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Genomic Survey and Cold-Induced Expression Patterns of *bHLH* Transcription Factors in *Liriodendron chinense* (Hemsl) Sarg.

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Abstract: *bHLH* transcription factors play an animated role in the plant kingdom during growth and development, and responses to various abiotic stress. In this current study, we conducted, the genome-wide survey of *bHLH* transcription factors in *Liriodendron chinense* (Hemsl) Sarg., 91 *LcbHLH* family members were identified. Identified *LcbHLH* gene family members were grouped into 19 different subfamilies based on the conserved motifs and phylogenetic analysis. Our results showed that *LcbHLH* genes clustered in the same subfamily exhibited a similar conservative exon-intron pattern. Hydrophilicity value analysis showed that all *Lc*bHLH proteins were hydrophilic. The Molecular weight (Mw) of *Lc*bHLH proteins ranged from 10.19 kD (*LcbHLH*15) to 88.40 kD (*LcbHLH*50). A greater proportion, ~63%, of *Lc*bHLH proteins had a theoretical isoelectric point (pI) less than seven. Additional analysis on the collinear relationships within species and among dissimilar species illustrated that tandem and fragment duplication are the foremost factors of amplification of this family in the evolution process, and they are all purified and selected. RNA-seq and real-time quantitative PCR analysis of *LcbHLH* members showed that the expression of *LcbHLH*35, 55, and 86 are up-regulated, and the expression of *LcbHLH*9, 20, 39, 54, 56, and 69 is down-regulated during cold stress treatments while the expression of *LcbHLH*24 was up-regulated in the short term and then later down-regulated. From our results, we concluded that *LcbHLH* genes might participate in cold-responsive processes of *L. chinense*. These findings provide the basic information of *bHLH* gene in *L. chinense* and their regulatory roles in plant development and cold stress response.

Keywords: *bHLH* transcription factor; cold stress; expression pattern; genome-wide identification; *Liriodendron chinense*

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1. Introduction

Globally out of all abiotic stress factors, cold, drought, and heat stresses are declared as the most complex ones affecting plant growth, survival, and crop productivity. Molecular regulation at the post-transcriptional level possesses a vital role for development, growth, nutrient allocation, and defensive mechanism in plants [1,2]. The *bHLH* family regulates growth and development, morphogenesis, and stress responses in plants [3–5], characterized by a helix-loop-helix (HLH) domain, with an approximated 15 amino acids N-terminal as the base region: known for recognizing and binding to specific DNA while, the C-terminal is the HLH region with about 50 amino acids [6–8]. The helix is also associated with DNA sequences that recognize protein-specific binding [9] and can form homodimer or heterodimer with other proteins [10]. On top of an α-helix near the N-terminal is another

α- helix [11]. The two α-helices are connected with a ring formed by amino acid chains to form an HLH structure.

Generally, *bHLH* transcription factors are known to act as transcriptional activators or inhibitors for seed germination and flowering regulation [9]. However, a study in Arabidopsis mutant srl2, *AtPIF4* (*AtbHLH*09) spectacled a specific role in the signaling network in phytochrome B (phyB) and in light regulation [12]: *AtPRE1* (At*bHLH*136) and ILI1 were also identified to regulate cell elongation by interacting with IBH1 (At*bHLH*158) under the action of brassinosteroids (BR) and gibberellin signals [13]. Moreover, *AtPRE1* (*AtbHLH*136) and *IBH1* (*AtbHLH*158) form regulatory system with *AtACE*1/2/3 (*AtbHLH*049/074/077) that competitively regulate cell growth. IBH1 (*AtbHLH*158) has also been shown to negatively regulate cell growth by interacting with the positive regulatory gene *AtACE*1/2/3 (*AtbHLH*049/074/077) [13]. Certain members of the *bHLH* transcription factor family have also been shown to enhance resistance to harsh conditions when plants retort to abiotic stresses [14,15]. For instance, overexpression of *AtICE*1 (*AtbHLH*116) and *AtICE*2 (*AtbHLH*33) can augment the expression of *CBF* promoter at low temperature and mend the stress resistance of transgenic plants [16,17]. Feng et al. [18] has also demonstrated that *MdCIbHLH*1 protein binds to the *MdCBF*2 promoter and upregulates the expression of *CBF*2 through the C-repeat-binding factor (CBF) pathway and promote the cold tolerance of transgenic apple plants. A study in trifoliate orange has also shown *PtrbHLH* to increase cold resistance by activating *PtrCA*T [19].

To date, research on different plant genomes has concurred that the *bHLH* transcription factor family is incessantly distinguished, with the structural characteristics and response profiles to various environmental stresses [10,20–22]. Nonetheless, few studies on the *bHLH* gene family of the forest tree species have been conducted with less on the *L. chinense*. *L. chinense* is a kind of tall deciduous tree, which is of economic, ornamental, medicinal, and ecological value [23,24]. The recent release of the *L. chinense* genome provided the opportunity for its *LcbHLH* gene family (which will be referred to as *Lc* in this study) to be analyzed [23]. In this current study we identified 91 *LcbHLH* transcription factors, which were further analysed using Bioinformatic approach for evolution, conserved motif arrangement, exon-intron patterns, and other physiochemical proprieties. Additionally, each subfamily of the *LcbHLH* gene family was shown to play imperative biological functions in abiotic stress responses. The identification and distinctive analysis of the *bHLH* transcription factor of *L. chinense* will assist in comprehending the structural characteristics of gene families in *L. chinense* and preliminarily predict the function of *bHLH* members, which will provide the gene resources for the improvement of *L. chinense* germplasm by genetic engineering technology in the future.

2. Materials and Methods

2.1. Identification and Physicochemical Properties Analysis of bHLH Family Members of Liriodendron chinense

The nucleic acid and protein sequences of *L. chinense* were collected from the local protein database [23]. The protein sequences of the bHLH family of *Arabidopsis* and rice were retrieved and downloaded from the plant transcription factor database (http://planttfdb.cbi.pku.edu.cn (accessed on 12 November 2021)) [25]. The bHLH protein sequences of *Arabidopsis* and rice were used as query sequences, while the candidate protein-containing bHLH/HLH domain was screened from the *L. chinense* database by local blastp program. Then, the HMMER model downloaded from the Pfam database was used to identify the candidate bHLH protein of *L. chinense* in a local protein database. Finally, proteins with the bHLH/HLH domain were taken as the final bHLH family members of *L. chinense*. The physical and chemical properties (including molecular weight, isoelectric point, and hydrophilicity) of LcbHLH family members were analyzed using the Protparamin EXPASY database.

2.2. Phylogenetic Analysis of LcbHLHs

ClustalX2 was used for multiple sequence alignment of the *bHLH* domain. The *bHLH* proteins of three plants, rice, Arabidopsis, and poplar, have been downloaded from National Center for Biotechnology Information (NCBI). The phylogenetic tree was constructed using MEGA7.0 with the Neighbor-Joining method [26,27]. The evolutionary distance was obtained through the p-distance method, with the distances employed to estimate the number of amino acids at each locus. The reliability of each phylogenetic tree was guaranteed by 1000 bootstrap sampling iterations.

2.3. Chromosome Location and Gene Replication of LcbHLHs

The data of the chromosomal location of *LcbHLH* members were obtained from annotated files in the Liriodendron genomic database, while the distribution of *LcbHLH* members was plotted using the biological software TBtools [28]. The gene replication events were analyzed according to the following three standard definitions: (1) the length of one shorter sequence is greater than 70% of that of the other longer sequence; (2) the similarity between the two sequences is greater than 70%; (3) two genes separated by five or fewer genes in a 100 kb chromosome segment are considered as tandem repeat genes [29]. To analyze the collinearity correlation between *LcbHLH*s and *bHLH*s in other species, the genome data of Arabidopsis and rice were downloaded from Ensemble (http://plants.ensembl.org/index.html (accessed on 13 November 2021)). The multicollinearity scanning tool MCsanX was employed to compare the whole genome sequence of Liriodendron with that of Arabidopsis and rice, respectively [30]. The visualization of chromosome distribution was obtained through the Circos in TBtools. The ratio of Ka/Ks was calculated by using KaKs_calculator to acquire the natural purification selection between target gene pairs [31].

2.4. Analysis of Gene Structure, Conserved Motifs and Cis-Regulation Elements of LcbHLHs

TBtools software was adopted to map the gene structure of *LcbHLH* members onto a diagram. MEME was used to predict and analyze the conservative motif of the *bHLH* protein in *L. chinense*. Cis-regulation elements of *LcbHLH* members were predicted by the software Plantcare (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/ (accessed on 25 November 2021)) and plotted by TBtools.

2.5. Analysis of Protein Interaction among LcbHLHs

The protein interaction network was generated using the STRING (www.string-db.org (accessed on 3 December 2021)) based on the high homology between *LcbHLH*s and *AtbHLH*s proteins. In addition, six *LcbHLH* proteins with high homology to *AtbHLH* were selected to map the extrafamilial protein interaction network using Cytoscape 3.8.2 [32].

2.6. Three-Dimensional Structure Modeling and Verification of bHLH Protein

The full-length atomic structures of *LcbHLH*24, *LcbHLH*72, and *At*ICE1 proteins were constructed based on the synthesis method on the Robetta online website. Homologous modeling was used for proteins with the sequence matching model, while the threading method was used for proteins with the sequence non-matching model. Then, the sequence was assembled to construct the protein structure. The reliability of their protein structures was further confirmed by ERRAT, PROVE, and Ramachandran on the online website Savesv6.0. VMD software was used for 3D modeling.

