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Keywords: L. chinense var. rubrum.; bHLH transcription factor; expression analyses; anthocyanin

Article

Genome-wide analysis of bHLH gene family in *Loropetalum chinense* var. *Rubrum*: identification, classification, evolution, and expression pattern diversity on cultivations

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Abstract: The basic helix-loop-helix (bHLH) transcription factor family is the second-largest transcription factor in plants. Members of this family are involved in the process of growth and development, secondary metabolic biosynthesis, signal transduction, and plant resistance. *Loropetalum chinense* var. *rubrum* is a critical woody plant with higher ornamental and economic values, which used as ornamental architecture and traditional Chinese herbal medicine plant. However, the bHLH transcription factor in the *L. chinense* var. *rubrum* (*Loropetalum chinense* var. *Rubrum*) have yet to be systematically demonstrated, and their role in anthocyanin biosynthesis remains secret. Here, we identified 165 potential *LcbHLHs* genes by two methods, and they were unequally distributed on chromosomes 1 to 12 of the *L. chinense* genome. Based on a phylogenetic comparison with proteins from *Arabidopsis*, these bHLH proteins were categorized into 21 subfamilies. Most *LcbHLHs* in a given subfamily had similar gene structures and conserved motifs. The gene ontology annotation and cis-elements predicted that the *LcbHLHs* had many molecular functions and were involved in plant growth processes, including flavonoid and anthocyanin biosynthetic processes, etc. Transcriptomic analysis revealed different expression patterns among different tissues and cultivars in *L. chinense*. Many *LcbHLHs* were expressed in leaves, and only a few genes were highly expressed in flowers. Six *LcbHLH* genes differentially expressed between species and periods in color variation, which may play a key role in anthocyanin synthesis. These further functional predictions of *LcbHLHs* were established by quantitative real-time PCR analysis and protein-protein interaction network. This study identified the six potential *bHLH* genes related to anthocyanin in *L. chinense* and created a solid framework for future research on the function and evolution of *bHLHs*.

Keywords: *L. chinense* var. *rubrum*.; bHLH transcription factor; expression analyses; anthocyanin

1. Introduction

Transcription factors (TFs) serve as a central regulators to regulate the expression of target genes and they can form intricate networks through protein–protein interactions to control or affect many biological processes at the transcription level [1]. Among the various TFs, the bHLH TFs family is a relatively large family of transcription factors, which have been widely found in various eukaryotes. The bHLH domain often makes up of 50-60 amino acids and has two functionally distinct regions: a length of 10-15 basic amino acids (The basic region) and a stretch of roughly 40 amino acids(the helix-loop-helix region), which can form two amphipathic α-helices separated by an intervening loop[2,3]. The basic region is located at the N-terminus of the bHLH domain and is the DNA binding region that allows the bHLH TFs to bind to the E-box (CANNTC), while the HLH region is located at the C-terminus of the bHLH domain and acts as a dimerization domain which can promote the formation of homodimers or heterodimers between proteins to alter the expression of target genes involved in various signaling pathways [4,5]. From the bHLH protein domain, only 19 amino acids are conserved and possesses a highly conservative H-E-R motif. Previous studies have typically classified members of the bHLH superfamily into subfamilies or subgroups based on these conserved motifs, evolutionary relationships, DNA binding specificity, and structural domains. Ledent and Atchley have classified the bHLH transcription factors in animals into six subgroups (A-F) based on sequence homology and phylogenetic relationships [3,6]. However, because of the relative independence of the bHLH transcription factors' genealogy between plant and animal, there are differences in the classification and so far no definite categories of plant bHLH transcription factor families criteria have been proposed[7]. Generally, the bHLH transcription factor family in plants has been divided into 15-26 groups [3,8,9]. 147 and 167 bHLH family members were identified from Arabidopsis and rice, respectively, and then classified into 21 and 25 subfamilies [9,10]. Subsequently, the various functions of the *bHLH* gene also have been validated.

Through previous studies, scientists have classified the function of *bHLH* genes in *Arabidopsis* and found that *bHLH* genes play different roles in different aspects [11]. In terms of mineral nutrition of plants, the *AtbHLH121, AtbHLH18, AtbHLH19, AtbHLH20, AtbHLH25* can regulate iron homeostasis by indirectly activating the FER-LIKE IRON DEFICIENCY-INDUCED TRANSCRIPTION FACTOR (FIT) [12]. In growth and development, the *AtLP1* and *AtLP2* can regulate longitudinal cell elongation[13]. Also, the *AtNFL* promotes flowering under short-day conditions in *Arabidopsis* [14]. The *PobHLH5* and *PobHLH8* of *P. ostreatus* infestation in *Arabidopsis* verified that *bHLH* genes play an important role in growth and development[15]. In addition, *bHLHs* are also involved in plant abiotic stresses, signal transmission and secondary metabolism, etc.[16] For example, the overexpression of *TabHLH39* improved drought tolerance, salt tolerance and frost resistance in transgenic *Arabidopsis*[17] and The *MdCIB1* in apple plays an active role in drought resistance[18]. Researchers also found that expression of *CpbHLH1* in transgenic model plants suppressed anthocyanin accumulation [19] and *NnTT8* in Lotus had been indicated to be involved in the positive regulation of anthocyanin biosynthesis [20]. It has been further shown that pomegranate fruit anthocyanins can be regulated by a combination of bHLHs and MYB [21], especially the formation of MYB-bHLH-WD40 ternary complex[22,23].

Loropetalum chinense var. *rubrum*（*L. chinense*）is a typical and vivid flowering and foliage plant in Asia, Europe, and America. Ornamental plants lovers deeply enjoy its beautiful foliage and pretty plant figure [24]. The ornamental value of *Loropetalum chinense* var.*rubrum* mainly includes leaf color, flower color and tree posture, among which leaf color is one of the essential ornamental values. Previous studies have shown that anthocyanin is a crucial compound for the leaf coloration of *Loropetalum chinense* var.*rubrum* [25]. Its synthesis is usually affected by the external environment, synthetic structural genes, and regulatory genes. Studies have also shown that short-wavelength light quality

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is more conducive to improving the activity of phenylalanine ammonia lyase (PAL) in the callosity of *Loropetalum chinense* var.*rubrum* and promoting the synthesis of anthocyanin in the callus[26]. High temperature and drought usually cause the regreening of *Loropetalum chinense* var.*rubrum* leaves, which is mainly due to the degradation of anthocyanins [27]. In addition, the researchers also further understood the coloration mechanism of *L.chinense* leaves from the molecular level, identified and cloned structural genes related to anthocyanin synthesis, including *LcCHS*, *LcDFR*, *LcCHI*, *LcANS* and so on[28]. *bHLH* gene is one of the essential regulatory genes in the anthocyanin synthesis pathway, which can encode the corresponding transcription factors and then activate or inhibit the spatial and temporal expression of structural genes by forming the MYB-bHLH-WD40 complex, thereby regulating the synthesis of plant anthocyanins. It has been reported that the genome sequencing and identification of the bHLH family have been completed in a variety of plants, such as Arabidopsis, potato, bamboo, and grape etc[11,29–31]. However, the characteristics of bHLH transcription factors and their roles in *L.chinense* are still unclear.

In this study, the bHLH gene family of *L. chinense* was systematically identified by using bioinformatics methods. Then, potential leaf color-regulated *bHLH* genes were filtered by a series of biological analyses and expression pattern analysis. These results gave us a further understanding of the structure, function, and evolution of the *L. chinense* bHLH family, and provided a potential basis for the regulatory network of leaf coloration in *Loropetalum chinense* var.*rubrum*.

