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Research article

Genome-wide characterization and expression profiling of the relation of the HD-Zip gene family to abiotic stress in barley (*Hordeum vulgare* L.)

Yuan Li[a], Huiyan Xiong[b], Duojie Cuo[a], Xiongxiong Wu[a], Ruijun Duan[a,c,*]

[a] College of Eco-environmental Engineering, Qinghai University, Qinghai, 810016, China
[b] College of Agriculture and Animal Husbandry, Qinghai University, Qinghai, 810016, China
[c] Qinghai Provincial Key Laboratory of Hulless Barley Genetics and Breeding, Qinghai University, Qinghai, 810016, China

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ABSTRACT

The homeodomain-leucine zipper (HD-Zip) gene family plays an important role in plant growth and environmental responses. At present, research on the HD-Zip gene family of barley is incomplete. In this study, 32 HD-Zip genes (*HvHD-Zip 1–32*) were identified from the barley genome and were subsequently divided into four subfamilies according to conserved structure and motif analysis. Whole genome replication events in barley and *Arabidopsis*, rice, and wheat HD-Zip gene families were analyzed, yielding 3, 14 and 25 gene pairs, respectively, but no segmental or tandem duplication events were identified in the barley HD-Zip gene family. Subsequently, quantitative real-time PCR (qRT-PCR) analysis revealed that the *HvHD-Zip* gene is sensitive to drought stress and that members of the HD-Zip I and HD-Zip IV subfamilies are generally more sensitive to abiotic stresses. Our results suggest a relationship between barley resistance and the potential key *HvHD-Zip* gene, which lay the foundation for further functional studies.

1. Introduction

Homeodomain-leucine zipper (HD-Zip) genes comprise an important transcription factor family, and exist only in higher plants, having many members that are relatively evolutionarily conserved (Ariel et al., 2007). The structural feature shared by the HD-Zip genes is the homeodomain (HD), which is a specific DNA binding site at the C-terminus, and the leucine-zipper (LZ) domain, which is closely associated with the former and is responsible for protein dimerization (Johannesson et al., 2001). Based on the DNA sequence and conserved motif, and combined with HD-Zip's physiological function, this family is divided into four subfamilies, HD-Zip I-IV (Ariel et al., 2007). The class I HD-Zip transcription factor (HD-Zip I) subfamily is very simple, containing only a highly conserved HD domain and the LZ domain, and the class II HD-Zip transcription factor (HD-Zip II) subfamily members contain a CPSCE (Cys-Pro-Ser-Cys-Glu) motif consisting of five conserved amino acids after the C-terminus of the LZ domain. The class III HD-Zip transcription factor (HD-Zip III) subfamily is the most complex (Elhiti and Stasolla, 2009).

HD-Zip proteins participate in a variety of processes during plant growth and development, and some members are involved in general responses to abiotic stress (Chew et al., 2013; Perotti et al., 2017). For example, previous studies performed functional analysis of two HD-Zip I subfamily genes, *OsHOX22* and *OsHOX24*, in rice, demonstrating that these genes were highly upregulated under various abiotic stress conditions, and over-expression of *OsHOX24* in transgenic *Arabidopsis* (*Arabidopsis thaliana*) exhibits increased sensitivity to hormonal and abiotic stresses (Bhattacharjee et al., 2016). Studies indicated that *Arabidopsis* HD-Zip II subfamily gene *ATHB2* is a positive regulator involved in plant shade-avoidance responses (Carabelli et al., 2013). Studies have shown that members of the class IV HD-Zip transcription factor (HD-Zip IV) subfamily are preferentially expressed in epidermal cells and are primarily involved in the development and maintenance of plant epidermal cell layers (Nakamura et al., 2006), regulation of anthocyanins, lipid transport and stress responses (Elhiti and Stasolla, 2009). One cotton HD-Zip IV subfamily member gene, *GaHDG11*, was shown to improve osmotic tolerance in transgenic *Arabidopsis* (Chen et al., 2017).

At present, the HD-Zip gene family has been extensively studied in various plants, including *Arabidopsis*, maize (*Zea mays* L.) (Mao et al., 2016), rice (*Oryza sativa* L.) (Agalou et al., 2008), wheat (*Triticum aestivum* L.) (Yue et al., 2018), tomato (*Solanum lycopersicum*) (Zhang et al., 2014a), peach (*Prunus persica* L.) (Zhang et al., 2014b), grape (*Vitis vinifera*) (Li et al., 2017a), soybean (*Glycine max*) (Chen et al., 2014a), citrus (*Citrus sinensis*) (Ge et al., 2015), cotton (*Gossypium arboretum*) (Chen et al., 2017), poplar (*Populus trichocarpa*) (Hu et al.,

* Corresponding author. College of Eco-environmental Engineering, Qinghai University, Qinghai, 810016, China.
E-mail address: ruijunduan@163.com (R. Duan).

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2012), cassava (*Manihot esculenta*) (Ding et al., 2017), and other species. Some HD-Zip family studies involved abiotic stress (Agalou et al., 2008; Yue et al., 2018; Ding et al., 2017), of which salt, cold and drought stress are the most common, in addition to abiotic factors that have a significant impact on the growth, yield and quality of cereal crops (Zhu, 2016). Barley (*Hordeum vulgare* L.) is one of the world's oldest food and feed crops in the world. Barley is an important crop in China that has high economic value and strong abiotic resistance (Janska et al., 2013). As a representative crop of abiotic stress resistance, the latest physical, genetic and functional sequence assembly of the barley genome was completed in 2012 (International Barley Genome Sequencing et al., 2012) and 2016 (Mascher et al., 2017), providing important reference materials for future research on crop breeding and quality improvement. However, only sporadic studies on individual *HD-Zip* genes in barley have been performed (Komatsuda et al., 2007). Therefore, this study identified and characterized HD-Zip transcription factor family members in barley, including their structural characteristics, evolution and gene duplication events. In addition, analysis of tissue expression patterns was also examined using existing RNA-seq (RNA sequencing) data and expression profiles in different barley tissues and the response patterns after stress based on qRT-PCR technology. The results of this study provide a foundation for further study of the biological and molecular functions of HD-Zip transcription factors in barley.

2. Materials and methods

2.1. Identification and chromosomal localization of HD-Zip transcription factor family members in barley

Arabidopsis and rice HD-Zip proteins sequences were downloaded as query sequences (seed sequences) from the TAIR database (*Arabidopsis* information resource, http://web.arabidopsis.org), the RGAP database (Rice Genome Annotation Project, http://rice.plantbiology.msu.edu/) and PlantTFDB v4.0 database (Plant Transcription Factor Database, http://planttfdb.cbi.pku.edu.cn/). All known query sequences were used to identify barley HD-Zip by BLASTP searches with a cutoff e-value (-10) in IPK database (Leibniz Institute of Plant Genetics and Crop Plant Research, http://www.ipk-gatersleben.de/). In addition, the barley HD-Zip hmm search was performed, all obtained protein sequences were integrated and redundancy was removed by manual curating. The SMART (Simple Molecular Agriculture Research Tool, http://smart.embl-heidelberg.de/) and EMBL-Pfam (https://pfam.xfam.org/) databases were used to identify conserved protein domains. The remaining coincident sequences were considered putative barley *HD-Zip* genes (*HvHD-Zip*).

The ExPASy database (https://www.expasy.org/) was used to calculate the biochemical parameters of HvHD-Zip proteins. Plant-mPLoc (http://www.csbio.sjtu.edu.cn/bioinf/plant-multi/) predicts subcellular parameters. Finally, *HvHD-Zip* gene chromosome localization was obtained from genome annotation information in IPK, and MapChart software was used for visualization.

