**Abstract**

**Background:** CCCH transcription factors are important zinc finger transcription factors involved in the response to biotic and abiotic stress and physiological and developmental processes. Barley (Hordeum vulgare) is an agriculturally important cereal crop with multiple uses, such as brewing production, animal feed, and human food. The identification and assessment of new functional genes are important for the molecular breeding of barley.

**Results:** In this study, a total of 53 protein-encoding CCCH genes unevenly dispersed on seven different chromosomes were identified in barley. Phylogenetic analysis categorized the barley CCCH genes (HvC3Hs) into eleven subfamilies according to their distinct features, and this classification was supported by intron–exon structure and conserved motif analysis. Both segmental and tandem duplication contributed to the expansion of CCCH gene family in barley. Genetic variation of HvC3Hs was characterized using publicly available exome-capture sequencing datasets. Clear genetic divergence was observed between wild and landrace barley populations in HvC3H genes. For most HvC3Hs, nucleotide diversity and the number of haplotype polymorphisms decreased during barley domestication. Furthermore, the HvC3H genes displayed distinct expression profiles for different developmental processes and in response to various types of stresses. The HvC3H1, HvC3H2 and HvC3H13 of arginine-rich tandem CCCH zinc finger (RR-TZF) genes were significantly induced by multiple types of abiotic stress and/or phytohormone treatment, which might make them as excellent targets for the molecular breeding of barley.

**Conclusions:** Overall, our study provides a comprehensive characterization of barley CCCH transcription factors, their diversity, and their biological functions.

**Keywords:** Barley, CCCH gene family, Genetic variation, Haplotype analysis, Expression pattern
histidine, and X for any amino acid) based on the number of amino acid spacers between the cysteine and histidine residues [4–6]. CCCH proteins are over-represented by a class of proteins that contains a plant-unique tandem CCCH zinc finger (TZF) domain preceded by an arginine (R)-rich region; hereafter named RR-TZF proteins [7].

Many studies have shown that CCCH-type zinc finger proteins play a role in cell fate specification and developmental processes in plants. For example, AtKHZ1 and AtKHZ2 are required for flowering and senescence in Arabidopsis [8]. AtC3H59/ZFWD3 plays an essential role in seedling development and seed germination and development by interacting with the PPPDE gene family protein Desil [9]. In rice, OsDOS and OsTZFI act as repressors of leaf senescence [10, 11]. OsGZF1 affects glutelin accumulation during seed development [12]. GmZF351 and GmZF392 in soybean are involved in the accumulation of lipid in seeds [13, 14]. The involvement of CCCHs in hormone signaling adds complexity to the plant growth and development regulatory network. AtTZF4/5/6 act as negative regulators of light and gibberellins (GAs) and act as positive regulators of abscisic acid (ABA)-mediated regulation of seed development, dormancy, and germination [15]. The CCCH-type zinc finger gene OsLIC is involved in the biosynthesis and/or signal transduction of brassinosteroids, which affects the architecture of rice plants [16]. In switchgrass, PvC3H69 is a negative regulator of leaf senescence by repressing ABA synthesis and the ABA signaling pathway [17].

Several CCCH genes are implicated in the response to biotic and abiotic stressors in plants. For example, OsC3H10, OsC3H47, and OsTZFS are involved in the regulation of tolerance to drought stress in rice [18–20]. Arabidopsis AtZFPI has been reported to confer salt tolerance by limiting oxidative and osmotic stress and maintaining an ionic balance [21]. The non-tandem CCCH-type gene AtC3H17 in Arabidopsis has pleiotropic effects in the salt stress response via an ABA-dependent signaling pathway [22]. Switchgrass PrvC3H72 was the first CCCH family gene identified to be involved in plant chilling and freezing tolerance, possibly through an ABA-mediated pathway [23]. Moreover, DgC3H1 confers cold tolerance in Chrysanthemum plants by regulating the osmotic and reactive oxygen species (ROS) system, as well as the expression of genes associated with the cold stress response [24]. In addition, CCCH genes are involved in other adaptive processes, such as resistance to bacterial blight disease [25], zinc homeostasis [26], hydrogen peroxide [11], and oxidative stress [27].

Genome-wide identification and characterization of CCCH genes have not been identified in barley, and their biological functions and evolutionary history remain poorly understood. This study aimed to genomically identify and characterize the barley CCCH genes (HvC3Hs). The phylogenetic relationships, distribution of motifs, intron–exon organization, and gene duplication events were comprehensively analyzed. Genomic variation, genetic diversity, and selection on these genes during barley domestication were also investigated using barley resequencing data (including wild and landrace barley accessions). Finally, we conducted RNA-seq and quantitative real-time polymerase chain reaction (qRT-PCR) analyses to determine the possible function of HvC3Hs. Our preliminary analysis provides new insight into the evolutionary history of CCCH genes and will aid future efforts to functionally characterize and genetically improve barley.

Results

Genome-wide identification and characterization of CCCH proteins in barley

The most updated barley Morex assembly was used for the identification of barley CCCH genes. A total of 53 high-confident HvC3Hs with complete open reading frames were identified, accounting for 1.62% of the total annotated protein-coding genes in barley (Table 1; Supplementary Table S1). Because there is no standard nomenclature for barley CCCH genes, the candidate HvC3Hs were designated as HvC3H1 to HvC3H53 according to their chromosomal number and location. A BLAST search against the barley ESTs indicated that 38 HvC3Hs possessed EST records, which supported the existence of the HvC3Hs. Analysis of the physicochemical properties of HvC3H proteins demonstrated that the amino acid length varied from 127 amino acids (HvC3H35) to 1,456 amino acids (HvC3H6), with an average length of 482.2 amino acids. The pI varied from 5.13 to 10.15, and the MW ranged from 14.407 kDa to 160.373 kDa. All of these CCCH proteins possessed negative GRAVY values (average value: -0.704), indicating the hydrophobic nature of HvC3Hs. Subcellular localization prediction showed that most of these proteins were located in the nucleus (45 HvC3Hs; 84.91%), which was consistent with their localization in Arabidopsis, rice, and wheat [4, 35].

