

Genome-wide identification and characterization of barley bHLH transcription factors and their expression in response to low nitrogen stress

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Abstract

Background: Improvement of low nitrogen (LN) tolerance or nitrogen use efficiency (NUE) in crops is imperative for environment-friendly agriculture development. The basic helix–loop–helix (bHLH) transcription factors are involved in multiple abiotic stress, suitable as the candidate genes for improving LN tolerance. Little research was done on characterization of *bHLH* gene family and their response to LN stress in barley.

Results: In this study, 168 *bHLH* genes were identified in barley through genome-wide analysis. Hv**bHLH** proteins were classified into 26 subfamilies based on phylogenetic analysis with bHLH proteins from *Arabidopsis thaliana* and rice. The analysis of conserved motifs and gene structures supported the evolutionary relationships among these Hv**bHLH** proteins. Further, analysis of stress-related *cis*-elements in the promoter regions showed that bHLH proteins in barley are probably involved in multiple stress responses. Finally, at least 16 bHLH genes were differentially expressed in two barley genotypes differing in LN tolerance under LN stress. Dynamic expression analysis showed that these differentially expressed genes (DEGs) differed between the two barley genotypes in response to LN stress.

Conclusion: It is the first genome-wide analysis of bHLH family genes in response to LN stress in barley. The results indicate the distinct difference among *HvbHLH* genes in response to various abiotic stresses. The *HvbHLHs* specifically expressed in the LN-tolerant barley genotype XZ149 identified herein may be valuable for future function analysis of *HvbHLH* genes under LN stress and breeding for barley cultivars with LN tolerance.

Introduction

Transcription factors (TFs) play important roles in the growth and development of plants and animals, and their responses to the external environment via regulating downstream gene expression. As one of the most critical factors regulating gene expression, TFs have been intensively concerned in the field of biological research. Among various TF families, the basic-helix-loop-helix (bHLH) TFs constitute one of the largest TF families [1]. The bHLH proteins are characterized by a conserved bHLH domain consisting of about 60 amino acids. Each domain has two functional regions, i.e. the basic region at N-terminus and the HLH region at the C-terminus. The basic region comprises about 15 amino acids including six basic residues, and has DNA-binding activity [2]. The HLH region, containing about 50 amino acids, is composed of two α -helices separated by a variable loop, and it participates in the formation of homodimers or heterodimers [3].

The bHLH TFs were first identified and characterized in mammals and then discovered gradually in other eukaryotic species. At present, genome-wide comprehensive analyses of plant bHLH proteins have been carried out in numerous species [4]. At least 162 and 167 *bHLHs* have been identified in model plants *Arabidopsis* and rice, respectively [5-7]. Recently, 225, 208, 155, 152, 124 and 142 bHLHs were identified in

wheat, maize, bean, tomato, potato, and cucumber, respectively [4, 8-12]. However, the similar research has been little done in barley, although barley is an important crop worldwide.

TFs are the excellent candidate genes for developing cultivars with improved tolerance to adverse stress [13-15]. In plants, bHLH TFs are involved in many biological processes, such as the regulation of flavonoid biosynthesis, morphology and the accumulation of fruit pigments [16-17]. Some bHLH TFs participate in regulation of biotic and abiotic stress responses [18-20]. For example, some bHLH TFs confer drought tolerance through regulating stomatal development, photosynthesis and growth [21], or by promoting root development and abscisic acid synthesis [22]. Some enhance plant salt stress [23], cold [24], or manganese [25] through their overexpression. And some could improve plant growth while subjected to nutrition deficiency, such as iron, Pi and N deficiency [26-28].

Environmental pollution caused by excessive application of nitrogen (N) fertilizers became a big issue. One of the efficient solutions is to develop cultivars with high N use efficiency /low nitrogen (LN) tolerance. It is reported that bHLH could improve plant tolerance to N deficiency [28]. Thus, *bHLH* genes may be used as suitable candidates for the genetic improvement of plant LN tolerance/high N use efficiency. However, up to date little has been known about the function of plant bHLH proteins. In this study, *bHLH* family genes in barley were identified and characterized at genome-wide level. It was found that *HvbHLH* genes were differentially expressed in the two barley genotypes (XZ149, LN tolerant and XZ56, LN sensitive) differing in LN tolerance [29-30] at transcription level under LN stress by RNA-seq. The major objectives of this study were to determine the characteristics of *HvbHLH* gene family, to explore the differences of *HvbHLH* gene expression between the two contrasting genotypes in response to LN stress, and to identify the *HvbHLH* genes useful for LN tolerance breeding.

Results

Identification of *bHLH* family genes in barley

Proteins containing bHLH domain in barley were identified by searching the whole barley genome. Totally 168 *bHLH* genes were obtained in barley genome after removing the redundant protein sequences (Additional file 1 and 2: Table S1 and S2). By EXPASY analysis, we found the amino acid length of HvbHLH proteins ranged from 36 to 938, accordingly the molecular weight (MW) ranging from 9.63 to 102.01 kDa (Additional file 1: Table S1). The isoelectric point (pI) varied greatly, from 4.63 to 11.9 (Additional file 1: Table S1). While the MW and pI of 11 HvbHLH proteins were not predicted, due to existence of some consecutive undefined amino acids (Additional file 1: Table S1).

Phylogenetic analysis of bHLH proteins

In order to analyze the evolutionary relationship of the *bHLH* genes between barley and other species, the unrooted phylogenetic tree was constructed using the full-length amino acid sequences of all bHLH in *Arabidopsis*, rice and barley by the neighbor-joining (NJ) method. The result showed that 450 bHLH proteins from *Arabidopsis*, rice and barley were divided into 27 subgroups (Subgroup 1 to 27; Fig. 1)

based on the classification of bHLH proteins in *Arabidopsis* and rice [6-7]. Except Subgroup 20, all members of *bHLH* family in barley could be found in all the other subgroups (Fig. 1). Subgroups 3 and 9 were the smallest, each only having three members in barley, rice and *Arabidopsis*. Subgroup 27 was the largest, having 20, 16 and 17 members in barley, rice and *Arabidopsis*, respectively. Another NJ phylogenetic tree was also constructed based on HvbHLH protein sequences, and HvbHLHs were classified into 25 subfamilies (Subfamily A to Y, Fig. 2a). Classification in HvbHLH was generally consistent with those in *Arabidopsis* and rice phylogenetic tree (Fig. 1 and 2a).

Analysis of motifs and exon/intron structures

Ten conserved motifs containing 11 to 57 amino acids were predicted in HvbHLH proteins through MEME software (Additional file 3: Fig. S1). HvbHLH proteins consist of 1- 5 motifs, varying with different subfamilies (Fig. 2b). However, all of them contained at least of the Motif 1 or 2, and the proteins in the same subfamily exhibited the similar motif composition (Figs. 2a and b). For example, Motifs 2, 3, and 10 were identified in all 6 members of Subfamily K, and Motifs 1, 2, and 7 were identified in 12 of the 15 members of Subfamily X. Furthermore, some motifs are unique to one or more Subfamilies. For instance, Motif 5 was specifically shared by each member in the Subfamily D, and motif 3 was only found in Subfamilies B, K and L. This conservation of the motif composition patterns in each subfamily might indicate their similar biological functions in the same subfamily [31].

The alignment of genome and coding sequences, and also exon/intron structure within the coding sequence in bHLH genes was performed using the TBtools [32]. The structure differed greatly among *HvbHLH* genes, but most members of the same subfamily had the similar exon/intron structure (Figs. 2a and c). For instance, the members in the Subfamily A had the relatively simple structure, possessing only 1-2 exons, while the members in the Subfamily T had 6-8 exons, being much more complex in its structure (Fig. 2c).

Chromosomal distribution and gene duplication of *HvbHLH* genes

According to their physical positions, 161 *HvbHLH* genes were nonrandomly mapped on seven barley chromosomes, with most located at the both ends of the chromosomes (Fig. 3). More than one-third of the *HvbHLH* genes were located on Chr3 (30 members) and Chr5 (26 members), and only 12 genes was located on Chr1 (Fig. 3). In addition, 7 *HvbHLHs* could not be conclusively localized to a chromosome (Fig. 3).

Totally 18 segmental duplication events were identified among *HvbHLHs* (Fig. 4, Additional file 4: Table S3). Among them, 16 events happened between diverse chromosomes, while only 2 events were detected within a same chromosome. Nine genes were identified as tandem duplication genes (Fig. 4, Additional file 4: Table S3), and the genes located on chromosome 3 showed the highest numbers (7) of tandem duplications, while two genes (HORVU4Hr1G087590 and HORVU4Hr1G087610) were tandem duplicated on Chr4 (Fig. 4). Each pair of duplication genes were derived from the same subfamily (Fig. 1 and 4).

Obviously, some *HvbHLH* genes were generated due to gene duplication, and the segmental duplication may play vital roles in the *HvbHLH* gene family expansion in barley genome.

Stress-related *cis*-elements in the promoter of *HvbHLH* genes

To understand the potential regulatory mechanisms of *HvbHLH* genes, *cis*-elements were analyzed using 1.5-kb upstream sequences from ATG through PlantCARE. Various *cis*-elements involved in abiotic stress responses were identified, and mainly they could be divided into two groups. One is elements responsive to hormones, including abscisic acid (ABRE), jasmonic acid (CGTCA and TGACG motif), auxin (AuxRR-core and TGA-element), gibberellins (GARE motif, TATC-box and P-box), salicylic acid (SARE and TCA-elements). The other one is responsive to environmental stress, including low-temperature responsive element (LTR), anaerobic induction element (ARE), MYB binding site involved in drought-inducibility (MBS), and defense and stress responsive elements (TC-rich repeats) (Fig. 5, Additional file 5: Table S4). All *HvbHLH* genes had light responsive *cis*-elements, belonging to the most commonly predicted *cis*-elements in the promoter of *HvbHLH* genes, and 155 (92.26%) *HvbHLH* genes contained the G-box elements. Most genes had more than one kind of *cis*-elements, suggesting that both groups could respond to multiple stresses.