2.2. Phylogenetic and gene duplication analysis

Multiple sequence alignments were generated using ClutsalX1.81 for barley, rice and *Arabidopsis* HD-Zip protein sequences. The phylogenetic tree was constructed using MEGA7.0 software with the N-J (Neighbor-joining) method, and the resulting graph was creating using the iTOL online service (https://itol.embl.de/).

Gene duplication was investigated following the method described by Gu et al. and Kong et al. (Gu et al., 2002; Kong et al., 2013). The MCScanx and Circos programs were used to retrieve and map collinearity between different plant genomes.

2.3. Gene structure and protein conserved motif analysis

Gene structure was displayed using the GSDS program (Gene Structure Display Server, http://gsds.cbi.pku.edu.cn/). Conserved motifs were identified using the MEME program (Multiple Em for Motif Elicitation, http://meme-suite.org/tools/meme), and the TBtools software was used for visual optimization.

2.4. Tissue expression profile analysis of HvHD-Zip genes

Barley RNA-Seq data were retrieved from the IPK database, and transcript abundance of *HvHD-Zip* genes was assessed using FPKM (Fragments Per Kilobase of exon model per Million mapped reads) values. Heat maps of *HvHD-Zip* gene expression were generated based on their FPKM values using Cluster and TBtools software.

2.5. Plant materials and abiotic stress treatments

Barley seeds were surface sterilized with 10% H_2O_2, germinating seeds were planted in 1.5 L pots, filled with improved Hoagland's nutrient solution (macronutrients: 0.5 mM $MgSO_4$, 2 mM $Ca(NO_3)_2$, 2 mM KNO_3, 0.5 mM $NH_4H_2PO_4$, 0.5 mM $(NH_4)_2HPO_4$ and 0.5 mM NaCl; micronutrients: 6.25 μM H_3BO_3, 0.125 μM $CuSO_4$, 0.5 μM $MnSO_4$, 0.4 μM $ZnCl_2$, 0.19 μM Na_2MoO_4 and 18 μM FeNaEDTA) and plants were grown in a growth chamber at 22 °C with a 16 h photoperiod (12000 lux) and 8 h dark period. The barley variety 'Morex' was subjected to drought stress by application of 18% polyethylene glycol to the nutrient solution, cold stress by treatment at 4 °C in the cryogenic incubator (OSTC Percival LT-36VL, China) and salt stress by application of 200 mM NaCl to the nutrient solution for 48 h each. Root and leaf tissues samples were taken three times before application of the stressor (14 days after germination), 2 days after the stressor and 2 days after recovery. All conditions were replicated three times.

2.6. Quantitative real-time PCR analysis

Total RNA was isolated using the Plant RNA isolation kit (Takara, Shiga-ken, Japan) according to the manufacturer's instructions. RNA quality and quantity were assessed using 1.0% (w/v) agarose gels stained with ethidium bromide (EB) and a NanoDrop® spectrophotometer. qRT-PCR was performed using a LightCycler® 96 System (Roche, Indianapolis, IN, USA) with *HvqActin* as the reference gene. Three biological replicates for each sample were used, and expression levels were evaluated using the $2^{-\Delta\Delta Ct}$ method (Udvardi et al., 2008).

3. Results

3.1. Identification and analysis of HvHD-Zip genes

Thirty-two non-redundant *HD-Zip* genes were identified in barley, designated as *HvHD-Zip 1–32*, and were used for subsequent analysis (Table 1). Because of the differences in genome versions reported between 2012 and 2016 in which individual data could not be fully matched, the subsequent analysis relied primarily on the 2016 genomic data, except for the gene structure analysis. The full-length coding sequences (CDS) of *HvHD-Zip* genes ranged from 594 bp (*HvHD-Zip I 7* and *HvHD-Zip I 12*) to 2949 bp (*HvHD-Zip III 3*), with deduced protein lengths of 198–983 amino acids. The predicted grand average of hydropathicity (GRAVY < 0) for HvHD-Zip proteins revealed hydrophilicity. The predicted sub-cellular localization of HvHD-Zip proteins showed that they all localize to the nucleus, representing characteristics of HD-Zip transcription factors.

3.2. Chromosome localization of HvHD-Zip genes

Distribution of *HvHD-Zip* genes was determined by their

Table 1
Characteristics of putative homeodomain-leucine zipper (HD-Zip) proteins in barley.

Gene name	Gene ID in 2012 barley genome database	Gene ID in 2016 barley genome database	Identifier in the barley Genome	chr	Location (cM)	Protein length (aa)	CDS length	Isoelectric point	Molecular weight (Da)	Instability index	Aliphatic index
HvHD-Zip I 1	AK358936	HORVU2Hr1G092710.2	morex_contig_57812	2H	80.9490085	261	783	5.32	28977.23	72.59	59.92
HvHD-Zip I 2	MLOC_80844.1	HORVU2Hr1G097940.2	morex_contig_93135	2H	91.00566572	224	672	9.08	25352.79	67.53	70.54
HvHD-Zip I 3	MLOC_55339.1	N/A	morex_contig_39872	3H	52.76203966	222	666	9.27	24729.11	70.05	65.54
HvHD-Zip I 4	MLOC_52115.1	HORVU4Hr1G075180.3	morex_contig_37292	4H	80.66572238	331	993	6.27	36442.79	67.91	58.13
HvHD-Zip I 5	MLOC_71555.2	HORVU4Hr1G078410.2	morex_contig_60056	4H	81.56916148	317	951	4.91	35067.69	63.72	58.71
HvHD-Zip I 6	AK354063	HORVU5Hr1G070260.1	morex_contig_1175226	5H	72.5	267	801	4.63	28726.76	61.05	57.9
HvHD-Zip I 7	MLOC_77955.2	HORVU5Hr1G067010.2	morex_contig_78074	5H	62.5	198	594	9.04	21518.23	76.64	61.31
HvHD-Zip I 8	AK356850	HORVU5Hr1G059410.2	morex_contig_50226	5H	49.65277778	296	888	5.02	32327.78	67.97	65.1
HvHD-Zip I 9	AK376953	HORVU5Hr1G081090.1	morex_contig_56855	5H	97.29166667	250	750	5.03	27694.81	59.16	56
HvHD-Zip I 10	MLOC_5668.1	HORVU6Hr1G072810.6	morex_contig_136532	6H	68.20113314	339	1017	4.62	37240.83	56.9	62.57
HvHD-Zip I 11	MLOC_66350.2	HORVU6Hr1G061390.1	morex_contig_51430	6H	59.63172805	252	756	5.58	28292.5	70.14	68.25
HvHD-Zip I 12	MLOC_56648.1	N/A	morex_contig_41023	6H	49.22096317	198	594	9.88	21508.24	49.43	79.44
HvHD-Zip I 13	AK359060	N/A	morex_contig_270956	N/A	N/A	279	837	9.25	29315.05	50.87	66.95
HvHD-Zip I 14	AK249428.1	HORVU1Hr1G082910.2	morex_contig_136615	N/A	N/A	298	894	7.12	32708.75	60.36	69.19
HvHD-Zip I 15	AK365238	HORVU7Hr1G018780.2	morex_contig_2425015	N/A	N/A	308	924	9.5	32244.21	54.93	64.25
HvHD-Zip I 16	MLOC_60848.4	HORVU4Hr1G065900.5	morex_contig_44850	N/A	N/A	281	843	7.67	30293.22	62.69	67.51
HvHD-Zip II 1	AK366966	HORVU1Hr1G055930.1	morex_contig_39504	1H	52.54957507	321	963	9.27	34425.82	74.19	66.39
HvHD-Zip II 2	N/A	HORVU3Hr1G000250.1	N/A	3H	N/A	211	633	9.11	23366.69	49.58	76.81
HvHD-Zip II 3	N/A	HORVU5Hr1G049790.1	N/A	5H	N/A	225	675	9.83	24867.16	62.87	66.74
HvHD-Zip II 4	N/A	HORVU7Hr1G098230.1	N/A	7H	N/A	225	675	9.83	24867.16	62.87	66.74
HvHD-Zip III 1	AK362009	HORVU1Hr1G041790.2	morex_contig_45665	1H	47.82772584	840	2520	5.65	92197.25	51.31	84.55
HvHD-Zip III 2	AK365312	HORVU3Hr1G026990.29	morex_contig_8318	3H	45.54994115	845	2535	5.77	91712.01	51.67	87.54
HvHD-Zip III 3	MLOC_58644.1	N/A	morex_contig_42852	5H	N/A	983	2949	7.57	106917.04	47.84	82.61
HvHD-Zip III 4	AK364215	N/A	morex_contig_241849	5H	120.0694444	867	2601	6.12	106917.04	48.44	84.07
HvHD-Zip IV 1	MLOC_52077.2	HORVU1Hr1G050620.18	morex_contig_37272	1H	48.0878187	884	2652	5.61	95624.2	49.48	76.99
HvHD-Zip IV 2	MLOC_65829.2	HORVU2Hr1G106970.2	morex_contig_50724	2H	112.3229462	777	2331	5.59	83628.5	46.75	82.84