2. Results

2.1. Identification of LcbHLHs gene members and Phylogenetic Analysis

A total of 165 *bHLH* members were acquired by the method of homologous blast and HMM rsearch from genomic data of *L. chinense* and named *LcbHLH1* to *LcbHLH165* according to the chromosomal localization **(Figure 1 Table S1).** Prediction of physicochemical properties by ExPasy revealed that the number of amino acids contained in the bHLH protein sequence of *L. chinense* **(Table S2)**, which ranged from 120 (LcbHLH96) to 1186aa (LcbHLH164), with an average of 375aa. The molecular weight of these protein ranged from 13460.46 (LcbHLH96) to 130429.73Da (LcbHLH84), with an average of 41508.43Da, and the theoretical isoelectric points ranged from 4.70 (LcbHLH62, LcbHLH102, LcbHLH103) to 10.12 (LcbHLH96), with 62.71% of them lower than 7, as predicted to be acidic. It was consistent with the previous study of the isoelectric point pattern in *Arabidopsis* and *Oryza sativa*. The Grand average of hydropathicity (GRAVY) of the proteins in the range of -1.037 to -0.102, showing that all LcbHLHs are hydrophilic. The instability index (II) ranged from 36.68 to 71.31, with only two manifested stable proteins (II < 40) and the aliphatic index was between 51.42 and 105.58. Most of the LcbHLHs were localized in the nucleus, and only a few were distributed in the cytoplasm, chloroplasts, plasmodesmata and Golgi apparatus. No signal peptide was found for any of the LcbHLHs by SignalP, indicating that they are non-secretory proteins.

In order to clarify the evolutionary relationship of *L. chinense* var. *rubrum*, a phylogenetic tree was constructed with *Arabidopsis* **(Figure S1)**. The presence of LcbHLHs in 21 of the 24 Arabidopsis bHLH subfamilies and only three subfamilies XIII, XIV and XV did not contain members of LcbHLHs **(Figure 2)**. Subfamily XII with 20 members, was the largest subfamily of LcbHLHs, while subfamilies IVd, VI and X were the smallest, each with only a member. In addition, compared with *A. thaliana*[5], *Ficus carica L[32]*, *P. persica[33]* **(Table 1)**, the III(a+b+c), the III(d+e), the IVa, the VII(a+b) and the XII subfamilies were considerably expanded.

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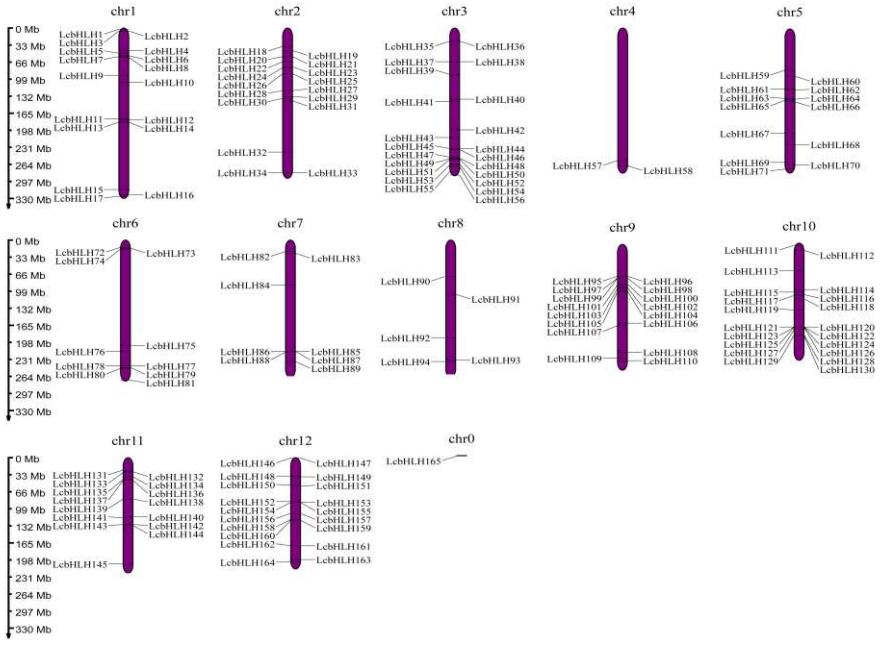

Figure 1. Chromosomal distribution of LcbHLHs. The left scale is used to assess chromosome size. All chromosomes contain the *LcbHLH* genes. The chr0 represents scaffold.

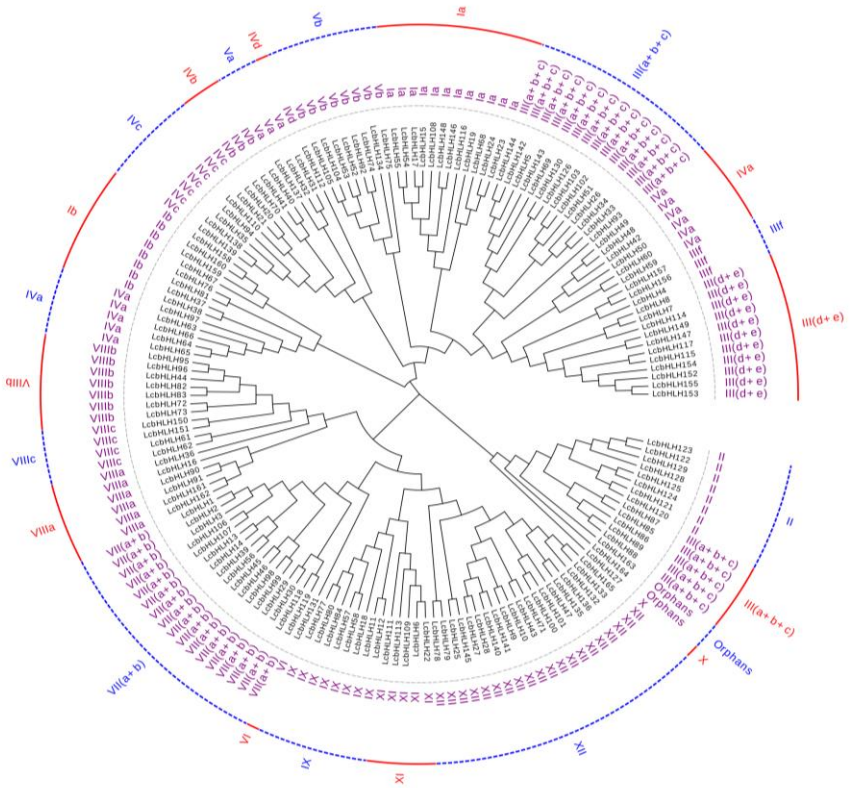

Figure 2. Phylogenetic analysis of bHLH proteins in *L. chinense*. The phylogenetic trees of *Arabidopsis thaliana* and *L. chinense* were constructed by using MEGA11 software with the neighbor-joining (NJ) method. The parameters are set as follows: method "p-distance", gaps date treatment "partial deleted", self-expansion value "Bootstrap=1000".

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Table 1. Quantitative distribution of each subfamily of bHLHs in *L. chinense*, *A. thaliana*, *Ficus carica L.* and *P. persica* Subfamily.

