Genome-wide identification of *PbrbHLH* family genes, and expression analysis in response to drought and cold stresses in pear (*Pyrus bretschneideri*)

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Abstract

Background: The basic helix-loop-helix (bHLH) transcription factors play important roles in many processes in plant growth, metabolism and responses to abiotic stresses. Although, the sequence of Chinese white pear genome (cv. ‘Dangshansuli’) has already been reported, there is still a lack of clarity regarding the bHLH family genes and their evolutionary history.

Results: In this work, a genome-wide identification of the *bHLH* genes in Chinese white pear was performed, and we characterized the functional roles of these *PbrbHLH* genes in response to abiotic stresses. Based on the phylogenetic analysis and structural characteristics, 197 identified *bHLH* genes could be well classified into 21 groups. Expansion of *PbrbHLH* gene family were mainly driven by WGD and dispersed duplication with the purifying selection from the recent WGD. The functional annotation enrichment showed that the majority of *PbrbHLHs* were enriched in the GO terms and KEGG pathways involved in responds to stress conditions as TFs. Transcriptomic profiles and qRT-PCR revealed that *PbrbHLH7*, *PbrbHLH8, PbrbHLH116*, *PbrbHLH128*, *PbrbHLH160* and *PbrbHLH195* were significantly up-regulated under cold and drought treatments. In addition, *PbrbHLH195*-silenced pear seedlings display significant reduced cold tolerance, exhibiting reduced chlorophyll content, as well as increased Electrolyte leakage and concentrations of malondialdehyde and H_2O_2.

Conclusion: For the first time, a comprehensive analysis identified the *bHLH* genes in Chinese white pear and demonstrated that *PbrbHLH195* is involved in the production of ROS in response to cold stress, suggesting that members of the *PbrbHLH* family play an essential role in the stress tolerance of pear.

Keywords: Chinese white pear, bHLHTF, gene family, evolution, VIGS, drought stress tolerance, cold stress tolerance

Background

Transcription factors (TFs) are protein molecules with special structure and function of regulating gene expression, which plays many crucial roles in plant growth and development [1]. The basic helix-loop-helix (bHLH) transcription factor family is the second largest family in plants. The members of this family are designated by a highly conserved domain called the bHLH which are able to bind and form DNA dimers [2]. The conserved bHLH domain consists of about 60 amino acids and has two functional segments, the basic region and the HLH region. The N-terminal basic region, which contains 13-17 major basic amino acids, serves as the DNA binding domain to identify and specifically bind to DNA motifs in the promoter of the target gene [3-6]. The HLH region is located at the C-terminus of the bHLH domain, which consists of two parental α-helixes, mainly composed of hydrophobic residues, connected by a relatively dispersed (length and primary sequence) loop region. The HLH domain promotes protein-protein interactions and allows the formation of homo-dimer or hetero-dimer complexes [7]. *bHLH* transcription factors are involved in many processes about plant growth and metabolism, such as stomata development [8], light signal transduction [9,10], flowering regulation [11], anthocyanin and secondary metabolism [12-14]. There have been reported that *bHLH* genes are mainly involved in abiotic stress in plants, such as the responses to drought, low temperature, salt, ABA and mechanical damage [15,16]. For example, *AtbHLH006, AtbHLH17, AtbHLH32, AtbHLH92, AtbHLH122, AtbHLH128* and *AtbHLH130* are directly or

indirectly involved in ABA signaling pathway to improve drought resistance in *Arabidopsis* [17]. The over-expression of *bHLH*TF MYC-type *ICE1*, *ICE2* and *CBF* enhanced the tolerance of *Arabidopsis* to low temperature stress [18]. In wheat, *TabHLH1* is able to regulate ABA-mediated stress tolerance pathway to improve plant adaptability to drought and salt stresses [19]. The *TabHLH39* gene is involved in regulating gene expression levels in stress responses, thereby increasing salt tolerance in over-expressing plants [20]. In rice, *OsbHLH148* and *OsbHLH006* (*RERJ1*) respond to drought stress through the jasmonic acid signaling pathway [21,22]. The bHLH transcription factor *RsICE1* can improve the cold tolerance of transgenic rice [23].The expression of the *PebHLH35* gene in populus increased during drought and ABA induction, and *PebHLH35* had an active regulatory effect under drought stress, which mentioned plant tolerance [24]. Similarly, it was shown that VabHLH1 and VvbHLH1 are positive regulators of response to low temperature stress in Chinese wild *Vitis amurensis* and *Vitis vinifera* cv. Cabernet Sauvignon, and able to confer enhanced low temperature tolerance to transgenic plants by regulating the expression level of cold regulated (*COR*) genes [25].