(*continued on next page*)

Table 1 (*continued*)

Gene name	Gene ID in 2012 barley genome database	Gene ID in 2016 barley genome database	Identifier in the barley Genome	chr	Location (cM)	Protein length (aa)	CDS length	Isoelectric point	Molecular weight (Da)	Instability index	Aliphatic index
HvHD-Zip IV 3	AK365794	HORVU5Hr1G080700.2	morex_contig_134844	5H	95.90277778	774	2322	6.06	83633	49.74	82.82
HvHD-Zip IV 4	MLOC_61420.1	HORVU6Hr1G065300.3	morex_contig_45477	6H	61.32081082	748	2244	5.62	81308.7	50.26	84.41
HvHD-Zip IV 5	MLOC_52316.1	HORVU7Hr1G073440.1	morex_contig_37438	7H	70.60906516	796	2388	5.99	85553.2	50.53	76.14
HvHD-Zip IV 6	MLOC_13375.1	HORVU3Hr1G077690.2	morex_contig_1565479	3H	N/A	665	1995	7.14	73331.5	51.34	81.97
HvHD-Zip IV 7	MLOC_35104.1	HORVU5Hr1G083940.3	morex_contig_245609	5H	N/A	776	2328	5.5	84589.2	38.55	83.23
HvHD-Zip IV 8	MLOC_15014.2	HORVU6Hr1G079410.4	morex_contig_1569621	6H	N/A	685	2055	5.51	86291.1	48.5	81.87

Note: N/A indicates that the gene contained incomplete data or was found not in another barley database. Protein stability is expressed according to the instability index. When the value is less than 40, the protein was stable, and a value greater than 40 indicates that the protein might be unstable. The hydrophilicity of the protein is expressed by the hydrophobic average. If the value is positive, it indicates the protein's hydrophobicity, and a negative value indicates hydrophilicity.

chromosomal position. Except for four members, *HvHD-Zip I 3*, *HvHD-Zip II 1*, *and HvHD-Zip III 3–4*, which lack physical location information, the remaining 27 *HvHD-Zip* genes were mapped onto barley's 7 chromosomes (Fig. 1). There are 3 or 4 *HvHD-Zip* genes distributed on all the other chromosomes, except for 7 genes on chromosome 5H, indicating that they are more uniform overall.

3.3. Classification and structural analysis of HvHD-Zip

To gain insight into the relationship between classification and structure, we determined conserved motif structures of HvHD-Zip protein and gene structures. A simple phylogenetic tree was generated with HvHD-Zip proteins, showing that the 32 members are divided into four subfamilies (Fig. 2A). The conserved motif analysis results revealed that the same subfamily of HvHD-Zip proteins appear to have similar motifs (Fig. 2B). Among these, conserved motifs encoding the HD and LZ domains were found in all HvHD-Zip proteins, with motif 3 specifying the HD domain and motif 1 corresponding to the LZ domain. Motif 2, motif 8, motif 9, motif 16, motif 17, and motif 19 correspond to the START (steroidogenic acute regulatory protein-related lipid transfer) domain, and motif 11 is related to the SAD (START-associated) domain, which was present in HD-Zip III and HD-Zip IV subfamily proteins. The HD-Zip I and HD-Zip II subfamilies exhibit a simple distribution of motifs that are completely different from the HD-Zip III and HD-Zip IV subfamilies, comprising fewer motifs, and this difference may be related to differences in gene structure between different subfamilies.

The gene structure of 29 *HvHD-Zip* genes demonstrated large differences in exon-intron arrangement (Fig. 3). Of note, the *HD-Zip II 1* gene has a longer 3′-UTR (untranslated region) than the 5′-UTR and has only one intron. In the HD-Zip I subfamily, most members have 2 to 4 exons, and their distribution is different with a relatively simple gene structure. Different from HD-Zip I and HD-Zip II subfamilies, the HD-Zip IV subfamily members have 5 to 11 exons, and the HD-Zip III subfamily has the most complex gene structure with the largest number of exons (17–20). The causes of these gene structure inconsistencies between subfamilies may be related to their unique functions.

3.4. Phylogenetic and synteny analysis of HvHD-Zip genes

Transcription factor families transitioned with plants' transition to land, and it is not difficult to understand the important role of transcription factors in plant development and evolution (Romani et al., 2018). To better understand the evolutionary relationship of *HD-Zip* genes among barley and other species, a complex phylogenetic tree was generated comprising barley, *Arabidopsis* and rice HD-Zip protein sequences (Fig. 4A). Phylogenetic analysis showed a total of 121 HD-Zip proteins were also separated in four groups, except the HD-Zip II subfamily members. The 34 HD-Zip IV subfamily members and 14 HD-Zip III subfamily members clustered separately. The 38 HD-Zip I subfamily members were clustered together, however, 9 genes are still mixed in the branch of the HD-Zip II subfamily with 26 members. According to this analysis, HD-Zip I and HD-Zip II subfamilies' conserved domains are similar and cannot be accurately distinguished.

Next, synteny analysis among barley with *Arabidopsis*, rice and wheat was conducted (Fig. 4B). Among the synteny analysis, there are 26 genome-wide collinear blocks between barley and *Arabidopsis* and 3 syntenic HD-Zip gene pairs (9.4%), among which 231 genome-wide collinear blocks exist between barley and rice, including 14 gene pairs (43.8%) and 499 collinear blocks between barley and wheat with 25 gene pairs (78.1%). Monocot rice, wheat and barley HD-Zip gene collinear rate is significantly higher than the dicot *Arabidopsis*. Interestingly, no segmental or tandem duplication events were identified in the HvHD-Zip gene family from the 1593 gene pairs in genome-wide duplication events. Hence, the numbers of barley HD-Zip gene families are further reduced by counting the number of HD-Zip transcription factors