Expression profiles of *HvbHLHs* in different plant tissues

To further understand the functions of *HvbHLHs*, the expression profiles of *HvbHLH* genes in 8 plant tissues of a barley cultivar 'Morex' were analyzed based on the available transcriptomic data [33]. The expression of 149 *HvbHLH* genes were identified in all 8 tissues, and their expression levels varied considerably with genes and tissues (Fig. 6 and Additional file 6: Table S5). Some *HvbHLHs* in the same subfamily showed the similar expression pattern, while some others displayed the different patterns (Fig. 6). Totally 128 (128/149, 85.91%), 125 (125/149, 83.89%), 118 (118/149, 79.19%), and 101 (101/149, 67.79%) *HvbHLH* genes expressed in four-day-old embryos, roots and shoots, and internodes of six-leaf-old seedlings, respectively (Fig. 6). In addition, 118 (118/149, 79.19%) and 110 (110/149, 67.79%) *HvbHLH* genes expressed in young fluoresce (5 mm and 1-1.5 cm) and developing grains (5- and 15-day post anthesis, DPA), respectively (Fig. 6).

Some *HvbHLHs* highly expressed in the specific tissue(s). For instance, the genes HORVU2Hr1G044230 and HORVU6Hr1G020520 showed higher expression in caryopsis (5 and 15 DPA) than other tissues (Fig. 6), suggesting their significant roles in grain growth and development. HORVU2Hr1G044230 and HORVU1Hr1G072810 had higher expression in embryos and roots, while HORVU2Hr1G044230, HORVU7Hr1G050530 and HORVU1Hr1G054260 had higher expression in shoots (Fig. 6), indicating these genes may participate in growth and development of vegetative organs. Notably, HORVU2Hr1G044230 displayed extremely high expression in all the tissues (Fig. 6).

Expression profiles of *HvbHLHs* under low nitrogen stress

The expression patterns of *HvbHLH* genes in response to LN stress were analyzed in the roots of the two barley genotypes (XZ149, LN tolerant and XZ56, LN sensitive) using RNA-seq (Additional files 7 and 8: Tables S6 and S7). Consequently, 16 differentially expressed genes (DEGs) encoding *HvbHLH* proteins were identified using pair-wise comparison for each genotype under LN stress (Fig. 7, Additional file 9: Table S8). Remarkably, no DEG was found in the two barley genotypes under LN stress at 12 d, suggesting *HvbHLHs* may play a regulating role at early stress. Furthermore, all the DEGs were changed only at one time point, except MLOC_74557 (HORVU6Hr1G064820) and MLOC_21066 (HORVU3Hr1G000150), which was down-regulated in XZ149 both at 6 h and 48h after stress (Fig. 7).

Eleven DEGs were down-regulated in their expression under LN stress, and their response patterns showed the marked difference. Six DEGs responded quickly to LN stress (at 6 h), and MLOC_37666 (HORVU2Hr1G108480), MLOC_72940 (HORVU2Hr1G117820), MLOC_21066 (HORVU3Hr1G000150) were down-regulated only in XZ149, while MLOC_67937 (HORVU5Hr1G002090) was down-regulated only in XZ56 (Fig. 7). The other five DEGs were changed at 48 h after LN stress, and among them MLOC_12832 (HORVU5Hr1G066530), MLOC_66385 (HORVU4Hr1G061760) were down-regulated only in XZ149, MLOC_64390 (HORVU3Hr1G027630) only in XZ56, while MLOC_62844 (HORVU2Hr1G066100) and MLOC_43080 (HORVU3Hr1G000150) in both XZ149 and XZ56 (Fig. 7).

Four DEGs were up-regulated under LN stress, and three of them responded at 6 h, with MLOC_72280 (HORVU2Hr1G114070) only in XZ149, and MLOC_36351 (HORVU3Hr1G108680) and MLOC_76486 (HORVU5Hr1G018100) in both XZ149 and XZ56. MLOC_55964 (HORVU4Hr1G075250) was up-regulated at 48 h in both XZ149 and XZ56 (Fig. 7). Notably, the change fold of all the up-regulated DEGs was larger in the tolerant genotype XZ149 relative to the sensitive genotype XZ56 (Fig. 7).

To confirm the transcriptomic results and understand the expression patterns of *HvbHLHs*, the dynamic response to LN stress were examined using real time PCR for the 6 identified DEGs at 8 time points after LN stress. The expression patterns of *HvbHLHs* at 6 h, 48 h and 12 d after LN stress were highly consistent with those obtained by the transcriptomic analysis. (Additional file 10: Fig. S2). Under LN stress, different *HvbHLHs* exhibited distinct dynamic expression patterns, suggesting that *HvbHLHs* may be involved in regulation of LN tolerance in different ways. For instance, MLOC_72280 (HORVU2Hr1G114070) was up-regulated during 1 h -2 d under LN stress, but down-regulated at 3 d -12 d in XZ149, while it was up-regulated at 1 h, 3 h, 6 h, and 2 d, and down-regulated at 24 h and 3 d - 12 d in XZ56 (Additional file 11: Fig. S3). For MLOC_37666 (HORVU2Hr1G108480), its expression generally showed the decrease with the time of exposed LN stress in XZ149, but increase at 1 h, 3 h, and 6 d under LN stress in XZ56 (Additional file 11: Fig. S3).

Discussion

The *bHLH* TFs in higher plants comprise a large family, involving in plant growth, development, and stress responses [2]. It is well documented that *bHLH* is responsible to many abiotic stresses such as cold, drought and salt stress [18, 34-35], and nutrients such as iron, Pi and N deficiency [20, 28, 36].

Therefore, it is imperative to make the comprehensive analysis of *bHLH*TFs family. N deficiency is a common issue in the agricultural production worldwide. Some studies found that bHLHs are involved in LN stress [28], nevertheless, their relationship/ roles of *bHLH* family genes in LN tolerance has not been clearly defined. In this study, genome-wide identification and characterization of the *bHLH* gene family in barley were carried out. Furthermore, the expression profiles of *bHLH* family under LN stress were determined by using two barley genotypes differing largely in LN tolerance.

As a result, totally 168 *bHLH* genes were identified in barley through genome-wide analysis (Table S1). These *HvbHLH* genes were distributed over the barley genome, mainly on both ends of the chromosomes (Fig. 3), similar to the distribution of *Arabidopsis* and rice *bHLH* genes on their chromosomes [6-7]. Gene duplication is an important event for the evolution of plant genome, leading to the generation of new genes [37-38]. There were at least 14 pairs of duplication genes in barley *bHLH* family, indicating that gene expansion occurred during the evolution of the barley genome. Moreover, the segmental duplication could be a predominant driving force for the enlargement of *HvbHLH* [39].

Based on phylogenetic analysis, HvbHLH proteins could be classified into 26 subfamilies (Fig. 1). Motif and gene structure analysis further supported the subfamily division, as members of the same subfamily showed the similar gene structure and motif (Fig. 2), indicating their similar evolutionary origins and biology functions. For instance, in the subfamily K, *AtbHLH100* (AT2G41240), *AtbHLH101* (AT5G041059), *AtbHLH38* (AT3G56970), and *AtbHLH39* (AT3G56980) played important roles in regulating iron homeostasis under Fe deficiency in *Arabidopsis* [19, 26]. In the same subfamily, OsbHLH056/OsIRO2 (Os01g72730) protein is an essential regulator of the genes involved in Fe uptake under Fe-deficient conditions in rice [40]. On the other hand, *HvbHLH*s displayed diverse expression patterns in plant tissues, and some genes within the same subfamily may have different expression patterns (Fig. 6), indicating that their functions have been differentiated even in the same subfamily or they may perform the same function in the different ways.

A great number of *cis*-elements associated with abiotic stress responses were discovered in promoter regions of *HvbHLH*s, suggesting that HvbHLHs may be highly associated with the regulation of abiotic stresses response in barley. To further explore the possible functions of the bHLH family in LN tolerance, the expression profiles of *HvbHLH* genes were analyzed using two barley genotypes differing in LN tolerance. A total of 16 DEGs encoding HvbHLH proteins were identified in the two genotypes after 6 h and 48 h treatments (Fig. 7), but no one was found at 12 d LN treatment, suggesting that HvbHLHs respond to LN stress only at early time.

There was a large difference in the transcription regulation of *HvbHLH* between the two barley genotypes. In detail, the change fold of five up-regulated DEGs was much higher in the LN tolerant genotype XZ149 than the LN sensitive genotype XZ56 under LN stress (Fig. 7). In particular, MLOC_72280 (HORVU2Hr1G114070) and MLOC_42946 (HORVU4Hr1G003210) were up-regulated in XZ149, but little changed or down-regulated in XZ56 (Fig. 7). Thus, it is worthy to determine the roles of these *HvbHLH* genes in XZ149. It has been reported that overexpression of *TabHLH1* in tobacco improved plant

tolerance to N deprivation via regulation of nitrate transporter (NRT) gene transcription [28]. In *Arabidopsis*, the expression of specific bHLH transcription factors was enhanced in response to N deficiency, accompanied by anthocyanin accumulation [41-42]. Thus, we may focus the candidate genes on the roles in anthocyanin synthesis pathway and regulation of NRT gene transcription under LN stress in the further study.

Conclusion

In summary, 168 bHLH genes were identified in barley by a genome-wide analysis, and their evolutionary relationships were clarified using phylogenetic, conserved motif, and exon/intron structures analyses. The expression profiles revealed that at least 16 *HvbHLH* genes may mediate LN stress in barley. The specifically expressed *bHLH* genes in XZ149 may be valuable for further functional characterizations of *bHLH* genes under LN stress and breeding of barley cultivars with LN tolerance.