Subfamilies	Number of *LcbHLHs*	Number of At-bHLHs	Number of FcbHLHs	Number of PpbHLHs
Ia	12	10	8	8
Ib	8	13	7	8
II	8	4	0	1
III(a+b+c)	19	10	9	10
III(d+e)	11	8	6	11
IIIf	3	4	1	2
IVa	11	4	4	3
IVb	3	3	4	0
IVc	7	4	3	0
IVd	1	2	1	2
Va	3	3	3	2
Vb	8	5	6	6
VI	1	2	2	0
VII(a+b)	17	15	7	7
VIIIa	6	4	3	2
VIII(b+c)	11	11	14	9
IX	8	6	5	6
X	1	10	14	2
XI	5	5	4	4
XII	20	17	13	12
XIII	0	3	4	0
XIV	0	3	0	0
XV	0	5	0	0
Orphans	2	8	0	0

2.2. Multiple Sequence Alignment, Motif, Domain and Structure Analysis

To further elucidate the structural features of LcbHLH proteins, a multiple sequence alignment analysis was performed on the bHLH structural domain. As shown in **Figure 3**, each LcbHLH protein displayed four conserved regions, including one basic region, one loop region and two helix regions. Most LcbHLHs proteins possessed hightly conserved basic region and two helixes, except the LcbHLH120, LcbHLH121, LcbHLH122, LcbHLH123, LcbHLH124 and LcbHLH125. LcbHLH122 and 123 had no loop region and two helix regions. LcbHLH120, LcbHLH121, LcbHLH124 and LcbHLH159 had no loop region and the second helix region. Multiple sequence alignment showed that 24 amino acid residues were highly conserved (> 60% consensus ratio), and two of those were conserved with a 100% consensus ratio. It is noteworthy that most LcbHLH proteins have the highly conserved H-ER-RR structures and Leu-27 in helix 1 and Leu-59 in helix 2 of the HLH region that are considered to play an important role in protein dimerization. Thus, we speculated that LcbHLH proteins may have the capacity to form protein complexes.

The conserved motifs, domain distribution and gene structure analysis of *L. chinense* were carried out to obtain more information about *bHLHs*. As is shown in **Figure 4**, there were ten motifs were identified and marked as 1-10 **(Table 2)**. The different motifs correspond to their bHLH superfamily domains. For example, we can clearly see that motif 8 represents the bHLH-MYC_N domain, and motif 1 and motif 2 can form various bHLH structural domains. In combination with protein sequence alignment, one basic region and one helix region were predicted in motif 1, and another helix region were predicted in motif 2. However, because of the less conserved protein sequence of loop region between helix 1 and helix 2, most of the LcbHLH proteins had a combination of motifs 1 and

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2, while LcbHLH122, LcbHLH123, LcbHLH129, LcbHLH128, LcbHLH131, LcbHLH81, LcbHLH76, LcbHLH159, LcbHLH55, LcbHLH54, LcbHLH108, LcbHLH148, LcbHLH146, and LcbHLH93 have only motif 1. The LcbHLH proteins clustered in the same subfamily often have similar motifs. For example, motifs1, 2, 5, 4, 9, 6 and 10 were identified in subfamily II, and Motifs 1, 2, and 8 were identified in subfamily III(d+e+f).

The evolutionary relationships of the members of gene families can be shown through gene structure analysis. The gene structure of *LcbHLHs* in the same supfamily always were consistent. The exon number of *LcbHLHs* varied from 1 to 13, whereas the exon-intron organizations were phylogenetically related **(Figure 4)**. The *LcbHLHs* with one exon were clustered in four subfamilies (III(d+e), VIIIb, Orphans and II) and all the members of subfamily VIIIb and Orphans only have one exon.

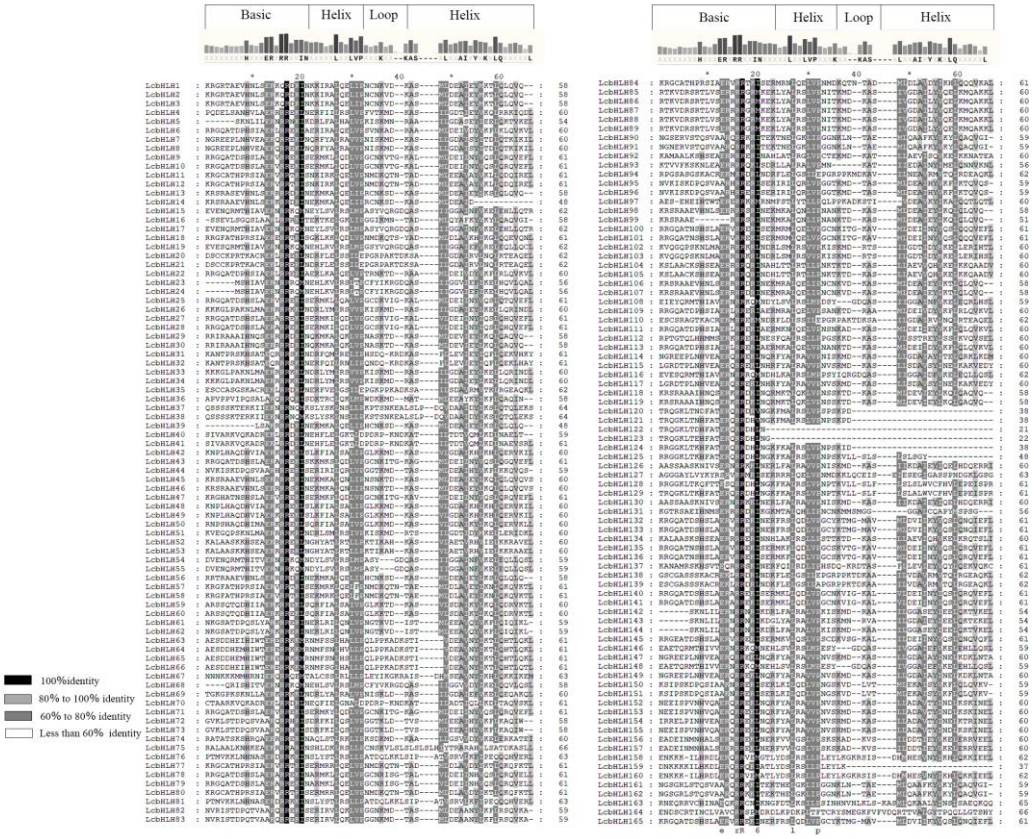

Figure 3. Multiple alignments of the bHLH domains in the LcbHLH family proteins. Conserved amino acids in the LcbHLHs domain. Sequence identity > 60 % in gray or black shades.