To date, based on the rapid development of genome sequencing, a number of plant bHLH TF genes have been identified and characterized in many species. There are 162, 167, 155, 124 and 188 *bHLHs* have been identified in *Arabidopsis*, rice, bean, potato and apple, respectively [26]. However, there have been no report about the *bHLH* family in pear. Pear is an important cash crop and widely distributed in the world. However, pears were suffered from abiotic stresses such as drought, low

temperature, and salt during the growth and development process, which not only restricts the cultivation area, but also affects their growth, development and yield. Therefore, investigating of pear bHLH transcription factors are necessary to elucidate the biological processes underlying pear stress responses.

In this study, we identified 197 pear *bHLH* (*PbrbHLH*) genes from the Chinese white pear genomic sequence and carried out phylogenetic analysis to determine the relationships among these genes. Analysis results of protein motifs and intron/exon structures support the classification of the *bHLH* family. At the same time, we identified duplication events that likely contributed to the expansion of the *bHLH* family. In addition, RNA-Seq data showed that the expression patterns of *PbrbHLHs* differed in response to drought and cold stress.The data from this study will increase our understanding of *PbrbHLH* functions associated with stress responses. Meanwhile,our systematic analysis provided a foundation for further mechanisms of cold-tolerance and drought-tolerance for *bHLH* genes in pear, especially for aiming to identify candidate genes that may be involved in the cold- and drought-tolerance of pears.

Results

Identification, classification and function annotation of *bHLH* genes in Chinese white pear

To identify the *PbrbHLH* genes, we performed local HMM-search with the HMM file (PF00010) against Chinese white pear genome, with default parameters. 200 putative PbrbHLH protein sequences were identified. SMART and NCBI Batch CD-Search tools were used to confirmed the existence of the conserved bHLH domain, and redundant sequences were removed. We finally obtained 197 sequences in pear *bHLH* family. According to the order of gene ID, these genes were named from *PbrbHLH1* to *PbrbHLH197* (Table 1 and Table S1). 168 *PbrbHLH* genes are randomly distributed on all 17 chromosomes ranging from 1 to 25 per chromosome, and the others were localized to 25 unanchored scaffolds (Table 1). Chromosome 15 has the most *PbrbHLHs* (25 genes), followed by chr 5 (21 genes) and chr 14 (15 genes).

The exact number of subgroup classifications for plant bHLH proteins is unknown, but is thought to be 15–32 [7,8,27]. To classify these genes and investigate their evolutionary relationships, phylogenetic tree was built applying the NJ method (Fig 1 and Fig 2a). The unrooted tree revealed that *PbrbHLH* gene family could be separated into 21 clades with the subfamily names A to U, which is the same number as those found in tomato [28] and *Phaseolus vulgaris* [29]. Unlike other clades, clade P and Q contained a single bHLH protein, respectively, meaning that *PbrbHLH32* and

PbrbHLH184 are unique. Except clade P and Q, the gene numbers of each clade varied wildly from 3 (clade L and M) to 22 (clade U). The results of gene structure analysis also showed that the *PbrbHLH* gene family have a broad range of exon numbers as well the gene structural diversity (Fig 2c), such as the fact that there is no characteristic distribution pattern of exons and UTRs within most of certain subfamilies. However, the distribution patterns of exons were relatively conserved in clade D, F, G, H, J, K and U, and genes in these clades have a high similarity in exon number, exon pattern and the length of each exon, such as *PbrbHLH73*, *PbrbHLH74*, *PbrbHLH75*, *PbrbHLH76* and *PbrbHLH180* in clade F and *PbrbHLH47* to *PbrbHLH77* in clade H.

The characteristics of the *PbrbHLH* family and their coding genes are shown in Table 1 and Table S1. The protein molecular weights of bHLHs were from 10.38 to 274.01 kD. Protein isoelectric points (PI) ranged from 4.24 to 10.62, and 120 of them were lower than 7 (Table 1). The grand average of hydropathy (GRAVY) for all bHLH proteins in pear was positive, suggesting that all PbrbHLHs were likely soluble proteins which are consistent with their potential functions as TFs.

