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Genome-wide identification of chitinase gene family in *Hordeum vulgare*: insights into stress response mechanisms and evolutionary dynamics

Irfan Ali Sabir[1†], Farhan Nabi[1†], Muhammad Aamir Manzoor[1], Fazal Ullah[2], Muhammad Saeed[2], Abeer Hashem[3], Jawaher Alkahtani[3], Elsayed Fathi Abd-Allah[4] and Muslim Qadir[1*]

Abstract

Background Chitinase, a key enzyme family within the pathogenesis-related (PR) protein, plays a crucial role in plant defense by degrading chitin, a major component of fungal cell walls. The HvCHT genes in barley are involved in responses to biotic and abiotic stresses, although their full range of functions is not yet fully understood.

Results In this study, we identified 24 potential HvCHT genes through a genome-wide analysis. The comparative synteny analysis showed conserved relationships between *HvCHT* genes and their homologs in *Sorghum bicolor*, *Oryza sativa*, and *Arabidopsis thaliana*. Chromosomal mapping, gene structure, characterization, protein motif analysis, and miRNA regulation were performed to gain insight into the genetic structures of these genes. Segmental duplication events observed in the HvCHT family suggest an important role in the evolutionary development of these genes. Additionally, *c*is-regulatory element analysis revealed the presence of light-responsive elements, and regulators for Abscisic acid, methyl jasmonate (MeJA), salicylic acid, and gibberellins, indicating potential involvement in stress responses. Transcriptomic data showed differential expression of HvCHT genes in response to salt stress, with distinct patterns observed in leaf and root tissues. Furthermore, the genes defensive responses to drought stress and *Fusarium* infection were characterized across multiple time points. Notably, qRT-PCR analysis confirmed the upregulation of *HvCHT1*, *HvCHT4*, and *HvCHT17*, highlighting their potential involvement in stress-related pathways.

Conclusion These findings provide a comprehensive overview of the HvCHT genes role in barley defense mechanisms, underlining their regulatory functions in biotic and abiotic stressors. The results lay the groundwork for future functional studies on HvCHT genes, with the potential to enhance stress tolerance in crops.

Clinical trial number Not applicable.

Keywords HvCHT gene family, Evolutionary analysis, *Hordeum vulgare*, Stress response mechanisms, Transcriptomic study

[†]Irfan Ali Sabir and Farhan Nabi contributed equally to this work.

*Correspondence:
Muslim Qadir
qadir_m@scau.edu.cn

Full list of author information is available at the end of the article

Introduction

Chitinases are enzymes within the pathogenesis-related (PR) protein family, classified under the glycosyl-hydrolase family. These enzymes catalyze the hydrolytic cleavage of chitin, a structural polysaccharide found in the cell walls of fungi, as well as in the exoskeletons of insects and crustaceans [1–4]. The hydrolysis of chitin results in the generation of chitin fragments which act as Pathogen-Associated-Molecular-Patterns (PAMPs). These PAMPs trigger PAMP-triggered immunity (PTI), a crucial component of the plant immune response [5]. The molecular mechanism of chitinase involves the cleavage of β-1,4-glycosidic bonds between N-acetyl-glucosamine residues within the chitin molecule. This enzymatic activity is essential for plant defense against fungal pathogens, as it not only breaks down the fungal cell wall but also initiates downstream immune signaling pathways [6]. Chitin fragments are recognized as PAMPs to be detected by plant receptors, which activate immune response to limit pathogen spread. Chitinases contribute to a positive response loop within the plant's defense system In addition to their direct antimicrobial action [7]. This dual role of chitinase directly breaking down fungal cell walls and modulating immune signaling pathways underlines its importance in plant defense against fungal pathogens [8]. Firstly, they contribute to the degradation of the fungal cell wall, thereby impeding fungal growth. Secondly, they facilitate the release of minute fragments by the fungal pathogens, which subsequently function as elicitors [9]. Moreover, chitinases show a key role in plants' defense mechanisms against stress, particularly heavy metal exposure [9–12]. Chitinases are induced at the transcriptional and protein levels in response to heavy metal stress, particularly in Pb^{2+} (lead), Hg^{2+} (mercury), and Cd^{2+} (cadmium), in crop species and heavy metal hyperaccumulator plants [13]. Interestingly, transgenic plants overexpressing chitinases demonstrate enhanced resistance to these metals. However, the response of plant chitinases can differ depending on the specific metal ion and the duration of exposure [14, 15]. In response to both biotic and abiotic stresses, these proteins are elicited, thereby categorizing them as integral components of a composite defense system within plants [16].