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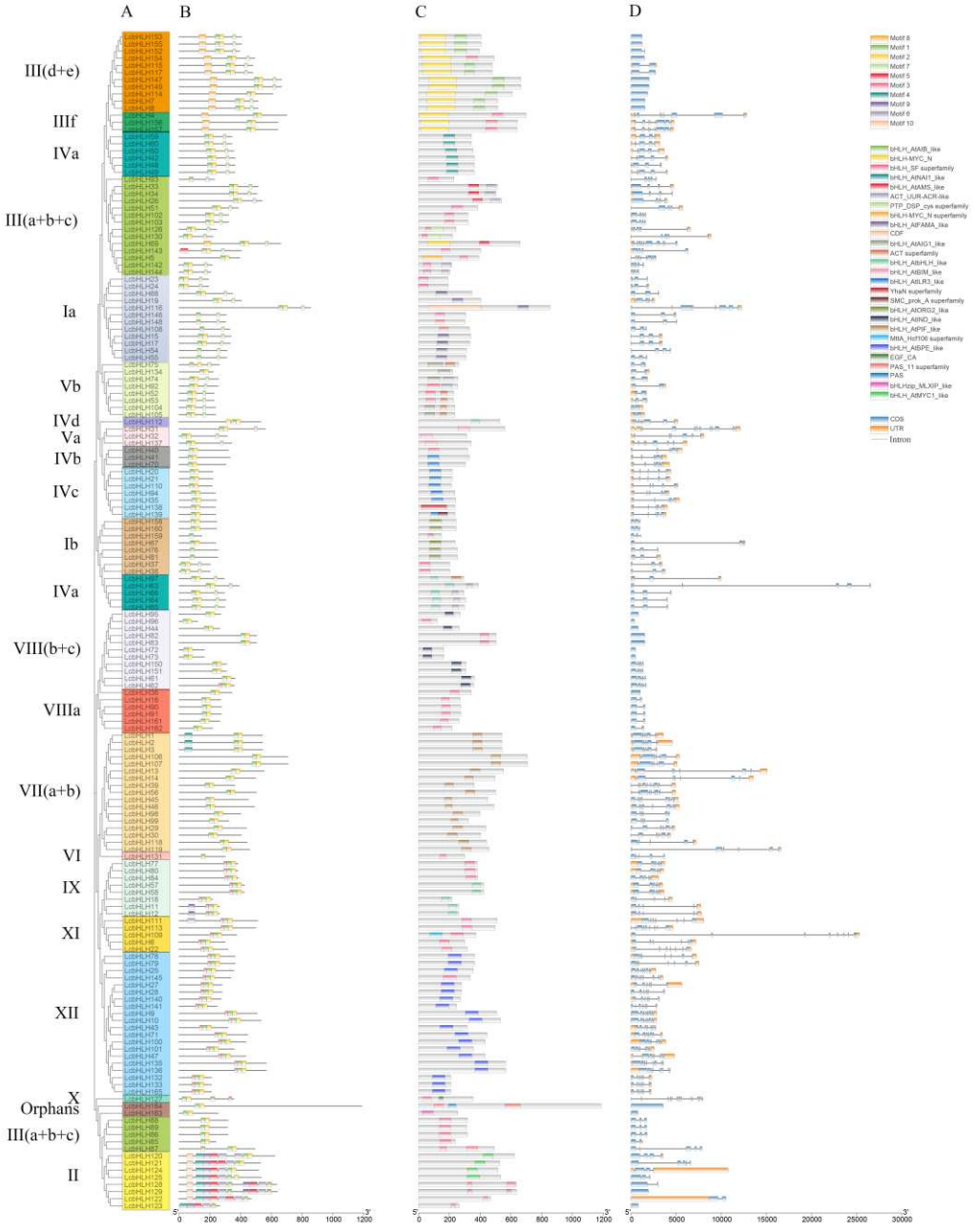

Figure 4. Phylogenetic relationships, family structural domains, conserved motifs, and gene structural analyses of the LcbHLHs. (**A**)Phylogenetic tree of LcbHLHs. (**B**)The family domain distribution and types of LcbHLH. (**C**)Conserved motifs of LcbHLHs. The blue rectangle represents the exon, the orange rectangle represents the UTR, and the black line represents the intron. The size of these genes is evaluated by the bottom coordinate. (**D**)Gene structure of LcbHLHs. The 10 predicted motifs are depicted by rectangles of various colors.

Table 2. Sequences of 10 predicted motifs of FcbHLH proteins.

Motif	Sequence
1	SHSLAERRRRERJNERFKALRSLVPNCSK
2	MDKASMLDEAIEYVKELQRQVQELSMKLE
3	EEPKSDYIHVRARRGQATD
4	QVMSFEQSNWDASVHEIQGMTSFEHPHNQDQQLHLLHEMQQNGHHHPQSF
5	FVQKPANFQTSLGFLGDLPTPDNASASSVLYDPLFHLNLPPQPPLFRDLF

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6	YNLPASRTASLFGGGIDEKEGSGGVYQNGVATQFDNGVLEFTGDIGGMGK
7	RJVSALEKLGLDIVHANVSTF
8	ERAKLAKSAGIRTLVCIPTASGVVELGSTEIIKEDLGLIQLIKSLF
9	NSSTLPDTSPYINNPPTQHLLNLFHLPRCSPSSNLLPNSSI
10	DNIRLSMEELSYHQNPHQEDDAALEQHLGFDMENCYNINNN

2.3. Gene Duplication and Collinear Correlation Analysis

Tandem and fragment chromosome replication are critical means of gene family amplification. The 165 *LcbHLHs* in our study were unequally distributed among 12 *L. chinense* var. *rubrum* chromosomes, with a maximum of 22 on chr3 and a minimum of 2 on chr4. Tandem replication is thought to occur when the distance between genes is less than 100 kb, and 7 pairs of *LcbHLHs* fell into that category **(Table S3)**. An intraspecific collinearity study revealed that fragment replication produced 56 pairs of *LcbHLHs* **(Table S4)**. The findings have shown that tandem replication and fragment replication are critical processes for expanding the *LcbHLH* gene family. In addition, the substitution rate ratio, Ka/Ks, is applicable criterion for gene duplication selective pressure. Ka/Ks value less than 1 often represents negative selection, equal to 1 represents a neutral selection, and greater than 1 represents a positive selection. Among the 56 pairs of fragment replication gene pairs and 7 pairs of tandem replication gene pairs, except that the pair of *LcbHLH29/30*, *LcbHLH140/141*, *LcbHLH142/144*, and *LcbHLH102/103* in the fragment replication pair were greater than 1, which showed positive selection. The others' Ka/Ks values were less than 1. It was speculated that it might have been purified and selected in the evolutionary history, indicating that most *bHLH* genes evolved slowly **(Table S5)**.

In order to better understand the genetic differentiation, gene replication and evolution among *bHLH* gene families of *L. chinense*, *Arabidopsis*, *Oryza sativa*, *Zea mays* and *Vitis vinifera*, MCScanX was used to analyze the homologous *bHLH* genes among these species. 89, 44, 41 and 123 pairs of orthologous *bHLH* genes were detected in three comparisons (*L. chinense* vs. *Arabidopsis*, *L. chinense* vs. *Oryza sativa*, *L. chinense* vs. *Zea mays* and *L. chinense* vs. *Vitis vinifera*) **(Figure 5 Table S6)**. Therefore, the *bHLH* genes between *L. chinense* and *Vitis vinifera* were considered to be more closely related than the *bHLH* genes among *L. chinense* and *Oryza sativa*, *Zea mays* or *Arabidopsis*.

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Figure 5. Syntenic analyses of *bHLH* genes in *L. chinense*, *Arabidopsis* (**A**), *Oryza sativa* (**B**), *Zea mays* (**C**) and *Vitis vinifera* (**D**).