2.7. Expression Analysis of LcbHLHs in Response to Cold Stress by RNA-seq and qRT-PCR

The somatic embryo-regenerated seedlings of hybrid Liriodendron with consistent growth were cultured in an incubator (23 °C, 16 h light, and 8 h dark) and then treated at 4 °C. Seedling leaves were sampled at 0 h, 6 h, 1 day, and 3 days with three biological replicates. The collected leaves were quickly frozen in the liquid nitrogen and put in a −80 °C refrigerator for storage. Transcriptome sequencing was performed on the above samples. Transcriptome data of *LcbHLH* members were extracted from the sequencing

results. The expression levels of each member at each period of cold stress treatment (the maximum expression value of each *LcbHLH* gene was set to 1, and then the expression values of the gene at other stress and growth stages were normalized to the maximum expression value) were normalized and displayed on the heatmap. The expression patterns of ten *LcbHLH* members were determined by quantitative RT-PCR analysis (qRT-PCR). The qRT-PCR was performed using SYBR-green in the Roche Light Cycler®480 real-time PCR system (Switzerland, Sweden). The relative expression abundance of *LcbHLH* was calculated with the ΔΔCT method. 18s rRNA was used as the internal reference. All qRT-PCR primers were designed by Primer5.0 and were listed in Table S1.

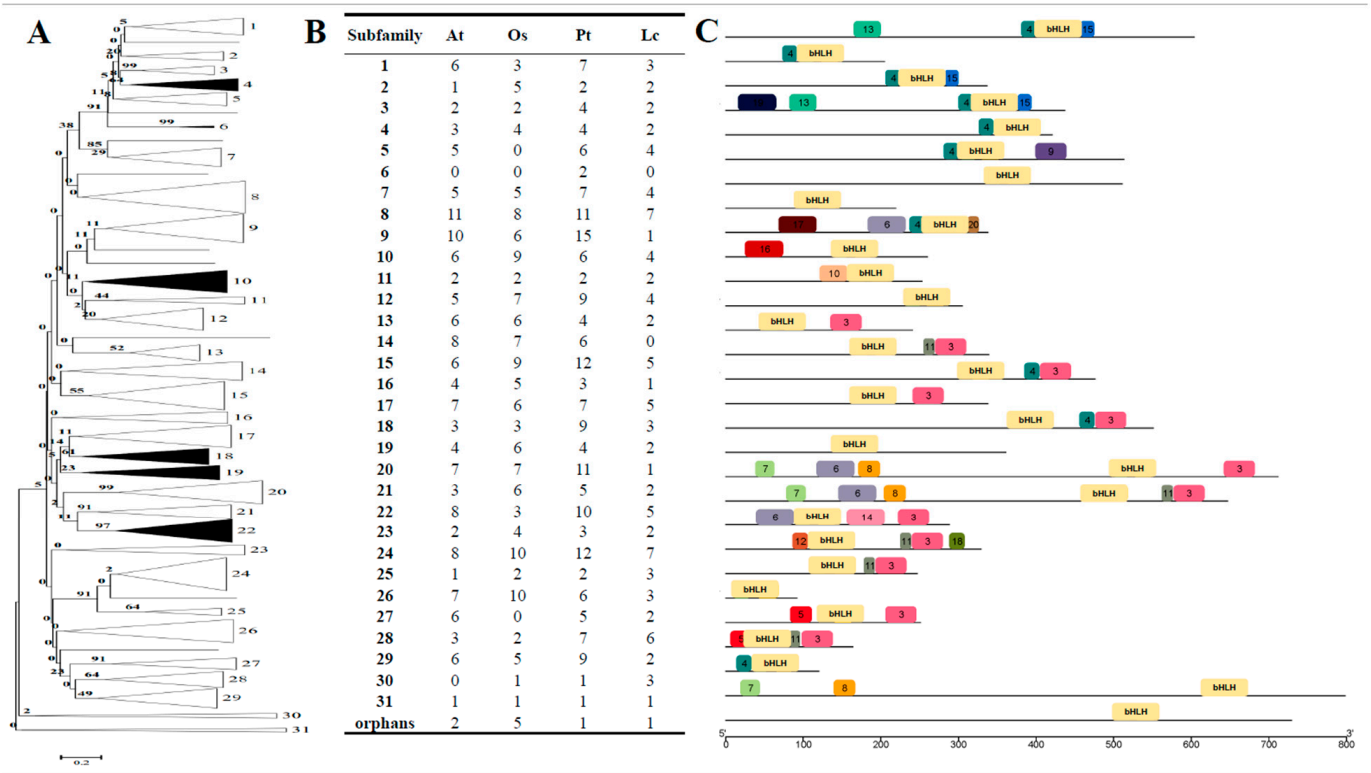
3. Results

3.1. Identification and Physiochemical Characteristics of LcbHLHs

Based on the search of the conserved bHLH domain (Pfam number: PF00010), 91 *LcbHLH* family members were recognized after further validation in the conserved domain database (CDD) and Pfam database. They were renamed as *Lc*bHLH 1~91 based on their chromosomal position. The physical and chemical properties of *Lc*bHLH members were computed. Analysis of the hydrophilicity value of all *Lc*bHLH proteins showed a negative total average value that ranged from −0.816 (*LcbHLH*47) to −0.143 (*LcbHLH*68), concluding that *Lc*bHLH proteins are hydrophilic. The Molecular weight (Mw) of *Lc*bHLH proteins ranged from 10.19 kD (*LcbHLH*15) to 88.40 kD (*LcbHLH*50), the majority (61%) were in the range of 21.41 kD to 48.85 kD, and the molecular weight of 24 members (about 26%) was in the range of 20 kD to 30 kD. Additionally, the theoretical isoelectric points (pI) of *Lc*bHLH proteins ranged from 4.59 (*LcbHLH*81) to 9.91 (*LcbHLH*53). Most *Lc*bHLH proteins (about 63%) were less than 7, and about 30% of *LcbHLH* proteins had a pI between 6 and 7 (Table S2).

3.2. Phylogenetic Characteristics of LcbHLHs

To fully comprehend the evolutionary relationship of the identified *Lc*bHLH protein sequences, *L. Chinense (Lc)*, *Arabidopsis thaliana* (*At*), *Oryza sativa* (*Os*), and *Populus. trichocarpa* (*Pt*), *bHLH* gene families were further compared and subjected in phylogenetic tree analysis (Figure 1A and Figure S1). A total of 581 bHLH protein sequences were obtained and divided into 31 groups, which were identified as evolutionary branches with high bootstrap values. Among the 31 subfamilies, 26 subfamilies were presented in all four species, signifying that the genes of these subfamilies had high homology in the four species and strong phylogenetic conservatism. Some *LcbHLH* genes in Arabidopsis and rice were clustered in the same subfamily. *LcbHLH* proteins were clustered in 29 subfamilies and an orphan sequence was observed. Subfamily 13 was clustered with *LcbHLH*14, *LcbHLH*15, *AtPRE*1/2/3/4/5, and *AtKDR*. Subfamily 17 was clustered with *LcbHLH*24, *LcbHLH*31, *LcbHLH*82, *LcbHLH*18, *At033SCRM*, and *At116ICE1* (Table S3). Subfamily 25 was clustered with *LcbHLH*16, *LcbHLH*69, *LcbHLH*78, *At*SPCH, and OsSPC1/2. Additionally, subfamily 6 was only found in Poplar, indicating individual evolution and functional diversity of Poplar (Figure 1B).



Subfamily	At	Os	Pt	Lc
1	6	3	7	3
2	1	5	2	2
3	2	2	4	2
4	3	4	4	2
5	5	0	6	4
6	0	0	2	0
7	5	5	7	4
8	11	8	11	7
9	10	6	15	1
10	6	9	6	4
11	2	2	2	2
12	5	7	9	4
13	6	6	4	2
14	8	7	6	0
15	6	9	12	5
16	4	5	3	1
17	7	6	7	5
18	3	3	9	3
19	4	6	4	2
20	7	7	11	1
21	3	6	5	2
22	8	3	10	5
23	2	4	3	2
24	8	10	12	7
25	1	2	2	3
26	7	10	6	3
27	6	0	5	2
28	3	2	7	6
29	6	5	9	2
30	0	1	1	3
31	1	1	1	1
orphans	2	5	1	1

Figure 1. Phylogenetic tree of four species proteins. (**A**) The phylogenetic tree of four species; *Liriodendron* (*Lc*), Rice (*Os*), *Arabidopsis* (*At*), *Poplar* (*Pt*). The branches with a bootstrap value greater than 50 were represented by black triangles, while those with a bootstrap value less than 50 were represented by white triangles, which are divided into 31 subfamilies. (**B**) Summary of each group plant-species member representation in phylogeny analysis, plant species, (*At*) Arabidopsis, (*Os*) Rice, (*Pt*) Poplar, and (*Lc*) Liriodendron, group presentation denoted relative to their group marked as subfamily. Orphan genes are shown in the bottom column denoted orphans. (**C**) The motif patterns of *Lc*bHLH subfamilies, showing the bHLH domain present in all protein sequence analysed and other motif.

3.3. Gene Structure and Conserved Motifs of LcbHLHs

Gene structure prediction plays an animated role in studying the evolution of gene family members. To further explore the phylogenetic relationships within the *LcbHLH* members, the intron/exon structures of the *LcbHLH* gene were analyzed based on the genomic annotation files of 91 *LcbHLH* members in combination with phylogenetic tree (Figure 2A). The number of introns in the *LcbHLH* gene ranged from 1 to 11. *LcbHLH* genes were clustered together by parallel exon/intron patterns in exon length and intron number (Figure 2B).