Materials And Methods

Identification of bHLH genes in barley

The protein sequences of bHLH in *Arabidopsis thaliana* and *Oryza sativa* were retrieved from TAIR (<http://www.arabidopsis.org>) and RGAP (<http://rice.plantbiology.msu.edu/>), respectively. Barley sequence data were download from IPK (https://webblast.ipk-gatersleben.de/barley_ibsc/) and Gramene (http://ensembl.gramene.org/Hordeum_vulgare/Info/Index). Totally 282 *Arabidopsis* and rice bHLH protein sequences (list in Additional file 12: Table S9) were used to search against the barley protein sequences using BlastP with a threshold of an e-value $< 1e^{-10}$ [43-44]. Moreover, the predicted HvbHLH proteins were also searched against the barley protein database employing HMMER program with Hidden Markov Model (HMM) profiles of the bHLH domain (PF00010), obtained from PFAM database (<http://pfam.xfam.org/>) [45]. After removing redundant protein sequences, the confirmation of the predicted bHLH proteins were performed through NCBI Conserved Domain Search (<http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>) and the Pfam (<http://pfam.sanger.ac.uk/search>) [46-47]. The biophysical properties of bHLH proteins were calculated using the ExPASy (<https://web.expasy.org/protparam/>) [48].

Phylogenetic analysis

Multiple alignment was performed based on the sequences of *Arabidopsis thaliana*, rice and barley using ClustalW (<http://www.genome.jp/tools/clustalw/>) [49]. The unrooted phylogenetic tree was constructed by MEGA 6.0 software employing the neighbor-joining (NJ) method, bootstrapping with 1000 replicates.

Exon/intron structure and motif analysis

The exon/intron structure of the barley *bHLH* genes were determined by alignment of the cDNAs and their corresponding genomic DNA sequences from the barley genome database. The conserved motifs were

predicted on the online MEME tool (<http://meme-suite.org/tools/meme>) [50]. And a diagrammatic sketch structure and the motif composition of *HvbHLHs* was mapped through TBtools software [32].

Chromosomal location and gene duplication

The distribution of *HvbHLH* transcription factors on chromosomes were mapped by MapGene2Chrom online software (http://mg2c.iask.in/mg2c_v2.1/) [51], according to the specific positions of barley genes obtained from IPK (https://webblast.ipk-gatersleben.de/barley_ibsc/). To identify duplication gene, all barley genome sequences were aligned using BLASTP with an e-value of $1e^{-10}$, and then segmental and tandem duplications were identified by MCScanX with default [52]. The synteny relationship of *HvbHLHs* were displayed with circos (<http://circos.ca/>) [53].

Cis-elements in promoter regions of *HvbHLHs*

To predict the *cis*-elements in the promoter regions of *HvbHLHs*, the 1500 bp upstream sequences from the start codon of each *HvbHLH* were retrieved. The *cis*-element distribution was then investigated in PlantCARE (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>) [54].

Plant materials and stress treatments

Two Tibetan wild barley genotypes XZ149 (LN tolerant) and XZ56 (LN sensitive) were used for transcriptome and real time PCR. The barley seedlings were cultivated with hydroponics in a plant growth chamber (22/18 °C, day/night) according to Quan et al. (2016) [55]. Then, three-leaf-stage seedlings were exposed to 0.2 mM N (LN stress) and 2 mM N (control).

Expression patterns analysis of *HvbHLH* genes

Transcriptomic expression level of *HvbHLH* genes in 8 different tissues (4-day embryos dissected from germinating grains, roots and shoots from seedling (10 cm shoot stage), developing inflorescence (5 mm and 1-1.5 cm), the third internode at six leaves visible stage, 5 and 15 days post-anthesis developing grain (bracts removed)) of the cultivar 'Morex' were obtained from EMBL-EBI (<https://www.ebi.ac.uk/gxa/plant/experiments>) [33].

The roots in XZ149 and XZ56 were taken for transcriptomic analysis at 6 h, 48 h and 12 d after LN stress. The threshold for screening DEGs was set as $FDR < 0.05$ and $FPKM \geq 1$ at least in one of the samples [56]. Differential expression analysis of *HvbHLHs* was displayed using FPKM (fragments per kilobase of exon per million fragments mapped reads) [57] and the expression levels are showed as the heatmaps created by TBtools software by a color gradient from low (blue) to high (red) [32].

To confirm the RNA-Seq results and to further explore the expression patterns of *HvbHLH* genes, the expression of *HvbHLH* genes in roots was analyzed at 1 h, 3 h, 6 h, 24 h, 48 h, 6 d and 12 d under LN stress by real time PCR. RNA extraction and the first strand cDNA synthesis were used FastPure Plant Total RNA Isolation Kit and Hiscript III Reverse Transcriptase (Vazyme, China), respectively. The specific-

gene primers for *HvbHLH* and internal control gene *HvGAPDH* were presented in Additional file 13: Table S10. The real time PCR was analyzed on a CFX96 system (USA) with three biological replicates. The relative expression was set as the fold change referred to the expression under control and calculated by the comparative CT method [58].

Abbreviations

bHLH, basic helix–loop–helix; DEG, differentially expressed gene; DPA, day post anthesis; FPKM, fragments per kilobase of exon per million fragments mapped reads; LN, low nitrogen; N, nitrogen; NRT, nitrate transporter; NUE, nitrogen use efficiency; TFs, transcription factors

Declarations

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the first author on reasonable request, and her email address is bio_quanxy@ujn.edu.cn.

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Contributions

XQ and WH designed the research. XQ, XL, CX, NY, NZ and HL performed research. XQ analyzed data and wrote the manuscript. WH revised the manuscript. All authors have read, edited and approved the current version of the manuscript.

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Ethics declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Supplementary Information

Additional file 1: Table S1 Characteristics of bHLH family genes in barley

Additional file 2: Fig. S1 Conserved motifs of bHLH domains in barley

Additional file 3: Table S2 Gene ID and sequences of 168 HvbHLH identified in this study

Additional file 4: Table S3 Duplication genes among *HvbHLH* genes

Additional file 5: Table S4 Predicted cis-elements in *HvbHLH* promoters

Additional file 6: Table S5 The expression profiles (FPKM) of the *HvbHLH* in barley cultivar ‘Morex’ under different tissues

Additional file 7: Table S6 Gene accession numbers and sequences of 16 *HvbHLH* DEGs in XZ149 and XZ56 under LN stress

Additional file 8: Table S7 The FPKM value of 16 *HvbHLH* DEGs in XZ149 and XZ56

Additional file 9: Table S8 DEGs at 6 h and 48 h after low N stress in XZ149 and XZ56

Additional file 10: Fig. S2 Real time PCR validation of 6 bHLH DEGs. a Transcript levels of 6 DEGs and the corresponding expression data of RNA-Seq. The bars represent SE (n = 3). The columns represent relative expression obtained by RNA-Seq, and solid lines represent relative expression obtained by qRT-PCR. b Comparison between the relative expression obtained from RNA-Seq data and qRT-PCR. The RNA-Seq value of the relative expression (y-axis) has been plotted against the developmental stages (x-axis).

Additional file 11: Fig. S3 Expression analysis of the *HvbHLH* genes in wild barley XZ149 and XZ56 at 8 time points after LN stress. Expression data were the values of LN/control at each time point. The color scale represents relative expression levels from high (red) to low (blue).

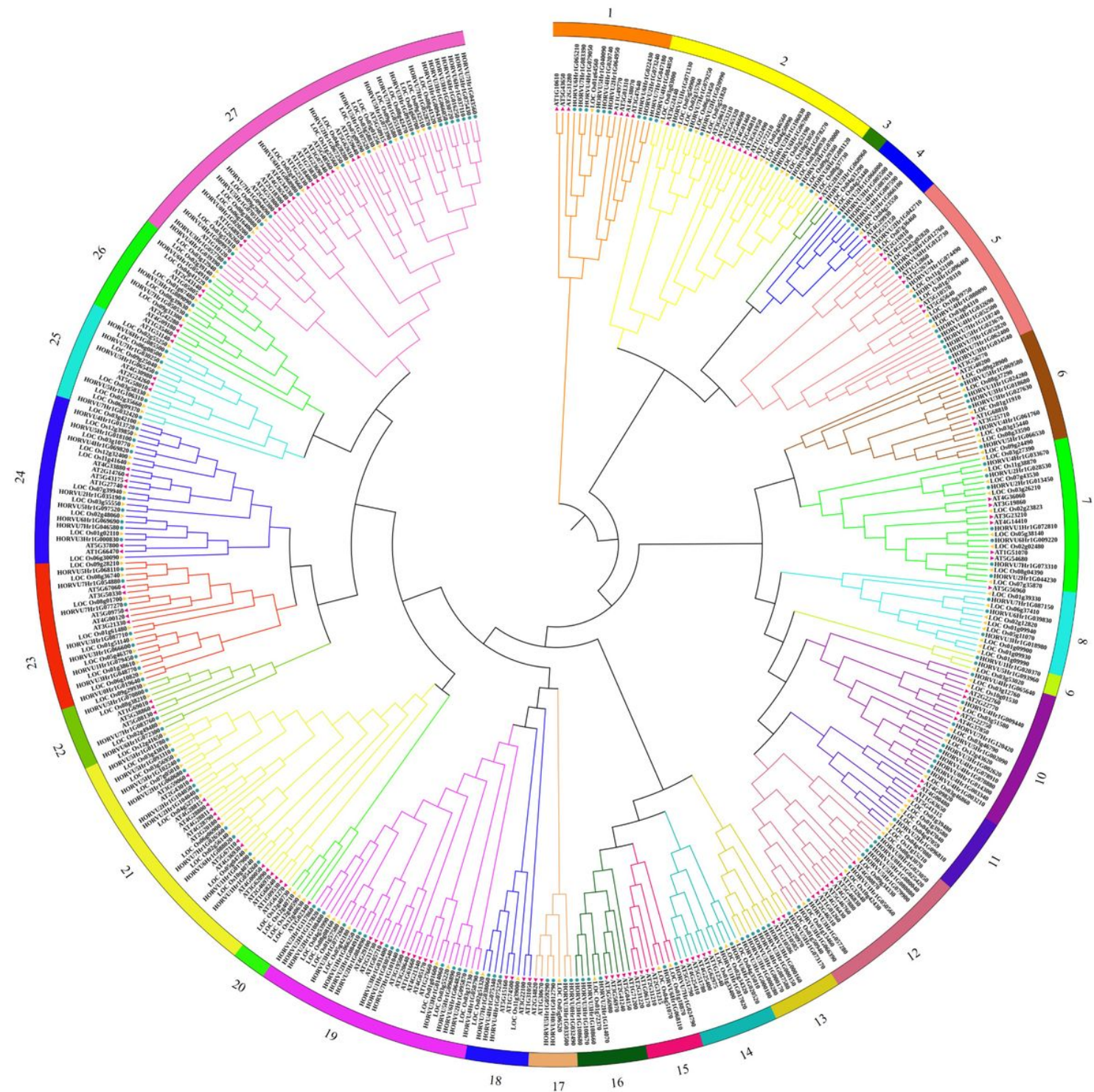
Additional file 12: Table S9 Gene ID and sequences of *bHLH* used for search in *Arabidopsis* and rice