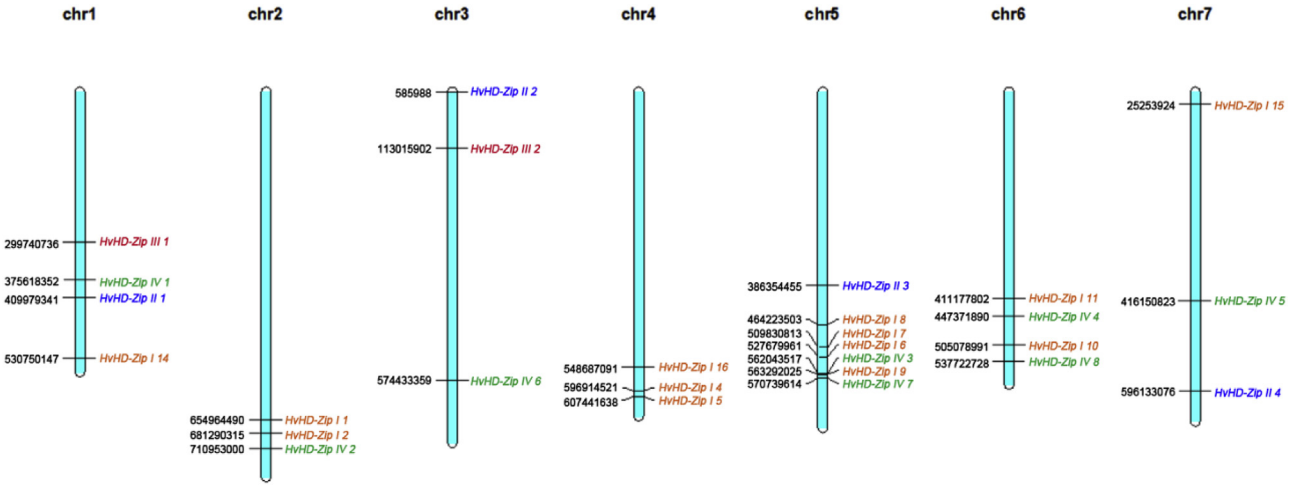

Fig. 1. Chromosomal localization of *HvHD-Zip* genes on the 7 barley chromosomes. Chromosome numbers are shown at the top of each vertical bar. Position data are shown on the left side of the chromosome, and corresponding *HvHD-Zip* gene names are connected by a short line on the right side, with different colors representing different subfamilies. chr, chromosomes. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

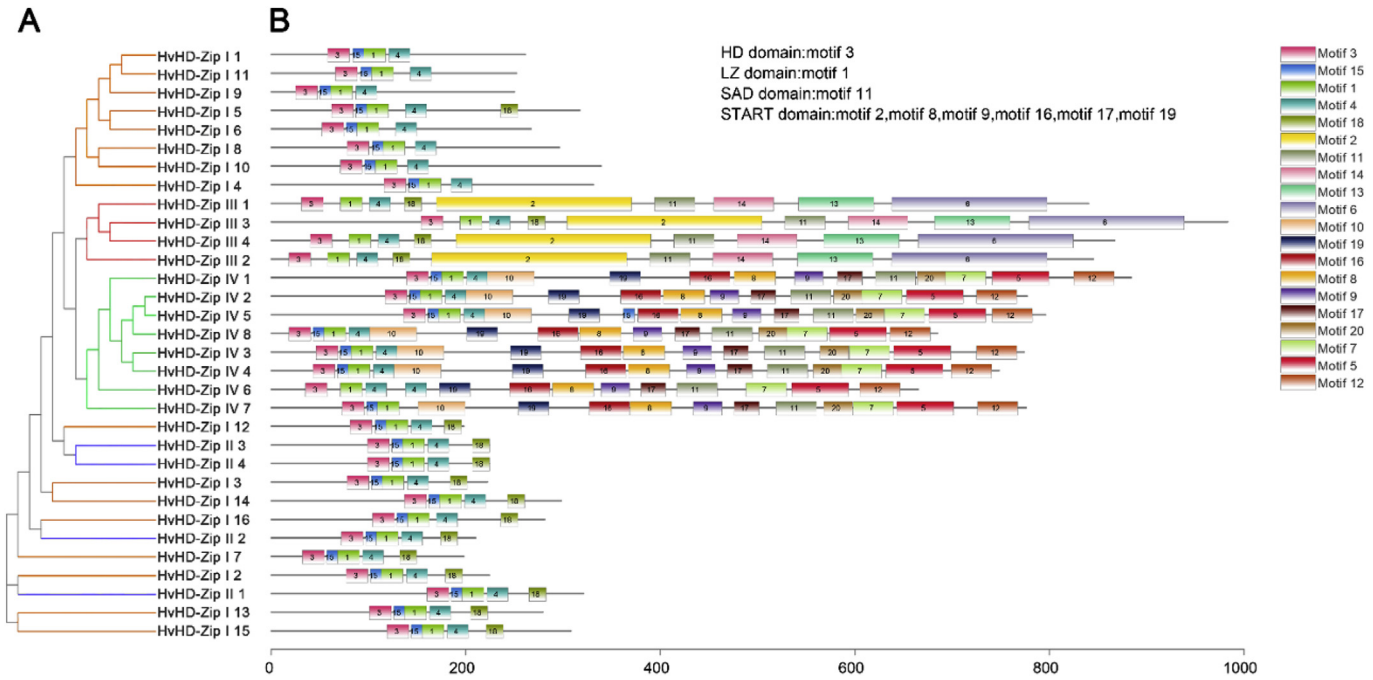

Fig. 2. Phylogenetic tree and protein structure of HvHD-Zip. (A) The phylogenetic tree was constructed by N-J method with 1000 bootstrap replicates. (B) Conserved motifs were identified by MEME. Motifs are indicated by different colored boxes with the motif number, while non-conserved sequences are represented by gray lines. Length of motifs is proportionally represented.

in 13 different species, including barley (Table 2).

3.5. Expression profiles of HvHD-Zip genes in different tissues

Based on FPKM values, a heatmap of *HvHD-Zip* genes was created (Fig. 5). These 32 genes have differential expression patterns and cluster on two branches, a and b. Branch a has 11 genes with high expression levels, and branch b contains the remaining 21 genes (67.7%) with lower expression levels in different tissues. Further analysis indicates that most of the *HD-Zip* genes in barley are expressed at low levels, but gene expression is still tissue-specific.

3.6. Expression profiles of HvHD-Zip genes in response to abiotic stress

In the natural environment, stresses on plant seedling growth and development are mostly temporary and fluctuate. To approximate the natural growth state of barley seedlings, short-term drought, cold and salt stress were applied to barley seedlings, and relatively quantitative analysis of *HvHD-Zip* gene expression in root and leaf tissue was performed. As expected, the response patterns of *HvHD-Zip* genes are very different under different stresses and in different tissues (Fig. 6, Fig. S1). Within the three stresses, *HvHD-Zip* gene response was the strongest in root tissues under drought stress in which expression of 17 genes (58.6%) was significantly different from that of controls (Fig. S1A). However, in root, only the *HvHD-Zip I 9* gene responded significantly to cold stress (Fig. S1B), and 9 genes responded significantly to salt stress (Fig. S1C). The *HvHD-Zip* genes are more sensitive to drought stress than barley root tissue, and in leaf tissue, the *HvHD-Zip* genes responded more significantly to cold stress. Overall, the *HvHD-Zip* genes are less responsive in leaf tissue than in root tissue. In addition, under