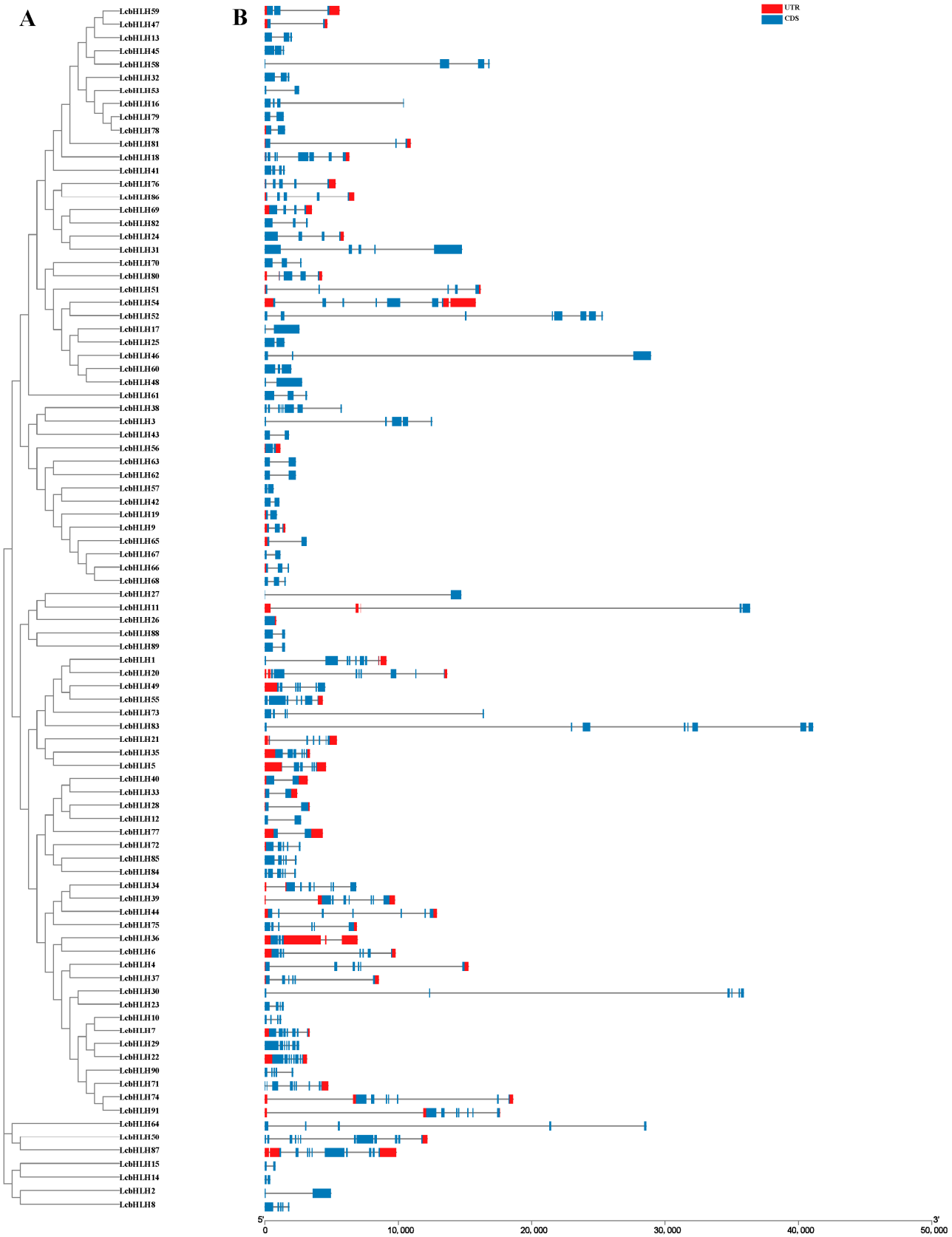


Figure 2. Phylogenetic relationships and exon/intron structures of LcbHLH protein. (**A**) The phylogenetic tree of LcbHLH protein. (**B**) Exon/intron structure analysis of LcbHLHs. Blue boxes represent CDS, red boxes represent UTR, and gray lines represent introns. The size of exons and introns can be estimated by the scale at the bottom.

In this study, the configuration of the *LcbHLH* conservative motif was discovered through the protein conservative theme sites predicted by online software MEME (Figure 1C and Table S4). *bHLH* conserved domain was constituted by motif 1 and motif 2 (Figure 1C). The meticulously connected *LcbHLH* proteins on immediate evolutionary branches of the phylogenetic tree had the same or comparable motif structures. Moreover, there were significant differences between dissimilar subfamilies, suggesting that members of the identical subfamily of *bHLH*s might play related roles in *L. chinense*. Seven subfamilies shared motif 11, eleven subfamilies shared motif 3, and nine subfamilies shared motif 4. Motif 19 only occurred in subfamily 4, motif 17 and motif 20 only occurred in subfamily 10, motif 16 only occurred in subfamily 11, motif 10 only occurred in subfamily 12.

3.4. Cis-Regulation Elements of LcbHLHs

The cis-regulatory element plays an imperative role in regulating the expression of stress response genes [33]. The presence of the cis-elements of the *LcbHLH* members in the promoter region (2000 bp upstream of the transcription initiation site) was predicted. Twenty-five typical elements with relatively robust functions were divided into three groups shown in Figure 3. Based on the functional annotations, cis-elements were categorized into three major classes: plant growth and development, phytohormone responsive, and abiotic and biotic stresses (Figure 3). Our findings showed that G-Box and ABRE were the most represented transcription factors in the *LcbHLH* gene family. Specifically, *LcbHLH7* had the most representation of G-Box and ABRE. 67 *LcbHLH* members had elements responsive to the methyl Jasmonate, including CGTCA-motif and TGACG-motif. Fifty-four members had gibberellin-responsive elements, including P-box and GARE-motif. Seventy-two members had salicylic acid responsiveness elements, TCA-element. Moreover, 45 members had auxin-responsive elements, including AuxRR-core and TGA-element. 52 *LcbHLH*s contained LTR elements that might be interrelated to the cold stress response of *L. chinense*.

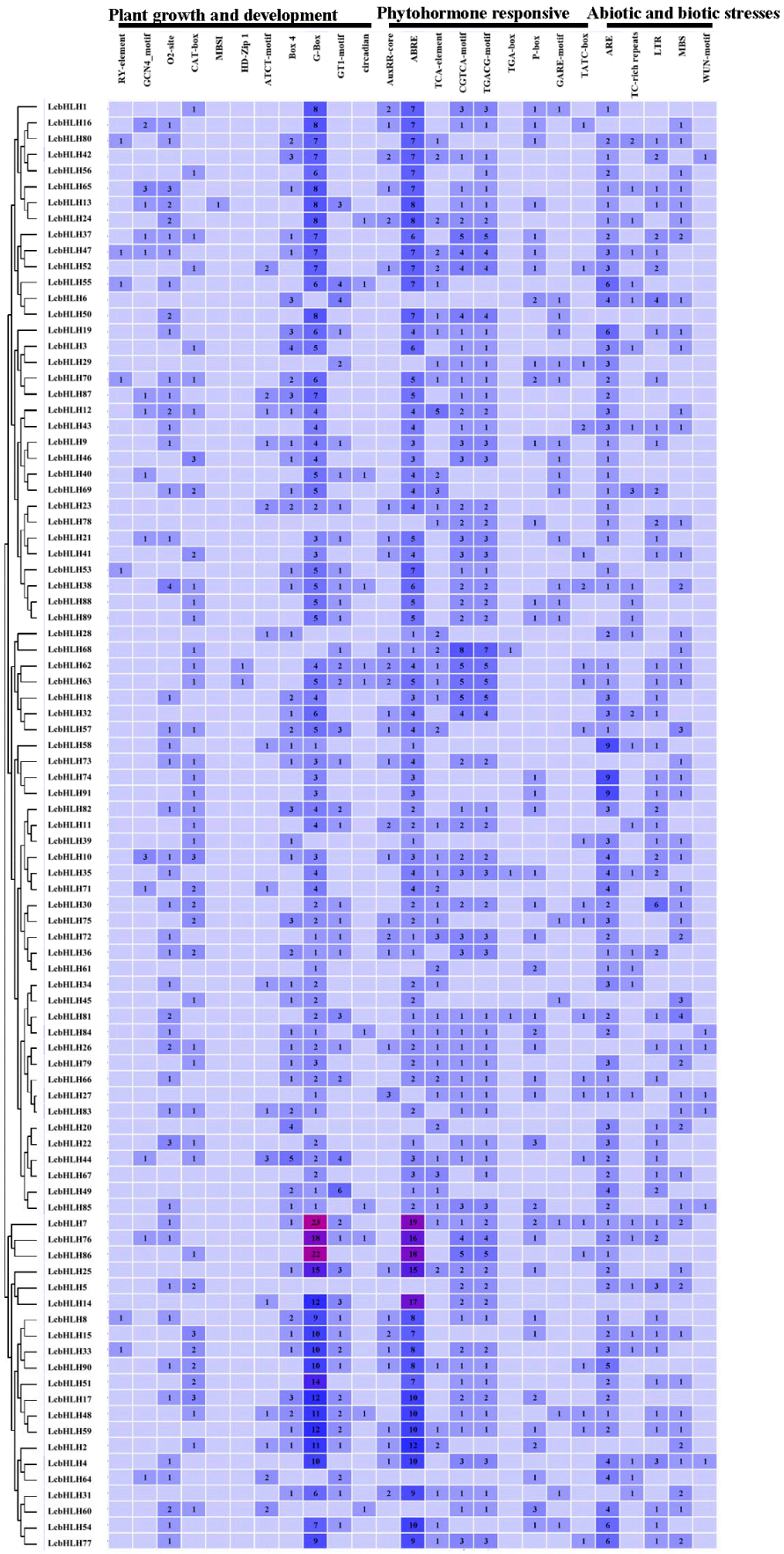


Figure 3. Cis-regulatory elements in the promoters of LcbHLHs.

3.5. Intergenomic Collinearity and Gene Replication of LcbHLHs

Amongst 91 *LcbHLH* genes, 89 were distributed on 19 chromosomes, and the other two were assigned to unassembled genomic contigs (Figure 4). The number of *LcbHLH* genes on each chromosome ranged from 1 to 9.