CCCH domain structure analysis of HvC3Hs

Significant differences in the domain organization of HvC3Hs were observed. A total of eleven domain
Table 1  Characteristics of CCCH transcription factor gene family in barley

| Gene Name | Gene ID          | Chr | Protein Length (aa) | Isoelectric Point | Molecular Weight (kDa) | Subcellular Location | Grand Average of Hydropathicity | ESTs Hit |
|-----------|------------------|-----|---------------------|-------------------|------------------------|----------------------|---------------------------------|----------|
| HvC3H1    | HORVU.MOREX. r2.1HG0011690 | chr1H | 609 | 8.16 | 64.492 | Nucleus | -0.324 | 109 |
| HvC3H2    | HORVU.MOREX. r2.1HG0067940 | chr1H | 278 | 5.45 | 29.946 | Nucleus | -0.375 | 60 |
| HvC3H3    | HORVU.MOREX. r2.1HG0072390 | chr1H | 304 | 9.15 | 34.276 | Nucleus | -1.163 | 11 |
| HvC3H4    | HORVU.MOREX. r2.1HG0074950 | chr1H | 402 | 9.57 | 42.999 | Nucleus | -0.305 | 2 |
| HvC3H5    | HORVU.MOREX. r2.1HG0074970 | chr1H | 327 | 9.42 | 35.232 | Nucleus | -0.389 | 6 |
| HvC3H6    | HORVU.MOREX. r2.1HG0076880 | chr1H | 1456 | 5.13 | 160.373 | Nucleus | -0.84 | 14 |
| HvC3H7    | HORVU.MOREX. r2.2HG0082780 | chr2H | 914 | 5.48 | 98.863 | Nucleus | -0.561 | 0 |
| HvC3H8    | HORVU.MOREX. r2.2HG0091490 | chr2H | 697 | 6.26 | 73.624 | Chloroplast | -0.384 | 25 |
| HvC3H9    | HORVU.MOREX. r2.2HG0110160 | chr2H | 668 | 6.33 | 71.328 | Nucleus | -0.468 | 48 |
| HvC3H10   | HORVU.MOREX. r2.2HG0140180 | chr2H | 341 | 10.15 | 38.974 | Nucleus | -1.009 | 22 |
| HvC3H11   | HORVU.MOREX. r2.2HG0166340 | chr2H | 304 | 9.38 | 31.487 | Nucleus | -0.623 | 33 |
| HvC3H12   | HORVU.MOREX. r2.2HG0176080 | chr2H | 695 | 5.49 | 77.804 | Nucleus | -1.149 | 11 |
| HvC3H13   | HORVU.MOREX. r2.3HG0196310 | chr3H | 379 | 7.51 | 40.767 | Nucleus | -0.53 | 57 |
| HvC3H14   | HORVU.MOREX. r2.3HG0200320 | chr3H | 224 | 9.13 | 24.591 | Nucleus | -0.81 | 0 |
| HvC3H15   | HORVU.MOREX. r2.3HG0200330 | chr3H | 232 | 6.17 | 24.956 | Nucleus | -0.616 | 1 |
| HvC3H16   | HORVU.MOREX. r2.3HG0209640 | chr3H | 165 | 8.88 | 18.115 | Extracellular | -0.54 | 0 |
| HvC3H17   | HORVU.MOREX. r2.3HG0210630 | chr3H | 1008 | 6.58 | 113.677 | Nucleus | -0.151 | 0 |
| HvC3H18   | HORVU.MOREX. r2.3HG0210880 | chr3H | 467 | 7.85 | 49.838 | Nucleus | -0.493 | 53 |
| HvC3H19   | HORVU.MOREX. r2.3HG0210900 | chr3H | 426 | 8.07 | 45.402 | Nucleus | -0.658 | 16 |
| HvC3H20   | HORVU.MOREX. r2.3HG0221360 | chr3H | 676 | 5.58 | 73.029 | Nucleus | -0.599 | 0 |
| HvC3H21   | HORVU.MOREX. r2.3HG0225190 | chr3H | 500 | 8.67 | 54.722 | Nucleus | -0.648 | 32 |
| HvC3H22   | HORVU.MOREX. r2.3HG028500 | chr3H | 384 | 8.6 | 42.042 | Nucleus | -0.419 | 9 |
| HvC3H23   | HORVU.MOREX. r2.3HG0230880 | chr3H | 281 | 9.59 | 32.735 | Chloroplast | -1.177 | 64 |
| HvC3H24   | HORVU.MOREX. r2.3HG0253280 | chr3H | 370 | 5.21 | 42.08 | Nucleus | -1.102 | 0 |
| HvC3H25   | HORVU.MOREX. r2.3HG0258540 | chr3H | 435 | 8.82 | 47.4 | Nucleus | -0.564 | 61 |
| HvC3H26   | HORVU.MOREX. r2.4HG0279920 | chr4H | 750 | 7.59 | 80.177 | Nucleus | -0.407 | 61 |
| HvC3H27   | HORVU.MOREX. r2.4HG0294950 | chr4H | 613 | 6.04 | 65.557 | Nucleus | -0.418 | 0 |
| Gene Name       | Gene ID          | Chr | Protein Length (aa) | Isoelectric Point | Molecular Weight (kDa) | Subcellular Location | Grand Average of Hydropathicity | ESTs Hit |
|-----------------|------------------|-----|---------------------|-------------------|------------------------|----------------------|--------------------------------|----------|
| HvC3H28 HORVUMOREX. r2.4HG0318770 | chr4H | 299 | 8.3 | 32.522 | Nucleus | -0.595 | 2 |
| HvC3H29 HORVUMOREX. r2.4HG0325540 | chr4H | 326 | 7.14 | 36.231 | Nucleus | -1.003 | 11 |
| HvC3H30 HORVUMOREX. r2.5HG0362710 | chr5H | 691 | 9.39 | 78.976 | Nucleus | -1.218 | 5 |
| HvC3H31 HORVUMOREX. r2.5HG0370720 | chr5H | 617 | 5.82 | 65.496 | Nucleus | -0.292 | 74 |
| HvC3H32 HORVUMOREX. r2.5HG0374920 | chr5H | 509 | 6.39 | 55.923 | Nucleus | -0.702 | 23 |
| HvC3H33 HORVUMOREX. r2.5HG0377520 | chr5H | 442 | 8.78 | 47.516 | Nucleus | -0.504 | 20 |
| HvC3H34 HORVUMOREX. r2.5HG03833720 | chr5H | 598 | 5.86 | 64.404 | Nucleus | -0.418 | 0 |
| HvC3H35 HORVUMOREX. r2.5HG0394250 | chr5H | 127 | 8.89 | 14.407 | Nucleus | -0.997 | 0 |
| HvC3H36 HORVUMOREX. r2.5HG0407060 | chr5H | 314 | 9.25 | 36.792 | Chloroplast | -1.241 | 36 |
| HvC3H37 HORVUMOREX. r2.5HG0429150 | chr5H | 752 | 7.39 | 85.417 | Nucleus | -1.256 | 5 |
| HvC3H38 HORVUMOREX. r2.5HG0439140 | chr5H | 404 | 8.81 | 46.699 | Nucleus | -1.655 | 0 |
| HvC3H39 HORVUMOREX. r2.6HG0469440 | chr6H | 337 | 5.82 | 36.132 | Nucleus | -0.461 | 0 |
| HvC3H40 HORVUMOREX. r2.6HG0475520 | chr6H | 211 | 9.17 | 23.087 | Nucleus | -0.813 | 0 |
| HvC3H41 HORVUMOREX. r2.6HG0475530 | chr6H | 342 | 8.03 | 36.041 | Nucleus | -0.357 | 0 |
| HvC3H42 HORVUMOREX. r2.6HG0475540 | chr6H | 358 | 8.63 | 38.145 | Chloroplast | -0.34 | 0 |
| HvC3H43 HORVUMOREX. r2.6HG0475550 | chr6H | 308 | 9.49 | 31.997 | Chloroplast | -0.541 | 39 |
| HvC3H44 HORVUMOREX. r2.6HG05005110 | chr6H | 433 | 7.88 | 46.635 | Extracellular | -0.222 | 0 |
| HvC3H45 HORVUMOREX. r2.6HG0505660 | chr6H | 359 | 6.66 | 40.211 | Nucleus | -0.46 | 75 |
| HvC3H46 HORVUMOREX. r2.6HG0515160 | chr6H | 1001 | 8.81 | 110.273 | Nucleus | -1.121 | 16 |
| HvC3H47 HORVUMOREX. r2.6HG0526270 | chr6H | 647 | 5.23 | 71.397 | Nucleus | -1.069 | 45 |
| HvC3H48 HORVUMOREX. r2.7HG0560290 | chr7H | 489 | 9.46 | 55.275 | Nucleus | -0.79 | 59 |
| HvC3H49 HORVUMOREX. r2.7HG0579580 | chr7H | 363 | 6.85 | 38.791 | Nucleus | -0.706 | 20 |
| HvC3H50 HORVUMOREX. r2.7HG0609090 | chr7H | 297 | 9.64 | 30.833 | Chloroplast Nucleus | -0.541 | 46 |
| HvC3H51 HORVUMOREX. r2.7HG0602740 | chr7H | 407 | 8.56 | 44.684 | Nucleus | -0.954 | 26 |
| HvC3H52 HORVUMOREX. r2.7HG0607870 | chr7H | 375 | 9.32 | 42.524 | Nucleus | -1.372 | 6 |
| HvC3H53 HORVUMOREX. r2.7HG0609970 | chr7H | 648 | 6.27 | 71.01 | Nucleus | -0.962 | 100 |
organizations of 141 CCCH motifs (C-X$_{5,17}$-C-X$_{4,6}$-C-X$_{3,7}$-H) were identified, with an average of 2.66 CCCH motifs per protein. CCCH proteins have been shown to have one to six CCCH motifs [4, 16, 39, 40], and the similar pattern was observed in our study (Fig. 1). Notably, HvC3H6 contained eight CCCH motifs, which was a kind of newly identified motif for CCCH-type zinc-finger protein. (Supplementary Table S2). Although different frequencies of CCCH motifs have been identified among barley CCCH proteins, two conventional CCCH motifs C-X$_{5}$-C-X$_{3}$-H and C-X$_{7}$-C-X$_{5}$-C-X$_{3}$-H were the two most common, suggesting that C-X$_{7,8}$-C-X$_{5}$-C-X$_{3}$-H might be ancestral to the other CCCH motifs (Supplementary Fig. S1) [41]. Additionally, a total of nine non-conventional CCCH zinc finger motifs, such as 6 C-X$_{7}$-C-X$_{5}$-C-X$_{3}$-H, 4 C-X$_{5}$-C-X$_{7}$-C-X$_{3}$-H, and 4 C-X$_{5}$-C-X$_{5}$-C-X$_{3}$-H, were observed, which were previously identified to be abundant non-conventional CCCH motifs in Arabidopsis and rice. Furthermore, a total of five HvC3H proteins (HvC3H1, -9, -13, -26, and -31) were assigned to the RR-TZF proteins with an arginine-rich (RR) region located in front of C-X$_{7,8}$-C-X$_{5}$-C-X$_{3}$-H-X$_{16/18}$-C-X$_{5}$-C-X$_{4}$-C-X$_{5}$-H (TZF) motif. Aside from the CCCH zinc finger motifs, some HvC3H proteins also contained several other known functional domains, such as KH, RRM, and RING. Five (HvC3H11, -41, -42, -43, and -50) and eight (HvC3H3, -8, -23, -27, -34, -36, -37, and -38) HvC3H members possessed the KH and RRM domains, respectively. An extra ankyrin (ANK) domain preceded the CCCH zinc finger motif was observed in HvC3H9, which was categorized as the ANK-RR-TZF protein.