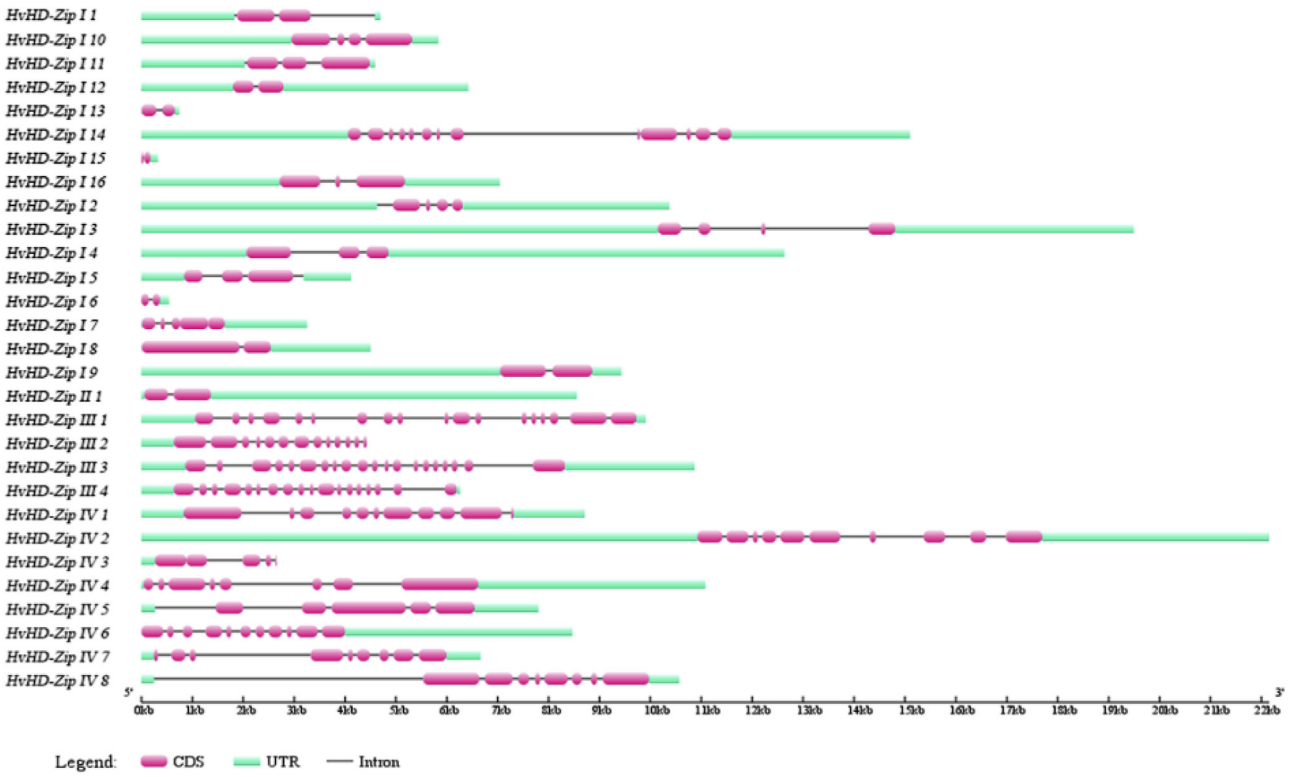

Fig. 3. Exon-intron gene structures of *HvHD-Zip*. Gene structure analysis was performed using GSDS. Length of exons and introns for each *HvHD-Zip* gene are proportionally displayed.

different stress treatments, the response levels of different subfamily members are also inconsistent. HD-Zip I and HD-Zip IV subfamilies responded significantly more than HD-Zip II and HD-Zip III subfamilies. Typically, the *HvHD-Zip III 3* gene does not respond to these three stresses, and combined with the function of this subfamily, it is speculated that the *HvHD-Zip III 3* gene is not sensitive to stress and may play a role in plant development.

There were 9 genes that responded in root tissue to the removal of drought stress. Eleven genes responded in leaf tissue, and 5 genes responded in root tissue to the removal of cold stress. Five genes respond in leaf tissue, and only one gene responded in root tissue in response to the removal of salt stress, while 8 genes responded in leaf tissue. A stronger response exists in leaf tissue than in root tissue in response to the removal of a stressor.

4. Discussion

As a traditional cultivated crop, barley exhibits good resistance to stress (Ligaba and Katsuhara, 2010), but there is an overall lack of research and no comprehensive reports on the HD-Zip transcription factor family in barley. In this study, 32 *HD-Zip* genes were identified in barley, fewer than in *Arabidopsis* (48), rice (41), wheat (66), maize (55), and soybean (88). This result may be related to the larger and more repetitive barley genome (5.1 Gb, Giga base pairs), and the other may be associated with the lack of segmental and tandem duplication events within barley *HD-Zip* genes. We speculate that there may be unknown parts within the barley genome, which will inevitably affect the quantitative statistics of the HvHD-Zip gene family. In addition, we did not find evidence of the HvHD-Zip gene family's expansion.

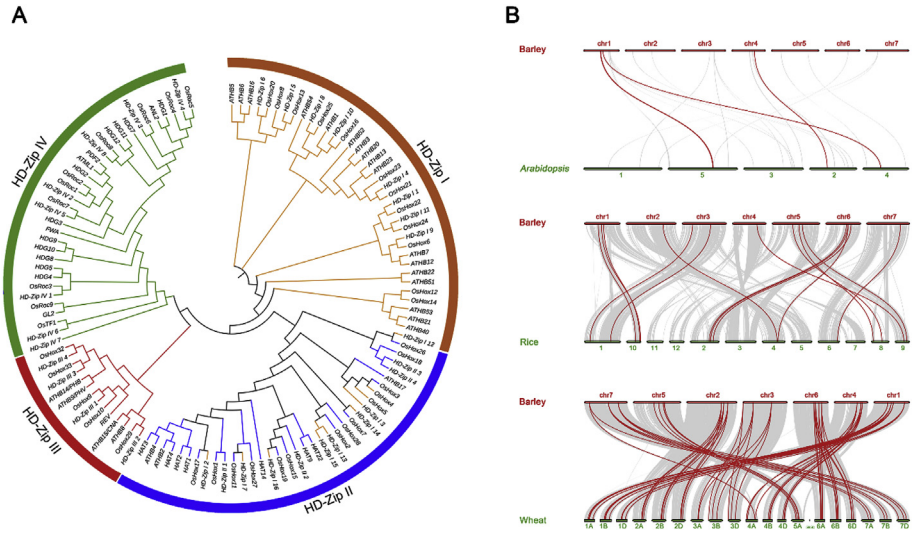

Fig. 4. Phylogenetic and synteny analysis of HD-Zip proteins. (A) Phylogenetic tree constructed in three species of barley (32), *Arabidopsis* (48), and rice (41). Different color blocks or lines represent different subfamily classifications. (B) Synteny analysis of *HvHD-Zip* genes. Colored bars connecting two chromosomal regions denote syntenic regions, gray lines represent orthologous gene pairs within genomes, and red lines represent orthologous gene pairs within the HD-Zip gene family. Chromosome numbers are shown at the top or bottom of each horizontal bar. chr, chromosomes. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 2
Number of *HD-Zip* genes in 13 different species.

Category	Barley	*Arabidopsis*	Rice	Wheat	Maize	*Setaria italica* L.	Soybean	citrus	peach	grapes	cassava	tea plant	Poplar
HD-Zip I	16	17	14	20	17	13	30	16	14	13	23	20	27
HD-Zip II	4	10	12	17	18	13	27	2	7	7	14	9	14
HD-Zip III	4	5	5	4	5	5	12	4	4	5	9	3	5
HD-Zip IV	8	16	10	5	15	16	19	5	8	8	11	1	17
Total	32	48	41	46	55	47	88	27	33	33	57	33	63