2.4. GO annotation and analyses of cis-regulatory elements

Highly divergent sequences outside the conserved structural domain of bHLH suggested that the LcbHLH protein may be involved in multiple biological processes to some extent. In order to clarify the particular functions of bHLH proteins, GO annotation of LcbHLH was performed. From **Table S7**, Most LcbHLH proteins are annotated to be associated with transcription factors and protein dimerization activity, regulation of transcription and developmental processes. For the cellular component, 139 of 158 *bHLH* genes from transcriptome data were located in the nucleus (139/158). Only a small number of genes were predicted distributions in organelles, such as the cytoplasm (4), cytosol (2), chloroplast envelope (1), nucleoplasm (1), vacuole (1), nuclear speck (2), and intracellular membrane-bounded organelle (1) etc. Also, few *LcbHLHs* are also located in less common membrane structures and complexes, including integral component of membrane (1), membrane (1), vacuolar membrane (1), transcription factor complex (1) and RNA polymerase II transcription factor complex (6). It was anticipated that practically most LcbHLH proteins were engaged in protein dimerization activity (152/158) according to the molecular functions. In addition, the LcbHLHs participated in various biological processes, and many LcbHLH proteins were annotated to be associated with DNA binding, growth regulation and response of stimuli. For example, 127 LcbHLHs associated with DNA binding TFs, part of LcbHLH proteins involved the growth and development including the regulation of growth (6), pollen development (10), anther wall tapetum development (11) and negative regulation of seed germination (6), and several LcbHLHs could react environmental stimulation like cold (7), iron (5) and light (2) etc.

Members of gene families can differ in their expression patterns as well as their function. We looked for cis-elements in the promoter regions of the various *LcbHLH* genes to see whether there were any distinctions in gene regulation. From this **Table S8**, Light response elements were the most common cis-elements in *LcbHLH* promoter sequences, including Box 4, Gap-box, G-Box, AE-box, I-box, GT1-motif, TCCC-motif and so on. In the promoter regions of *LcbHLH* genes, cis-elements participated in growth and development and related to different stress responses were also discovered, such as MSA-like involved in cell cycle regulation, motif I and RY-element involved in seed-specific regulation, GCN4 motif involved in endosperm development, circadian involved in circadian control etc. Also, the cis-elements related to different stress responses are various, including the abiotic response elements which involved in abscisic acid (ABRE), auxin (AuxRR-core, TGA-element, TGA-box, AuxRE), MeJA (CGTCA-motif, TGACG-motif), gibberellin responsiveness (GARE-motif, TATC-box P-box) and the biological response elements related to salt stress (DRE) and low temperature responsiveness (LTR) etc. We also identified some cis-regulatory elements with MYB recognition sites, which can regulate plant flavonoid synthesis (MBSI) by binding to MYB-related genes, and conduct drought induction (MBS) and light response (MRE).

2.5. Expression profiles of LcbHLHs and Gene Expression Analysis by qRT -PCR

The expression pattern of *LcbHLHs* was analyzed based on transcriptome data of different *Loropetalum chinense* cultivars. New varieties of *Loropetalum chinense* mainly include 'Xiangnong-Nichang'(XNNC), 'Xiangnong-Fengjiao'(XNFJ), 'Xiangnong-Xiangyun'(XNXY) and 'Xiangnong-Xiaojiao'(XNXJ) **(Table S9, Figure 6A)**. In order to better observe the difference in *LcbHLHs* expression, we standardized the FPKM value and removed the *LcbHLHs* with FPKM value less than 1, which is usually considered an invalid expression. From that, an expression pattern map of 105 *LcbHLHs* was established **(Figure 6B)**.

In **Figure 6B**, it was found that the expression levels of *LcbHLHs* in flowers, young leaves (I-period) and mature leaves (II-period) displayed significant differences. Five groups of *LcbHLHs* were divided according to the cluster analysis. Genes in group IV, V were highly expressed in the flower

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and lowly expressed in the leaves, mainly predicted to involve floral organ formation and development. For example, *LcbHLH133/145/25/102/69/43* in group IV was up-regulated in the flowers of XNXY and XNFJ, while its corresponding leaves showed lower expression levels. *LcbHLH84* in group V was highly expressed in flowers of all varieties, *LcbHLH43* was significantly expressed in XNFJ flowers, and *LcbHLH22/51* was highly expressed in XNXY flowers and lowly expressed in leaves. According to GO enrichment analysis, *LcbHLH84* may be involved in photoperiod and flowering processes, *LcbHLH69* can regulate pollen development, *LcbHLH109* plays a vital role in double-forming fertilization azygote and endosperm and multicellular organismal development. In addition, *LcbHLH51/25/102/43* can participate in gibberellin-related anabolism and mediate brassinosteroid signaling pathway. Cis-element analysis showed that all of these genes have light responsive elements and some hormone response elements, such as gibberellin, jasmonic acid, salicylic acid, auxin and abscisic acid. Previous studies have shown that plant flowering is regulated by various plant hormones. Among them, gibberellin plays an essential role in flowering, and other hormones such as abscisic acid, auxin, salicylic acid, and jasmonic acid are also involved in the regulation of flowering. These results were consistent with GO enrichment and transcriptome analysis to some extent.

Since the ornamental value of *L. chinense* was mainly reflected in leaf color, three genes (*LcbHLH4/156/157*) from IIIf subfamily were selected to involve in anthocyanin and flavonoid biosynthesis based on structure and function prediction. We performed a preliminary analysis of the gene expression profiles of different leaf color varieties in period I and II **(Figure 6B)**. *LcbHLH4* was found distinctly more highly expressed in purple leaves (XNNC) than in green leaves (XNXY) and significantly down-regulated as leaf color becomes green from period I to period II. On the other hand, *LcbHLH156* and *LcbHLH157* were highly expressed in period I of different dark leaf varieties (XNFJ, XNXJ, XNNC) and decreased in expression as the leaf color became green during II period. In addition, it is also worth noting that *LcbHLH149/114* was also highly expressed in XNFJ and *LcbHLH117* also showed high expression in dark leaf varieties (XNNC). These expression levels of these genes showed similar trends in leaf phenotype color changes. Thus, we speculated that their function is related to the synthesis of anthocyanins.

To further validate the relationship between *LcbHLHs* and *L. chinense* leaf color, we demonstrated the expression levels of candidate genes of XNFJ species from transcriptomic data using qRT-PCR technique. We also investigated the gene expression levels of three different colors of leaves in the natural state of HYJM1. These genes had consistent expression trends at two developmental periods (I and II) **(Figure 7 Table S10)**. We could clearly see that the expression of these six candidate genes was higher in the young leaf stage (I) and significantly decreased in the leaf maturation stage (II), which was consistent with the phenotypic trend. In addition, we compared the gene expression levels of HYJM1 in the three types of leaves using green leaves (GL) as a control group **(Figure 8A)**. The results showed that these six genes were differentially up-regulated in both mixed leaves (ML) and purple leaves (PL), especially the expression was most significantly up-regulated in ML, with the most significant difference in *LcbHLH4/156/157* expression **(Figure 8B Table S11)**.