**Phylogenetic relationships, gene structure, and conserved domain organization of HvC3H genes**

To determine the evolutionary relationships among HvC3Hs, a Maximum Likelihood (ML) phylogenetic tree was constructed based on the full-length CCCH protein sequences of barley. According to the criteria proposed by Wang and Peng et al. with slight modifications [4, 6], the HvC3Hs were classified into eleven subfamilies (group I to group XI) (bootstrap values > 60%) (Fig. 2A). Twenty-six HvC3Hs formed thirteen sister gene pairs, twelve of which possessed high bootstrap support (> 98%). The number of CCCH proteins varied greatly for different subfamilies; subfamilies I and II rank the largest clusters with seven members, followed by the subfamilies VII (6 HvC3Hs) and XI (5 HvC3Hs). Notably, four RR-TZF genes (HvC3H1, -13, -26, and -31) were classified into subfamily XI, whereas the phylogenetic relationships of the ANK-RR-TZF gene (HvC3H9) remained ambiguous because of its low bootstrap values. We also constructed another phylogenetic tree based on the alignment of 188 CCCH proteins from Arabidopsis (68), rice (67), and barley (53) (Supplementary Fig. S2). The phylogenetic tree revealed an alternating distribution of monocot and eudicot CCCH genes in certain of the subfamilies.