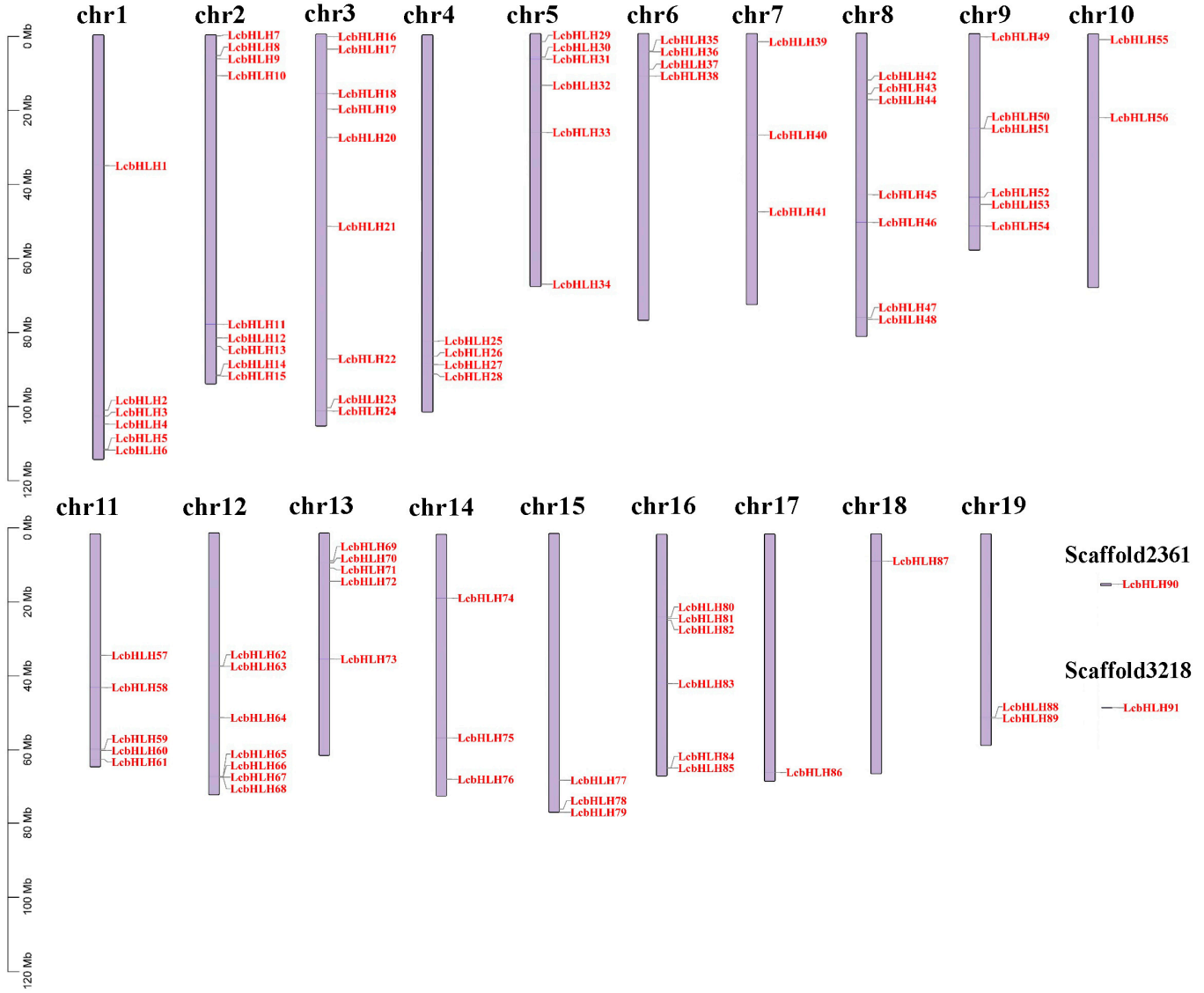


Figure 4. Chromosome distribution of *LcbHLH* gene. Ninety-one genes were labeled on 19 chromosomes and two scaffolds. Positional information for each *LcbHLH* gene is displayed on each chromosome (chr). The left scale represents the length of the chromosome.

The analysis of genome-wide replication, fragment replication, and tandem replication of gene family has a significant role in explaining the process of gene family expansion. In this analysis, intraspecies comparisons of *L. chinense* and *A. thaliana*, *L. chinense,* and rice were implemented at the genome-wide level (Figure 5). A total of 24 pairs of replication genes were found in the *LcbHLH* family, and 21 pairs of gene clusters with high similarity were institute in *LcbHLH*s (Figure 5A). For example, the protein sequences of *LcbHLH*88 and *LcbHLH*89 shared 99.23% resemblance. The similarity between *LcbHLH*63 and *LcbHLH*62 was 99.65%, respectively.

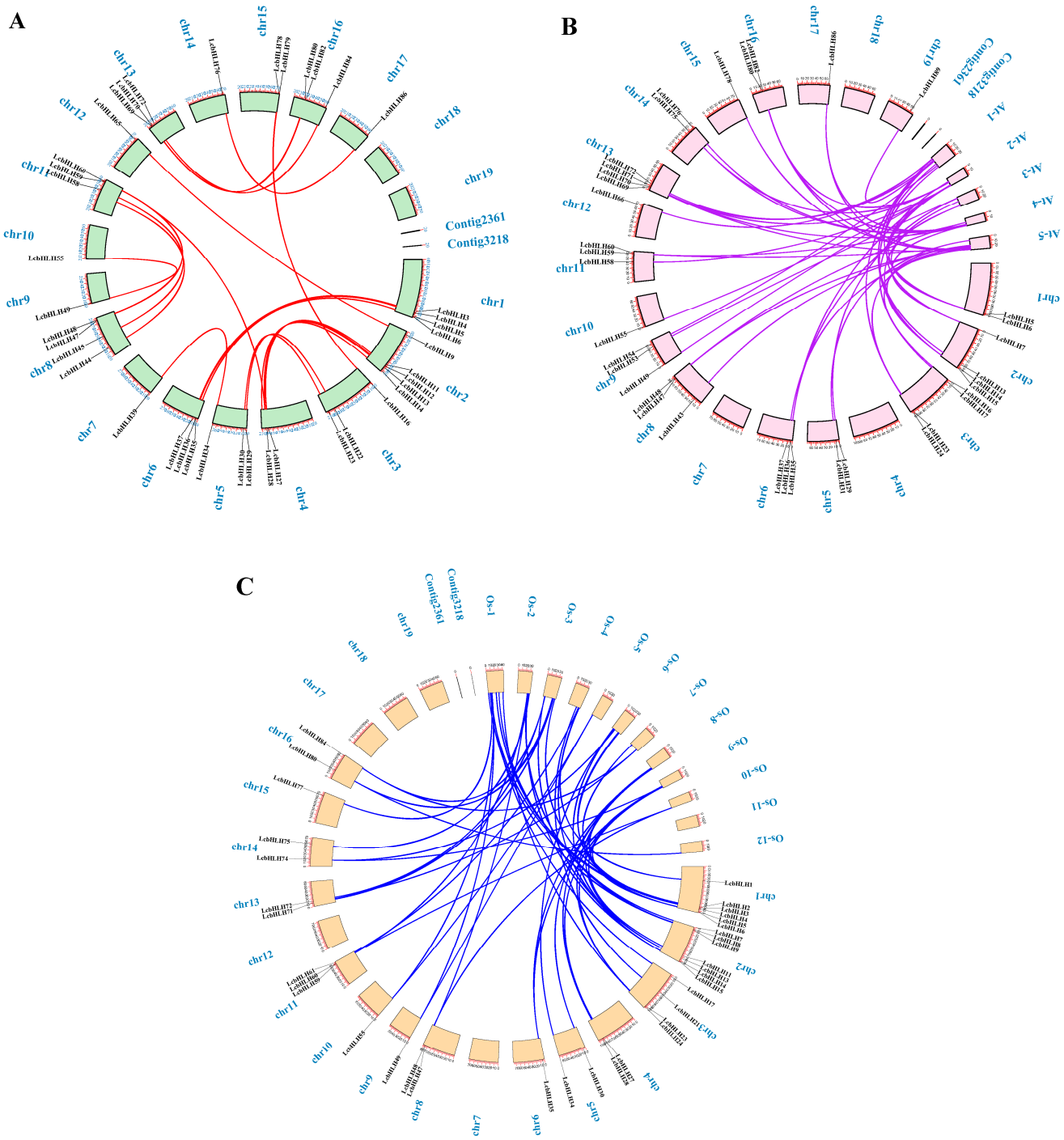


Figure 5. Fragment replication and chromosome distribution of *bHLH* genes in *Liriodendron chinense*. (**A**) Nineteen chromosomes were represented by green segments, red lines connected with homologous genes. (**B**) Collinearity analysis of *Liriodendron chinense* and *Arabidopsis thaliana*; (**C**) Collinearity analysis of Liriodendron chinense and Rice. The gene pairs between them are represented by purple lines and blue lines respectively.

Additionally, tandem repeat genes comprised the same number of exons due to closely related imitation associations. The tandem repeat genes *LcbHLH*14 and *LcbHLH*15 and *LcbHLH*62 and *LcbHLH*63 had a similar two exon and intron-exon structure pattern. Likewise, *LcbHLH*84 and *LcbHLH*85 had a similar intron structure pattern. Remarkably, as revealed in Figure 5A, there were four pairs of fragment-repetitive genes: *LcbHLH*3, *LcbHLH*4 and *LcbHLH*36, and *LcbHLH*37; *LcbHLH*12, *LcbHLH*13 and *LcbHLH*27, and *LcbHLH*28;

*LcbHLH*69, *LcbHLH*70 and *LcbHLH*80, and *LcbHLH*82; *LcbHLH*47, *LcbHLH*48 and *LcbHLH*59, and *LcbHLH*60. Together, these results show that the *LcbHLH* gene family was amplified by fragment replication and tandem replication of the *LcbHLH* genes.

The tandem repeated *bHLH* gene has a related gene structure, motif composition, and expression. The tandem repeated and intra-and inter-chromosome repeated regions of *LcbHLH* members were examined in the present study. Our results showed that greater than 38% (15 tandem and 22 fragment-repeat genes) of the *LcbHLH*s might have evolved from some genomic replication event. The substitution rate (Ka/Ks) between nonsynonymous and synonymous was an operative quantity of selection pressure after gene replication [34]. Consequently, the Ka/Ks of the *LcbHLH* repeat gene was premeditated (Table S5). For all tandem repeat pairs, the Ka/Ks values were well below one, which indicated that there were purification options during amplification. Besides, for gene pairs with fragment repeats, all Ka/Ks were less than one, indicating that there was strong purification selection pressure during evolution.

With genome-wide comparison and analysis of *L. chinense*, *A. thaliana*, and rice, it was established that most *LcbHLH*s were positively homologous in rice and *A. thaliana* (54% and 60%), respectively (Figure 5B,C, Tables S6 and S7). The Ka/Ks ratios of *L. chinense* to rice and *A. thaliana* were 0.175 and 0.186, respectively. These results indicate that *bHLH* gene pairs underwent strong purification selection and that there was a close correlation between them before. In brief, gene replication events, including tandem and fragment repeats, appeared to be essential for the expansion of the *bHLH* gene family in Liriodendron, as well as for the functional preservation and differentiation.

