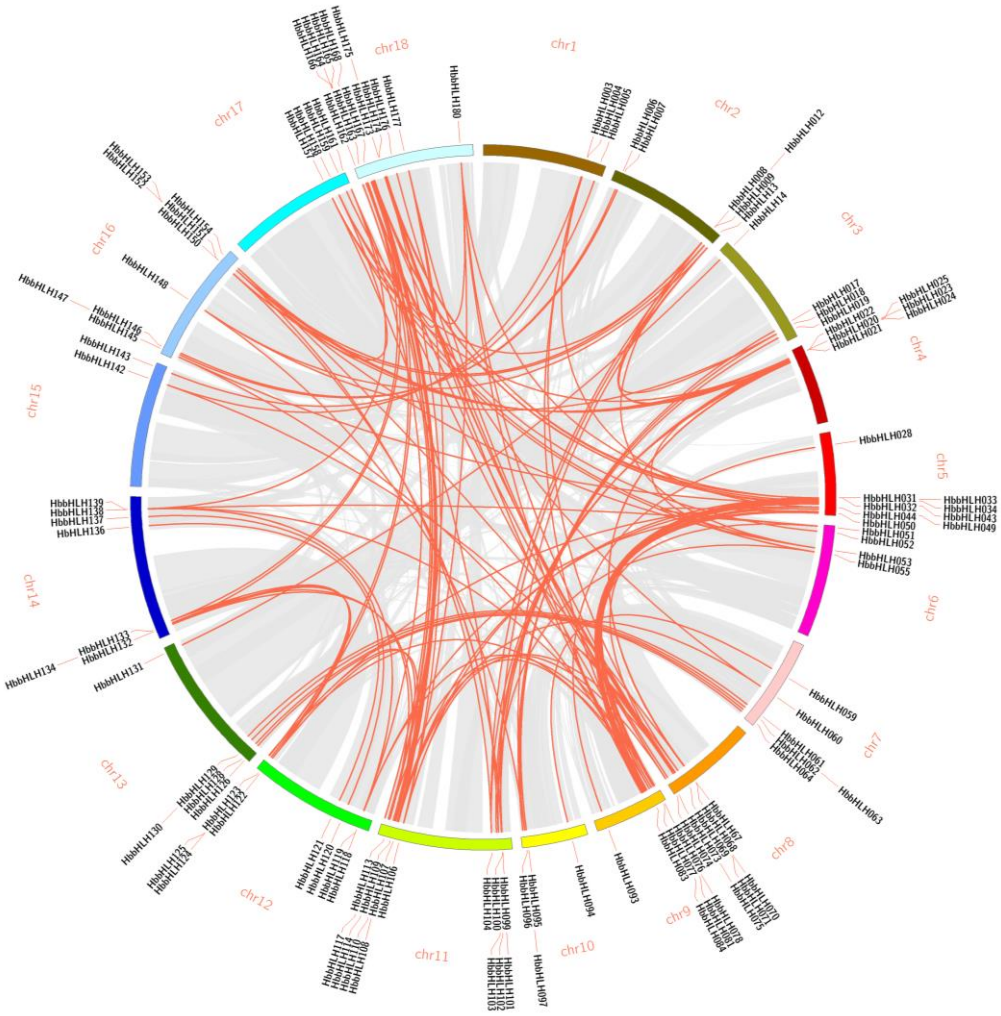

Figure 5. Collinear analysis of *HbbHLH* genes. Gray lines in the background indicate all collinear blocks within the rubber tree genome, whereas red lines indicate collinear gene pairs of *bHLH* genes.

3.4. Gene Ontology and Interaction Network Analysis of Hevea bHLH

Based on previous analyses of the *HbbHLH* gene structure and motif diversity, as well as literature mining, it is speculated that *HbbHLH* genes are involved in a range of biological processes. To further investigate the function of these genes, Gene Ontology

(GO) enrichment analysis was performed. The results showed that 94 *HbbHLH* genes were enriched among 134 GO terms. Specifically, all nine molecular function (MF) terms were associated with DNA binding and RNA polymerase II specificity, whereas the two cellular component (CC) terms were related to transcription regulation complexes. More importantly, enrichment in 123 biological processes (BP) highlights the broad functional repertoire of *HbbHLH* genes, with notable processes including positive regulation of RNA polymerase II-mediated transcription, plant development and differentiation, light response, and morphogenesis (Figure 6). These findings suggest that *HbbHLH* genes are integral to the coordination of growth and development of rubber trees.