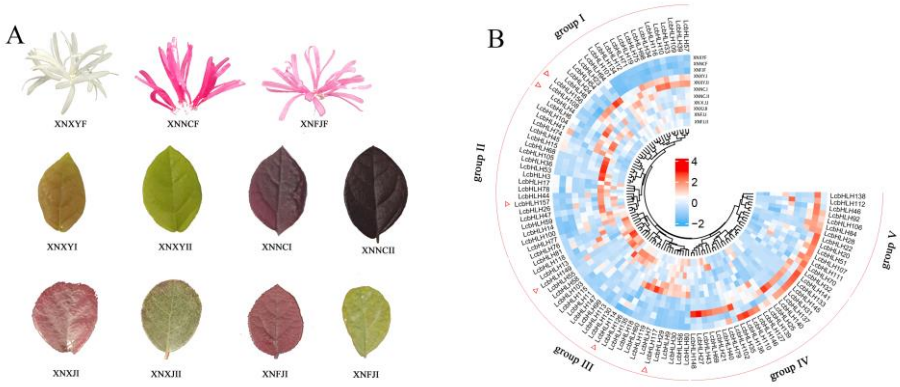

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Figure 6. Expression pattern of the *LcbHLHs* (**A**) Four varieties with different colored leaves and flowers. The "F" after the name indicates the flower, and"I" and "II" represent the first period and the second period leaves respectively. The first period is the young leaf, the second period indicates the mature leaf period. (**B**) Expression pattern of the LcbHLHs. Expression profiles of *LcbHLHs* in different tissues. "XNFJ", "XNXY","XNXJ" and "XNNC" are the names of our selected materials.

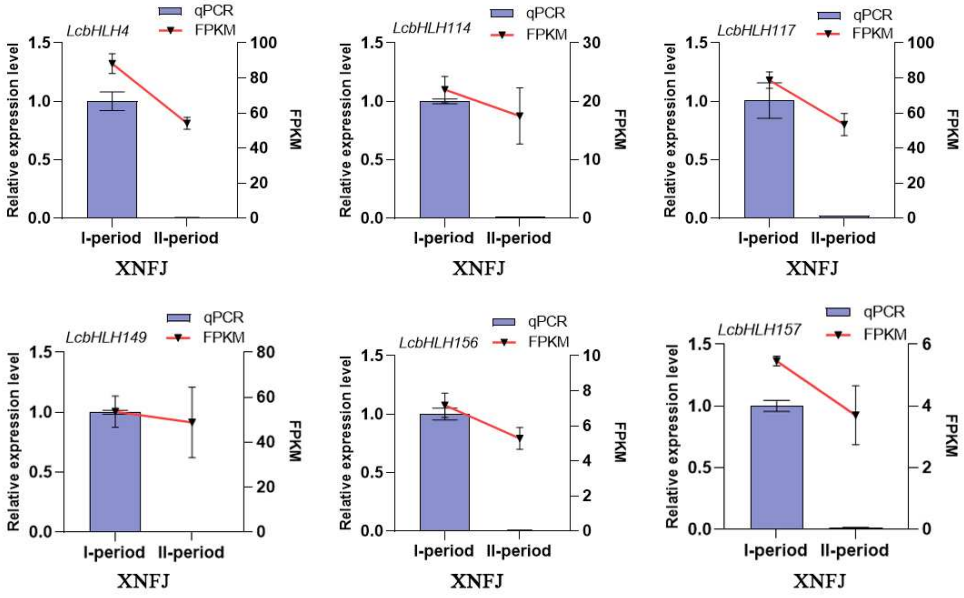

Figure 7. Expression of six differential *LcbHLHs* in XNFJ variety during I and II period.

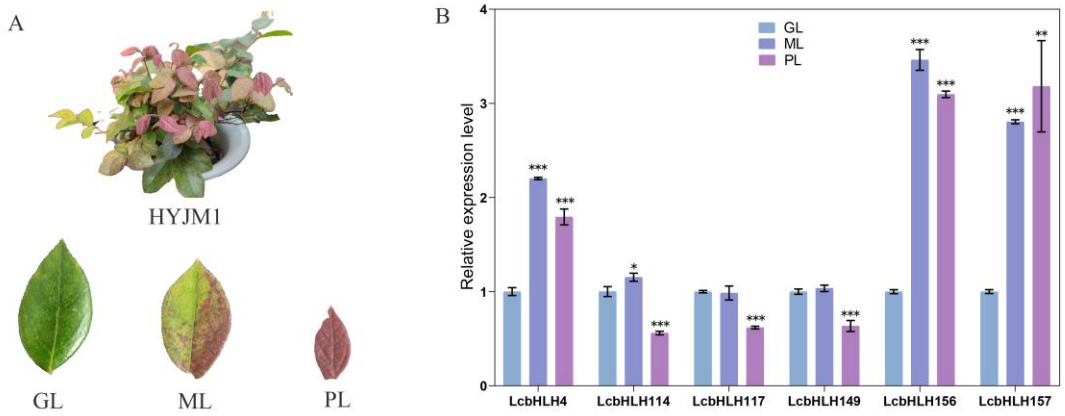

Figure 8. Expression of six differential *LcbHLHs* in different color leaves (**A**) HYJM1 varieties with different colored leaves. GL represents green leaves, ML represents green and purple mixed leaves, and PL represents purple leaves. (**B**) Expression of six *LcbHLHs* in different color leaves (GL, ML, PL). green leaves (GL) as a control group.

2.6. The protein-protein interaction network of candidate gene.

Based on the above results, it was preliminarily speculated that the six candidate genes *LcbHLH4/156/157/114/117/149* were most likely to participate in anthocyanin biosynthesis. To clarify their functions, we further constructed protein interaction network via *Arabidopsis* protein database **(Figure 9)**. As shown in Fig. 9, LcbHLH4 (TT8 in *Arabidopsis*) could coordinate with TTG1 and TT2 to make DIHYDROFLAVONOL 4-REDUCTASE (DFR) and BANYULS (BAN) correctly expressed and then regulated the flavonoid pathway[23,34,35]. Furthermore, LcbHLH156/157(GL3 in *Arabidopsis*)

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could interact with TTG1 to regulate trichome development[36]. It was identified as an essential regulator of the anthocyanin pathway in *Arabidopsis*, together with TTG1 and MYB75 transcription factors, regulating specific genes in the anthocyanin synthesis pathway[37]. LcbHLH117/149 (MYC2 in *Arabidopsis*) could involve in the jasmonic acid (JA) signaling pathway and control the biosynthesis of anthocyanin by regulating the expression of genes encoding positive regulators such as MYB Domain Protein 75/Production of Anthocyanin Pigment 1 (MYB75/PAP1) and Enhancer of Glabra 3 (EGL3).[38–41]. LcbHLH114 (AT1G01260 in *Arabidopsis*) is an ABA-inducible BHLH-type transcription factor, which also involved in JAs signal transduction through the interaction of COI1, JAZ and MYC2 to regulate anthocyanin synthesis indirectly[42]. The protein-

protein interaction networks of LcbHLHs further suggested that these six bHLH proteins were involved in anthocyanin biosynthesis in different ways.