3.6. Protein Interaction Network of bHLHs

Diverse *bHLH* proteins bind to specific DNA and regulate the downstream target's transcription by forming homodimer or heterodimer mediated by their α-helix near the N-terminal [10]. Hence, protein interaction analysis is essential to fully review the function of *LcbHLH* proteins (Figure 6). It can be speculated that *LcbHLH*s might have played a role in forming protein complexes and attempted to construct an interaction network of *LcbHLH*s. In this current study, the interaction network within the *LcbHLH* gene family was constructed based on the orthogonal analysis of *AtbHLH*s (Figure 6A and Table S8). The protein interaction network indicated that most *LcbHLH* proteins could interact with more than one *bHLH* protein. More than a quarter of *LcbHLH* proteins can interact with four or more other *bHLH* proteins. Numerous imperious interactions were predicted, such as how *CIB*1 (*LcbHLH7*) can participate in the regulation of flowering time [35]. *ICE*1 (*LcbHLH*24, 31) interacts with *FMA* (*LcbHLH*32), *SPCH* (*LcbHLH*78, 79, 16) and *MUTE* (*LcbHLH*53) to regulate stomatal diversity [34]. *LRL*1 (*LcbHLH*75) and *RDH*6 (*LcbHLH*8) can interact with *RSL*2 (*LcbHLH*85 and 86) and contribute to the regulation of root hair development. These protein interaction networks further ascertained that the *LcbHLH* genes exerted their diverse biological functions through interaction and coordination with other members.

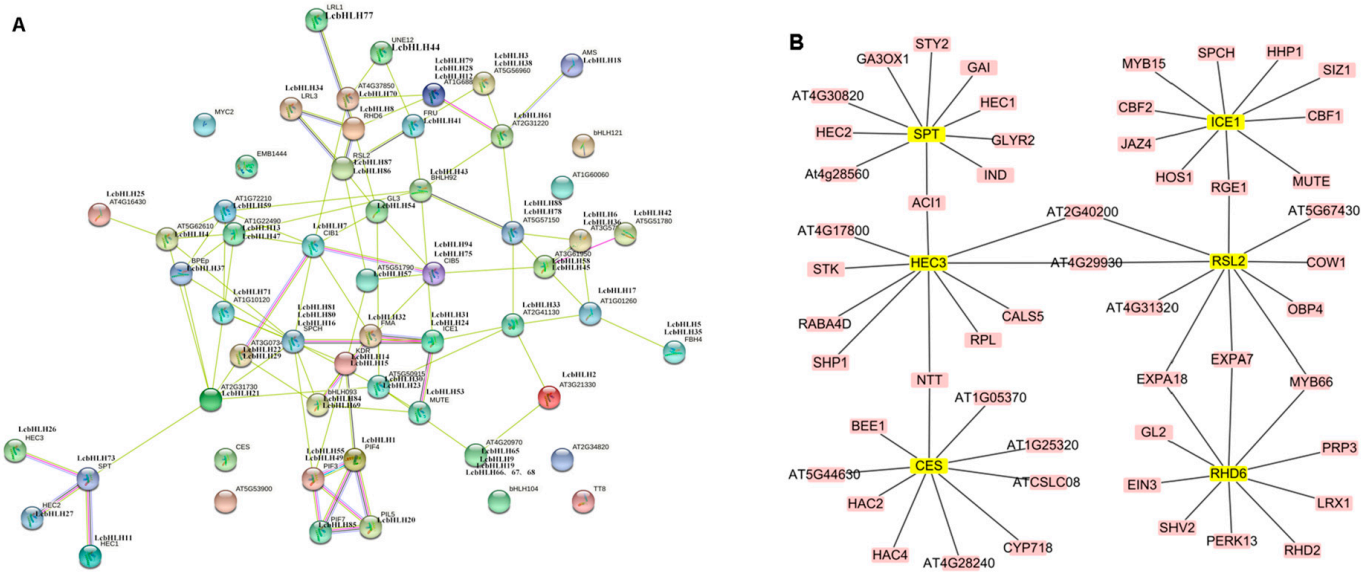


Figure 6. Prediction of LcbHLH protein interaction network based on Arabidopsis orthologs. (**A**) The protein interaction analysis in the LcbHLH family is predicted according to the homology with Arabidopsis thaliana by using a string online website, and the name of LcbHLH protein is marked next to Arabidopsis thaliana orthologous. (**B**) With Cytoscape software, six LcbHLH proteins with high gene homology with Arabidopsis thaliana were predicted and analyzed for extracellular protein interaction prediction according to String website.

3.7. Structural Modeling of LcbHLH Protein

The *bHLH* transcription factor family plays a vital role in plant response to abiotic stress by forming dimer and its helical structure [36]. *ICE*, one of the *bHLH* families, activates CBF via transcription and persuades its expression, playing a central role in cold response and signal transcription [16,37–41]. The amino acid sequence of *LcbHLH*24 in *L. chinense* is extremely homologous to that of *ICE*1 in *A. thaliana*. For that reason, this research predicted that these two protein structures, *LcbHLH*24 and *LcbHLH*72 (homologous gene of *AtRSL*2), interacted with *LcbHLH*24 through *RGE*1 in the protein network (Figure 7A). The structure of *LcbHLH*24 consisted of 14 α-helices and 19 loops (Figure 7A), and the model of *LcbHLH*72 had ten α-helices and eight loops (Figure 7B). The three-dimensional structure of *AtICE*1 protein consisted of 14 α-helices and 12 loops (Figure 7C).

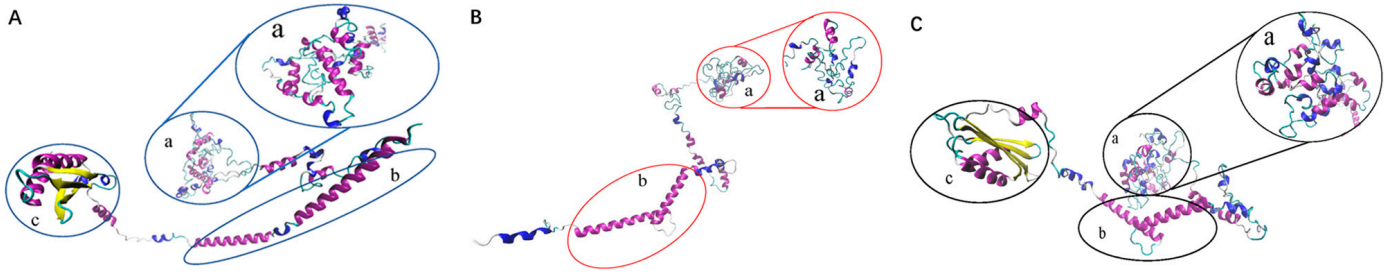


Figure 7. Three-dimensional structure of *bHLH* protein. a, b and c represent same protein regions in three different protein structures, respectively. (**A**) Three-dimensional structure of the protein of *LcbHLH*24; (**B**) Three-dimensional structure of the protein of *LcbHLH*72; (**C**) Three-dimensional structure of the protein of *At*ICE1.

In the 3D model of *LcbHLH*24, the structural model could be roughly divided into three regions, exposed as a, b, and c. *LcbHLH*72 could be divided into two regions, designated as a and b. Three structural regions could be found in *At*ICE1, in which region b was similar to the structure of the other two proteins. Nevertheless, region a of *LcbHLH*24

and *At*ICE1 is a little richer than that of *LcbHLH*72. According to the homology modeling of SWISS-MODEL and the prediction of the conserved domain of NCBI (CDD), region b is the *bHLH* conserved domain of three proteins. The conserved structural region b of *LcbHLH*24 and *AtICE*1 was predicted by SWISS-MODEL to have the domain characteristics of the MYC2 subfamily. Alternatively, region b of *LcbHLH*72 showed high consistency with MITF/CLEAR box structure. Interestingly, special structural region Berninger c was only identified in *LcbHLH*24 and *AtICE*1, and region c in *LcbHLH*24 was almost identical to *At*ICE1. In summary, comparative analysis of *LcbHLH*24 and *AtICE*1 protein sequences, region c is a highly conservative Zipper domain.

3.8. Cold Stress-Induced Expression Pattern of LcbHLHs

The expression patterns of *LcbHLH*s under cold stress in transcriptome data were analyzed (Figure 8) to understand the responses of *LcbHLH*s to cold stress, and 78 *LcbHLH* genes were examined to express in the seedling leaves of *L. chinense*. During the cold stress treatment, the expression patterns of *LcbHLH* members were coarsely defined by constant up-regulations and down-regulations (Figure 8). The expression patterns under the cold treatment of 20 *LcbHLH* genes (22.2%) showed a constant up-regulation trend, 15 *LcbHLH* genes (16.7%) were incessantly down-regulated; 28 of the total *LcbHLH*s (31.1%) were up-regulated and then subsequently down-regulated with the extension of cold treatment time, and only four genes (4%) showed the down and then increased trends.

To further verify the expression pattern of *LcbHLH*s under cold stress, ten *LcbHLH*s (*LcbHLH*9, 20, 24, 35, 39, 54, 55, 56, 69, 86) were chosen to quantify the expression abundance in *L. chinense* by qRT-PCR. As shown in Figure 8B, the expression trends of these ten genes were almost consistent with their transcriptomic patterns. Three *LcbHLH* genes (*LcbHLH*35, 55, 86) showed an up-regulation trend in response to cold stress, six *LcbHLH* genes (*LcbHLH*9, 20, 39, 54, 56, 69) displayed a down-regulation trend, and the expression profile of one *LcbHLH* gene (*LcbHLH*24) was up-regulated at 1d and then down-regulated at 3d.