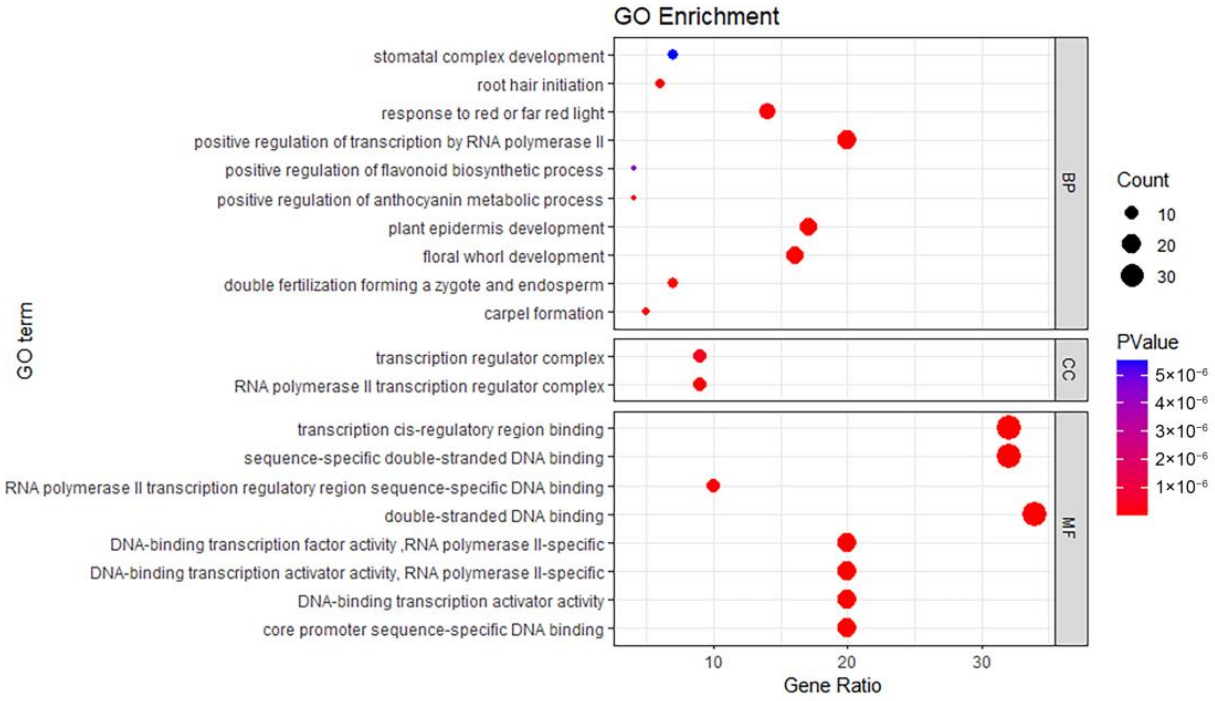

Figure 6. The Gene Ontology enrichment analysis of *HbbHLH* genes in rubber trees. Categorized into biological processes (BP), (only the top ten processes are shown), molecular functions (MF), and cellular components (CC).

Furthermore, various biological processes, including reproductive development, seed and seedling development, hormone biosynthesis (for example brassinosteroids and gibberellins), metabolism, secondary metabolism (mainly flavonoids), pigment metabolism, iron ion homeostasis, metabolism, and photosynthesis regulation were enriched (Table S2). These findings indicate that bHLH transcription factors are broadly involved in critical biological processes in rubber tree reproduction, growth, development, and stress responses, highlighting their significant roles.

In *A. thaliana*, the functions and interactions of various bHLH proteins have been well characterized by mapping *Hevea* bHLH proteins onto their *Arabidopsis* homologues, and an interaction network for 65 *HbbHLH* genes has been inferred. STRING analysis revealed extensive interactions among these *HbbHLH* proteins (Table S3), consistent with the characteristic ability of the bHLH family to form homodimers or heterodimers [57]. For example, homologues of *SCRM* (*HbbHLH004*), *MUTE* (*HbbHLH126*), *FAMA* (*HbbHLH062*), and *SPCH* (*HbbHLH049*) interact to regulate stomatal development [58]. Additionally, *IDT* (*HbbHLH063*), *PYE* (*HbbHLH038*), and *ILR3* (*HbbHLH061*) interact with each other and contribute to iron homeostasis [59]. These findings provide valuable insights into the potential functions of the bHLH genes in rubber trees.

The predicted interactions between *HbbHLH* proteins and other genes revealed three major functional clusters: one involving iron metabolism, with interactions including *BTS*,

FRO2, *ZIF1*, and *NAS4*; another related to plant growth and development, particularly brassinosteroid signaling (for example, *IBH1*, *BZR1*), and light responses (for example, *PAR1* and *CRY2*); and a third cluster associated with epidermal and stomatal development, involving genes such as *EPFL4* and *EPF2* (Figure 7). These interaction patterns suggest that the bHLH gene family plays a significant regulatory role in metal homeostasis, hormone signalling pathways, light-responsive processes, and epidermal and stomatal development in rubber trees.