Chitinases can be classified into Glycosyl-Hydrolase families 18 and 19 (GH18 and GH19) based on the analysis of amino acid sequence homology within the catalytic domains [17] and further these families are categorized into 5 classes (I to V). In the taxonomic classification of Glycoside-Hydrolases (GH), GH18 is categorized within enzyme classes III and V, with distribution observed in bacteria, fungi, animals, viruses, and higher plants. Conversely, GH19 is situated within classes I, II, and IV, predominantly occurring in higher plants [8]. The chitinase gene family in *Hordeum vulgare* (barley) is characterized by the presence of domains such as Chitin_bind_1, Glyco_hydro_19, chitinase_GH19, and Lyz-like superfamily. These domains collectively confer functional attributes associated with chitinase genes in the context of *H. vulgare*, as elucidated by the current research findings. These entities hold the potential to contribute to stress responses elicited by salt, drought, or pathogens and may exert regulatory influence over growth and development processes [18].

Chitinases play a crucial role in plant responses to both biotic and abiotic stressors, including drought, excessive salinity, cold, injury, heavy metal pollution, and ultraviolet light [19]. In pepper plants, knocking down the chitinase gene *CaChiIV1* increased susceptibility to *Phytophthora capsici* infection, and mannitol treatment led to a significant decrease in root physiological activity, such as reduced water uptake and root growth [20]. This reduction in root activity was found associated with a substantial reduction in total chlorophyll content and an increase in electrolyte leakage, underlining the role of chitinase in the response to drought stress. Additionally, the overexpressing *LcCHI2*, a Class II chitinase from *Leymus chinensis*, in tobacco and maize reduced Na + and MDA levels and lowered electrical conductivity under salt stress, suggesting a protective role against oxidative damage and ion imbalance [21]. Wintersweet, bromegrass, and rye include chitinase genes (*CpCHT1*, *BiCHT1*, *CHT9*, and *CHT46*) that produce antifreeze proteins to prevent freezing injury [22, 23]. Overexpressing *CHIT33* and *CHIT42* from *Trichoderma harzianum* in tobacco plants increased tolerance to heavy metals, such as Cu, Hg, and Cd [13]. Similarly, exposure to heavy metals like Pb, Cd, and As (arsenic) induced the production of various chitinase isoforms in crops such as faba bean, soybean, pea, barley, dwarf sunflower, and maize [9, 15]. Additionally, SA, UVC radiation, and wounding increase the accumulation of IF3 mRNA and the protein it encodes in lupine [24]. Plants use chemical substances such as ABA (Abscisic acid), JA (Jasmonic acid), SA (Salicylic acid), ETH (Ethylene), systemin, and bioelectrical impulses as defense signals [25, 26]. Chitinase genes, which play a role in plant defense, are frequently triggered by phytohormones in response to wounding or stress. ABA and JA, plant stress hormones, significantly increase *AtChiC* expression in Arabidopsis with expression levels showing a notable fold change compared to control plants [27]. SA significantly upregulated the strawberry chitinase gene *FnCHIT2*. Ectopic expression of *FnCHIT2* in Arabidopsis increased resistance to *Colletotrichum higginsianum* and *Pseudomonas syringae* pv. tomato DC3000 [28]. MeJA treatment in kiwifruit increased *AcCHI* activity and reduced damage caused by *Botryosphaeria dothidea* [29]. Overexpressing the cotton chitinase gene *GhChi6* in Arabidopsis plants enhanced

resistance to aphids by increasing transcript levels of key genes involved in the SA signaling pathway. However, the expression levels of genes involved in the JA and ETH signaling pathways decreased compared to wild-type plants [30].