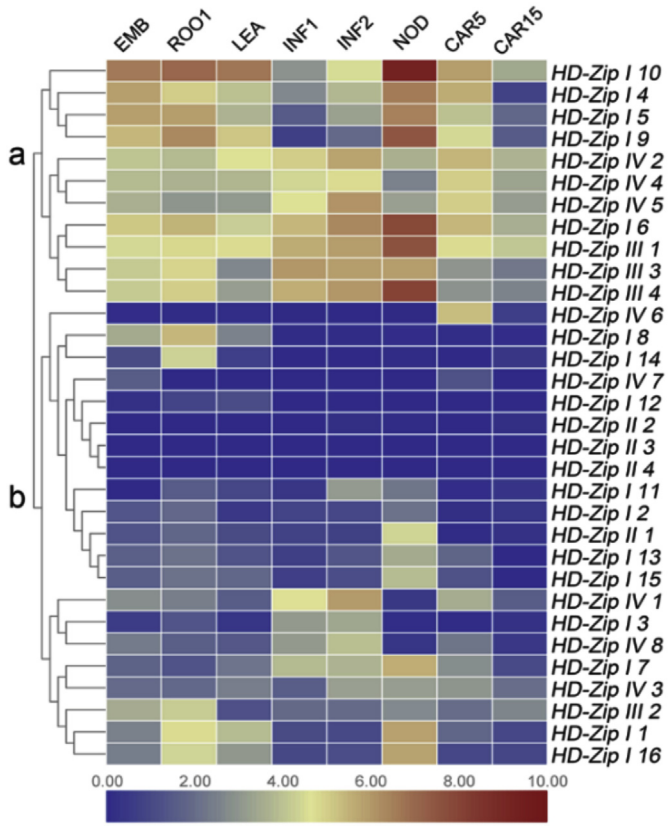

Fig. 5. Expression profiles of *HvHD-Zip* genes in different tissues. RNA-seq data from eight tissues at different developmental stages of barley seedlings. EMB: 4-day embryos; ROO1: Roots from seedlings (10 cm shoot stage); LEA: Shoots from seedlings (10 cm shoot stage); INF1: Young developing inflorescences (5 mm); INF2: Developing inflorescences (1–1.5 cm); NOD: Developing tillers, 3rd internode (42 DAP); CAR5: Developing grain (5 DAP); CAR15: Developing grain (15 DAP) was used to analyze tissues expression patterns. Expression level is shown using color as the scale with red representing high expression level and blue representing low expression level. a and b represent two different branches. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Gene and protein structure may also be related to the control of gene expression patterns in tissues or in response to abiotic stress (Elhiti and Stasolla, 2009). In general, most of the conserved domains and motifs in the same subfamily were similar, which might be related to a specific function and phylogenetic clustering. Further analysis of the structure, conserved motifs and phylogenetic tree of the four HvHD-Zip gene subfamilies revealed its significant family classification features (Fig. 2). Although the HD-Zip I subfamily contains only HD and LZ domains and lacks a conserved N-terminus compared to the HD-Zip II subfamily, the HvHD-Zip I gene family structure is not significantly different from the HvHD-Zip II subfamily. However, the 32 *HvHD-Zip* genes were clearly divided into four clades (Fig. 2A). Interestingly, the phylogenetic tree shows that these genes are clearly paired into 11 gene pairs, but analysis of tissue expression patterns in barley revealed that there is not a completely single positive or negative correlation between phylogenetic relationships and tissues expression patterns (Fig. 5). Identically to *HvHD-Zip I 13-HvHD-Zip I 15*, *HvHD-Zip III 3-HvHD-Zip III 4*, and *HvHD-Zip IV 2-HvHD-Zip IV 5*, these three gene pairs have very similar tissue expression patterns. However, the *HvHD-Zip I and 1-HvHD-Zip I 11* gene pair has a completely inconsistent tissue expression pattern, while *HvHD-Zip I 1* and *HvHD-Zip I 16* are not a gene pair but have highly consistent tissue expression patterns. Therefore, tissue expression regulation is a complex system, and close evolutionary relationships are not necessary for similar tissue expression patterns between genes. Similar findings have been reported in grapes (Li et al., 2017b).

Many crop studies have proven that when plants are stimulated by the environment, transcription factors typically upregulate gene expression to cope with stress (Yamaguchi-Shinozaki and Shinozaki, 2006), and the HD-Zip protein family is a key regulatory transcription factor, unique to higher plants, that has been widely studied in many crop plants. At present, homeodomain-leucine zipper I class genes, *vrs1 and hox2,* have been studied in barley, and they played a key role in barley domestication (Komatsuda et al., 2007). However, the rigor of domain validation in this study indicated that these two genes are not involved. Highland barley transcriptome studies have shown that HD-Zip transcription factors may play an important role in highland domestication, especially in highland stress adaptation (Duan et al., 2015; Chen et al., 2014b), but there are no other barley HD-Zip studies, particularly in stress research. In this study, barley *HvHD-Zip* gene expression levels in response to drought, cold and salt stress conditions were verified by qRT-PCR (Fig. 6, Fig. S1). Seventeen (root tissue) and 4 (leaf tissue) stress response genes responded to drought. One (root tissue) and 7 (leaf tissue) genes responded to cold stress, and 9 (root tissue) and 4 (leaf tissue) genes responded to salt stress. Some genes that are persistently and significantly differentially expressed under stress and removal of stress may act in a long-lasting or slow-acting manner in response to the plant stress tolerant process and may be less sensitive to stress. Hence, these genes should be considered candidate genes for future non-biological functional studies.

Three orthologous *HD-Zip* gene pairs were identified by synteny analysis between barley and *Arabidopsis* (Fig. 4B), *HvHD-Zip I 16-HAT9*, *HvHD-Zip II 1-HAT4* and *HvHD-Zip IV 1- HDG5* (HD-Zip IV subfamily). *HvHD-Zip I 16*, *HvHD-Zip II 1* and *HvHD-Zip IV 1* genes showed decreased expression levels in different barley tissues (Fig. 5), and these three genes have a tissue expression specificity similar to that of *HvHD-Zip* genes. After three stressor treatments, *HvHD-Zip I 16* and *HvHD-Zip II 1* genes were detected only in root tissues under drought stress, suggesting that they may play a regulatory role during drought stress in barley roots. The *HvHD-Zip IV 1* gene showed a wide range of differential expression after treatment with different stressors, especially in barley root tissue. Three orthologous genes, in which the *HAT4* gene in *Arabidopsis* belongs to the HD-Zip II subfamily with the *HAT9* gene and shares a common evolutionary history, have also been reported in earlier studies that darkness and *HAT4* gene overproduction may act in a synergistic manner to promote plant growth in the light (Schena et al., 1993). Of course, overexpression mutants also exhibit obvious phenotypic changes, such as early flowering (Bou-Torrent et al., 2012; Kollmer et al., 2011), suggesting that some genes may not only respond to abiotic stress but may also play an important role in regulating plant

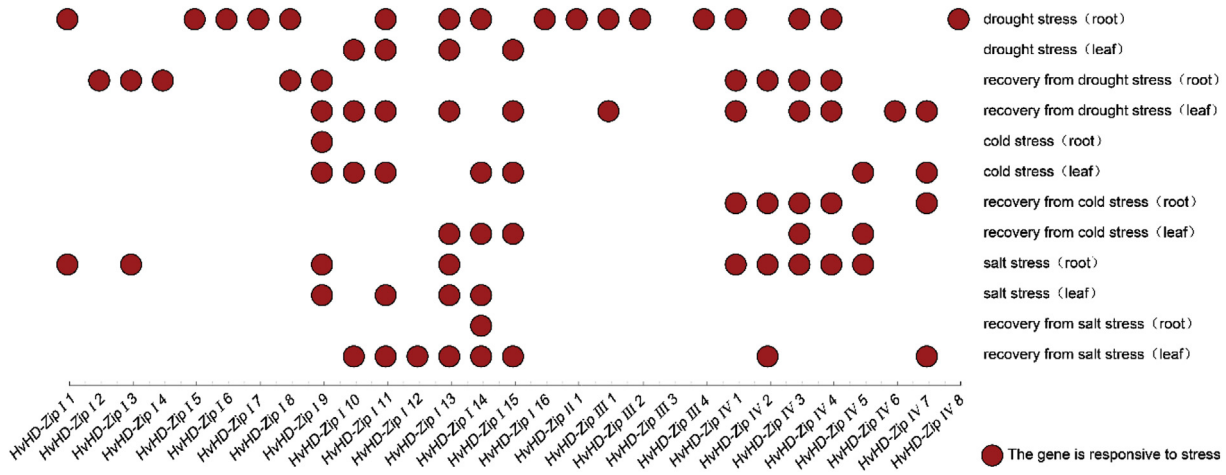

Fig. 6. Expression profiles of *HvHD-Zip* genes in response to different stressors. Solid red circles indicate that expression levels of the gene are significantly different from that of the control in response to the stressor. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

growth and development.