The intron–exon gene structure provides potential insight into the functional diversification during evolution [42]. Unlike other TF family genes, which tend to lack introns, the average intron number of HvC3Hs was 4.08 (ranging from 0 to 13) (Fig. 2B). In general, genes within the same subfamily had a similar structure of introns and exons. For example, genes from subfamily XI tended to be intron-less; subfamilies VII, VIII, and X were nearly identical in their intron/exon lengths and structural organization.

Consistent with the patterns in intron–exon gene structure, HvC3H proteins within the same subfamily tended to have a similar organization of motifs, and the patterns were highly variable among different phylogenetic clades (Fig. 2C). For example, the HvC3Hs in subfamily X possessed one CCCH motifs and one RRM motif, whereas subfamily I tended to have two or more CCCH motifs and one RRM motif. HvC3Hs in subfamily IV contained the RING domain. The variation in gene structure and motif composition among subfamilies suggests prior sub-functionalization or neofunctionalization of these HvC3Hs.

**Chromosomal distribution and gene duplication**

Chromosome location analysis revealed that the HvC3Hs were unevenly located on the seven barley chromosomes, and chromosome 3H possessed the most abundant CCCH genes (thirteen HvC3Hs) (Supplementary Fig. S3). By contrast, chromosomes 4H had only four CCCH genes. Chromosome 5H and 6H both contained nine CCCH genes, and chromosome 1H, 2H, and 7H both had six.

Gene duplication is considered one of the primary drivers of gene family expansion in plants and plays an important role in the evolution of new gene functions and adaptation [43, 44]. A total of six duplicated gene pairs were identified (Fig. 3). Two gene pairs (HvC3H14/HvC3H15 and HvC3H41/HvC3H42)
Fig. 1 (See legend on previous page.)
were tandemly duplicated genes, the rest four gene pairs were designated as segmentally duplicated genes. According to the phylogenetic tree, these duplicated genes were clustered in the same clade. For example, \( \text{HvC3H14}/\text{HvC3H15} \) were clustered in subfamily VI, \( \text{HvC3H27}/\text{HvC3H34} \) were assigned to subfamily X, and \( \text{HvC3H45}/\text{HvC3H48} \) belonged to subfamily IV. The \( \text{Ka}/\text{Ks} \) ratios of the segmentally duplicated genes were all lower than 1, indicating purifying selection (Supplementary Table S3) [7].

Syntenic relationships with six other representative species, including three monocots (\textit{Zea mays}, \textit{Oryza}}
sativa, and Triticum aestivum) and three dicots (Brassica rapa, Solanum lycopersicum, and Glycine max), were further analyzed to determine the mechanisms underlying the evolution of HvC3Hs (Fig. 4). A total of 65, 27, and 20 orthologous gene pairs between barley and Triticum aestivum, Zea mays, and Oryza sativa were identified, respectively. Sixteen HvC3H genes were orthologous to three copies of CCCH genes in wheat, which might stem from the fact that the heterologous hexaploid wheat contained three distinct ancestral genomes, namely A, B, and D [45]. By contrast, the number of orthologous gene pairs between barley and three dicots (Glycine max, Brassica rapa, and Solanum lycopersicum) was ten, eight, and three, respectively, which was much lower than those between barley and three monocots. This finding is consistent with the observed phylogenetic relationships between barley and these species. HvC3Hs are phylogenetically closer to CCCHs in Triticum aestivum, Zea mays, and Oryza sativa than CCCHs in Glycine max, Brassica rapa, and Solanum lycopersicum. The overall Ka/Ks ratios between barley and the monocots (Triticum aestivum: 0.2729, Oryza sativa: 0.1840, and Zea mays: 0.1912) were significantly larger than that between barley and the dicots (Brassica rapa: 0.0646, Solanum lycopersicum: 0.0434, and Glycine max: 0.0295), suggesting the degenerated syntenic relationships after the separation of monocot and dicot (Supplementary Table S4).

**Cis-element analysis of HvC3H genes**
Cis-elements play important roles in the transcriptional regulation of genes throughout the life cycle of plants. A total of 52 functional cis-elements were identified and grouped into five categories. A large number of light-responsive elements were detected in the promoter regions of HvC3Hs, which accounted for most of the putative cis-elements (Supplementary Table S5, Supplementary Fig. S4). We also obtained a total of 11 types of hormone-responsive regulatory elements, such as auxin-responsive elements (AuxRR-core, TGA-box, and TGA-element), gibberellin-responsive elements (P-box, GARE-motif, and TATC-box), salicylic acid-responsive elements (TCA-element), and MeJA-responsive elements (CGTCA-motif and TGACG-motif). Several types of biotic and abiotic stress-related regulatory elements were observed in HvC3H promoters. Anaerobic induction elements (74 ARE and 45 GC-motif) were detected in 43 HvC3Hs. A
Fig. 4 Synteny relationships analysis of HvC3Hs between barley and three Monocotyledons, three Dicotyledons. A Oryza sativa. B Triticum aestivum. C Zea mays. D Glycine max. E Solanum lycopersicum. F Brassica rapa.
total of 36 low temperature-responsive elements (LTR) and 39 drought-responsive elements (MBS, myeloblastosis binding site) were detected in 25 and 25 HvC3Hs, respectively. Fourteen HvC3H genes possessed wound-responsive elements (WUN-motif). Additionally, eight types of plant organogenesis-related cis-elements were identified, such as the meristem expression-related element CAT-box (15 genes), zein metabolism regulation-related element O2-site (13 genes) and endosperm expression-related element GCN4-motif (seven genes). These findings suggest that HvC3Hs might play an important role in barley plant growth and development, hormone signal transduction, and the response to biotic and abiotic stress.