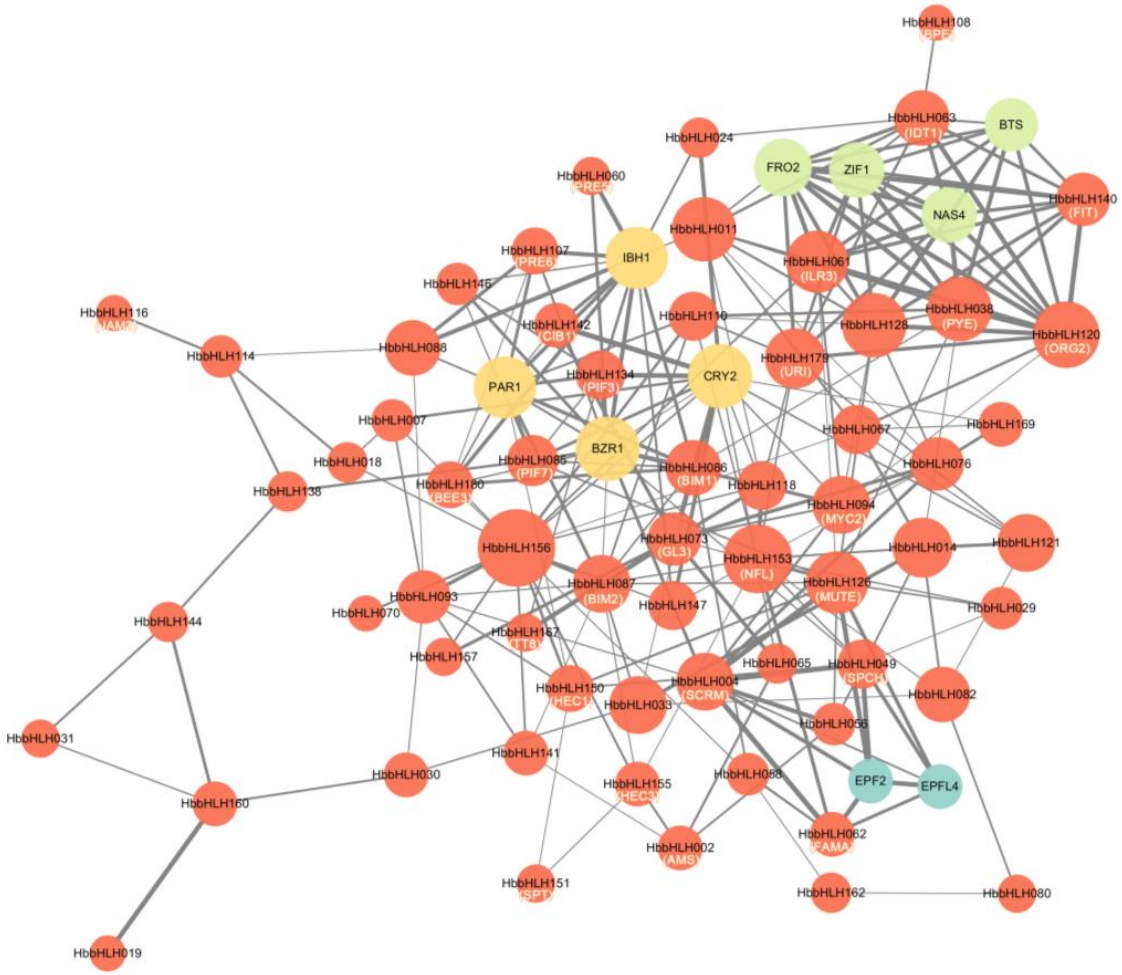

Figure 7. Protein interaction network of *HbbHLH* genes mapped to *Arabidopsis* genes. The circle size indicates the number of interaction partners, with larger circles representing more extensive interaction networks. The thickness of the connecting lines reflects the combined interaction scores, with thicker lines denoting stronger interactions. Orange circles highlight rubber tree bHLH genes, with black text indicating rubber tree gene ID and white text (in parentheses) showing their corresponding *Arabidopsis* homologues. Non-orange circles represent non-bHLH proteins labelled with *Arabidopsis* homologue names. Different colors denote distinct functional classifications.

3.5. Expression Patterns of Hevea bHLH in Diverse Environmental and Physiological Events

To investigate the spatiotemporal expression patterns of *HbbHLH* genes, expression levels (FPKM) from different tissues and treatments of 16 rubber tree varieties were downloaded from the HeveaDB database [51] (Table S4). A total of 39 *HbbHLH* genes with expression levels below 2 in all samples, such as *HbbHLH009*, *HbbHLH010*, and *HbbHLH019*, were filtered out, and a heatmap was created (Figure 8a).