The annotation information of *PbrbHLHs* from GO and KEGG databases were able to depict potential function of these genes. As shown in Fig S1a, these genes were mainly enriched in the terms of binding, cell part, cellular process, metabolic process and some regulation function, and all of these functions and processes are closely related to TFs. In addition, the KEGG enrichment result showed that these genes were largely enriched in circadian rhythm, MAPK signaling and plant hormone

signal transduction pathways (Fig S1b), all of which are the main mechanisms by which *bHLH* family TFs regulate the expression of downstream genes.

Synteny analysis of *PbrbHLHs*

The gene duplication events, such as WGD/segmental duplication, tandem duplication and transposition events, are the main causes for gene family expansion and affect the evolution of protein-coding gene families [30]. By using the MCScanX package, we detected the duplication events of *bHLH* gene family, and each of genes was assigned to one of five different duplication types: singleton, WGD/segmental, tandem, proximal or dispersed. Five duplication types were all detected driving the expansion of the *PbrbHLH* genes (Table 3 and Table S2). The results showed that 58.9% of*bHLH* genes in Chinese white pear were duplicated and retained from WGD/segmental events, and almost one quarter (23.9%) of *PbrbHLHs* were belonged to dispersed type.

To explore the evolutionary process behind the *PbrbHLH* genes, we performed intragenomic synteny analysis to identify conservation chromosome blocks within Chinese white pear. The landscape of ortholog *PbrbHLH* genes pairs were shown in Fig 3 and their chromosomal distribution was random. The evolutionary date of WGD/segmental duplication events could be estimated by the Ks value (synonymous substitutions per site) [31]. As the previous reports, based on Ks values, the genome of pear had undergone two genome-wide duplication events: the ancient WGD (Ks

~1.5–1.8) that took place ~140 MYA [32] and the recent WGD (Ks ~0.15–0.3) occurred at 30–45 MYA [33] in pear. Therefore, we used Ks values to estimate the evolutionary date of the gene duplication events among the *PbrbHLH* gene family. The Ks values implied that most *PbrbHLH* genes were duplicated from a date around the recent WGD event, and some of others were originated from the ancient WGD (Table 2). The selection intensity and direction could be represented by Ka/Ks ratio, Ka/Ks value of one indicates neutral evolution, positive selection was indicated by a Ka/Ks value greater than one, and purifying selection was indicated by a Ka/Ks value less than one [34]. The Ka/Ks ratios of almost all homologous *PbrbHLH* genes were less than one (except the gene pair *PbrbHLH110-PbrbHLH152*), which implying that *PbrbHLHs* mainly evolved under purifying selection (Table 2).

Conserved motif analysis of *PbrbHLH* gene family

The types and composition of inner motifs mainly determine the protein function, and the evolutionary relationships among these PbrbHLH proteins were also determined by analyzing their conserved motifs. To further identify motif constructions of the PbrbHLH proteins, the online MEME program was used in this study to detect motif patterns. As showed in Fig 2b, 20 conserved motifs with low E-values were recognized. The details of motif patterns were shown in Table S3. These composition patterns were nearly consistent with the phylogenetic analysis results, which were similar within the same group, but varying greatly between groups. Among

PbrbHLHs, although pattern [#1,2] was detected in all members as the conserved motif pattern for bHLH TF in Chinese white pear, some of the other motifs were present only in certain groups, including motif #8 in group B, I and K; motif #10 in group F and O; motif #11 in group N; motif #9 in group S and motif #19 in T. However, some unique motifs patterns also only could be detected in specific subfamilies. Such as the pattern [#13,12,10,1,2,6,3,14] in clade F, the pattern [#15,7,5,18,1,2,6,3] in clade K and the pattern [#1,2,6,3,20] in clade J. Although we found that many subfamilies had relatively certain motif composition and there were significant differences among each other, there was more than one pattern or no conserved pattern detected in some clades, indicating that *PbrbHLHs* in these groups were not conservative in the evolutionary process, and the division among groups might have occurred in an early period.

Expression profile and patterns of *PbrbHLH* genes in response to drought and cold stresses

Previous transcriptome analyses of Chinese white pear revealed the expression patterns of candidate *PbrbHLH* genes in response to drought stress and cold stress, respectively (Fig 4). Overall, the results indicated that although the background expression of some *PbrbHLH* genes was rarely detected, that of others was significantly different among these investigated time points. Several differentially expressed genes showed up-regulation trend under both two stress conditions, to

varying degrees, such as *PbrbHLH119* and *PbrbHLH120* in clade E, *PbrbHLH7* and *PbrbHLH160* in clade G and *PbrbHLH128* to *PbrbHLH80* from clade K. This suggested that these genes may be involved in some close-related pathways in response to drought and cold stresses. Interestingly, compared to the expression of these genes in cold treatment, the peak expression of them under drought condition was showed at a relatively late time point. In contrast, some other *PbrbHLHs* showed different (or even opposite) expression patterns, indicating that their responses might vary according to the different stress conditions.