Genetic variation of CCCH genes
We analyzed the sequence diversity of HvC3H genes at the population level based on exome-captured sequencing datasets. The average read coverage was 72.80% per sample per gene with the great majority (75.16%) that larger than 60% (Supplementary Table S6). The single nucleotide polymorphism (SNP)-calling pipeline generated 388 high-confident SNPs, 172 of which were in HvC3H32, followed by HvC3H21 (42) and HvC3HSl1 (23) (Supplementary Table S7; Supplementary Table S8). Most HvC3H-related SNPs were located within the intron regions (362); the rest of the SNPs were non-synonymous (13) and synonymous (13) SNPs. There were 314 InDels ranging from 1 to 55 bp in length, and short InDels 1 to 4 bp (76.54%) in length were more common than long InDels (Supplementary Table S9). Similarly, most HvC3H-related InDels were enriched in introns, which might be explained by the fact that the reading frame-independent variants were under weaker negative selection than frame-change variants.

To investigate the relatedness among the landraces and wild barley accessions worldwide, we carried out principal component analysis using HvC3H-related SNPs (Fig. 5A and B; Supplementary Table S10). The first principal component was correlated with the biological differentiation between landrace from wild barley and explained 22.11% of the total genetic variance; the second and third principal components captured 5.31% and 5.02% of the genetic variance, respectively, and revealed geographical differentiation in barley accessions. The phylogenetic tree further revealed genetically divergent clusters associated with the contrast between barley wild accessions versus landraces rather than their geographical origins (Fig. 5C). ADMIXTURE analysis confirmed these findings (Fig. 5D). When K = 2, two groups coinciding with landraces and wild barley were observed. Increasing K to 4 provided additional insights. Within barley landraces, we detected two geographically distributed components from Europe and Africa, whereas the rest of the landraces from Mediterranean areas displayed signs of genetic admixture. Within wild barley accessions, accessions from the Southern and Northern Levant regions formed two distinct groups.

Genetic diversity and haplotypes of HvC3Hs in wild and domesticated barley populations
Population-based nucleotide diversity was calculated to assess the occurrence of prior genetic bottlenecks of HvC3H genes during barley domestication. The total genetic diversity of HvC3H genes decreased by ~ 29.65% from the wild (π = 0.1050) to domesticated (π = 0.0739) barley population (Fig. 6A).

We further constructed the haplotype network for each HvC3H gene using their SNPs. A total of 922 non-redundant haplotypes belonging to 31 HvC3H genes were observed, with an average of 29.74 haplotypes per gene (Supplementary Fig. S5; Supplementary Table S8). Specific haplotypes represented in more than half of wild or landrace populations were defined as dominant haplotypes. Eleven HvC3H genes had no dominant haplotype, whereas 13 HvC3H genes had the same dominant haplotype in both wild and landrace populations. Nevertheless, clear genetic differentiation in haplotypes between wild and domesticated barley accessions was observed (Fig. 6B). HvC3H30 in wild barley mainly had the AAA AGGGGGTTTTTGCC haplotype, but domesticated barley mainly had the AAAAGGGGGATTTTTGCC haplotype. The dominant haplotype of HvC3H49 in wild barley was AAGTTTTCCCTTGGGAA, but haplotype AAGTTTTCTTTTGGGTT was the most common in domesticated barley. Some rare haplotypes were also observed for specific HvC3H genes, such as HvC3H49, HvC3HS1, and HvC3HS2. The appearance of novel allelic variants greatly increased the degree of haplotype polymorphism of HvC3Hs. The rare haplotypes were mainly observed in the wild barley group, which was consistent with the results of the genetic diversity analysis. These results indicated that these genes experienced a severe genetic bottleneck during barley domestication and that
Fig. 5 (See legend on previous page.)
the haplotype diversity decreased in domesticated barley relative to the wild population.

**Temporal-spatial and stress-induced expression pattern analysis**

Analysis of tissue-/stage-specific expression profiles provided valuable insights into the potential functions of genes in plant species. Distinct expression patterns were observed for the *HvC3Hs* by using the publicly available RNA-seq data from 16 different tissues (Fig. 7). The expression levels of *HvC3Hs* in group I were lower than those of genes in the other groups; eight genes were not expressed in most of the tissues/stages. By contrast, a total of thirteen genes in group III were highly expressed in most of the studied tissues/stages. *HvC3H25* was predominantly expressed in LOD, CAR15, and EPI, whereas *HvC3H3* and *HvC3H18* showed high expression in INF2 and LOD, respectively. Genes in cluster II showed a medium expression level. Within this cluster, *HvC3H7, -8, -13, -34, -43, and -52* tended to be expressed in INF1 and INF2. These findings indicate that these *HvC3Hs* might be associated with the development of these tissues in barley.

We analyzed the expression of *HvC3Hs* in response to different types of environmental stresses. Under cold treatment, four *HvC3H* genes displayed increased expression patterns (>2.0-fold change) (Fig. 8A). Among these genes, *HvC3H28, HvC3H2*, and *HvC3H30* exhibited their highest level of expression under cold treatment, showing fold-changes of 8.23, 4.57, and 2.17, respectively. Salt stress induced differential expression patterns of *HvC3H* genes in the three root regions (Fig. 8B). Compared with the unstressed control, a total of three, four, and two *HvC3H* genes were highly expressed in the meristematic, elongation, and maturation zones, respectively, especially *HvC3H22*, which exhibited a 10.96- and 6.67-fold increase in expression in the elongation and meristematic zones relative to the unstressed control. *HvC3H2* was up-regulated in all tissues; its expression was increased 2.38-, 3.95-, and 3.21-fold in the meristematic, elongation, and maturation zones, respectively. Under metal ion stress, the expression of *HvC3H2, -5, -13, -16, and -28* was significantly up-regulated, and the up-regulation of *HvC3H2* was induced by copper and cadmium treatment (Fig. 8C). Under zinc and cadmium stress, *HvC3H16* was up-regulated with 2.09- and 7.98-fold change, respectively.
Expression of HvC3Hs under drought, salt, cold, and ABA treatment by qRT-PCR analysis

To further investigate the expression of HvC3H genes in response to multiple treatments, 26 HvC3Hs were randomly subjected to qRT-PCR analysis. Under drought treatment, nine HvC3Hs were up-regulated at all time
points (Supplementary Fig. S6), and the expression of six of the nine HvC3Hs (HvC3H3, -8, -10, -18, -37, and -50) peaked at 24 h. The expression of HvC3H3 was approximately 54-fold larger than that of the control at 24 h.