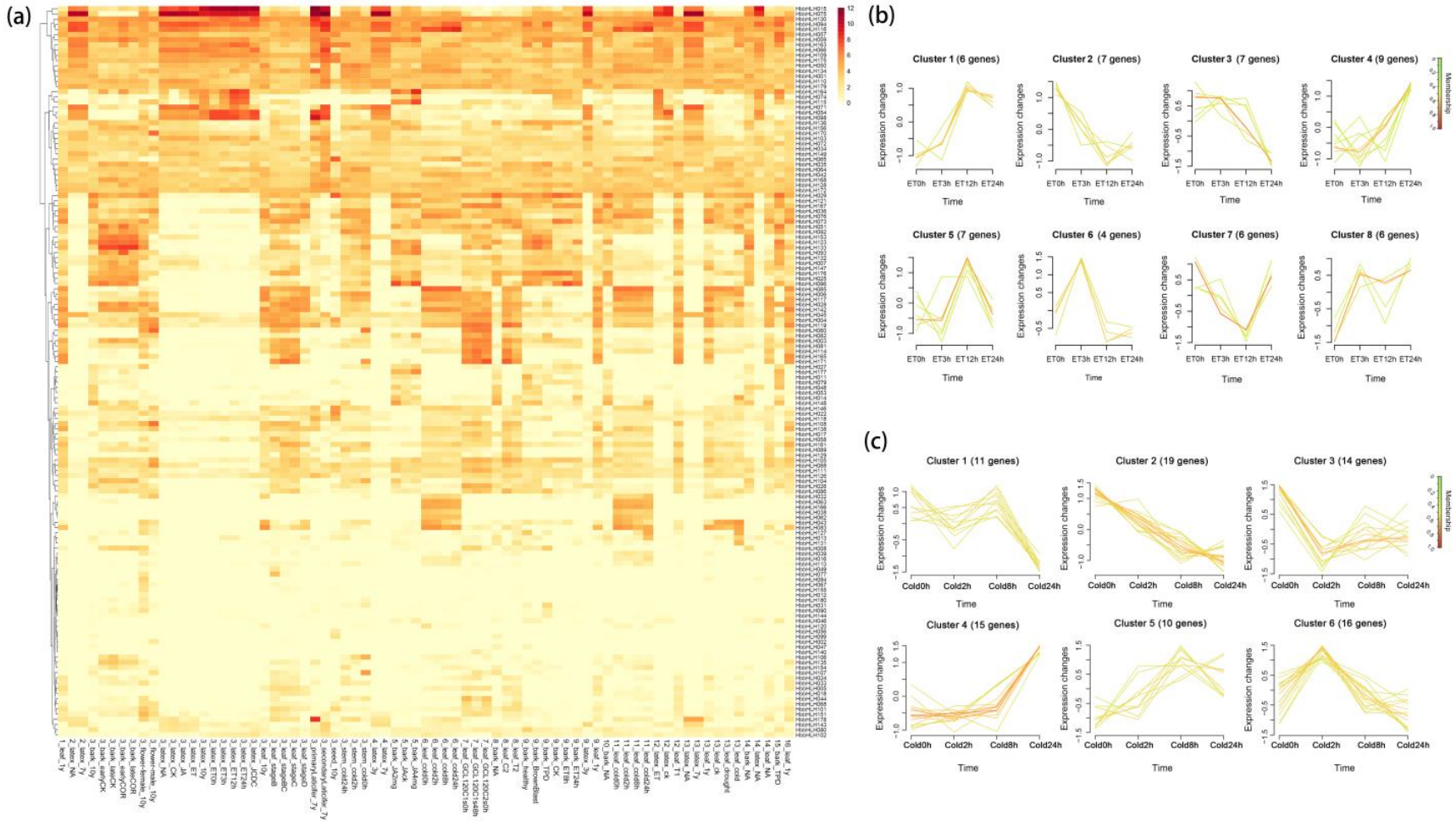

Figure 8. Temporal and spatial expression patterns of *HbbHLH* genes in 16 rubber tree varieties. (**a**) Heatmap of *HbbHLH* gene expression patterns in rubber trees. Each row represents an *HbbHLH* gene and the column names are formatted as variety_tissue_treatment. The variety numbers represent the following rubber tree varieties: 1: BT3410, 2: CATAS7-20-59, 3: CATAS7-33-97, 4: CATAS8-79, 5: CATAS88-13, 6: CATAS93-114, 7: FX3864, 8: GT1, 9: PR107, 10: PR255, 11: REKEN501, 12: RRII105, 13: RRIM600, 14: RRIM928, 15: TB1, and 16: Wencang11. (**b**,**c**) The series of diagrams illustrates the patterns of dynamic changes in *HbbHLH* DEGs during ethylene treatment and cold exposure, respectively, using Mfuzz.

Most *HbbHLH* genes exhibit significant tissue-specific expression in various tissues. For example, *HbbHLH015* and *HbbHLH075* showed significantly higher expression in latex than in other tissues; *HbbHLH060*, *HbbHLH170*, and *HbbHLH108* were highly expressed in male flowers, and *HbbHLH167*, *HbbHLH123*, and *HbbHLH096* were highly expressed in the bark. Genes within the same subfamily displayed similar expression patterns. Except for *HbbHLH177* and *HbbHLH178*, genes in the IIId and IIIe families were significantly expressed in latex. Most genes highly expressed in male flowers belonged to the XII family, whereas those highly expressed in the bark were mainly from the Ib family.

In rubber trees, jasmonic acid (JA) and ethylene are crucial for promoting laticifer differentiation [60] and latex production [61]. Previous studies have indicated that certain bHLH transcription factors respond to JA signals and integrate into an ethylene-regulated signalling network. In this study, we examined the expression patterns of *HbbHLH* genes under JA and ethylene stimulation using transcriptome data. The analysis revealed that most *HbbHLH* genes that were highly expressed in latex were sensitive to JA and ethylene. For example, *HbbHLH015*, *HbbHLH074*, and *HbbHLH164* were significantly upregulated by ethylene and JA treatment. Additionally, as treatment duration increased, these *HbbHLH* genes exhibited specific expression trends, revealing the dynamic characteristics of gene expression under hormone regulation.

To further understand the dynamic changes in gene expression during ethylene treatment, Mfuzz was used to group all differentially expressed genes at different time points into eight clusters (Figure 8b). Clusters 1 and 2 showed continuous upregulation and downregulation, respectively, after 12 h of ethylene treatment. Changes in Clusters 3 and 4 occurred at approximately 3 h, with significant downregulation and upregulation observed afterwards, respectively. Clusters 5 and 6 exhibited significant upregulation, followed by downregulation at approximately 12 h and 3 h, respectively. Cluster 7 displayed a general downregulation trend before 12 h and significant upregulation afterwards. Cluster 8 was significantly upregulated before 3 h of treatment, followed by fluctuations between downregulation and upregulation.

Given the significance of cold resistance mechanisms in rubber tree research and the role of bHLH transcription factors in response to cold, a similar time series analysis was conducted on *HbbHLH* genes under cold treatment. Mfuzz divided them into six clusters (Figure 8c), revealing that the genes in clusters 1 and 4 were significantly downregulated and upregulated after 8 h, respectively. Genes in cluster 2 were consistently downregulated throughout the cold stress period; cluster 3 showed significant downregulation between 0 and 2 h; cluster 6 showed significant upregulation between 0 and 2 h, followed by significant downregulation; and cluster 5 exhibited an overall upregulation trend.