To further verify the functions of these identified *PbrbHLHs*, eight differentially expressed *PbrbHLH* genes (*PbrbHLH119* from clade E, *PbrbHLH7*, *PbrbHLH8* and *PbrbHLH160* from clade G, *PbrbHLH80*, *PbrbHLH128*, *PbrbHLH161* and *PbrbHLH195* from clade K) were selected to examine the expression in response to drought and cold stresses, respectively (Fig 5). Comparing with the expression at 0 hpt (hours post treatment), except*PbrbHLH8*and *PbrbHLH80*in drought treatment as well *PbrbHLH7* and *PbrbHLH119* under cold stress (data not shown), expressions for all other genes were significantly altered in the early stage of drought or cold treatment. Their responses tended to be more rapid under drought conditions, usually changing within the first 12 hours. Under cold stress, expression of *PbrbHLH8*, *PbrbHLH116*, *PbrbHLH128* and *PbrbHLH160*initially showed down-regulating trend at first before being up-regulated as well as the expression of *PbrbHLH7* and *PbrbHLH195* under drought stress. The opposite trends between cold and drought stresses were noted for *PbrbHLH128* and *PbrbHLH160*. Under drought stress, both

were up-regulated at first and then down-regulated, whereas, under cold stress, their expression initially decreased before increasing. These results indicate that *PbrbHLH* genes were indeed involved in the responses to drought and cold stresses, and the pathways they taken part in under these stresses condition seemed to be different.

Silencing of *PbrbHLH195* reduced cold tolerance of *P. betulaefolia*

To understand whether *PbrbHLHs* is required for cold tolerance in pear, the VIGS system was employed to silence *PbrbHLH195*, which is significantly up-regulated under cold condition, in *P. betulaefolia*. The transcript abundance of *PbrbHLH195* in the positive plants was substantially reduced by 50–90 %, compared with that of WT (Fig 6j, k). The positive silent plants (p-TRV1, p-TRV2 and p-TRV3) and WT plants were morphologically indistinguishable under normal growing conditions (Fig 6a, d). However, upon exposure to 0℃ for 8 days, the silent plants displayed more severe damage in comparison with WT (Fig 6a). The electrolyte leakage (EL) and malondialdehyde (MDA) concentrations in silent plants were significantly higher than those in WT under cold stress(Fig 6b, c). Meanwhile, when they were subjected to cold treatment, Chl fluorescence in silent plants were prominently repressed, accompanied by significantly lower Fv/Fm ratio and Chl content, in comparison with WT (Fig 6d-g). In addition, compared with silent plants, WT had lower H_2O_2 content (Fig 6h, i). In situ accumulation of H_2O_2 was histochemically stained with DAB. In the presence of low temperature, the staining became darker, but silent plants staining

was deeper and stronger than that of WT (Fig 6h), which was further confirmed by quantitative measurement (Fig 6i), which means that silencing plants accumulate more reactive oxygen species than WT. These results indicated that silencing of *PbrbHLH195* promotes cold susceptibility in *P. betulaefolia*.

Discussion

After the release of the Chinese white pear genome sequencing data, there were many TF genes at the whole-genome level have been identified and characterized, including NAC-TF, BAM-TF and WRKY-TF et.al. [22,46,47]. bHLH transcription factors are involved in many pathways in plant growth and metabolism [12]. However, no such detailed studies have been done with the *bHLH* family, and only a few examinations have been made of *PbrbHLHs* in pear. Here, we identified 197 *PbrbHLH* genes in Chinese white pear. Results of the phylogenetic analysis, gene structure and protein conserved motif analysis enable us to classify these PbrbHLH proteins into 21 groups, which is the same number reported in tomato and apple [48,49], even though those organisms have fewer *SlbHLHs* (159) and *MdbHLHs* (188) than the members of *PbrbHLHs* in pear. On the basis of phylogenetic analysis, the unrooted tree showed that *PbrbHLHs* were well separated into 19 clades with the wildly varied gene numbers from 3 (clade L and M) to 22 (clade U) and two one-gene clade P and Q. The gene and protein structure analysis showed that *PbrbHLH* family also has a broaddiversity in intron/exon organizations as well the protein motif patterns. Though,