The economic significance of hexaploid wheat and barley, ranked as the second and fourth most produced cereal crops globally in 2004, respectively (http://http://faostat.fao.org/), highlights the need for improving yield and quality. Addressing this demands a comprehensive understanding of the genetic factors that govern critical traits [31]. In particular, the chitinase gene family in barley, which plays a pivotal role in defense against both biotic and abiotic stresses remains underexplored. A systematic genome-wide study on this family is important to understand how it barley responds to pathogen attacks and mitigates stressors such as salinity, drought, and heat. Specifically, chitinase genes like *HvCHT1* and *HvCHT4* are key players in barley resistance mechanisms against fungal pathogens such as *Fusarium* and *Blumeria*, as well as in improving tolerance to environmental stresses. Understanding these gene functions will provide critical insights into barley resilience, paving the way for strategies to enhance global barley production and sustain food security.

Materials and methods

Database search, sequence retrieval, and physiochemical properties

The amino acid sequences related to the chitinase gene family were retrieved from the Phytozome (https://phytozome-next.jgi.doe.gov/) and Ensembl (https://www.ensembl.org/index.html) databases. This protein family comprises three domains: the Lyz-like superfamily, which houses the chitinase_GH19, and Glyco_hydro_19; STKc_IRAK. For the acquisition of chitinase gene family sequences of *Oryza sativa*, and *Sorghum bicolor*, the Phytozome platform (https://phytozome.jgi.doe.gov/) was employed in conjunction with the BLAST-P tool (Protein Basic Local Alignment Search Tool) [32]. Proteins lacking any of the specified domains were filtered out using the Motif Finder tool (https://www.genome.jp/tools/motif/) with default parameters. Additional details, such as chromosome number, base pair location, orientation, and amino acid length, were obtained from Phytozome. The physicochemical properties, including isoelectric point (pI), molecular weight, and instability index, were retrieved using the ProtParam tool (https://web.expasy.org/protparam/).

Comparative phylogenetic analysis of chitinase genes

The protein sequences of *Arabidopsis thaliana* were retrieved from the TAIR database (https://www.A.thaliana.org/). Sequence alignment was conducted using the MUSCLE tool within Mega-11 software. A phylogenetic tree was constructed employing the neighbor-joining method in MEGA-11, incorporating the Poisson model, pairwise deletion, and 1,000 bootstrap replicates as parameters [33]. For improved visualization and customization, the phylogenetic tree was further refined using the iTOL platform (https://itol.embl.de/) [34].

Cis-element and conserved motif domain analysis

The 1000-bp upstream promoter sequences associated with the chitinase gene family were retrieved from the Phytozome database. *C*is-acting regulatory elements were identified using the PlantCare database (https://bioinformatics.psb.ugent.be/webtools/plantcare/html/) [35]. A heatmap was accomplished using TBtools. For the analysis of conserved domains, the NCBI Conserved Domain Database (CCD) tool (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi), was employed. Conserved motif analysis was conducted using the MEME Suite (https://meme-suite.org/meme/) with default settings for a maximum of 20 motifs [36].

Gene structure, gene ontology, and sub-cellular localization

Gene structure analysis, focusing on exon-intron organization, was conducted using the GSDS 2.0 tool (http://gsds.gao-lab.org/) [37]. Gene ontology analysis was conducted using ShinyGO v0.741 (http://bioinformatics.sdstate.edu/go74/) [38]. Sub-cellular localization predictions for the chitinase gene family in *Hordeum vulgare* were obtained from the WoLF PSORT database (https://wolfpsort.hgc.jp).