Regarding how HD-Zip transcription factors regulate gene expression, it has been reported that *OsTF1L* in rice is a key regulatory gene in the drought stress response mechanism. *OsTF1L* directly binds to the promoters of lignin biosynthesis and drought-related genes, enhancing drought tolerance through lignin biosynthesis and stomatal closure in rice (Bang et al., 2019). Similarly, the HD-Zip transcription factors are directly involved in the regulation of abscisic acid homeostasis and signaling (Sessa et al., 2018). *PpHB22* was identified and characterized from 47 *HD-Zip* genes in the pear genome and is a negative regulator of plant growth associated with the ABA response pathway, which functions upstream of *PpDAM1* (dormancy associated MADS-box 1) (Yang et al., 2018). Another study demonstrates that stress-induced expression of *AtHDG11* improved drought and salt tolerance through upregulation of known stress responsive genes in peanut (Banavath et al., 2018). These findings provide an important reference for future functional research of barley HD-Zip transcription factor genes.

In summary, this study systematically identifies and characterizes the levels of the barley HD-Zip gene family in the entire genome. Thirty-two barley *HD-Zip* genes belonging to groups I to IV were identified and showed a uniform distribution across the seven barley chromosomes. Genome-wide gene duplication event analysis found no segmental or tandem duplication events in the barley HD-Zip gene family. Analysis of RNA-Seq data revealed that the *HvHD-Zip* genes exhibited low expression levels in different tissues. The response patterns to drought, cold and salt stress showed that the HvHD-Zip gene family exhibited significant responses under drought stress in root tissues and that HD-Zip I and HD-Zip IV subfamily members were generally more sensitive to abiotic stresses. Overall, this study lays the foundation for future research on the molecular mechanisms of barley stress adaption.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Author contribution

Ruijun duan and huiyan xiong conceived the research plan and designed the experiments. Yuan li was the primary author involved in performed the experiments and writing the original draft of the paper. Duojie cuo and xiongxiong wu contributed to review and editing of the paper, providing helpful comments and discussions. All authors read and approved the final manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.05.026.

References

Agalou, A., Purwantomo, S., Overnas, E., Johannesson, H., Zhu, X., Estiati, A., de Kam, R.J., Engstrom, P., Slamet-Loedin, I.H., Zhu, Z., Wang, M., Xiong, L., Meijer, A.H., Ouwerkerk, P.B., 2008. A genome-wide survey of HD-Zip genes in rice and analysis of drought-responsive family members. Plant Mol. Biol. 66, 87–103.

Ariel, F.D., Manavella, P.A., Dezar, C.A., Chan, R.L., 2007. The true story of the HD-Zip family. Trends Plant Sci. 12, 419–426.

Banavath, J.N., Chakradhar, T., Pandit, V., Konduru, S., Guduru, K.K., Akila, C.S., Podha, S., Puli, C.O.R., 2018. Stress inducible overexpression of *AtHDG11* leads to improved drought and salt stress tolerance in peanut (*Arachis hypogaea* L.). Front. Chem. 6, 34. https://doi.org/10.3389/fchem.2018.00034.

Bang, S.W., Lee, D.K., Jung, H., Chung, P.J., Kim, Y.S., Choi, Y.D., Suh, J.W., Kim, J.K., 2019. Overexpression of *OsTF1L*, a rice HD-Zip transcription factor, promotes lignin biosynthesis and stomatal closure that improves drought tolerance. Plant Biotechnol. J 17, 118–131. https://doi.org/10.1111/pbi.12951.

Bhattacharjee, A., Khurana, J.P., Jain, M., 2016. Characterization of rice homeobox genes, *OsHOX22* and *OsHOX24*, and over-expression of *OsHOX24* in transgenic *Arabidopsis* suggest their role in abiotic stress response. Front. Plant Sci. 7, 627. https://doi.org/10.3389/fpls.2016.00627.

Bou-Torrent, J., Salla-Martret, M., Brandt, R., Musielak, T., Palauqui, J.C., Martinez-Garcia, J.F., Wenkel, S., 2012. *ATHB4* and *HAT3*, two class II HD-ZIP transcription factors, control leaf development in *Arabidopsis*. Plant Signal. Behav. 7, 1382–1387.

Carabelli, M., Turchi, L., Ruzza, V., Morelli, G., Ruberti, I., 2013. Homeodomain-Leucine Zipper II family of transcription factors to the limelight: central regulators of plant development. Plant Signal. Behav. 8, e25447.

Chen, X., Chen, Z., Zhao, H., Zhao, Y., Cheng, B., Xiang, Y., 2014a. Genome-wide analysis of soybean HD-Zip gene family and expression profiling under salinity and drought treatments. PLoS One 9, e87156.

Chen, X., Long, H., Gao, P., Deng, G., Pan, Z., Liang, J., Tang, Y., Tashi, N., Yu, M., 2014b. Transcriptome assembly and analysis of Tibetan Hulless Barley (*Hordeum vulgare* L. var. *nudum*) developing grains, with emphasis on quality properties. PLoS One 9, e98144.

Chen, E., Zhang, X., Yang, Z., Wang, X., Yang, Z., Zhang, C., Wu, Z., Kong, D., Liu, Z., Zhao, G., Butt, H.I., Zhang, X., Li, F., 2017. Genome-wide analysis of the HD-ZIP IV transcription factor family in *Gossypium arboreum* and *GaHDG11* involved in osmotic tolerance in transgenic *Arabidopsis*. Mol. Genet. Genom. 292, 593–609.

Chew, W., Hrmova, M., Lopato, S., 2013. Role of Homeodomain leucine zipper (HD-Zip) IV transcription factors in plant development and plant protection from deleterious environmental factors. Int. J. Mol. Sci. 14, 8122–8147.

Ding, Z., Fu, L., Yan, Y., Tie, W., Xia, Z., Wang, W., Peng, M., Hu, W., Zhang, J., 2017.