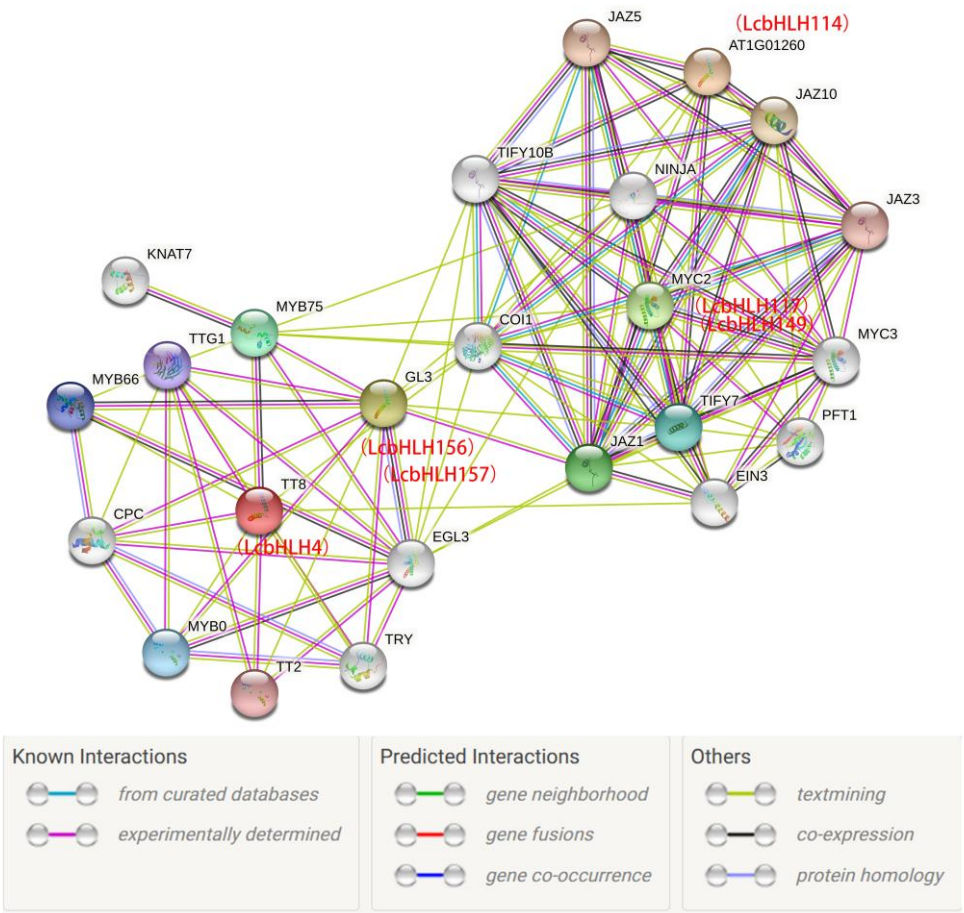

Figure 9. Interaction network analysis for LcbHLHs. The predicted results in parentheses are based on homologous genes in *Arabidopsis*.

3. Discussion

3.1. Systematic and comprehensive genome-wide detection of LcbHLHs in L. chinense

With the completion of whole genome sequencing of a large number of species, a large number of studies have been conducted on the *bHLH* family of various species. Such as *Arabidopsis* (n=162)[8,9], rice (n=167)[10] , fig (n=118)[32], strawberry (n=113)[43], lotus (n=115)[44], peach (n=95)[45], bamboo (n=448)[30], potato(n=124)[31], grape (n=94)[31] and maize(n=208)[45] etc (The "n"

here refers to the number of *bHLH*). Compared with the number of these species, the number of *LcbHLHs* is appropriate. However, the quantity is much smaller than bamboo and maize. Changes in the number of *bHLH* genes between species were often thought to be possibly associated with gene duplication events or genome size[46]. Our study identified 165 *bHLH* genes of *L. chinense* var. *rubrum*. According to phylogenetic analysis, these 165 *bHLHs* were divided into 21 subfamilies, and the number distribution of these subfamilies was similar to that of *Arabidopsis thaliana*. Protein multi-sequence alignment also confirmed that LcbHLHs had a typical bHLH domain. Further structural analysis revealed that most LcbHLHs were linked to DNA-binding and homodimer formation functions. Additionally, the analysis of conserved motifs discovered that most of the LcbHLH family members had the respective conserved motifs (motif1 and motif2), constituting the bHLH structural domain [5]. These results further confirmed the accuracy of *bHLH* gene family analysis in *L.chinense* var. *rubrum* and provided a basis for further application.

3.2. *Functional prediction of LcbHLHs*

As the second largest gene family in plants, *bHLHs* always play an irreplaceable role in plant growth and development survival[5,11]. In our study, *LcbHLHs* are predicted to be widely involved in the growth and development of plants, including the synthesis of floral organs, the regulation of circadian rhythm and the response to various stresses etc. In addition, a few *LcbHLHs* are closely related to the synthesis and metabolism of some hormones, such as gibberellin, jasmonic acid, auxin and so on. It is predicted that LcbHLHs also play an important role in regulating the synthesis of secondary metabolites, including the biosynthesis of anthocyanins and brassinosteroids. To further explore the accuracy of our functional analysis, we analyzed the transcriptome data and validated some of them by Real-time Quantitative PCR Detecting System.

Many *LcbHLHs* performed differently in different tissues. Some *LcbHLHs* were highly expressed in the flowers of the three varieties. *LcbHLH106* was annotated as PIF3, which is a phytochrome-interacting factor necessary for photoinduced signal transduction that can further regulate flowering time[47]. *LcbHLH13* was highly expressed in leaves, NR annotated as PIL5 **(Table S12)**, which can indirectly regulate DELLA protein content and further participate in gibberellin pathway to regulate leaf elongation and other growth and development processes[48,49]. Moreover, the expression patterns of *LcbHLHs* in different periods and color leaves were analyzed. We found that the three genes（*LcbHLH156/LcbHLH157/4*）selected by phylogenetic analysis and GO annotation did show high expression patterns in dark leaves and *LcbHLH114/117/149* also were up-regulated in dark leaf varieties. Further qRT-PCR results of the six genes showed the same trend in XNFJ, they all expressed higher in I period than II period that matched the transcriptomic results and leaf color characteristics of varieties. In addition, through the qPCR results of HYJM1 leaves with different colors, we also found that the expression trends of *LcbHLH4/156/157* genes in HYJM1 leaves with different colors were generally consistent, and were significantly up-regulated in mixed and purple leaves.

In order to further demonstrate our hypothesis, we further analyzed the interaction relationship of these six bHLH proteins through the protein interaction network diagram. It is found that these proteins have mainly involved in two pathways. LcbHLH4/156/157 play an irreplaceable role in anthocyanin synthesis and trichome development, and have a predicted interaction with TT2/TTG1/MYB75, while LcbHLH117/149/114 mainly involves JA signal transduction. Previous studies have showed the bHLH in subfamily III(d+e) participate in the JA signal pathway, leading to the accumulation of anthocyanin in apples[50,51]and the regulation of plant defense by *Arabidopsis thaliana*[52–54]. In *Arabidopsis*, JAZ proteins directly interact with bHLH TFs (GL3, EGL3, and TT8) and MYB TFs (MYB75 and GL1). These bHLH and MYB TFs are essential components of the MBW complex to mediate anthocyanin accumulation [39]. In our study, LcbHLH4/156/157 candidate proteins directly regulated the composition of related complexes in the anthocyanin synthesis pathway as well as the expression of structural genes, while LcbHLH114/117/149 were related to the JAZ protein family and mainly mediated the JA signal pathway, and their roles in anthocyanin synthesis deserve further investigation.

4. Materials and Methods

4.1. Plant materials and data resources

Tissue specimens of *L. chinense* were obtained from the Floral Experimental Station of the College of Horticulture, Hunan Agricultural University, Changsha, China. These experimental materials included the flowers and leaves of some new *Loropetalum chinense* cultivars 'Xiangnong Xiangyun' (XNXY), 'Xiangnong Fengjiao' (XNFJ), 'Xiangnong Nichang' (XNNC), 'Xiangnong Xiaojiao' (XNXJ) and 'The NO.1 *Loropetalum chinense*' (HYJM1). The leaf material of HYJM1 is mainly three different colors of leaves in their natural state, while the leaf material of other species was mainly collected at the following developmental stages: a new leaf period (I-period): the leaf is soft and brightly colored, and a mature period (II-period): the leaf is leathery and darkly colored. Samples were obtained as three biological replicates from the four new varieties and immediately frozen in liquid nitrogen for transcriptome sequencing. The sample RNA preparation and library construction are consistent with the previous studies[55,56].