Additionally, *HbbHLH* genes exhibited significant expression dynamics at various stages of leaf growth and development in rubber trees. Specifically, *HbbHLH077* exhibited the highest expression during Stage B, whereas *HbbHLH171* peaked during Stage BC. The expression levels of *HbbHLH083*, *HbbHLH076*, *HbbHLH006*, etc., gradually increased with leaf maturation. Conversely, *HbbHLH045* and *HbbHLH133* showed decreasing trends throughout the developmental stages. Notably, most *HbbHLH* genes were abundantly expressed during Stages B, BC, and C but significantly decreased at Stage D. Furthermore, members of the *HbbHLH* family also responded to the disease. During tapping panel dryness (TPD), a common stress condition encountered by rubber trees due to frequent tapping for latex collection, genes such as *HbbHLH156*, *HbbHLH176*, and *HbbHLH086* were upregulated, whereas *HbbHLH096* and *HbbHLH167* were downregulated. When confronted with Brown Blast infection, the expression of genes such as *HbbHLH094* and *HbbHLH163* decreased, but *HbbHLH059*, *HbbHLH123*, *HbbHLH133*, and others were upregulated, while under drought stress, the expression of genes such as *HbbHLH057*, *HbbHLH164*, and *HbbHLH167* was inhibited, while genes such as *HbbHLH029*, *HbbHLH051*, and *HbbHLH176* were upregulated. These observations strongly support the functional diversity of bHLH transcription factors, indicating their extensive involvement in regulating plant growth and development, hormone responses, and stress adaptation.

Genes that were significantly highly expressed in all tissues or showed differential expression in at least one of the five tissues were selected for RT-qPCR validation of the expression levels in different tissues (bark, leaves, latex, female flowers, and male flowers). The qPCR results were largely consistent with the transcriptome data. *HbbHLH130* showed a relatively uniform expression across all tissues. *HbbHLH015* was highly expressed in the latex. *HbbHLH060* was highly expressed in the male flowers. *HbbHLH083* was significantly highly expressed in the leaves and almost undetectable in other tissues. *HbbHLH123* showed higher expression levels in bark and flowers (Figure 9).

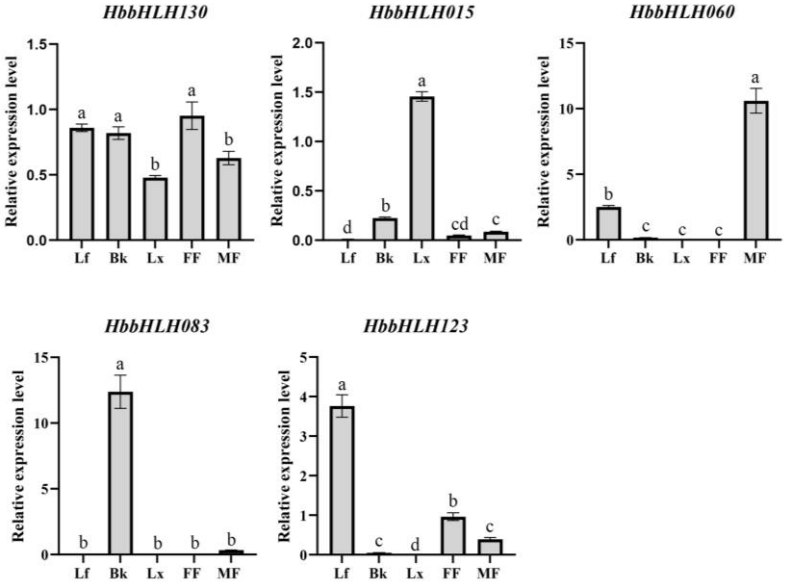

Figure 9. Transcriptional analysis of five *HbbHLH* genes across different tissues in the rubber tree with error bars representing the standard deviation of three technical replicates. Bk, bark; Lf, leaf; Lx, latex; FF, female flower; MF, male flower. Statistical significance was determined using one-way ANOVA and Tukey's multiple comparison test, with differences denoted by lowercase letters.

Based on the transcriptome data and previous research, 11 genes were selected for RT-qPCR to further elucidate the expression patterns of *HbbHLH* genes under ethylene and methyl jasmonate treatments. Some of these selected gene homologues have been shown in other plants to be involved in responses to ethylene and methyl jasmonate.

The results indicated that most of the selected genes responded to ethylene and methyl jasmonate treatment. Under ethylene treatment, *HbbHLH015*, *HbbHLH115*, *HbbHLH163*, and *HbbHLH164* displayed an initial upregulation, followed by downregulation, with a peak at 8 h post-treatment. *HbbHLH098*, *HbbHLH109*, and *HbbHLH116* were significantly upregulated within the first 4 h, followed by downregulation, with expression levels rebounding 12 h post-treatment. *HbbHLH059*, *HbbHLH074*, *HbbHLH075*, and *HbbHLH094* exhibited fluctuations in expression during the early stages of treatment, peaking at 24 h (Figure 10a). Under methyl jasmonate treatment, most genes were significantly upregulated, peaking at 8–12 h. Notably, *HbbHLH094*, *HbbHLH163*, *HbbHLH074*, and *HbbHLH115* demonstrated similar expression patterns under JA treatment (Figure 10b). Additionally, *HbbHLH075* and *HbbHLH163* showed similar transcriptional changes after treatment with ethylene and methyl jasmonate. However, the quantitative results for individual genes did not always align with the transcriptional changes observed in transcriptome data. This discrepancy may be due to sample heterogeneity, experimental variability, or differences in the data normalization methods.