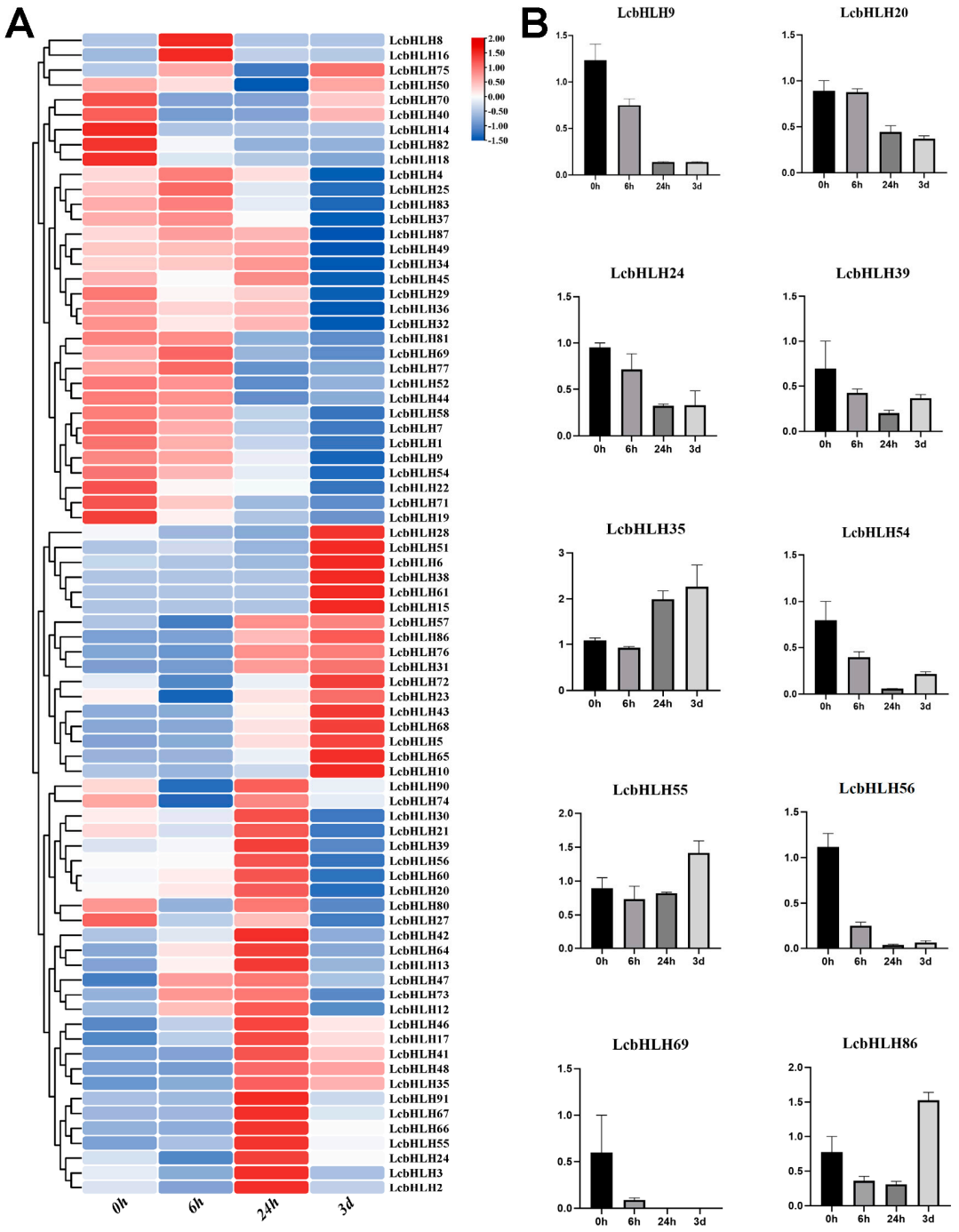


Figure 8. Expression analyais of *LcbHLH* genes in response to cold stress. (**A**) Transcriptomic expression analysis of *LcbHLH* genes. (**B**) Expression analysis of *LcbHLH* genes by qRT-PCR. 0h, 6h, 24h and 3d represent the treatment times of cold stress.

4. Discussion

Given the significant character and diverse functions in biological processes, the *bHLH* transcription factors have attracted more and more attention in recent years [21,42,43]. In this current study, members of the *bHLH* family identified from the genome of *L. chinense* had analogous structural characteristics to those of other species, especially the *bHLH* domain. That was highly conservative with 19 amino acid residues, of which five were base regions, five were distributed in the first helix, one in the loop, and eight in the second helix [44]. However, typical conserved sites were found in the domain of the *L. chinense*

bHLH gene family, like the *AtbHLH* families. This indicated that *LcbHLH*s might have DNA-binding activity like that of *A. thaliana* [45].

We constructed a phylogenetic tree to better understand the evolutionary relationship of *bHLH* gene families between different species, *L. chinense*, *A. thaliana*, rice, and poplar. Interestingly, genes with the same functions were clustered into the same clade. For example, *LcbHLH*78, *LcbHLH*79 and *AT5G53210* (*AtSPCH*), *Os*02g15760 (*OsSPCH2*) and *Os*02g33450 (*OsSPCH*1) were clustered into subfamily 25. We used this evolutionary clustering on the same branch to speculate the functional importance of identified *LcbHLH*s. Previous studies in *A. thaliana* have shown that *At*SPCH can regulate the formation of stomata together with *AtMUTE* and *AtFAMA* [46]. In rice, *SPCH* and *MUTE* have also been shown to exhibit the same functional importance in stomatal formation [47]. Hence, it is reasonable to speculate that *LcbHLH*78 and *LcbHLH*79 are imperative genes regulating the stomatal switch in *L. chinense*. Furthermore, the *LcbHLH*24 and *LcbHLH*31 were also clustered into the same subfamily (subfamily 17) as *AT1G12860* (*AtICE*1), *AT3G26744* (*AtICE*2), *Os*11G32101 (*OsICE*1), and *Os*01G0310 (*OsICE*2). *AtICE*1 and *AtICE*2 are the main transcription factors found in *A. thaliana* responding to low-temperature stress [17]. *OsICE*1 can be phosphorylated by *OsMAPK*3, thus enhancing the activation of *OsbHLH* to its target gene *Os*TPP1 in response to low-temperature stress [48]. So, it is reasonable to speculate that *LcbHLH*24 and *LcbHLH*31 are most likely to participate in the signal transduction of *L. chinense* in response to low-temperature stress.

Similarly, exon-intron patterns and similar conservative motif arrangements are consistent with the subfamily classification. It is known that genes with few or no introns have low levels of expression in plants [49]. However, a gene structure with compact exons may facilitate rapid expression in response to both endogenous and exogenous stimuli [50]. We observed that the exon structures of *LcbHLH*5 and *LcbHLH*35 were relatively tight, and they both belonged to the subfamily 29. According to the transcriptomic data, the expression of these two genes under low-temperature stress was increasing in response to an increase in the duration of treatment exposure.

Genomic replication events occur throughout plant evolution, often leading to the expansion of gene families [51,52]. Tandem and fragment gene replication events are two major replication patterns common in the evolution of angiosperms [34,53] and play an essential role in gene family extension [51,54]. In the present study, several distinct gene clusters of *LcbHLH*s were distributed in the different chromosomes. Therefore, gene duplication might be an important reason for the large number of *LcbHLH*s. Gene replication is a common phenomenon in many organisms, which can regulate gene expression, improve genetic and environmental adaptability, and serve as a steppingstone in the evolution of new biological functions [55,56]. The relatively strong sequence diversity besides the *bHLH* domain suggests that the *bHLH* family has undergone extensive domain reorganization after gene replication [57]. More than 20 different conserved motifs with different arrangements were found in the *bHLH* family of *L. chinense*. Thus, extensive domain reorganization occurred in the protein structure of the *bHLH* members. This phenomenon implies that the evolutionary position of Liriodendron is difficult to determine accurately [23].

Time-specific expression patterns of genes in plant growth usually reflect variances in biological functions of gene family members and interactions among related pathways [58,59]. In transcriptional expression profiles, the diverse expression patterns of *LcbHLH* genes under cold stress inferred that each *LcbHLH* member might participate in the various cascades of signal transduction in *L. chinense* in response to cold stress. By predicting the cis-regulation elements of these *LcbHLH* genes, we observed regulatory elements responsive to temperature stress, including LTR, TCA, and AT-rich. The low-temperature responsive element LTR, with CCGAC as the core sequence, demonstrated diverse expression patterns under low-temperature stress, suggesting that LTR plays a key role in responding to low-temperature stresses [60,61]. CRT/DRE element is an important low-temperature response element in the *bHLH* family. *CBF* transcription factor can bind to CRT/DRE sequence and induce the expression of the *COR* gene to improve the cold resistance of plants [62,63].

Numerous proteins in the *bHLH* family are intricate in the tolerance to low-temperature stress, and *ICE*1 is a typical transcription factor that can regulate cold-responsive signal transduction in plants [37,64]. Two members (*LcbHLH*24 and *LcbHLH*31) were found to be highly homologous to *AtICE*1 and *AtICE*2 in the genome of *L. chinense*. The expression of *LcbHLH*31 was continuously up-regulated under cold stress, while the expression *LcbHLH*24 was continuously increased during one day but decreased after three-day treatment, but its abundance was still higher than that of the control. This indicated that two genes, *LcbHLH*31, and *LcbHLH*24, participated in the response of *L. chinense* to low-temperature stress. Over the comparative analysis of the protein sequences of *LcbHLH*24 and *AtICE1*, it can be inferred that *LcbHLH*24 has the characteristics of the typical *ICE* gene family, which contains an S-rich region and disulfide bonds. They can preserve the stability of its gene, but not in *LcbHLH*72. Consequently, it can be reasonably inferred that the stability of *LcbHLH*24 protein is stronger than that of *LcbHLH*72. Region c, which is found in the structure of *LcbHLH*24, shares the same characteristic with the structure of Zipper found in *ICE* of *A. thaliana* and other species. It can be expected and assumed that the special zipper protein structure of *LcbHLH*24 may be beneficial for further exploring and analysing the response of the *bHLH* family to low-temperature stress in *L. chinense*.