304 the distribution pattern of exons and UTRs in clade D, F, G, H, J, K and U were
305 relatively conserved, there was a broad range of exon numbers and structural diversity
306 in many other clades, which is similar to the results of protein motif pattern analysis.
307 By using online MEME software, 20 conserved protein motifs were detected among
308 PbrbHLHs with low E values, and pattern [#1,2] were existed in all bHLHs which
309 was regarded as the characteristic pattern for PbrbHLH TF. Meanwhile, some other
310 motifs were present only in certain groups, including the motif #8 in group B, I and K
311 and motif #10 in group F and O. Furthermore, three unique motif patterns only could
312 be detected in specific subfamilies, respectively, such as the pattern
313 [#13,12,10,1,2,6,3,14] in clade F, the pattern [#15,7,5,18,1,2,6,3] in clade K and the
314 pattern [#1,2,6,3,20] in clade J. These results suggested that the *PbrbHLH* gene
315 family may play diverse roles in the adaptive evolution to environmental stresses, and
316 the division among groups might have occurred in an early period.

317 Gene duplication analysis revealed that the main driving force for the expansion
318 of *PbrbHLH* family was WGD/segmental events, which is same as the case in apple.
319 For instance, by applying MCScanX, 58.9% of *bHLH* genes in Chinese white pear
320 were categorized into WGD/segmental type. Although pear was undergone the recent
321 WGD events, almost one quarter of *bHLH* genes were duplicated from dispersed
322 envents. This may be due to the high ratio of self-incompatibility and the
323 domestication process of pear. These results showed that WGD/segmental and
324 dispersed gene duplications play critical roles in the expansion of the *bHLH* gene
325 family in Chinese white pear. Furthermore, Ks values analysis implied that almost all

WGD type *PbrbHLH* genes were duplicated from a date around the recent WGD event, and the Ka/Ks ratios indicated that *PbrbHLHs* evolved mainly under purifying selection and they seem to be necessary for adaptation to the current environment in their evolutionary history.

The function enrichment analysis showed that PbrbHLH genes were mainly enriched in the functions and processes closely related to TF, and the pathways they classified in were the main mechanisms by which *bHLH* family TFs regulate the expression of downstream genes, such as circadian rhythm, MAPK signaling and plant hormone signal transduction pathways. For example, *OsbHLH148* and *OsbHLH006* (REJ1) can improve drought stress by jasmonic acid signaling pathway in rice. Under salt and drought stress, in grapes *VvbHLH1* confers a dominant effect on salinity and drought tolerance thought increasing the accumulation of flavonoids and ABA signaling in transgenic *Arabidopsis thaliana*. In addition, bHLH protein is also involved in plant stress resistance. *Arabidopsis AtbHLH112* gene improves drought tolerance by increasing osmotic substances, eliminating ROS content and reducing water diversion. The results indicated that *PbrbHLHs* might play roles as other *bHLHs*.

By analyzing previous transcriptome data, we revealed the expression patterns of *PbrbHLHs* under cold and drought stress conditions. The results showed that, except some genes, the expression of most *PbrbHLHs* was significantly altered. For example, under both two stress, *PbrbHLH* genes including *PbrbHLH7*, *PbrbHLH119*, *PbrbHLH120*, *PbrbHLH160* and *PbrbHLH128* to *PbrbHLH80* in clade K had a

up-regulation trend, which suggested that these genes might play similar roles in some close-related pathways in response to drought and cold stresses. Comparing with cold treatment, the peak expression of them under drought condition was showed at a relatively late time point, indicating that the responses of *PbrbHLHs* varied according to the treatment applied. To verify whether *PbrbHLHs* were involved in the response to cold or drought stresses, we performed stress treatments and qRT-PCR analysis. The results showed that the expression of all tested genes was significantly altered in the early stage of drought or cold treatment, however, the responses of same gene between two treatment could be diverse. For instance, under cold treatment, expression of *PbrbHLH7*, *PbrbHLH8*, *PbrbHLH116*, *PbrbHLH128*, *PbrbHLH160* and *PbrbHLH195* showed down-regulating trend at first before being up-regulated, whereas, under drought stress, *PbrbHLH128* and *PbrbHLH160* were up-regulated at first and then down-regulated. Furthermore, as a high up-regulated gene induced in both cold and drought stress conditions, the interference of *PbrbHLH195* in transcription level significantly reduced the cold tolerance of the RNAi pear seedlings. These results indicate that *PbrbHLH* genes were involved in the responses to drought and cold stresses in pear, and the pathways they involved in seemed to be different under various stress conditions.