After salt treatment, the expression of HvC3H19 was suppressed compared with the control at all time points; the expression of 21 genes was significantly up-regulated, and the expression of these genes peaked at different times (Supplementary Fig. S7). For example, the expression of HvC3H3 peaked at 3 h and was up-regulated 43-fold, whereas the expression of HvC3H8, -10, and -18 was initially slightly up-regulated and peaked at 24 h.

The expression levels of HvC3Hs after cold treatment were analyzed, and the expression of six genes (HvC3H6, -8, -11, -30, -40, and -43) was inhibited compared with the control; the other HvC3Hs was up-regulated at specific time points (Supplementary Fig. S8). The expression of three HvC3Hs was up-regulated at 1 h (HvC3H36, -47, and -49), 3 h (HvC3H10, -45, and -50), and 6 h (HvC3H3, -25, and -33), suggesting that these HvC3H3s might primarily function in the initial stage in the response to cold injury. The expression of the other HvC3H genes peaked at 12 h or 24 h.

Plant CCCH proteins have been shown to be effective regulators of ABA-mediated stress responses [46]. qRT-PCR analysis showed that ABA treatment had a pronounced effect on the expression patterns of HvC3Hs, and a complex expression profile was observed (Supplementary Fig. S9). For example, the expression of HvC3H3 was significantly up-regulated at 1 h and 3 h but
down-regulated thereafter. By contrast, the expression of HvC3H37 was down-regulated before 12 h but significantly up-regulated at 24 h. Except for HvC3H8, -19, -30, -32, and -47, whose expressions were suppressed relative to the control, the maximum expression levels of the other HvC3Hs peaked at different time points.

Discussion

Identification of CCCH genes in barley

CCCH domain-containing proteins are involved in various processes, including plant growth, development, and adaptation. Barley is the most important temperate crop in modern society [38]. Herein, 53 highly confident CCCH zinc finger genes were identified in the barley genome through a comprehensive search. The number of CCCH proteins in barley was slightly lower than those identified in Arabidopsis (67), rice (68), maize (68), and grape (69); approximately half of those in poplar (91), Brassica rapa (103), and switchgrass (103), suggesting that the species origin and genome size are not directly associated with the number of CCCH genes.

The phylogenetetic tree using the CCCH proteins from barley, rice, and Arabidopsis showed that the HvC3H genes displayed closer relationships with their orthologues than their paralogs. For example, CCCH genes in groups VII, XII, XX, and XXI showed a one-to-one-to-one orthologous pattern referred to a barley gene with one unique counterpart in Arabidopsis and rice. In contrast, groups II, IV, IX, XV, and XXVI did not contain any CCCH genes from barley. These rice and/or Arabidopsis-specific clades suggested that a presumed barley-specific loss of CCCH genes may have occurred after the divergence of barley and other species.

Genetic variation and haplotype polymorphism of HvC3Hs during the domestication of barley

The genetic divergence of the HvC3H genes between wild and domesticated barley populations associated with domestication bottleneck from wild barley to cultivated barley and might be domestication-related genes.

Domestication is a plant-animal co-evolutionary process driven by the human demands for certain morphological and physical characteristics of crops [51]. This results in a severe genetic bottleneck that reduces allele nucleotide diversity [52]. The haplotype networks indicated that the haplotype composition of the HvC3H family in wild barley was rich compared with that in cultivated barley, indicating that initial human selection was focused on the retention of specific haplotypes by screening out a large number of undesirable haplotypes during domestication.

HvC3Hs might play a role in plant growth, abiotic stress, and phytohormone responses

The expression patterns of HvC3Hs provide insight into their possible functions. For example, HvC3H11, a KH domain-containing CCCH zinc finger gene, tended to be highly expressed in young inflorescences. In plants, the KH domain-containing genes FLK (Flowering Locus KH Domain) and PEP (PEPPER) regulate flowering by negatively and positively modulating the FLC expression, respectively [53, 54]. HvC3H22 was highly expressed in NOD, PAL, and LEM. Its orthologous gene AtC3H14 is the direct target of the MYB domain TF MYB46 and a master switch for cell elongation in Arabidopsis [55, 56].

Several studies have shown that CCCH proteins are involved in stress tolerance in plants [57]. For example, Arabidopsis AtSZF1 and AtSZF2, two closely related CCCH zinc finger genes, negatively regulate the expression of salt-responsive genes and modulate the tolerance to salt stress [58]. OsTZF1 negatively regulates leaf senescence under salt conditions and confers stress tolerance by delaying stress-responsive phenotypes, possibly through post-transcriptional control of the RNA metabolism of the salt stress-responsive genes [11]. In this study, the RR-TZF protein HvC3H13 was homologous with these genes, and its expression was significantly induced under salt stress according to the RNA-seq and qRT-PCR analyses, suggesting that HvC3H13 may have similar functions in the salinity stress response. HvC3H28 displayed the most upregulated pattern at 1, 6, and 12 h of drought stress. Homology analysis revealed that its orthologous gene OsC3H47 is involved in drought tolerance through its elevated sensitivity to ABA [19]. Another CCCH-tandem zinc finger protein OsTZF5, whose homologous gene was the RR-TZF protein HvC3H1, promotes drought avoidance and drought tolerance in rice [20]. Several MBS cis-acting elements associated with drought inducibility within the promoter regions of HvC3H28 and HvC3H1 were predicted, suggesting that these genes might play a potential role in the
response to drought stress. In *Chrysanthemum*, *DgC3H1* enhances low-temperature tolerance by regulating the ROS system and the expression of downstream cold-related genes [24]. However, the expression of *HvC3H43*, which is orthologous to *DgC3H1*, was not induced by cold stress according to RNA-seq and qRT-PCR analyses. No *cis*-acting element, such as LTR, was involved in low-temperature responsiveness within the *HvC3H43* promoter region. These results indicate that *HvC3H43* may have functionally diversified in barley. The expression of *HvC3H13* was significantly upregulated at 1, 3, and 6 h compared to the control under cold stress. Its orthologous gene *PtC3H72* acts as an added signaling component by regulating the ICE1-CBF-COR regulon and ABA-responsive genes during the switchgrass response to cold stress [23]. Notably, another RR-TZF protein *HvC3H2* is involved in several stressors, such as salt, low temperature, copper, and cadmium treatments. *HvC3H2* is homologous to *AtZFP1*, which encodes a CCCH-type zinc finger protein induced by salt stress in *Arabidopsis*. Overall, several candidate *CCCH* genes that could be targets for subsequent genetic isolation and functional investigation in barley as well as in other cereal crops.