Protein-protein interaction analysis predicted interacted relationship among *LcbHLH*s, which of them were confirmed by previous reports. *ICE*1 [16], *ICE*2 [65], and *MYB*15 [66] have been recognized as regulatory factors that induce CBF expression. In response to low temperature, *ICE*1 can be sumoylated by SIZ1, thus promoting the binding of *ICE*1 and increasing *CBF*s expression [67]. In addition, *SCRM2* plays an important role in regulating the stomatal development of *SPCH*, *MUTE*, and *FAMA* [36]. Evidence suggests that there may be a relationship between transcriptional regulation of environmental adaptation and stomatal development in plants [68].

5. Conclusions

This comprehensive genome-wide study systematically identified and functionally analyzed the *bHLH* gene family in *L. chinense*. A total of 91 *LcbHLH* family members were identified and divided into 31 subfamilies, which were unevenly distributed on 19 chromosomes of *L. chinense*. The reported gene structures, conservative motifs, and phylogeny further supported the characteristics of the phylogenetic trees. The amplification of the *LcbHLH* gene was due to duplication during evolution, suggesting that this gene family may play an important role in polyploid plants. Cis-regulation elements responding to low temperature were found in the upstream region of the *LcbHLH* gene, which indicated that the *LcbHLH*s might play an important role in response to cold stress. RNA-seq and qRT-PCR analysis showed that members of the *LcbHLH* genes had various expression patterns during cold treatments. These results may contribute to further functional studies of *LcbHLH* genes and may provide gene resources for the genetic improvement of *L. chinense*.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/f13040518/s1, Figure S1. Members of the bHLH family from four species: Arabidopsis thaliana (blue triangle), rice (red quadrangle), poplar (green circle), and Liriodendron chinense (purple square). The number on the right indicates their grouping; Figure S2. Logo of 10 conservative motifs of LcbHLH. Table S1. Basic protein information of LcbHLH family members. Table S2. The primers used in the qRT-PCR. Table S3. The segmental and tandem duplication events of LcbHLHs. Table S4. The Ka/Ks ratios between *L. chinenese* and *Arabidopsis thaliana*. Table S5. The Ka/Ks ratios between *L. chinenese* and *Oryza sativa*. Table S6. LcbHLH cis-regulation elements. Table S7. Phylogenetic Analysis and Classification of LcbHLH TF Family. Table S8. Detailed information of interaction network of LcbHLHs. Table S9. Detailed information of interaction network of LcbHLHs with other genes.

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visualization, supervision and funding acquisition, Y.L. and J.C. All authors have read and agreed to the published version of the manuscript.

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References

1. Gali, K.K.; Sackville, A.; Tafesse, E.G.; Lachagari, V.B.R.; McPhee, K.; Hybl, M.; Mikić, A.; Smýkal, P.; McGee, R.; Burstin, J.; et al. Genome-Wide Association Mapping for Agronomic and Seed Quality Traits of Field Pea (*Pisum sativum* L.). *Front. Plant Sci.* **2019**, *10*, 1538. [CrossRef] [PubMed]
2. Asim, A.; Gokce Ozturk, Z.N.; Bakhsh, A.; Cayli, I.T.; Aksoy, E.; Caliskan, S.; Caliskan, M.E.; Demirel, U. Individual and combined effect of drought and heat stresses in contrasting potato cultivars over expressing miR172b-3p. *Turk. J. Agric. For.* **2021**, *45*, 651–668. [CrossRef]
3. Ludwig, J.P.; Hull, C.N.; Ludwig, M.E.; Auman, H.J. Food habits and feeding ecology of nesting double-crested cormorants in the upper Great Lakes, 1986–1989. *Jack-Pine Warbler* **1989**, *67*, 115–126.
4. Goossens, J.; Mertens, J.; Goossens, A. Role and functioning of bHLH transcription factors in Jasmonate signalling. *J. Exp. Bot.* **2017**, *68*, 1333–1347. [CrossRef] [PubMed]
5. Buti, S.; Hayes, S.; Pierik, R. The bHLH network underlying plant shade-avoidance. *Physiol. Plant.* **2020**, *169*, 312–324. [CrossRef]
6. Murre, C.; McCaw, P.S.; Baltimore, D. A new DNA binding and dimerization motif in immunoglobulin enhancer binding, daughterless, MyoD, and myc proteins. *Cell* **1989**, *56*, 777–783. [CrossRef]
7. Ferré-D'Amaré, A.R.; Pognonec, P.; Roeder, R.G.; Burley, S.K. Structure and function of the b/HLH/Z domain of USF. *EMBO J.* **1994**, *13*, 180–189. [CrossRef]
8. Heim, M.A.; Jakoby, M.; Werber, M.; Martin, C.; Weisshaar, B.; Bailey, P.C. The basic helix-loop-helix transcription factor family in plants: A genome-wide study of protein structure and functional diversity. *Mol. Biol. Evol.* **2003**, *20*, 735–747. [CrossRef]
9. Feller, A.; Machemer, K.; Braun, E.L.; Grotewold, E. Evolutionary and comparative analysis of MYB and bHLH plant transcription factors. *Plant J.* **2011**, *66*, 94–116. [CrossRef]
10. Toledo-Ortiz, G.; Huq, E.; Quail, P.H. The Arabidopsis basic/helix-loop-helix transcription factor family. *Plant Cell* **2003**, *15*, 1749–1770. [CrossRef]
11. Aggarwal, P.; Das Gupta, M.; Joseph, A.P.; Chatterjee, N.; Srinivasan, N.; Nath, U. Identification of specific DNA binding residues in the TCP family of transcription factors in Arabidopsis. *Plant Cell* **2010**, *22*, 1174–1189. [CrossRef]
12. Huq, E.; Quail, P.H. PIF4, a phytochrome-interacting bHLH factor, functions as a negative regulator of phytochrome B signaling in Arabidopsis. *EMBO J.* **2002**, *21*, 2441–2450. [CrossRef] [PubMed]
13. Ikeda, M.; Fujiwara, S.; Mitsuda, N.; Ohme-Takagi, M. A triantagonistic basic helix-loop-helix system regulates cell elongation in Arabidopsis. *Plant Cell* **2012**, *24*, 4483–4497. [CrossRef] [PubMed]
14. Zhang, Y.; Zhang, M.; Hu, H.; Yang, J.; Cui, J.; Xu, J. Cloning and cold-resistance analyses of CfICE1 gene in Cryptomeria fortunei. *Plant Physiol. Biochem.* **2021**, *162*, 456–467. [CrossRef] [PubMed]
15. Zheng, P.F.; Yang, Y.Y.; Zhang, S.; You, C.X.; Zhang, Z.L.; Hao, Y.J. Identification and functional characterization of MdPIF3 in response to cold and drought stress in Malus domestica. *Plant Cell Tissue Organ Cult. (PCTOC)* **2021**, *144*, 435–447. [CrossRef]
16. Chinnusamy, V.; Ohta, M.; Kanrar, S.; Lee, B.H.; Hong, X.; Agarwal, M.; Zhu, J.K. ICE1: A regulator of cold-induced transcriptome and freezing tolerance in Arabidopsis. *Genes Dev.* **2003**, *17*, 1043–1054. [CrossRef] [PubMed]
17. Fursova, O.V.; Pogorelko, G.V.; Tarasov, V.A. Identification of ICE2, a gene involved in cold acclimation which determines freezing tolerance in Arabidopsis thaliana. *Gene* **2009**, *429*, 98–103. [CrossRef]
18. Feng, X.; Zhao, Q.; Zhao, L.L.; Qiao, Y.; Xie, X.B. The cold-induced basic helix-loop-helix transcription factor gene MdCIbHLH1 encodes an ICE-like protein in apple. *BMC Plant Biol.* **2012**, *12*, 22. [CrossRef]
19. Geng, J.; Wei, T.; Wang, Y.; Huang, X. Overexpression of PtrbHLH, a basic helix-loop-helix transcription factor from Poncirus trifoliata, confers enhanced cold tolerance in pummelo (Citrus grandis) by modulation of H_2O_2 level via regulating a CAT gene. *Tree Physiol.* **2019**, *39*, 2045–2054. [CrossRef]
20. Rushton, P.J.; Bokowiec, M.T.; Han, S.; Zhang, H. Tobacco transcription factors: Novel insights into transcriptional regulation in the Solanaceae. *Plant Physiol.* **2008**, *147*, 280–295. [CrossRef]