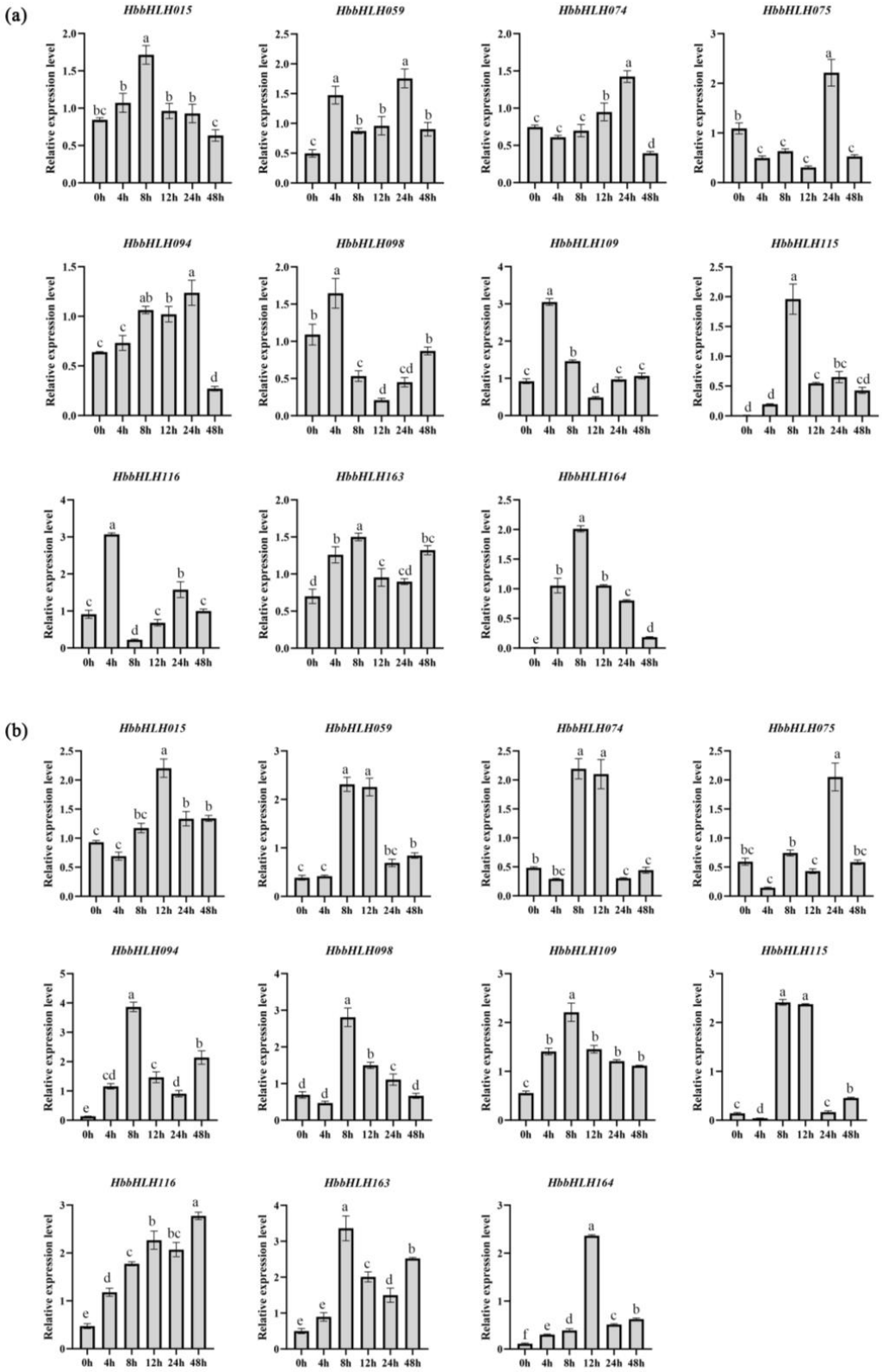

Figure 10. Transcriptional analysis of 11 *HbbHLH* genes in latex following treatment with ethylene (**a**) and methyl jasmonate (**b**). The x-axis labels denote ethylene (ET) and methyl jasmonate (JA). Error bars represent the standard deviation of three technical replicates. Statistical significance was assessed using one-way ANOVA and Tukey's multiple comparison test, with differences indicated by lowercase letters.

Similarly, based on transcriptome data and previous research, 12 *HbbHLH* genes were selected to validate changes in their expression patterns under cold conditions. The results showed that genes such as *HbbHLH094*, *HbbHLH076*, and *HbbHLH163* were significantly upregulated under cold conditions, whereas *HbbHLH043*, *HbbHLH051*, and *HbbHLH179* were significantly downregulated. Interestingly, *HbbHLH094* and *HbbHLH163*, which exhibited similar expression trends under methyl jasmonate treatment, also displayed similar expression patterns under cold conditions (Figure 11).