The raw transcriptome data involved in this paper were uploaded in the Genome Sequence Archive (Genomics, Proteomics & Bioinformatics 2021) at the National Genomics Data Center (Nucleic Acids Res 2022) and the Chinese National Center for Biological Information at the Institute of Genomics (GSA: CRA009284 and CRA009285). They are available at https://ngdc.cncb.ac.cn/gsa (accessed on May 10, 2023).

4.2. Identification and Physicochemical Characterization of the bHLH gene family

Genomic data and gene annotation information of *L. chinense* were obtained from the research group of *L. chinense* at Hunan Agricultural University[57]. First, downloaded the seed profile of the bHLH signature domain (PF00010) from the PFAM database(https://pfam.xfam.org/) and made use of a Hidden Markov Model HMMER (3.0) to screen the bHLH candidate protein with the E-value cut-off set to 10-5. Then, the *Arabidopsis* bHLH protein homology blast method was used to search extensively for candidate bHLH proteins. The protein sequences of *Arabidopsis* were downloaded from TAIR(https://www.arabidopsis.org/). The LcbHLHs candidate proteins were obtained by a combination of two methods. These proteins containing the bHLH domain were screened further in the databases of PFAM(Pfam: Home page (xfam.org) and SMART(https://smart.embl.de/). The subcellular localization of LcbHLHs was predicted by the online website Wolf-psort(https://wolfpsort.hgc.jp/). The sequences of LcbHLHs were analyzed bioinformatically, and the proteins' physicochemical parameters were calculated using ExPASy(https://web.expasy.org/compute_pi/).

4.3. Chromosomal Localization, Tandem Duplication and Collinearity of the bHLH genes

The chromosome distribution was performed on the online website GSDS(http://gsds.gaolab.org/). Gene duplication analyses for *L. chinense* var. *rubrum* was finished by using the Multiple Collinearity Scan Toolkit (MCScanX). Also, through the tools of TBtools [58] and MCScanX, the interspecies collinearity analysis of *bHLHs* between *L. chinense* and *Arabidopsis*, *Oryza sativa*, *Zea mays* and *Vitis vinifera* were performed. All of these genomic data were obtained from EnsemblPlants (https://plants.ensembl.org/). The non-synonymous replacement rate (Ka) and synonymous replacement rate (Ks) of the replicated gene pairs were calculated by KaKs Calculator 2.0, and environmental selection pressure was analyzed by Ka/Ks ratio.

4.4. Phylogeny and Multiple-Sequence Alignment of LcbHLHs

DNAMAN software was used for protein multiple sequence alignment, and SnapGene software was used to plot the amino acid site distribution of the bHLH protein conserved domain. The phylogenetic trees of Arabidopsis thaliana and L. chinense were constructed by using MEGA11 software.

4.5. Analysis of Gene Structure, Conserved Motif and Family Structural Domains

The intron/exon gene structure maps were obtained based on GFF3 files via the online website the Gene Structure Display Server (GSDS) (http://gsds.gao-lab.org/). The analysis of conserved motifs through the MEME online website(https://meme-suite.org/meme/doc/meme.html), and the number of motifs was 10, other parameters were default. The final result graph was showed by TBtools software.

4.6. GO Annotation, Analysis of Cis-acting Components and Protein-Protein Interaction of LcbHLHs

The NCBI database was selected as a reference database for further GO analysis of *LcbHLHs* by using the Blast2GO program[59]. Then, a 2000 bp promoter upstream of the *bHLH* gene family member of *L. chinense* was extracted by TBtools software and uploaded to the online website PlantCARE (https://bioinformatics.psb.ugent.be/webtools/plantcare/html/) for homeopathic element prediction. To better understand the regulation of LcbHLHs on protein-protein interaction networks, STRING database (STRING: functional protein association networks (string-db.org)) was used to predict the protein interaction network of the candidate *bHLH* gene family in *Loropetalum chinense* var. *rubrum*.

4.7. Gene expression patterns and quantitative real-time PCR analysis

All raw transcriptome data were mainly presented by FPKM values (the fragments per kilobase of transcript per million mapped reads). Homogenize raw FPKM values and visualize heatmap using R package.

Plants material RNA was extracted using FastPure Universal Plant Total RNA Isolation Kit. The first standard cDNA was synthesized by Evo M-ML RT Kit for qPCR and stored in a -40°C refrigerator. Primers were designed by Beacon Designer 8. **(Table S13)**, and qRT-PCR was performed on a Bio-Rad CFX384TM with 2X SYBR Green Pro Taq HS Premix (AG, Hunan, China). The cDNA was diluted to 500 ng, and the system was set to 10μl to be prepared as three technical replicates. Each reaction with a 1μl template. The conditions for qRT-PCR are as follows: 95°C for 5 minutes, followed by 40 cycles of 95°C for 15 seconds, 60°C for 1 minute, and 72°C for 5 minutes. Quantitative PCR expression level was calculated by $2-\Delta\Delta CT$ method and expression values of three replicates were normalized using *LcActin* as the internal control. The error bar represents standard errors from three biological replicates.

5. Conclusions

In summary, we analyzed the physicochemical properties, phylogeny, gene structure, Ka/Ks value and collinearity of different species of bHLH family members of L. chinense var. rubrum. In addition, we also predicted the function of LcbHLHs by using cis-elements, phylogenetic tree clustering and GO annotation. Six candidate genes related to anthocyanin synthesis were selected and verified the function prediction of LcbHLHs based on transcriptome data. Then, further qPCR detection was performed on leaves with different leaf colors in HYJM1. All these results strengthen our understanding of the bHLH gene family in Loropetalum chinense var. rubrum, and provide a reference for further understanding the regulation of anthocyanin synthesis.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Figure S1: Phylogenetic tree constructed with sequences of the *LcbHLH* and *AtbHLH*; Table S1: Gene accession number of all *LcbHLH* genes; Table S2: Physicochemical properties table; Table S3: The gene tandem replication on *L. chinense* var. *rubrum* chromosome; Table S4: The gene fragment replication on *L. chinense* var. *rubrum* chromosome; Table S5: The Ka/Ks ratios for duplicate pairs of LcbHLHs; Table S6: The synteny regions of bHLH genes in diverse species; Table S7: GO annotation of the bHLH proteins in *L. chinense*; Table S8: Cis-elements analyses of the bHLH proteins in *L. chinense*; Table S9: Transcriptome data FPKM value of LcbHLHs; Table S10: qPCR data of period I and II of XNFJ; Table S11: qPCR data of GL ML PL; Table S12: Nr annotation of LcbHLHs; Table S13: List of primers used for qRT-PCR.

Author Contributions: X. X, X.Y., and Y.L. (Yanlin Li) proposed the design of the article, performed the experiments, and authored or reviewed drafts of the article; Y.L. (Yang Liu) and L.L. performed the data analysis; Y.L.

(Yang Liu) and Q.M. prepared the figures and/or tables; W.L. and D.Z. authored or reviewed drafts of the article Y.L. (Yang Liu) completed the manuscript together. All authors have read and agreed to the published version of the manuscript.

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