Genome-wide characterization and expression profiling of HD-Zip gene family related to abiotic stress in cassava. PLoS One 12, e0173043.
Duan, R., Xiong, H., Wang, A., Chen, G., 2015. Molecular mechanisms underlying hull-caryopsis adhesion/separation revealed by comparative transcriptomic analysis of covered/naked barley (*Hordeum vulgare* L.). Int. J. Mol. Sci. 16, 14181–14193.
Elhiti, M., Stasolla, C., 2009. Structure and function of homodomain-leucine zipper (HD-Zip) proteins. Plant Signal. Behav. 4, 86–88.
Ge, X.X., Liu, Z., Wu, X.M., Chai, L.J., Guo, W.W., 2015. Genome-wide identification, classification and analysis of HD-ZIP gene family in citrus, and its potential roles in somatic embryogenesis regulation. Gene 574, 61–68.
Gu, Z., Cavalcanti, A., Chen, F.C., Bouman, P., Li, W.H., 2002. Extent of gene duplication in the genomes of Drosophila, nematode, and yeast. Mol. Biol. Evol. 19, 256–262.
Hu, R., Chi, X., Chai, G., Kong, Y., He, G., Wang, X., Shi, D., Zhang, D., Zhou, G., 2012. Genome-wide identification, evolutionary expansion, and expression profile of homeodomain-leucine zipper gene family in poplar (*Populus trichocarpa*). PLoS One 7, e31149.
International Barley Genome Sequencing, C., Mayer, K.F., Waugh, R., Brown, J.W., Schulman, A., Langridge, P., Platzer, M., Fincher, G.B., Muehlbauer, G.J., Sato, K., Close, T.J., Wise, R.P., Stein, N., 2012. A physical, genetic and functional sequence assembly of the barley genome. Nature 491, 711–716.
Janska, A., Hodek, J., Svoboda, P., Zamecnik, J., Prasil, I.T., Vlasakova, E., Milella, L., Ovesna, J., 2013. The choice of reference gene set for assessing gene expression in barley (*Hordeum vulgare* L.) under low temperature and drought stress. Mol. Genet. Genom. 288, 639–649.
Johannesson, H., Wang, Y., Engstrom, P., 2001. DNA-binding and dimerization preferences of *Arabidopsis* homeodomain-leucine zipper transcription factors in *vitro*. Plant Mol. Biol. 45, 63–73.
Kollmer, I., Werner, T., Schmulling, T., 2011. Ectopic expression of different cytokinin-regulated transcription factor genes of *Arabidopsis thaliana* alters plant growth and development. J. Plant Physiol. 168, 1320–1327.
Komatsuda, T., Pourkheirandish, M., He, C., Azhaguvel, P., Kanamori, H., Perovic, D., Stein, N., Graner, A., Wicker, T., Tagiri, A., Lundqvist, U., Fujimura, T., Matsuoka, M., Matsumoto, T., Yano, M., 2007. Six-rowed barley originated from a mutation in a homeodomain-leucine zipper I-class homeobox gene. Proc. Natl. Acad. Sci. U. S. A. 104, 1424–1429.
Kong, X., Lv, W., Zhang, D., Jiang, S., Zhang, S., Li, D., 2013. Genome-wide identification and analysis of expression profiles of maize mitogen-activated protein kinase kinase kinase. PLoS One 8, e57714.
Li, Z., Zhang, C., Guo, Y., Niu, W., Wang, Y., Xu, Y., 2017a. Evolution and expression analysis reveal the potential role of the HD-Zip gene family in regulation of embryo abortion in grapes (*Vitis vinifera* L.). BMC Genomics 18, 744.
Li, Y., Zhu, Y., Yao, J., Zhang, S., Wang, L., Guo, C., van Nocker, S., Wang, X., 2017b. Genome-wide identification and expression analyses of the homeobox transcription factor family during ovule development in seedless and seeded grapes. Sci. Rep. 7, 12638.
Ligaba, A., Katsuhara, M., 2010. Insights into the salt tolerance mechanism in barley (*Hordeum vulgare*) from comparisons of cultivars that differ in salt sensitivity. J. Plant Res. 123, 105–118.
Mao, H., Yu, L., Li, Z., Liu, H., Han, R., 2016. Molecular evolution and gene expression differences within the HD-Zip transcription factor family of *Zea mays* L. Genetica 144, 243–257.
Mascher, M., Gundlach, H., Himmelbach, A., Beier, S., Twardziok, S.O., Wicker, T., Radchuk, V., Dockter, C., Hedley, P.E., Russell, J., Bayer, M., Ramsay, L., Liu, H., Haberer, G., Zhang, X.Q., Zhang, Q., Barrero, R.A., Li, L., Taudien, S., Groth, M., Felder, M., Hastie, A., Simkova, H., Stankova, H., Vrana, J., Chan, S., Munoz-Amatriain, M., Ounit, R., Wanamaker, S., Bolser, D., Colmsee, C., Schmutzer, T., Aliyeva-Schnorr, L., Grasso, S., Tanskanen, J., Chailyan, A., Sampath, D., Heavens, D., Clissold, L., Cao, S., Chapman, B., Dai, F., Han, Y., Li, H., Li, X., Lin, C., McCooke, J.K., Tan, C., Wang, P., Wang, S., Yin, S., Zhou, G., Poland, J.A., Bellgard, M.I., Borisjuk, L., Houben, A., Dolezel, J., Ayling, S., Lonardi, S., Kersey, P., Langridge, P., Muehlbauer, G.J., Clark, M.D., Caccamo, M., Schulman, A.H., Mayer, K.F.X., Platzer, M., Close, T.J., Scholz, U., Hansson, M., Zhang, G., Braumann, I., Spannagl, M., Li, C., Waugh, R., Stein, N., 2017. A chromosome conformation capture ordered sequence of the barley genome. Nature 544, 427–433.
Nakamura, M., Katsumata, H., Abe, M., Yabe, N., Komeda, Y., Yamamoto, K.T., Takahashi, T., 2006. Characterization of the class IV homeodomain-Leucine Zipper gene family in *Arabidopsis*. Plant Physiol. 141, 1363–1375.
Perotti, M.F., Ribone, P.A., Chan, R.L., 2017. Plant transcription factors from the homeodomain-leucine zipper family I. Role in development and stress responses. IUBMB Life 69, 280–289.
Romani, F., Reinheimer, R., Florent, S.N., Bowman, J.L., Moreno, J.E., 2018. Evolutionary history of HOMEODOMAIN LEUCINE ZIPPER transcription factors during plant transition to land. New Phytol. 219, 408–421.
Schena, M., Lloyd, A.M., Davis, R.W., 1993. The *HAT4* gene of *Arabidopsis* encodes a developmental regulator. Genes Dev. 7, 367–379.
Sessa, G., Carabelli, M., Possenti, M., Morelli, G., Ruberti, I., 2018. Multiple links between HD-zip proteins and hormone networks. Int. J. Mol. Sci. 19, 4047. https://doi.org/10.3390/ijms19124047.
Udvardi, M.K., Czechowski, T., Scheible, W.R., 2008. Eleven golden rules of quantitative RT-PCR. Plant Cell 20, 1736–1737.
Yamaguchi-Shinozaki, K., Shinozaki, K., 2006. Transcriptional regulatory networks in cellular responses and tolerance to dehydration and cold stresses. Annu. Rev. Plant Biol. 57, 781–803.
Yang, Q., Niu, Q., Li, J., Zheng, X., Ma, Y., Bai, S., Teng, Y., 2018. *PpHB22*, a member of HD-Zip proteins, activates *PpDAM1* to regulate bud dormancy transition in 'Suli' pear (*Pyrus pyrifolia* White Pear Group). Plant Physiol. Biochem. 127, 355–365. https://doi.org/10.1016/j.plaphy.2018.04.002.
Yue, H., Shu, D., Wang, M., Xing, G., Zhan, H., Du, X., Song, W., Nie, X., 2018. Genome-wide identification and expression analysis of the HD-zip gene family in wheat (*Triticum aestivum* L.). Genes 9, 70.
Zhang, Z., Chen, X., Guan, X., Liu, Y., Chen, H., Wang, T., Mouekouba, L.D., Li, J., Wang, A., 2014a. A genome-wide survey of homeodomain-leucine zipper genes and analysis of cold-responsive HD-Zip I members' expression in tomato. Biosci. Biotechnol. Biochem. 78, 1337–1349.
Zhang, C.H., Ma, R.J., Shen, Z.J., Sun, X., Korir, N.K., Yu, M.L., 2014b. Genome-wide analysis of the homeodomain-leucine zipper (HD-ZIP) gene family in peach (*Prunus persica*). Genet. Mol. Res. 13, 2654–2668.
Zhu, J.K., 2016. Abiotic stress signaling and responses in plants. Cell 167, 313–324.