Additional file 13: Table S10 Primers used in real time PCR

Figures

Figure 1

Phylogenetic tree of bHLH families in barley, Arabidopsis and rice. The different-colored arcs indicate different subgroups of the bHLHs. The unrooted neighbor-joining phylogenetic tree was constructed using MEGA6.0 with full-length amino acid sequences of 450 bHLHs, and the bootstrap test replicate was



set as 1000 times. The circles, purple and yellow triangles represent the bHLH proteins from barley, Arabidopsis and rice, respectively.

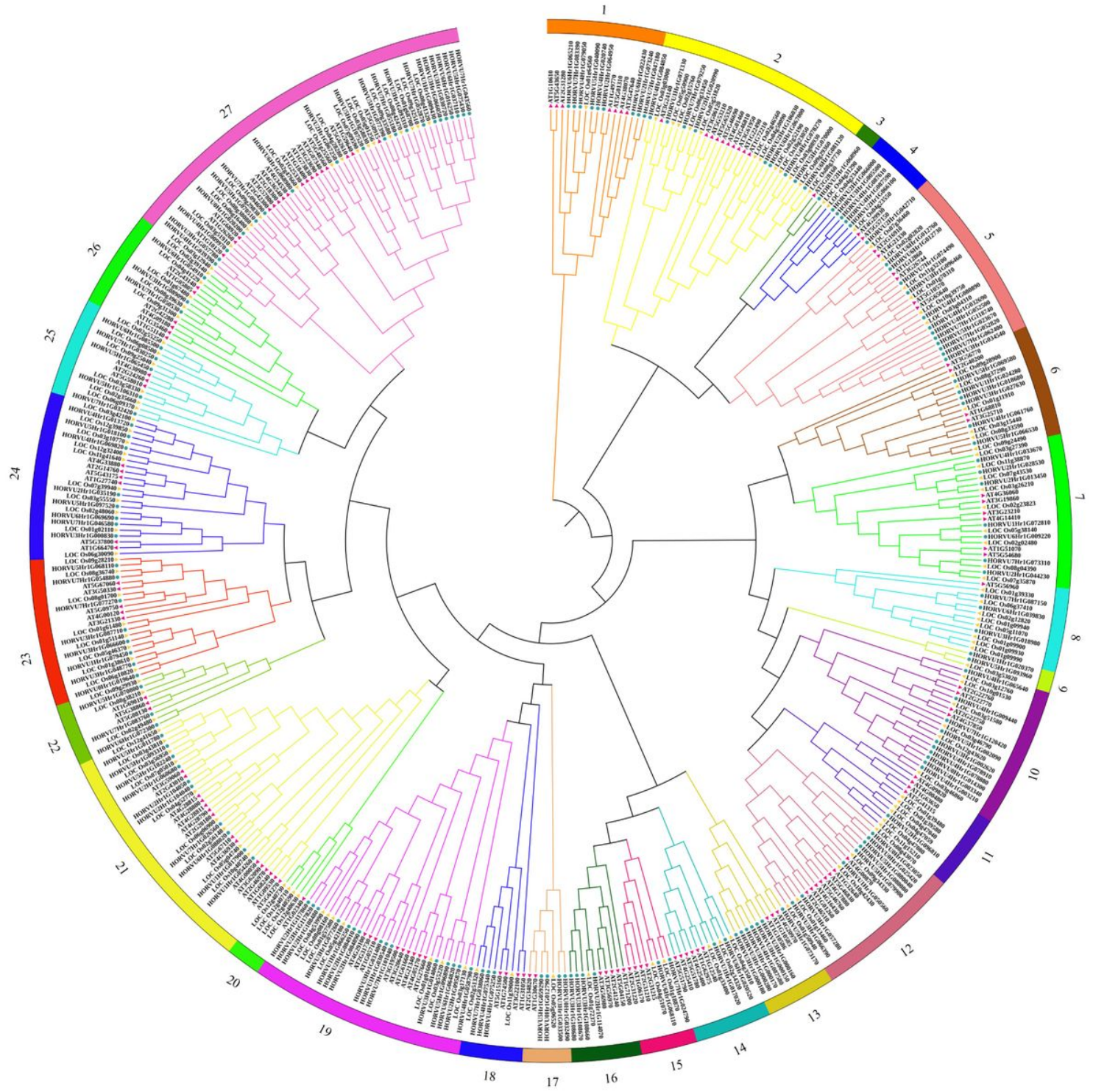


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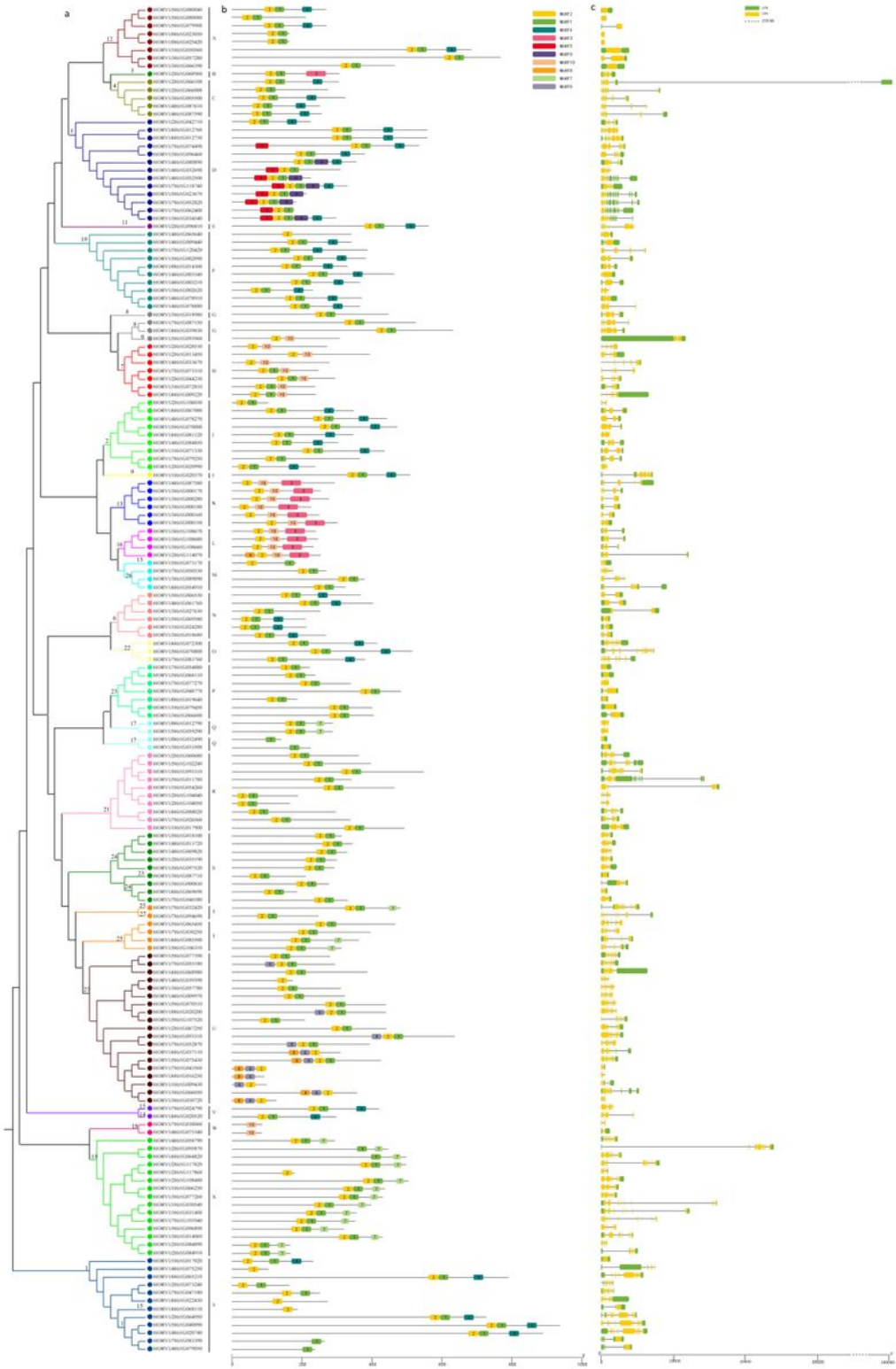


Figure 2

Phylogenetic relationships, conserved motifs and gene structure of the bHLH genes from barley. (a) Phylogenetic tree of 168 HvbHLH proteins. The unrooted neighbor-joining phylogenetic tree was constructed with MEGA6.0 using full-length amino acid sequences of 168 HvbHLH proteins, and the

bootstrap test replicate was set as 1000 times. (b) The motif composition of HvbHLH proteins. The motifs, numbers 1-10, are displayed in different colored boxes. The sequence information for each motif is showed in Fig. S1. The length of protein can be estimated using the scale at the bottom. (c) Exon/intron structure of HvbHLH genes. Yellow boxes represent exons and black lines represent introns. The upstream/downstream region of HvbHLH genes are indicated in green boxes. The length of exons can be inferred by the scale at the bottom.