Gene mapping, syntenic, and gene duplication analysis

The comparative synteny analysis of *Hordeum vulgare* was performed with *Arabidopsis thaliana*, *Oryza sativa*, and *Sorghum bicolor*. The synteny relationships of *HvCHT* genes were visualized using the circos module in TBtools. Paralogous genes within the chitinase protein family were identified through sequence alignment followed by phylogenetic analysis. TBtools software was used to calculate Ka/Ks ratios, which provide insights into synonymous (silent) and non-synonymous (amino acid changing) substitution rates. Divergence times for gene duplication events were calculated using Ks values, with the formula T = Ks / 2r, where 'r' represents the divergence rate ($r = 6.5 \times 10^{-9}$) [39]. A Ka/Ks ratio less than 1.0 suggests purifying (negative) selection, indicating that the gene is undergoing evolutionary pressure to eliminate harmful mutations. A ratio of approximately is indicative of neutral selection, where mutations do not affect the genes function and their spread in the population is governed by gene point. A Ka/Ks ratio greater than suggests positive selection, which implies that mutation

confers an adaptive advantage to the organism leading to the spread of beneficial genetic changes [40].

Protein-protein interaction and MiRNA

In order to predict protein-protein interactions, the STRING database (https://string-db.org/) was utilized [41], allowing for the exploration and prediction of potential interactions among proteins associated with HvCHT genes. For the identification of microRNA (miRNA) targets within the coding DNA sequences (CDS) of HvCHT genes, the psRNATarget database (https://www.zhaolab.org/psRNATarget/) was employed using transcriptomic data from H. vulgare cultivar "Morex" from the NCBI GEO source. This analysis aimed to elucidate potential interactions between miRNAs and HvCHT gene sequences [42].

Transcriptomic analysis of HvCHT genes

Gene expression profiling in response to salt stress

The RNA-seq dataset, accessed by the National Center for Biotechnology Information Gene Expression Omnibus (NCBI GEO) database (accession number GSE230751) (https://www.ncbi.nlm.nih.gov/geo/), was used to investigate gene expression patterns in response to salt stress in barley. The samples of leaf and root were collected at three time points: 1 h, 24 h, and 10 days post-treatment. Three biological replicates were performed for each time point, with each replicate consisting of pooled samples from 5 plants. The barley cultivar Nasonijo was used for gene expression profiling under salt stress conditions.

Gene expression to drought stress correlated with Fusarium inoculation

The dataset for this study, sourced from the NCBI GEO database (accession number GSE223521) (https://www.ncbi.nlm.nih.gov/geo/), was used to investigate the expression profiling of HvCHT genes. The transcriptional expression analysis involved the inoculation of two *Fusarium* strains, *F. avenaceum* and *F. culmorum*, into *H. vulgare* cultivar under drought and irrigated conditions. Expression data were recorded at two time points, 48 and 96-hour post-inoculation to assess the enhancement of stress response in barley under these conditions. The drought conditions were induced by adding 160 mM NaCl to the growth medium, while control plants were irrigated under normal conditions.

Transcriptome analysis of barley root meristem under heavy metal

The study also includes aluminum stress treatment in barley root meristems. A concentration of 110 µM ammonium ($AlCl_3$) was used to induce aluminum toxicity. This concentration is commonly used in plant stress studies to evaluate the impact of aluminum on plant growth and development. The dataset for this analysis, retrieved from the NCBI GEO repository (accession number GSE167271) https://www.ncbi.nlm.nih.gov, was used to study the expression profiling of *HvCHT* genes in response to low pH and aluminum stress in barley root meristems. The study, conducted on the Sebastian cultivar, utilized short-term (24 h) and long-term (7 days) hydroponic experiments. Each experiment included three replicates for each experimental condition (low pH = 4 and aluminum-treated with pH = 4), with each replicate consisting of root meristems from at least 8 plants, averaging 5 meristems per plant.