**Conclusions**

Despite the importance of *CCCH* genes in plant growth and development, the response to biotic and abiotic stress, and disease resistance, the precise roles of *CCCH* gene family members in barley have not yet been elucidated. Here, our genome-wide identification and characterization of *HvC3H* genes revealed the physical–chemical properties, phylogeny, intron–exon structure, and expansion patterns of these genes. The population structure based on the most recently released exome capture sequencing data revealed a deep phylogenetic split in the *HvC3Hs* between wild and domesticated barley. The nucleotide and haplotype diversity of most *HvC3Hs* indicated that these genes have undergone a severe genetic bottleneck during the transition from wild relatives to domesticated barley populations. The results of the expression profiling analysis suggested that *HvC3H* members might be associated with multiple physiological, metabolic, and developmental processes, especially in response to various types of biotic stresses. Overall, these findings will aid future studies examining the evolutionary history of *HvC3Hs* as well as functional studies of candidate *HvC3H* genes for molecular breeding in barley.

**Methods**

**Identification of CCCH proteins in barley**

The genomic proteins of barley Morex V2 were downloaded from the IPK database (https://doi.org/10.5447/ipk/2019/8). The CCCH protein sequences of *Arabidopsis* and rice were used as queries to search against the barley proteins with Basic Local Alignment Search Tool (BLAST) software (e-value<1e-5). The Hidden Markov Model (HMM) of CCCH conserved domain (PF00642) was used as a query to search against the barley proteins by HMMER v3.0 with the e-value<0.001. The candidate CCCH proteins were further verified by Simple Modular Architecture Research Tool (SMART) (http://smart.embl.de/), National Center for Biotechnology Information—Conserved Domains Database (NCBI-CDD) (https://www.ncbi.nlm.nih.gov/cdd/) and PFAM (http://pfam.xfam.org/) online databases. Putative proteins without CCCH domain were removed. A BLASTN search against barley expressed sequence tags (ESTs) was conducted to detect the existence of CCCH proteins with the following criteria: e-value<1e-5, identity>70% and coverage>70%. The computational physical and chemical properties of CCCH family members, including molecular weight (MW), theoretical isoelectric point (pI), instability index (II), and grand average of hydropathicity (GRAVY) were evaluated using the online tool ExPASy (http://web.expasy.org/protparam/). The subcellular location was predicted using the cello software (http://cello.life.nctu.edu.tw/).

**Phylogeny, gene structure and conserved motif analysis**

The ClustalX v2.1 software was used to perform multiple alignments using the full-length CCCH protein sequences with default parameters. ML tree was constructed with IQ-TREE v2.1.3, using the best-fitting substitution model (VT+F+R3) selected automatically with bootstrap value of 1000 replications [59]. The intron–exon organization of *HvC3H* genes was generated by Gene Structure Display Server (GSDS) (http://gsds.cbi.pku.edu.cn/) based on the gene annotation Gene Transfer Format (GTF) file [60]. The conserved domains were identified using the online SMART tools. The upstream 1.5 kb genomic sequences of *HvC3H* genes were extracted and then submitted to the PlantCARE online database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) to detect the potential *cis*-acting regulatory elements in the promoter regions.

**Chromosome localization and gene synteny analysis**

The chromosomal locations of *HvC3H* genes were obtained from IPK database (https://doi.org/10.5447/ipk/2019/8), and the chromosome maps were visualized using MapChart v2.32. The MCScanX software [61] was employed to analyze the synteny relationships of *HvC3Hs* in rice (*Oryza sativa*), wheat (*Triticum aestivum*), maize (*Zea mays*) soybean (*Glycine max*), tomato (*Solanum lycopersicum*), and *Brassica rapa*. The gene
duplication events of HvC3Hs were identified according to the genomic comparison. Tandem duplicated genes were defined based on the following criteria (1) located within the same chromosome; (2) <1 intervening gene [42]. The syntenic and duplicated gene pairs were visualized by the Circos v0.67 tool. The non-synonymous substitution (Ka) / synonymous substitution (Ks) ratio was calculated to estimate genes evolutionary rate using the PAL2NAL online tools (http://www.bork.embl.de/pal2nal/) [62]. Ka/Ks > 1, =1 and <1 represent positive, neutral, and purifying selection, respectively. The divergence time of syntenic and duplicated gene pairs was calculated based on the formula $T = (K_s / 2\lambda) \times 10^{-6}$ million years ago (MYA) ($\lambda = 6.5 \times 10^{-9}$) [63]. The BLAST and orthoVeen2 software were employed to analyze the homologous genes between barley and other related species [64].