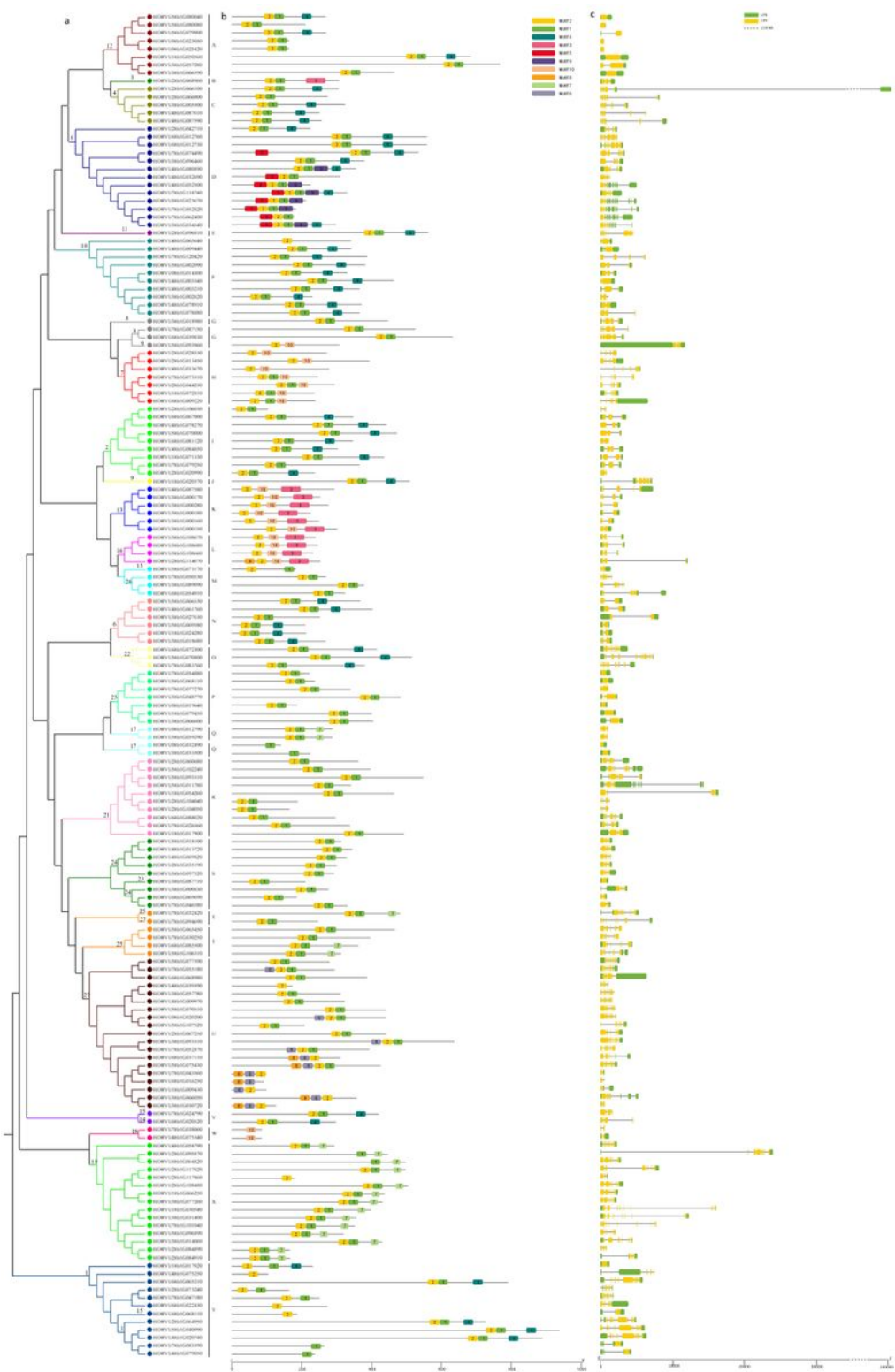


Figure 2

Phylogenetic relationships, conserved motifs and gene structure of the bHLH genes from barley. (a) Phylogenetic tree of 168 Hv bHLH proteins. The unrooted neighbor-joining phylogenetic tree was constructed with MEGA6.0 using full-length amino acid sequences of 168 Hv bHLH proteins, and the bootstrap test replicate was set as 1000 times. (b) The motif composition of Hv bHLH proteins. The motifs, numbers 1-10, are displayed in different colored boxes. The sequence information for each motif is showed in Fig. S1. The length of protein can be estimated using the scale at the bottom. (c) Exon/intron structure of Hv bHLH genes. Yellow boxes represent exons and black lines represent introns. The upstream/downstream region of Hv bHLH genes are indicated in green boxes. The length of exons can be inferred by the scale at the bottom.

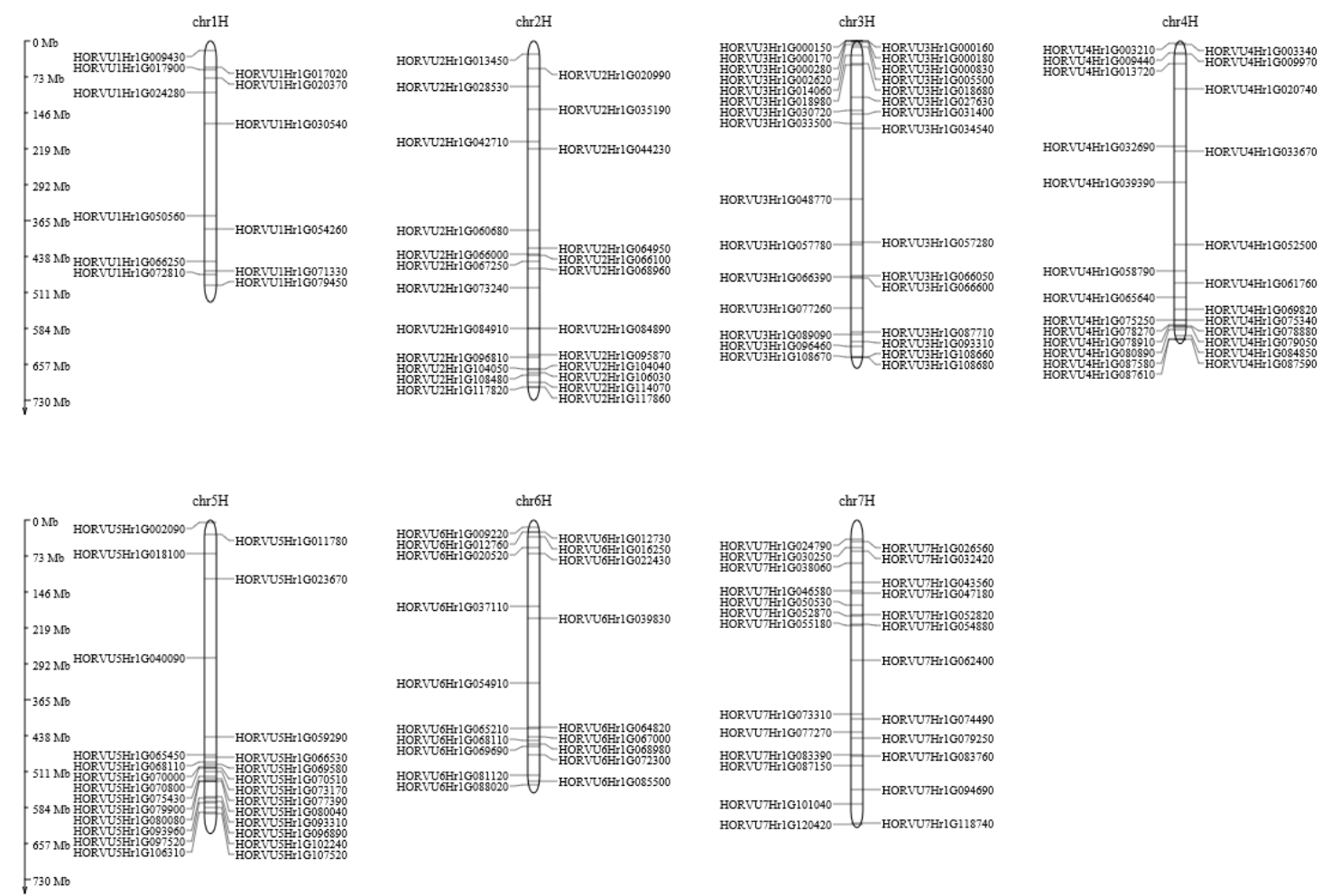


Figure 3

Chromosomal distribution of bHLH genes in barley. The chromosomes are numbered between 1 and 7 and shown at the top of each chromosome. Chromosomal distances are given in Mb, and left number represent physical location on chromosomes of Hv bHLHs.