Our works in this study highlight the importance of bHLH TF in the cold and drought tolerance of Chinese white pear. This is the first study to identify the *PbrbHLH* genes and examine their expression patterns in pear. QRT-PCR analysis showed that *PbrbHLH* is involved in stress tolerance pathways and functional

analysis showed that *PbrbHLH195* plays an important role in pear abiotic stress tolerance. However, further investigation will be required to understand the roles of *PbrbHLHs* in the stress response pathways, and the characterization of key (even the marker) bHLH TFs under each stress condition was also crucial to the revealing of the functional mechanisms of bHLH in pear.

Conclusions

In this study, we identified 197 *PbrbHLH* genes from Chinese white pear and carried out phylogenetic analysis to determine the relationships among these genes. Based on the results of protein motifs and intron/exon characteristics and phylogenetic analysis, *PbrbHLH* family were classified into 21 groups. According to the analysis of collinearity, WGD and dispersed duplication might have a role in the evolution of the *PbrbHLH* family. In addition, RNA-seq data, qRT-PCR and VIGS results revealed that *PbrbHLH* genes might have important roles in response to abiotic stresses, and the expression patterns of them differed in response to drought and cold stresses. The underlined collected data from this study provided a foundation for advanced studies to evaluate the mechanisms of cold-tolerance and drought-tolerance for *bHLH* genes in pear.

Methods

Plant materials and bacterial strains

The pear seedlings were grown in the greenhouse under a 16 h/8 h light/dark photoperiod, 75% relative humidity and 25 °C. *A. tumefaciens* GV3101 was grown in LB media supplemented with kanamycin and Rif at 28 °C in an orbital shaker at 200 rpm and harvested during the log phase of growth for infiltration.

Identification and functional annotation of *bHLH* gene family in Chinese white pear

To identify the *bHLH* genes in Chinese white pear, we performed multiple database-based searches. We downloaded all needed sequences and annotation file of Chinese white pear from Pear Centre of Nanjing Agricultural University (http://peargenome.njau.edu.cn/) and the seed file of bHLH conserved domain (PF00010) was downloaded from Pfam (http://pfam.sanger.ac.uk/). By using HMMER (Hidden Markov Model, HMM) software with default parameters E-value <0.05. Then we checked the predicted bHLH transcription factors by using the NCBI Batch CD-Search tools (Batch CD-Search: https://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi) based on CDD v3.18 and SMART v6.0 databases to verify the existence of bHLH domain (Table S1). The proteins with E-values greater than $1e^{-6}$ or without a bHLH domain were removed. The relevant gene ID of *PbrbHLH* genes were shown in Table 1. The annotation information for Chinese white pear was extracted from the GFF file, and the results was visualized by a R script.

Structure and conserved motif analysis of the *PbrbHLH* genes

The Gene Structure Display Server (GSDS 2.0) (http://gsds.cbi.pku.edu.cn/) was used to analyze the structures of the *bHLH* genes by aligning the cDNA sequences with their corresponding genomic DNA sequences. Conserved motif analysis of bHLH proteins was performed by online Multiple Expectation Maximization for Motif Elicitation (MEME) (http://meme.nbcr.net/meme/cgibin/meme.cgi). Maximum number parameter of motifs was seted as 20.

Phylogenetic analysis

The phylogenetic tree was built with Neighbor-Joining (NJ) method and a bootstrap of 1000 in MEGA7.0 (http:// www.megasoftware.net/). The p-distance was used and the optional parameters for pairwise deletion were considered.

Chromosomal localization and synteny analysis

The chromosomal localization information was extracted from the GFF file. The same procedure used in the PGDD (http://chibba.agtec.uga.edu/duplication/) was performed to analyze the synteny among the *PbrbHLHs*. Primarily, the local all-vs-all BLASTP searches among identified *PbrbHLH* genes were conducted ($E < 1e^{-10}$). Afterward, MCScanX was employed for the determination of syntenic gene pairs with the BLASTP result and gene location information used as input files [35]. The downstream analysis tool (duplicate_gene_classifier) in the MCScanX package was employed for the identification of tandem, proximal dispersed, and segmental/whole-genome duplications (WGD) of *PbrbHLH* family genes. The results were visualized using circos-0.69 software [36]. The Ka and Ks values were analyzed

via KaKs_Calculator 2.0 [37]. For the estimation of the date of segmental duplication events, the succeeding pairs of homologous genes within 100 Kb on all sides of the *PbrbHLH* genes, considered for the mean Ks calculation.