21. Carretero-Paulet, L.; Galstyan, A.; Roig-Villanova, I.; Martínez-García, J.F. Genome-wide classification and evolutionary analysis of the bHLH family of transcription factors in Arabidopsis, poplar, rice, moss, and algae. *Plant Physiol.* **2010**, *153*, 1398–1412. [CrossRef] [PubMed]
22. Bailey, P.C.; Martin, C.; Toledo-Ortiz, G.; Quail, P.H. Update on the basic helix-loop-helix transcription factor gene family in Arabidopsis thaliana. *Plant Cell* **2003**, *15*, 2497–2502. [CrossRef] [PubMed]
23. Chen, J.; Guang, X.; Zhao, C.; Wang, P. Liriodendron genome sheds light on angiosperm phylogeny and species-pair differentiation. *Nat. Plants* **2019**, *5*, 18–25. [CrossRef] [PubMed]
24. Hao, Z.; Liu, S.; Hu, L.; Shi, J.; Chen, J. Transcriptome analysis and metabolic profiling reveal the key role of carotenoids in the petal coloration of Liriodendron tulipifera. *Hortic. Res.* **2020**, *7*, 70. [CrossRef] [PubMed]
25. Jin, J.; Tian, F.; Yang, D. PlantTFDB 4.0: Toward a central hub for transcription factors and regulatory interactions in plants. *Nucleic Acids Res.* **2017**, *45*, D1040–D1045. [CrossRef]
26. Saitou, N.; Nei, M. The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **1987**, *4*, 406–425.
27. Hall, B.G. Building phylogenetic trees from molecular data with MEGA. *Mol. Biol. Evol.* **2013**, *30*, 1229–1235. [CrossRef]
28. Chen, C.; Chen, H.; Zhang, Y. TBtools: An Integrative Toolkit Developed for Interactive Analyses of Big Biological Data. *Mol. Plant* **2020**, *13*, 1194–1202. [CrossRef]
29. Zhu, Y.; Wu, N.; Song, W.; Yin, G. Soybean (Glycine max) expansion gene superfamily origins: Segmental and tandem duplication events followed by divergent selection among subfamilies. *BMC Plant Biol.* **2014**, *14*, 93. [CrossRef]
30. Wang, Y.; Tang, H.; Debarry, J.D. MCScanX: A toolkit for detection and evolutionary analysis of gene synteny and collinearity. *Nucleic Acids Res.* **2012**, *40*, e49. [CrossRef]
31. Zhang, Z.; Li, J.; Zhao, X.Q. KaKs_Calculator: Calculating Ka and Ks through model selection and model averaging. *Genom. Proteom. Bioinform.* **2006**, *4*, 259–263. [CrossRef]
32. Shannon, P.; Markiel, A.; Ozier, O.; Baliga, N.S. Cytoscape: A software environment for integrated models of biomolecular interaction networks. *Genome Res.* **2003**, *13*, 2498–2504. [CrossRef] [PubMed]
33. Wittkopp, P.J.; Kalay, G. Cis-regulatory elements: Molecular mechanisms and evolutionary processes underlying divergence. *Nat. Rev. Genet.* **2011**, *13*, 59–69. [CrossRef] [PubMed]
34. Lynch, M.; Conery, J.S. The evolutionary fate and consequences of duplicate genes. *Science* **2000**, *290*, 1151–1155. [CrossRef]
35. Liu, Y.; Li, X.; Li, K.; Liu, H. Multiple bHLH proteins form heterodimers to mediate CRY2-dependent regulation of flowering-time in Arabidopsis. *PLoS Genet.* **2013**, *9*, e1003861. [CrossRef]
36. Qi, X.; Torii, K.U. Hormonal and environmental signals guiding stomatal development. *BMC Biol.* **2018**, *16*, 21. [CrossRef]
37. Kashyap, P.; Deswal, R. Two ICE isoforms showing differential transcriptional regulation by cold and hormones participate in *Brassica juncea* cold stress signaling. *Gene* **2019**, *695*, 32–41. [CrossRef]
38. Zhou, L.; He, Y.J.; Li, J.; Li, L.Z. An eggplant SmICE1a gene encoding MYC-type ICE1-like transcription factor enhances freezing tolerance in transgenic Arabidopsis thaliana. *Plant Biol.* **2020**, *22*, 450–458. [CrossRef]
39. Verma, R.K.; Kumar, V.; Yadav, S.K.; Kumar, T.S. Overexpression of Arabidopsis ICE1 enhances yield and multiple abiotic stress tolerance in indica rice. *Plant Signal. Behav.* **2020**, *15*, 1814547. [CrossRef]
40. Liu, L.; Duan, L.S.; Zhang, J.C.; Zhang, Z.X. Cucumber (*Cucumis sativus* L.) over-expressing cold-induced transcriptome regulator ICE1 exhibits changed morphological characters and enhances chilling tolerance. *Sci. Hortic.* **2010**, *124*, 29–33. [CrossRef]
41. Lin, Y.; Guo, H.; Liu, C.X. Molecular cloning and expression analysis of cold-resistant transcription factor ICE1 from *Eucalyptus camaldulensis* L. *Plant Physiol. Commun.* **2011**, *47*, 488–494.
42. Song, X.M.; Huang, Z.N.; Duan, W.K.; Ren, J. Genome-wide analysis of the bHLH transcription factor family in Chinese cabbage (*Brassica rapa* ssp. *pekinensis*). *Mol. Genet. Genom.* **2014**, *289*, 77–91. [CrossRef] [PubMed]
43. Niu, X.; Guan, Y.; Chen, S.; Li, H. Genome-wide analysis of basic helix-loop-helix (bHLH) transcription factors in *Brachypodium distachyon*. *BMC Genom.* **2017**, *18*, 619. [CrossRef] [PubMed]
44. Atchley, W.R.; Fitch, W.M. A natural classification of the basic helix-loop-helix class of transcription factors. *Proc. Natl. Acad. Sci. USA* **1997**, *94*, 5172–5176. [CrossRef]
45. Shimizu, T.; Toumoto, A.; Ihara, K.; Shimizu, M. Crystal structure of PHO4 bHLH domain-DNA complex: Flanking base recognition. *EMBO J.* **1997**, *16*, 4689–4697. [CrossRef]
46. Chater, C.; Caine, R.S.; Fleming, A.J.; Gray, J.E. Origins and Evolution of Stomatal Development. *Plant Physiol.* **2017**, *174*, 624–638. [CrossRef]
47. Wu, Z.; Chen, L.; Yu, Q. Multiple transcriptional factors control stomata development in rice. *New Phytol.* **2019**, *223*, 220–232. [CrossRef]
48. Zhang, Z.; Li, J.; Li, F. *OsMAPK3* Phosphorylates *OsbHLH002/OsICE1* and Inhibits Its Ubiquitination to Activate *OsTPP1* and Enhances Rice Chilling Tolerance. *Dev. Cell* **2017**, *43*, 731–743.e5. [CrossRef]
49. Ren, X.Y.; Vorst, O.; Fiers, M. In plants, highly expressed genes are the least compact. *Trends Genet.* **2006**, *22*, 528–532. [CrossRef]
50. Jeffares, D.C.; Penkett, C.J.; Bähler, J. Rapidly regulated genes are intron poor. *Trends Genet.* **2008**, *24*, 375–378. [CrossRef]
51. Cannon, S.B.; Mitra, A.; Baumgarten, A.; Young, N.D. The roles of segmental and tandem gene duplication in the evolution of large gene families in *Arabidopsis thaliana*. *BMC Plant Biol.* **2004**, *4*, 10. [CrossRef] [PubMed]

52. Mehan, M.R.; Freimer, N.B.; Ophoff, R.A. A genome-wide survey of segmental duplications that mediate common human genetic variation of chromosomal architecture. *Hum. Genom.* **2004**, *1*, 335–344. [CrossRef] [PubMed]
53. Vision, T.J.; Brown, D.G.; Tanksley, S.D. The origins of genomic duplications in Arabidopsis. *Science* **2000**, *290*, 2114–2117. [CrossRef] [PubMed]
54. Xie, T.; Chen, C.; Li, C.; Liu, J. Genome-wide investigation of WRKY gene family in pineapple: Evolution and expression profiles during development and stress. *BMC Genom.* **2018**, *19*, 490. [CrossRef] [PubMed]
55. Andersson, D.I.; Hughes, D. Gene amplification and adaptive evolution in bacteria. *Annu. Rev. Genet.* **2009**, *43*, 167–195. [CrossRef] [PubMed]
56. Zhang, J. Evolution by gene duplication: An update. *Trends Ecol. Evol.* **2003**, *18*, 292–298. [CrossRef]
57. Morgenstern, B.; Atchley, W.R. Evolution of bHLH transcription factors: Modular evolution by domain shuffling? *Mol. Biol. Evol.* **1999**, *16*, 1654–1663. [CrossRef]
58. Hu, C.H.; Wei, X.Y.; Yuan, B.; Yao, L.B. Genome-Wide Identification and Functional Analysis of NADPH Oxidase Family Genes in Wheat During Development and Environmental Stress Responses. *Front. Plant Sci.* **2018**, *9*, 906. [CrossRef]
59. Zhao, Y.; Zhou, M.; Xu, K.; Li, J. Integrated transcriptomics and metabolomics analyses provide insights into cold stress response in wheat. *Crop J.* **2019**, *7*, 857–866. [CrossRef]
60. Brown, A.P.; Dunn, M.A.; Goddard, N.J.; Hughes, M.A. Identification of a novel low-temperature-response element in the promoter of the barley (*Hordeum vulgare* L.) gene *blt101.1*. *Planta* **2001**, *213*, 770–780. [CrossRef]
61. Maruyama, K.; Todaka, D.; Mizoi, J.; Yoshida, T.; Kidokoro, S. Identification of cis-acting promoter elements in cold- and dehydration-induced transcriptional pathways in Arabidopsis, rice, and soybean. *DNA Res.* **2012**, *19*, 37–49. [CrossRef] [PubMed]
62. Liu, Q.; Kasuga, M.; Sakuma, Y.; Abe, H. Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought- and low-temperature-responsive gene expression, respectively, in Arabidopsis. *Plant Cell* **1998**, *10*, 1391–1406. [CrossRef] [PubMed]
63. Kasuga, M.; Liu, Q.; Miura, S.; Yamaguchi-Shinozaki, K. Improving plant drought, salt, and freezing tolerance by gene transfer of a single stress-inducible transcription factor. *Nat. Biotechnol.* **1999**, *17*, 287–291. [CrossRef] [PubMed]
64. Hwarari, D.; Guan, Y.; Ahmad, B. ICE-CBF-COR Signaling Cascade and Its Regulation in Plants Responding to Cold Stress. *Int. J. Mol. Sci.* **2022**, *23*, 1549. [CrossRef] [PubMed]
65. Kim, Y.S.; Lee, M.; Lee, H.J.; Park, C.M. The unified ICE-CBF pathway provides a transcriptional feedback control of freezing tolerance during cold acclimation in Arabidopsis. *Plant Mol. Biol.* **2015**, *89*, 187–201. [CrossRef] [PubMed]
66. Agarwal, M.; Hao, Y.; Kapoor, A.; Dong, C.H. A R2R3 type MYB transcription factor is involved in the cold regulation of CBF genes and in acquired freezing tolerance. *J. Biol. Chem.* **2006**, *281*, 37636–37645. [CrossRef] [PubMed]
67. Miura, K.; Jin, J.B.; Lee, J.; Yoo, C.Y. *SIZ1*-mediated sumoylation of *ICE1* controls *CBF3/DREB1A* expression and freezing tolerance in Arabidopsis. *Plant Cell* **2007**, *19*, 1403–1414. [CrossRef]
68. Kanaoka, M.M.; Pillitteri, L.J.; Fujii, H.; Yoshida, Y. *SCREAM/ICE1* and *SCREAM2* specify three cell-state transitional steps leading to arabidopsis stomatal differentiation. *Plant Cell* **2008**, *20*, 1775–1785. [CrossRef]