Plant material preparation, stress treatment, and qRT-PCR analysis

Seeds of barley Morex were obtained from the South China Agricultural University (SCAU), Guangzhou, Guangdong, and used as the experimental material. Barley seeds were surface sterilized with 5% NaClO (sodium hypochlorite) for 5 min and washed with distilled water. Sterilized seeds were placed on wet filter paper in Petri dishes at 25 °C for 5 days to germinate. Seeds were transferred to hydroponic growth plates containing half-strength Hoagland nutrient solution when seedlings reached 4–5 cm and then grown in a growth chamber at 20 °C (day) and 15 °C (night) with a 16/8-hour light/dark cycle and 50% humidity until the trifoliate leaf stage. At the trifoliate leaf stage, the seedlings were subjected to stress treatments with 160 mM NaCl for salt stress or 110 µM ABA to the culture medium as an ABA-induced stress control. ABA plays an important role in mediating stress responses, particularly under drought and salt stress conditions. This regulates various stress-responsive pathways, including stomatal closure and gene expression, which help plants survive with abiotic stress.

Root and leaf samples were collected at 0 h, 6 h, and 12 h post-treatment to assess the stress responses. Control seedlings were grown without stress agents. The samples were immediately frozen in liquid nitrogen, and stored at − 80 °C. RNA was extracted using the RNAprep Pure Plant Kit (Tiangen) and reverse transcription was performed with the PrimeScript RT reagent Kit (TaKaRa) using 1 µg RNA. Gene-specific primers were designed with Beacon Designer 7.9. qRT-PCR was performed using SYBR premix ExTaq II (TaKaRa), with Actin (1) as the reference gene [43]. The thermal cycling conditions were: at 95 °C for 30 s followed by 40 cycles of 95 °C for 15 s, and 60 °C for 20 s. Data were analyzed using the $2-\Delta\Delta CT$ method [44].

Subcellular localization analyses

The complete coding sequences of *HvCHT1* and *HvCHT4* (excluding the termination codon) were

integrated into the pGreen-35 S-GFP vector to create recombinant plasmids (primer details are provided in Table S2). Subsequently, these recombinant constructs, pGreen-35 S-HvCHT1-GFP and pGreen-35 S-HvCHT4-GFP, were individually introduced into *Agrobacterium tumefaciens* strain GV3101 (pSoup-p19), sourced from Weidi Biotechnology in Shanghai, China. Concurrently, a control vector, pGreen-35 S-GFP, was also prepared. The Agrobacterium strains harboring the specific constructs were introduced into the leaves of *Nicotiana benthamiana* plants, together with At1g22590-RFP (used as a marker). After 48 h of incubation, leaf samples were collected, and the fluorescence signals of GFP and RFP were observed using a fluorescence microscope (ZEISS LCM-800, Oberkochen, Germany) [45].

Results

Genome-wide identification of *HvCHT* genes

In this study, we identified and characterized 24 HvCHT genes in barley through a comprehensive genome-wide analysis. We analyzed their physiochemical properties, including molecular weight (Mw), isoelectric points (pI), chromosomal location, coding sequence length, Grand Average of Hydropathy (GRAVY), and Aliphatic and Instability (II) index, utilizing multiple databases. The HvCHT genes exhibited significant diversity in peptide length. *HvCHT15* had the longest peptide sequence with 680 amino acids, while *HvCHT7* had the shortest at 107 amino acids (Table 1). This variation in peptide length suggests diverse functional roles for the HvCHT proteins, likely reflecting their distinct mechanisms in stress responses. Additionally, the lengths of the CDS varied, with *HvCHT15* over having the longest CDS at 2046 base pairs.

The genes were distributed across several chromosomes (chrUn, chr1H, chr2H, chr3H, chr4H, chr5H, and chr7H), with both forward and reverse orientations observed in equal proportions.