Expression analysis of *PbrbHLH* genes under drought and cold stress conditions

Published transcriptomic data characterizing the total RNA of drought treatment samples (D0, D1, D3 and D6 indicating the samples harvested at 0 hpt (hour post treatment), 1 hpt, 3 hpt and 6 hpt under drought stress) were downloaded from Li et al. (2016) [38]; cold treatment samples (C0, C5, C12 and C24 indicating the samples harvested at 0 hpt, 5 hpt, 12 hpt and 24 hpt under cold stress) were downloaded from Yang and Huang (2018) [39]. We determined the expression patterns of *PbrbHLH* family genes under drought and cold stress conditions. TBtools v1.068was used to visualize the results [40].

For the qRT-PCR analysis, 9-week-old pear seedlings were treated with drought and cold, respectively. The leaves were cryopreserved with liquid nitrogen at 0hpt, 1hpt, 3hpt, 6hpt, 12 hpt and 24 hpt after drought stress treatment as well the leaves with cold treatment at 0hpt, 1hpt, 3hpt,9 hpt, 12 hpt and 24 hpt. Total RNA extraction and the synthesis of cDNA were according to the instructions of RNA kit (Tiangen, Beijing, China) and PrimeScript RT reagent Kit (Trans Gen). Specialized primers of the constitutive *TUB* and eight tested *PbrbHLH* genes were designed via NCBI online tool Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome)

with the Specificity Parameters Organism option set as *Pyrus bretschneideri* (taxid:225117) (Table S4). The qRT-PCR assays were conducted with three technical copies. QRT-PCR reactions (20μl per hole) were performed as previously reported [41]. The expression was evaluated for each sample via the $2^{-\Delta\Delta Ct}$ method, and Duncan's multiple range test was conducted. A p-value of less than 0.05 was the considerable variation and the differentially expressed genes were identified with $|\log 2^{FC}| > 1$.

Generation of silenced plants

Virus-induced gene silencing (VIGS)-mediated suppression of *PbrbHLH195* was performed according to previous methods [42,43]. A 182 bp fragment of *PbrbHLH195* open reading frame (ORF) was inserted into *EcoR* I and *BamH* I sites of tobacco rattle virus-based vector 2 (TRV2) to generate the pTRV2-*PbrbHLH195* construct. The vectors pTRV1, pTRV2 and pTRV2-*PbrbHLH195* were transformed into *A. tumefaciens* strain GV3101 by heat shock. The bacterial cells (OD600 = 1.0) containing pTRV1 were mixed with pTRV2-*PbrbHLH195* or pTRV2 in a 1: 1 volume ratio in 2-(Nmorpholino) ethanesulfonic acid (MES) buffer (10 mM $MgCl_2$, 200 mM acetosyringone, and 10 mM MES, pH 5.6) and kept slowly shaking in dark for 4 h at room temperature. The bacterial mixtures were injected into the leaves of seedlings and rinsed with water, grown in soil pots and transferred to a controlled growth chamber. Two weeks later, un-injected leaves were collected from each plant and subjected to genomic PCR and qRT-PCR analyses, and the VIGS plants exhibiting similar magnitude of *PbrbHLH195* suppression were used for further analyses.

Physiological analysis

EL was measured by Dahro et al. (2016) [44]. MDA and H_2O_2 were detected according to the instructions using the corresponding detection kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Chl fluorescence was measured by Imaging PAM CHL fluorometer, Fv/FM ratio was calculated by imaging Winge software (Walz, Germany). Chl was extracted and analyzed according to Liu et al. (2008) [45].

Abbreviations

bHLH: basic helix-loop-helix; TF: Transcription factor; *COR* gene: cold regulated gene; PI: protein isoelectric points; GRAVY: grand average of hydropathy; Ks: synonymous substitutions per site; hpt: hours post treatment; EL: electrolyte leakage; MDA: malondialdehyde; HMM: Hidden Markov Model; GSDS: Gene Structure Display Server; MEME: Multiple Expectation Maximization for Motif Elicitation; NJ: Neighbor-Joining; WGD: whole-genome duplications; VIGS: Virus-induced gene silencing; ORF: open reading frame; MES: 2-(Nmorpholino) ethanesulfonic acid.