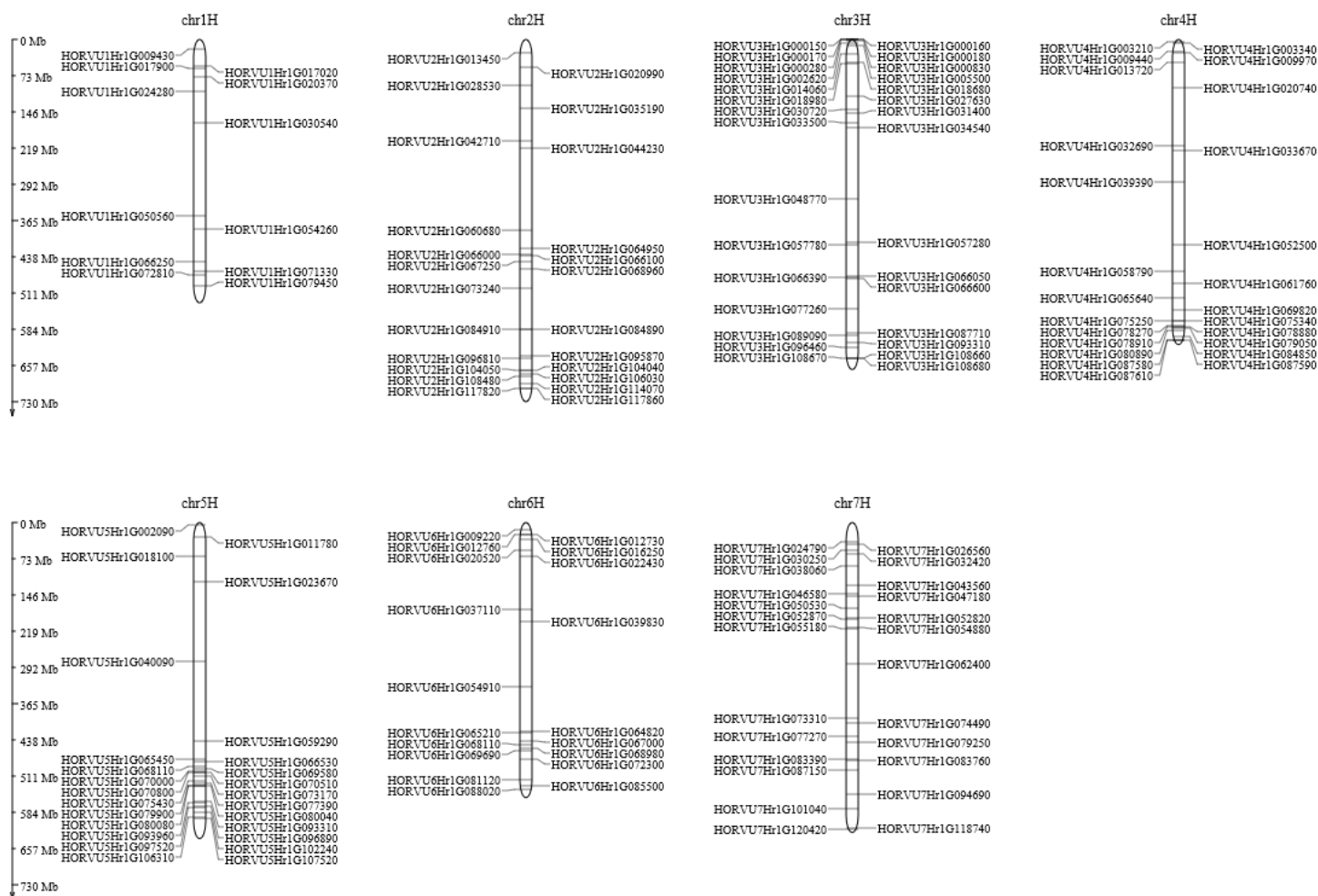


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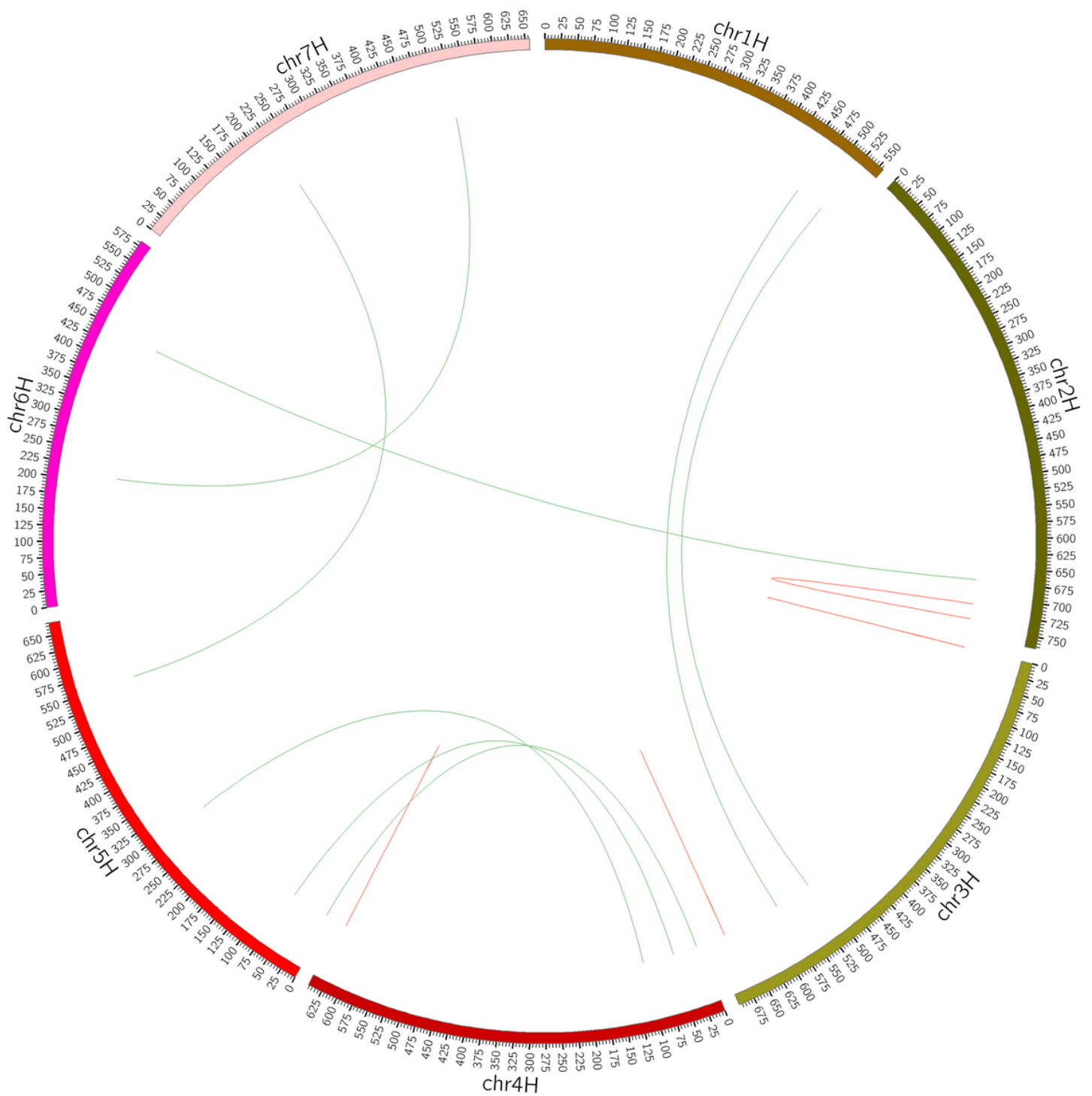


Figure 4

The synteny analysis of HvbHLH family in barley. Gray lines indicate all synteny blocks in the barley genome, and the red lines indicate duplicated HvbHLH gene pairs. The chromosome number is indicated at the bottom of each chromosome.