The molecular weights of the identified HvCHT proteins varied, with *HvCHT15* having the highest molecular weight (MW) of 73,947.94 kDa, while *HvCHT14* with the lowest at 16,910.08 kDa. However, in certain cases, MW data was unavailable due to incomplete sequence information, which was noted for genes like *HvCHT7*, *HvCHT10*, and *HvCHT24*. The Instability Index (II) was calculated to assess protein stability. Proteins with an II value below 40 are classified as stable, and those above 40 are considered unstable. Our analysis indicated that *HvCHT1*, *HvCHT9*, *HvCHT11*, *HvCHT12*, *HvCHT13*, and *HvCHT15* are predicted to be unstable due to their higher II values. The GRAVY score, which indicates protein polarity, showed that *HvCHT11* and *HvCHT17* had positive values, demonstrating their hydrophilic nature, whereas the other HvCHT proteins had negative scores, indicating hydrophobicity. The isoelectric points (pI) of the HvCHT proteins varied from 7.32 in *HvCHT14* to 9.35 in *HvCHT17*. This variation suggests that these proteins have different net charges at physiological pH, potentially affecting their interactions and functions. Lastly, the aliphatic index, an indicator of protein thermodynamic stability, ranged from 51.87 in *HvCHT14* to 80.63 in *HvCHT15*, indicating that *HvCHT15* is likely the most thermodynamically stable.

Comparative phylogenetic and domain analysis of the chitinase gene

In this investigation, we systematically curated genetic sequences of 24 genes from *H. vulgare*, 17 *from Sorghum bicolor*, 20 from *Oryza sativa*, and 15 from *Arabidopsis thaliana*. A total of 76 chitinase gene sequences were employed to construct a comprehensive phylogenetic tree utilizing the maximum joining method. The phylogenetic analysis classified the HvCHT genes into three major clades (Clades 1, 2, and 3). Clade 1 contained the highest number of HvCHT genes (9), followed by Clade 3 (8 genes) and Clade 2 (7 genes), respectively (Fig. 1). The motif analysis reveals conserved motifs and their corresponding domains in each HvCHT gene. Motifs analysis revealed conserved motifs across HvCHT genes, with motifs 2, 3, 6, and 8 showing significant conservation. All of these harbor the conserved Chitin_bind_1 domain along with the underlying sub-domain Lyz-like superfamily (Figure S1). The identified HvCHT genes exhibit variability in the number of motifs, ranging from a maximum of 10 motifs in *HvCHT5*, *HvCHT10*, *HvCHT13*, *HvCHT22*, and *HvCHT23* to a minimum of 2 motifs in *HvCHT12*.

Structural feature, mapping, and prediction of *C*is-acting regulatory elements

The genomic architecture and intron-exon distribution across various gene loci are depicted in Fig. 2A, as per the findings presented in this investigation. Notably, among the identified HvCHT genes, eleven exhibit a single structure of exons devoid of introns, while seven harbor two exons. Notably, a subset of HvCHT genes, namely *HvCHT4*, *HvCHT15*, *HvCHT16*, *HvCHT17*, *HvCHT19*, and *HvCHT22* manifests the maximal exon count of three (Fig. 2A). The biochemical pathways involved in the catabolism of various sugars, macromolecules, and the metabolic process regulating chitin are controlled by the discovered HvCHT genes. These genes govern essential biological processes, including chitin binding, chitinase activity, and hydrolase activity directed at glycosidic linkages (Figure S2). Gene mapping revealed the genomic distribution of HvCHT genes across multiple chromosomes. The highest concentration of HvCHT genes was found on chromosome 7 H, which contained six genes.