Population genetics analysis of HvC3H-related variants

The exome-captured resequencing data of 220 geographically-referenced barley accessions were retrieved from the NCBI SRA database (PRJEB8044/ERP009079) [47]. Raw reads were trimmed using Trimmomatic v0.36 with default parameters [65]. The high-quality reads were mapped to the reference genome of barley Morex V2 using BWA-MEM v0.7.13r1126. The Bedtools v2.18 was employed to calculate the reads coverage per sample per gene. The single nucleotide polymorphism (SNP) and insertion–deletion (InDel) were identified using the Picard-GATK pipeline [66]. The following criteria was used for SNPs filtration. (1) minor allele frequency (MAF) > 0.05 and <0.95; (2) a maximum missing rate <0.1; (3) biallelic alleles. SNP and InDel were annotated using the SnpEff v4.3 according to the barley genome GTF file [67]. To better reveal the evolutionary relationships, barley accessions with SNP missing rate larger than 0.1 were excluded. Totally, the final collection included 95 landraces and 51 wild barley accessions (Supplementary Table S6). Only SNPs that located within the HvC3H genes were extracted for phylogenetic tree, population structure and principal component analysis (PCA). The HvC3H-related SNPs were used to construct ML tree with the IQ-TREE v2.1.3. The phylogenetic tree was visualized by Figtree v1.4.4. The population structure was quantified using ADMIXTURE v1.3.0 with predefined K values ranging from 2 to 4. The PCA was performed using the smartpcga sub-package implemented in EIGENSOFT v4.2. The nucleotide diversity (n) was estimated using vcftools v0.1.16. The DNAsp v6.12.01 was employed to calculate the haplotypes for each HvC3H genes. Finally, the media-joining haplotype networks were constructed using the PopART v1.7 [68].

Expression patterns of HvC3H gene members

In order to explore the expression patterns of HvC3Hs, the publicly available 142 RNA-seq samples were downloaded from the NCBI Sequence Reading Archive (SRA) database, including different developmental stages and tissues and different biotic and abiotic stresses. The accession number and sample information of RNA-seq were listed in Supplementary Table S11. The fragments per kilobase per million (FPKM) value was calculated by the HISAT2 v2.1.0 and StringTie v1.3.5 pipeline. The R package pheatmap was used to visualize the expression profiles using the log2 transformed FPKM value.

Plant material, stress treatment, RNA extraction, and qRT-PCR analysis

Seeds of barley Morex were obtained from the College of Agronomy, Northwest A&F University, and were used as the experimental material. The barley seeds were hydroponically grown in growth chamber under controlled conditions (23±1 °C, 16-h light/8-h dark cycle). The seedlings were processed for stress treatments at the three-leaf stage. For salt, drought, cold, and ABA treatments, the plants were incubated in 150 mM NaCl, 19.2% (w/v) PEG-6000, 4 °C and 100 μM ABA for 0, 1, 3, 6, 12, and 24 h, respectively. Seedlings without any treatment at the same time point were considered as the control. Leaves of all these samples were randomly collected with three biological replications. The collected samples were immediately frozen in liquid nitrogen and stored at 80 °C for RNA extraction.

To further reveal the possible functions of HvC3Hs, a total of 26 HvC3Hs were randomly selected to investigate their expression profile under various stresses by quantitative real-time PCR (qRT-PCR) analysis. HvACTIN (HORVU.MOREX.r2.5HG0378970) was used as the internal reference gene and the detail information of all the primers was listed in Supplementary Table S12. The total RNA was extracted by Plant RNA Kit reagent (Omega BioTek, USA), and cDNA synthesis was performed using 5X All-in-one RT MasterMix (ABM, Canada) according to the manufacturer’s instructions. The TB-Green™Premix Ex Taq™ II kit (Takara, Dalian, China) was used to conduct qRT-PCR amplification in the Quant Studio™ Real-Time PCR system (Thermo Fisher, USA). The thermal cycling protocol was as follows: 95 °C temperature for 30 s, followed by 40 cycles of 95 °C for 3 s, and 30 s at 60 °C. The relative expression level was calculated by the $2^{-\Delta\DeltaCT}$ method [69]. Three technical replications were applied for each treatment. The T-test was conducted to evaluate the significance of the results using R. One asterisk (*) indicates 0.05 significance level
and double asterisk (**) indicates 0.01 significance level, respectively.

**Abbreviations**

ABA: Abscisic Acid; ABRE: Abscisic Acid Responsive Elements; ANK: Ankyrin; ARE: Anaerobic Induction Elements; BLAST: Basic Local Alignment Search Tool; ERE: Ethylene Responsive Element; EST: Expressed Sequence Tag; FPKM: Fragments Per Kilobase per Million; GA: Gibberellins; GRAY: Grand Average of Hydropathicity; GSDS: Gene Structure Display Server; GTF: Gene Transfer Format; HMM: Hidden Markov Model; HvC3H: barley CCCH genes; IBS: International barley Sequencing Consortium; I2: Instability Index; InDel: Insertion-Deletion; Ks: Non-synchronous substitution; Ks: Synonymous substitution; LTR: Low-Temperature Responsive; MAF: Minor Allele Frequency; MBS: Myeloblastosis Binding Site; MEME: Multiple Expectation Maximization for Motif Elicitation; MW: Molecular Weight; MYA: Million Years Ago; NCBI-CDD: National Center for Biotechnology Information—Conserved Domains Database; ML: Maximum Likelihood; PCA: Principal Component Analysis; p: Isolecitic Point; qRT-PCR: Quantitative real-time PCR; ROS: Reactive Oxygen Species; RR-TZF: Arginine-Rich Tandem CCCH Zinc Finger; SMART: Simple Modular Architecture; TF: Transcription Factor; π: Nucleotide diversity.

**Supplementary Information**

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**Authors' contributions**

YL and LC designed and supervised the project. QA and WP performed the data analysis. WP and YZ performed the experiments. QA and LC drafted the manuscript. All authors contributed to the article and approved the submitted version.

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**Additional file 1.**

**Additional file 2.**

**Additional file 3.**

**Additional file 4.**

**Additional file 5.**

**Additional file 6.**

**Additional file 7.**

**Additional file 8.**

**Additional file 9.**

**Additional file 10.**

**Declarations**

**Ethics approval and consent to participate**

The barley (*Hordeum vulgare*) cultivar Morex was grown and collected by College of Agronomy, Northwest A&F University (Yangling, China), and all samples from this cultivar was adopted for all experiment. These plant materials don’t include any wild species at risk of extinction. No specific permits are required for sample collection in this study. We comply with relevant institutional, national, and international guidelines and legislation for plant study.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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