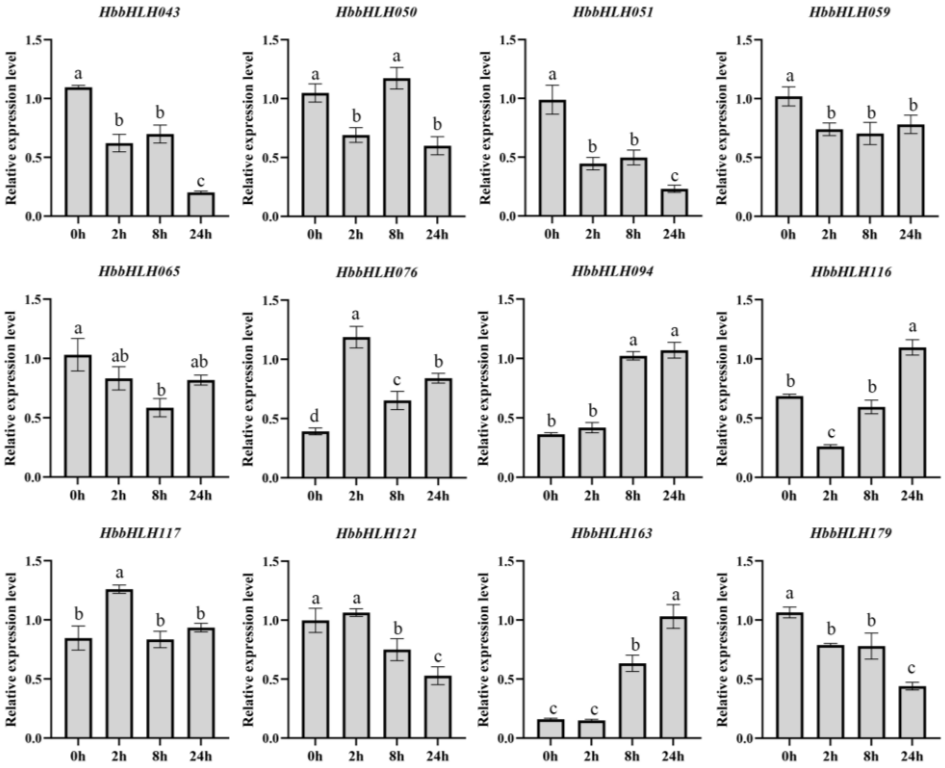

Figure 11. Transcriptional analysis of 12 *HbbHLH* genes in leaves at low temperatures (4 °C). Error bars represent the standard deviations of three technical replicates. Statistical significance was assessed using one-way ANOVA and Tukey's multiple comparison test, with differences indicated by lowercase letters.

4. Discussion

In most species, despite varying selective pressures, the number of bHLH genes remains relatively consistent. For example, the 119.1 Mb genome of *A. thaliana* has 162 bHLH genes [7]. In contrast, the 385.7 Mb genome of rice (*Oryza sativa*) contains 167 genes [9]. With a 392.2 Mb genome, *Populus trichocarpa* Torr. & Gray has 183 bHLH genes [8]. A total of 197 of these genes were found in *Pyrus* × *bretschneideri* Rehd [62], which has a genome size of 509.1 Mb. Finally, *H. brasiliensis* has 180 bHLH genes despite having a larger genome (1.9 Gb). This stability suggests that bHLH transcription factors play essential roles in fundamental biological processes, and that the family has undergone considerable evolutionary conservation.

In the rubber tree, the bHLH genes were categorized into 23 subfamilies; the number of members in most subfamilies was proportional to that in *Arabidopsis*.

Genes with close phylogenetic relationships and protein domains often have similar functions [63]. Previous studies have shown that in *Arabidopsis*, three bHLH proteins from the Ia family, *SPCH*, *MUTE*, and *FAMA*, along with their heterodimeric partners *SCRM*, collectively mediate cell-state transitions and induce stomatal development [64,65]. In rubber trees, homologous genes *HbbHLH049*, *HbbHLH126*, *HbbHLH062*, and *HbbHLH004* are likely to have similar functions. This conclusion is supported by the GO enrichment

analysis, which showed that these four genes, along with other members of the Ia family, are enriched in the biological process of stomatal complex development. Similarly, the IX subfamily in *Arabidopsis* includes *AtbHLH80* (*FBH*) genes, which regulate flowering time via the CO pathway [15]. In *Hevea*, the IX subfamily members *HbbHLH076*, *HbbHLH118*, and *HbbHLH139* were enriched in flowering-related GO terms (GO:0048573), suggesting similar regulatory roles. Additionally, the Ib subfamily members *HbbHLH038*, *HbbHLH120*, and *HbbHLH137* may regulate iron homeostasis in a manner similar to *AtbHLH38* (*ORG2*) and *AtbHLH39* (*OGR3*) [18].

Protein–protein interaction analysis identified interactions between bHLH genes and other functionally characterized genes. For example, *HbbHLH156* interacts with 16 other bHLH proteins across various subfamilies, suggesting that it is involved in important regulatory networks. However, research on this gene remains limited and further studies are warranted to explore its role in rubber tree physiology.