Declarations

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Availability of data and materials

All needed genome sequences and genome annotation files of Chinese white pear were obtained from the Nanjing Agricultural University pear genome project website (http://peargenome.njau.edu.cn). All data generated or analysed during this study are included in this published article and its supplementary information files.

Authors' contributions

HZD, YQD and WJH designed and carried out the experiments and QMC carried out all bioinformatics analysis and wrote the manuscript. XSH and SLZ directed and revised the manuscript. All authors read, reviewed and approved the final manuscript.

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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Figure legends

Fig 1 Unrooted phylogenetic tree of PbrbHLH proteins.

MEGA 7 was used to construct the phylogenetic tree based on the protein sequences. iTOL was used to annotate and review the phylogenic tree. The proteins were clustered into 21 groups. Different background colors indicate the different group of the PbrbHLH proteins.

Fig 2 Schematics of the gene structure and the conserved motifs in the *PbrbHLH* family.

A Conserved motif analysis. 20 distinct motifs were identified with MEME suite and each motif was represented with different color. b Gene structural analysis.

Fig 3 Distribution and collinearity of the *PbrbHLHs*.

Red lines along the circumference of the circle mark the positions of genes on chromosomes. The lines in different colors inside the circle indicate collinearity relationships among *PbrbHLH* genes.

Fig 4 Expression profile of *PbrbHLHs* under drought and cold stresses.

744 Expression analyses of *PbrbHLHs* using previous published transcriptome data under
745 cold and drought stress conditions.
746
747 **Fig 5 Expression analysis of *PbrbHLHs* under cold and drought stresses.**
748
749 A Relative expression of *PbrbHLH8*, *PbrbHLH80*, *PbrbHLH128*, *PbrbHLH160*,
750 *PbrbHLH161* and *PbrbHLH195* with cold treatment. b Relative expression of
751 *PbrbHLH7*, *PbrbHLH119*, *PbrbHLH128*, *PbrbHLH160*, *PbrbHLH161* and
752 *PbrbHLH195* with drought treatment. The pear *Actin* was used as internal reference
753 for the normalization. The statistical analyses were performed using student's t-test (*
754 $p < 0.05$).
755
756 **Fig 6 Cold tolerance assay of *PbrbHLH195*-silenced *Pyrus betulaefolia* plants.**
757
758 a-c Phenotype of 1-month-old *PbrbHLH195*-silenced plants before and after cold
759 treatment for 8 days (a). Electrolyte leakage (EL) (b) and malondialdehyde (MDA)
760 concentrations (c) after cold treatment. d-g Chlorophyll fluorescence imaging of
761 silenced plants and WT plants(d), Fv/Fm ratios (e), Chl content (f) and phenotype (g)
762 of WT and *pTRV-PbrbHLH195* silencing plants (pTRV-1, pTRV-2 and pTRV-3) at
763 the end of the chilling stress. h-i In situ accumulation of H_2O_2 of WT and silencing
764 plants, as revealed by histochemical staining with 3, 3-diaminobenzidine (DAB) (h)
765 after cold treatment. Quantitative measurement of H_2O_2 (i) levels after cold treatment.

766 The expression of *PbrbHLH195* was detected by RT-PCR (j) and qRT-PCR (k) at 8d
767 after cold treatment.
768
769 **Table 1** Characteristics of identified PbrbHLH proteins.
770 **Table 2** The duplicate mode and estimation of absolute date for large-scale
771 duplication events for *PbrbHLHs*.
772
773 **Table 3** Numbers of *bHLH* genes from different origins in Chinese white pear.
774
775 **Additional file 1.xlsx:**
776
777 **Table S1**Table S1 Detailed characteristics of *PbrbHLHs*.
778
779 **Table S2**Duplication type of *PbrbHLH* genes in Chines white pear.
780
781 **Table S3**Sequence informations of 20 detected motifs in MEME analysis.
782
783 **Table S4**The primers of *PbrbHLHs* for qRT-PCR and vector construction.
784
785 **Additional file 2.pdf:**
786
787 **Fig S1** Functional annotation enrichment analysis.

a GO (Gene ontology) term enrichment analysis of PbrbHLH proteins. b KEGG enrichment analysis of PbrbHLH proteins.