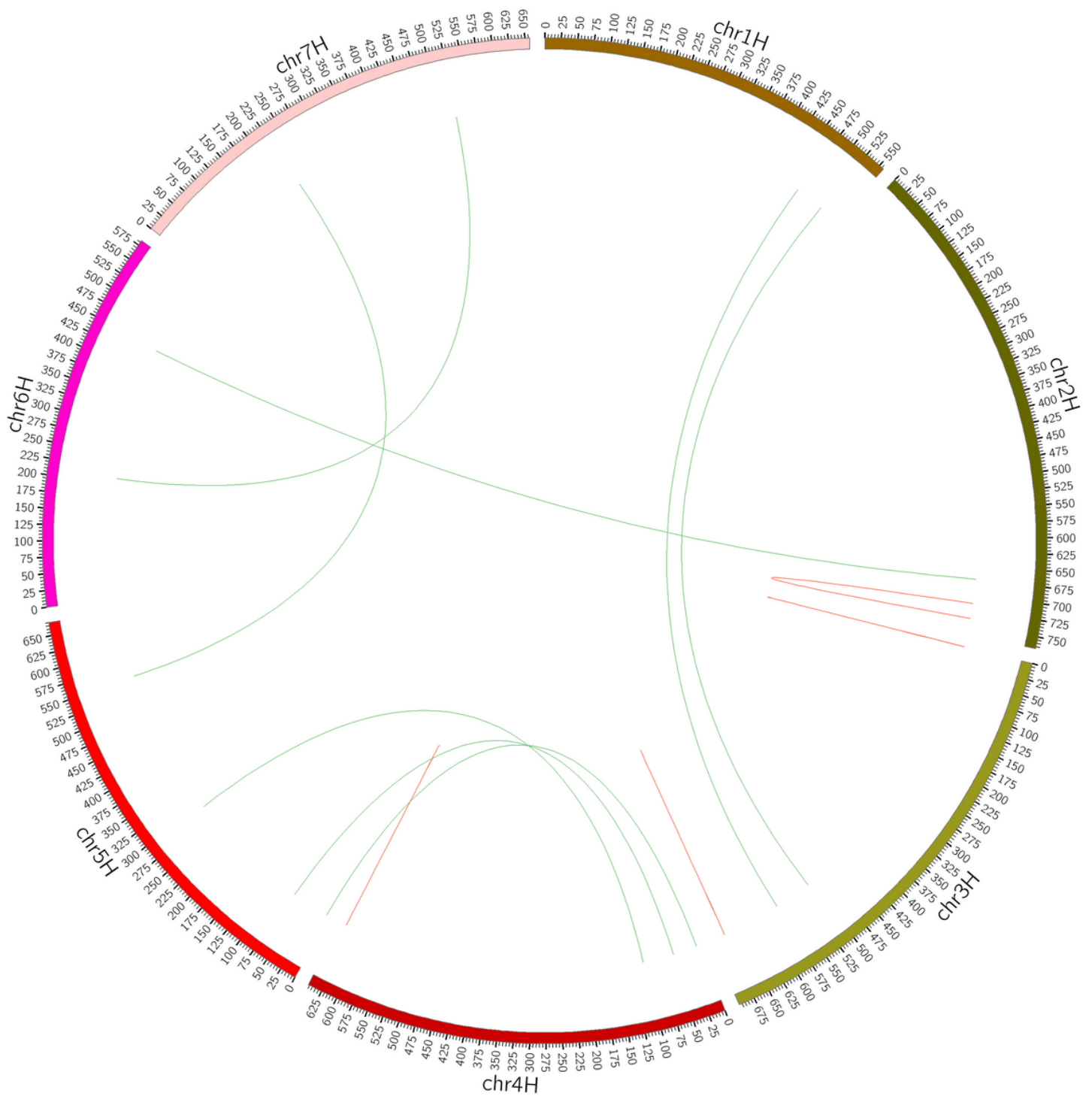


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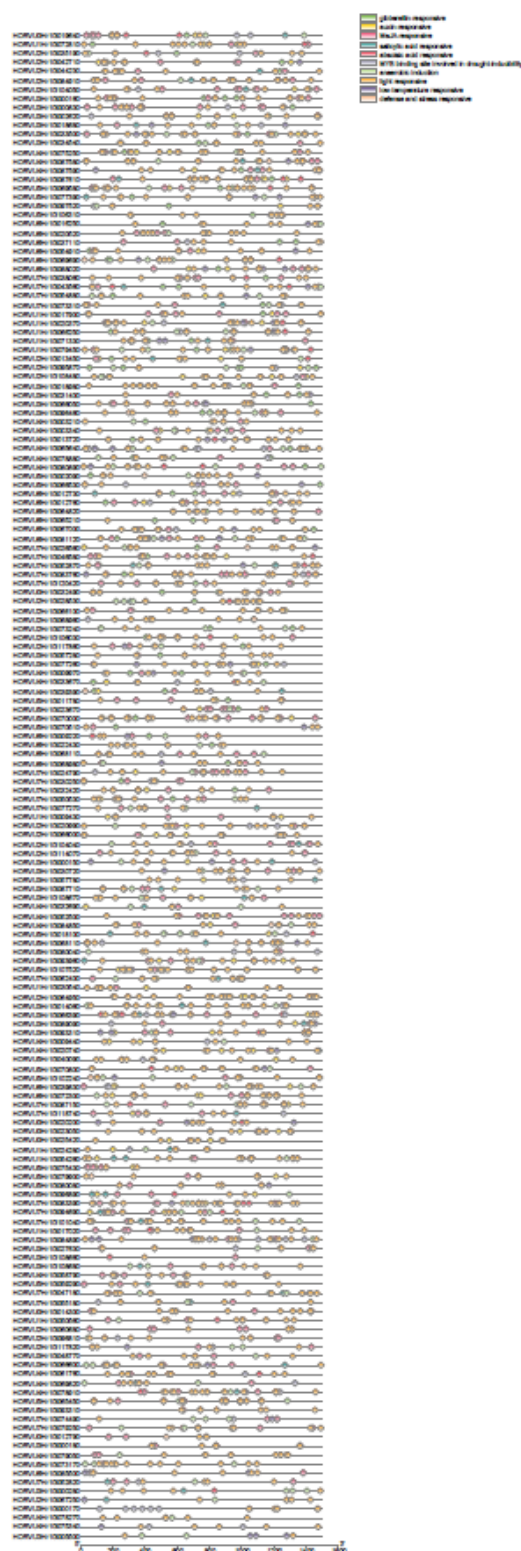


Figure 5

Predicted cis-elements in HvbHLH promoters. Promoter sequences (-1500 bp) of 168 HvbHLH genes were analyzed by PlantCARE. The upstream length to the translation starting site can be inferred according to the scale at the bottom.

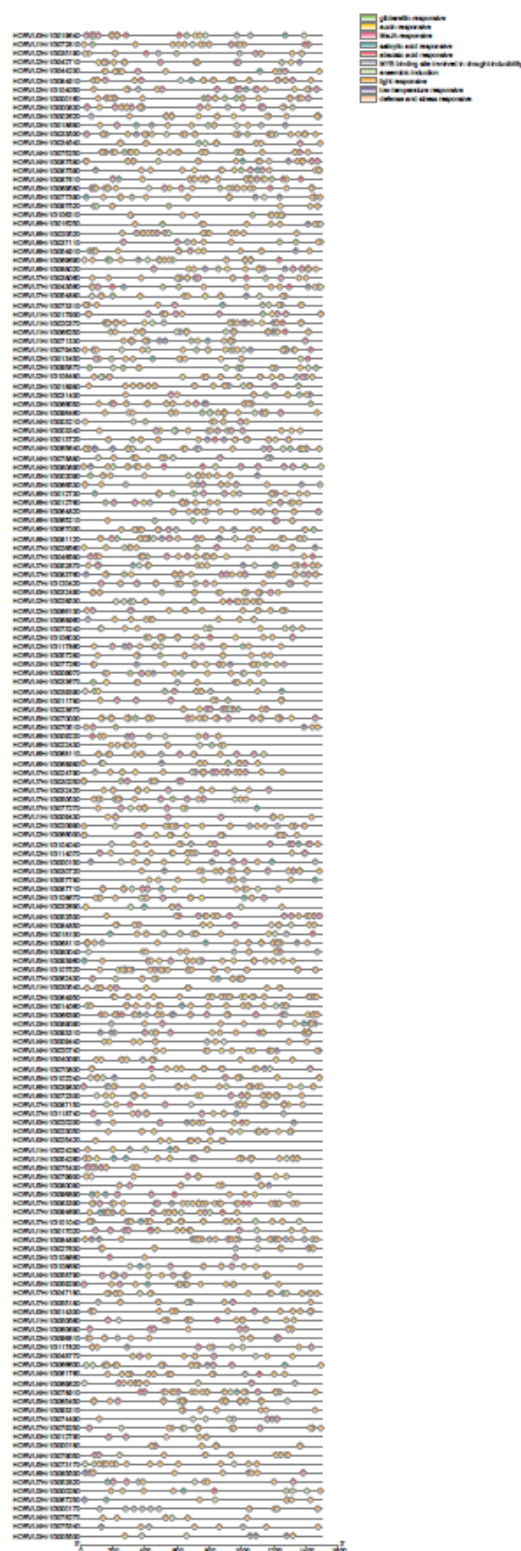


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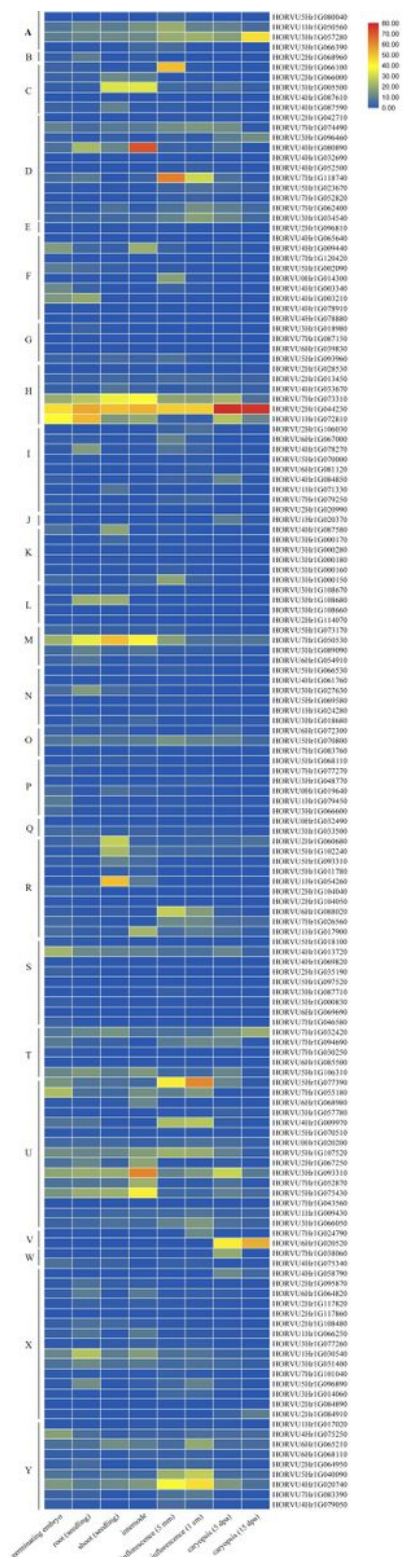


Figure 6

Expression profiles (FPKM) of the HvBHLH genes in different tissues of barley cultivar Morex. The color scale represents relative expression levels from high (red) to low (blue).