Jasmonic acid (JA) plays a crucial regulatory role in latex drainage in rubber trees [60,66], with MYC transcription factors from the bHLH family serving as key components of the JA signalling pathway [67]. In this study, we identified two subfamilies, IIId and IIIe, which are specifically and highly expressed in latex and show significant responsiveness to JA treatment. Phylogenetic analysis revealed that these genes closely cluster with the MYC family in *A. thaliana*. *AtMYC2*, *AtMYC3*, and *AtMYC4* regulate a range of JA-dependent functions, with *AtMYC2* being pivotal to the COI1-JAZ-MYC2 module [68]. In rubber trees, the HbCOI1–HbJAZ3–MYC2 module has been shown to regulate rubber biosynthesis [69]. Additionally, previous research has demonstrated that *HbMYC* can transcriptionally activate *HbPSK* to promote laticifer differentiation [70], thereby increasing rubber yield, a process that is also regulated by JA. In our study, we found that the rubber tree genes *HbbHLH059* and *HbbHLH094*, belonging to the IIIe subfamily, share high sequence similarity with the previously identified *HbMYC2b* [32] and *HbMYC2* [71] genes, which have been reported to mediate the upregulation of the rubber biosynthesis-related genes *HbFPS* and *HbSRPP*. Furthermore, under JA treatment, members of the IIId subfamily, *HbbHLH074*, *HbbHLH163*, and *HbbHLH115*, exhibited transcriptional changes similar to those of *HbbHLH094* and *HbbHLH059*. Therefore, we hypothesized that these genes may also positively regulate the transcription of rubber biosynthesis-related genes.

Phylogenetic analysis revealed that the IIId subfamily in rubber trees has undergone significant expansion compared to *Arabidopsis*, with four times as many members (Figure 3). Expression analysis showed that nearly all IIId subfamily members are highly and specifically expressed in latex, particularly *HbbHLH015* and *HbbHLH075*, whose expression levels in latex are more than tenfold higher compared than in other tissues. Jasmonic acid-responsive elements were identified in the promoter regions of *HbbHLH116*, *HbbHLH109*, *HbbHLH169*, *HbbHLH015*, and *HbbHLH098*, whereas ethylene-responsive elements were found in the promoters of *HbbHLH116*, *HbbHLH064*, and *HbbHLH075*. The expression levels of these genes showed significant changes under both the JA and ethylene treatments. Given that ethylene and JA have been shown to promote latex drainage in rubber trees, we speculated that these genes may also be involved in regulating rubber biosynthesis.

Several *HbbHLH* genes, including *HbbHLH043*, *HbbHLH051*, *HbbHLH076*, *HbbHLH094*, *HbbHLH116*, and *HbbHLH163*, showed significant transcriptional changes after cold treatment. Additionally, multiple cold-responsive elements were identified in the promoter regions of *HbbHLH043*, *HbbHLH121*, and *HbbHLH117*, suggesting that these genes may be involved in the cold tolerance response of rubber trees. According to the GO enrichment analysis, these genes are involved in multiple subfamilies and are implicated in a variety of biological processes. This suggests that these genes may participate in indirect pathways related to cold stress, such as light signalling, stomatal movement, or hormone response pathways.

Previous studies have shown that *MYC2* mediates cold stress responses through the JA signalling pathway in apples [72]. In *Hevea*, *HbbHLH094* exhibited significant transcriptional changes under both low-temperature and JA treatments, suggesting that it might also

participate in JA-mediated cold stress responses. In contrast, the segment duplication gene *HbbHLH059* did not display similar changes in expression, likely because of differences in *cis*-regulatory elements. Notably, *HbbHLH094* and *HbbHLH163* displayed similar transcriptional patterns in response to both methyl jasmonate and cold treatments, indicating that they may jointly participate in JA pathway regulation of cold stress responses.

5. Conclusions

This study identified 180 bHLH genes in *H. brasiliensis*, highlighting the evolutionary conservation and functional diversity of the bHLH gene family. Through the integration of extensive transcriptomic data, GO enrichment analysis, and protein interaction analysis, we demonstrated that bHLH genes in *Hevea* are involved in a broad spectrum of biological processes including growth and development, photomorphogenesis, iron ion homeostasis, secondary metabolite synthesis and metabolism, and responses to abiotic stress and hormonal signals. We hypothesized that *HbbHLH094*, *HbbHLH059*, *HbbHLH163*, *HbbHLH074*, and *HbbHLH115* may play a positive regulatory role in latex biosynthesis-related genes. *HbbHLH094* and *HbbHLH163* are possibly involved in cold stress responses through the JA signalling pathway. However, because the current analysis is primarily based on computational predictions, transcriptomic and enrichment analyses offer valuable insights into potential gene functions. However, direct experimental validation is required to confirm the roles of these *HbbHLH* genes. Additionally, the precise molecular mechanisms by which these genes influence rubber biosynthesis remain unclear and present a critical avenue for future research. Overall, the findings of this study provide a solid foundation for a more comprehensive understanding of the bHLH gene family in *Hevea brasiliensis* and offer important clues for the functional characterization of individual members.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/f15112027/s1, File S1: Amino acid sequences of 180 *HbbHLH* proteins. File S2: CDS of 180 *HbbHLH* genes. Supplemental Figures: Figure S1: Gene structure and conserved motif analysis of 180 *HbbHLH* genes. Figure S2: Analysis of cis-regulatory elements in the promoter regions of 180 *HbbHLH* genes. Table S1: Characterization of 180 *HbbHLH* genes. Table S2: GO enrichment analysis of 180 *HbbHLH* genes. Table S3: Protein interaction network of *HbbHLH* genes mapped to *Arabidopsis*. Table S4: Raw data for transcriptomic analysis of 180 *HbbHLH* genes. Table S5: The primers used in RT-qPCR analysis.

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Conflicts of Interest: The authors declare no conflicts of interest.

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