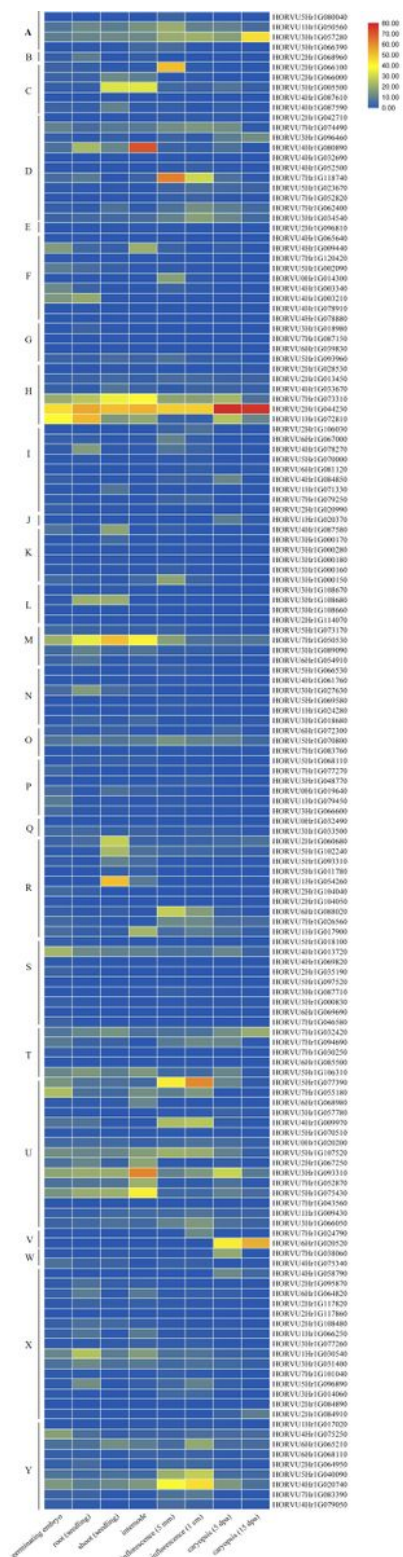


Figure 6

Expression profiles (FPKM) of the HvHLH genes in different tissues of barley cultivar Morex. The color scale represents relative expression levels from high (red) to low (blue).

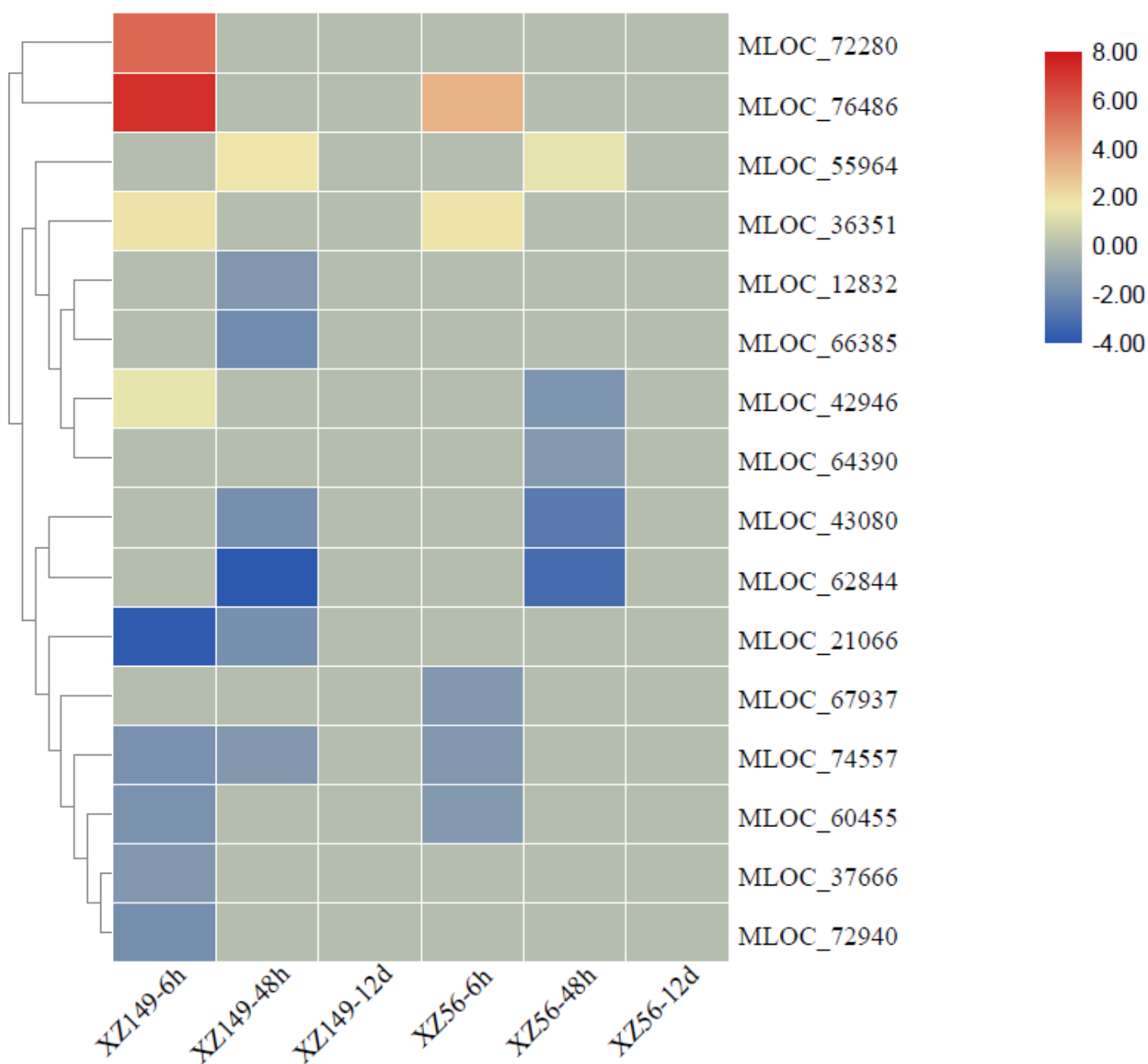


Figure 7

Expression profiles of the HvbHLH genes in wild barley XZ149 and XZ56 at 6 h, 48 h, and 12 d under LN stress. Expression data were the values of LN/control at each time point. The color scale represents relative expression levels from high (red) to low (blue).

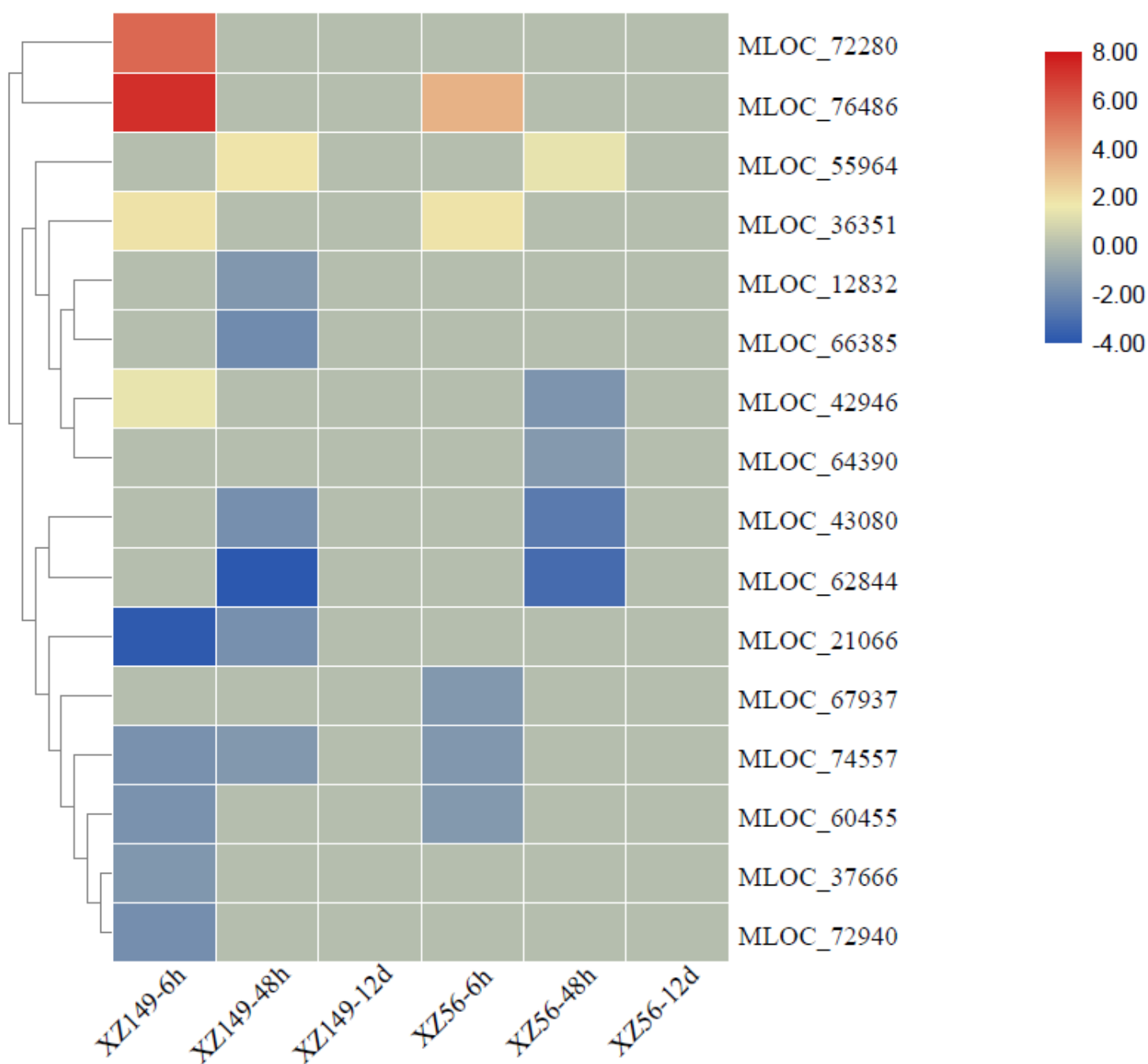


Figure 7

Expression profiles of the HvbHLH genes in wild barley XZ149 and XZ56 at 6 h, 48 h, and 12 d under LN stress. Expression data were the values of LN/control at each time point. The color scale represents relative expression levels from high (red) to low (blue).

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