Table 1 This illustrates the physiochemical properties of identified *HvCHT* genes

Phytozome ID	Gene ID	Chr No	Start	End	Direction	CDS	Peptide	Mw	pI	II	AI	GRAVY
HORVU0Hr1G016310	*HvCHT1*	chrUn	86,914,109	86,915,460	Reverse	897	298	33706.78	10.72	69.77	91.01	-0.39
HORVU2Hr1G085270	*HvCHT2*	chr2H	616,911,312	616,919,740	Reverse	813	270	28558.01	8.69	23.17	54.33	-0.302
HORVU1Hr1G017670	*HvCHT3*	chr1H	60,972,623	60,974,125	Reverse	903	300	33375.33	5.18	38.8	64.17	-0.363
HORVU1Hr1G052430	*HvCHT4*	chr1H	388,627,044	388,643,679	Forward	759	252	26601.63	6.09	25.77	67.14	-0.151
HORVU1Hr1G062030	*HvCHT5*	chr1H	447,425,688	447,427,360	Reverse	1023	340	35814.4	8.28	38.74	63.62	-0.159
HORVU1Hr1G078050	*HvCHT6*	chr1H	519,071,261	519,118,868	Reverse	687	228	23115.05	8.23	26.63	35.31	-0.263
HORVU1Hr1G078070	*HvCHT7*	chr1H	519,101,087	519,101,687	Forward	324	107	N/A	N/A	16.9	11.03	-0.399
HORVU2Hr1G085280	*HvCHT8*	chr2H	617,146,749	617,148,154	Reverse	507	196	20464.97	8.04	37.03	62.86	-0.236
HORVU2Hr1G126720	*HvCHT9*	chr2H	764,378,804	764,380,707	Reverse	327	108	10995.65	8.48	47.91	68.8	0.078
HORVU2Hr1G126740	*HvCHT10*	chr2H	764,571,006	764,574,276	Forward	966	322	N/A	N/A	32.79	59.47	-0.155
HORVU2Hr1G126760	*HvCHT11*	chr2H	764,637,920	764,638,247	Forward	327	108	10995.65	8.48	47.91	68.8	0.078
HORVU3Hr1G027030	*HvCHT12*	chr3H	113,173,687	113,175,060	Reverse	492	365	40585.48	12.17	84.61	77.29	-0.835
HORVU3Hr1G064470	*HvCHT13*	chr3H	491,894,155	491,895,847	Forward	963	320	33642.56	7.41	41.51	57.09	-0.21
HORVU3Hr1G089420	*HvCHT14*	chr3H	628,355,426	628,356,316	Reverse	501	166	16910.08	6.01	27.72	51.87	-0.03
HORVU4Hr1G081590	*HvCHT15*	chr4H	619,752,839	619,755,607	Forward	2046	680	73947.94	6.22	50.23	80.63	-0.198
HORVU5Hr1G039990	*HvCHT16*	chr5H	292,466,129	292,467,632	Forward	960	319	35217.82	7.16	31.95	63.61	-0.333
HORVU5Hr1G074340	*HvCHT17*	chr5H	542,981,415	542,984,571	Forward	999	209	23125.34	9.35	33.8	90.57	0.391
HORVU5Hr1G098470	*HvCHT18*	chr5H	607,875,827	607,877,174	Reverse	939	312	33366.5	7.32	35.1	59.26	-0.178
HORVU7Hr1G051580	*HvCHT19*	chr7H	194,170,649	194,172,694	Reverse	915	304	33423.68	6.44	38.12	64.31	-0.307
HORVU7Hr1G106900	*HvCHT20*	chr7H	621,060,168	621,061,170	Forward	642	213	21881.82	8.09	34.26	44.65	-0.196
HORVU7Hr1G107000	*HvCHT21*	chr7H	621,634,993	621,636,172	Reverse	579	192	19521.93	7.32	30.56	42.34	-0.235
HORVU7Hr1G113270	*HvCHT22*	chr7H	636,588,301	636,590,301	Forward	972	323	34089.59	7.36	38.97	63.59	-0.067
HORVU7Hr1G121850	*HvCHT23*	chr7H	654,817,538	654,819,565	Forward	990	330	34966.27	8.63	34.95	54.52	-0.205
HORVU7Hr1G121860	*HvCHT24*	chr7H	654,821,505	654,822,312	Forward	807	269	N/A	N/A	33.31	63.64	-0.055

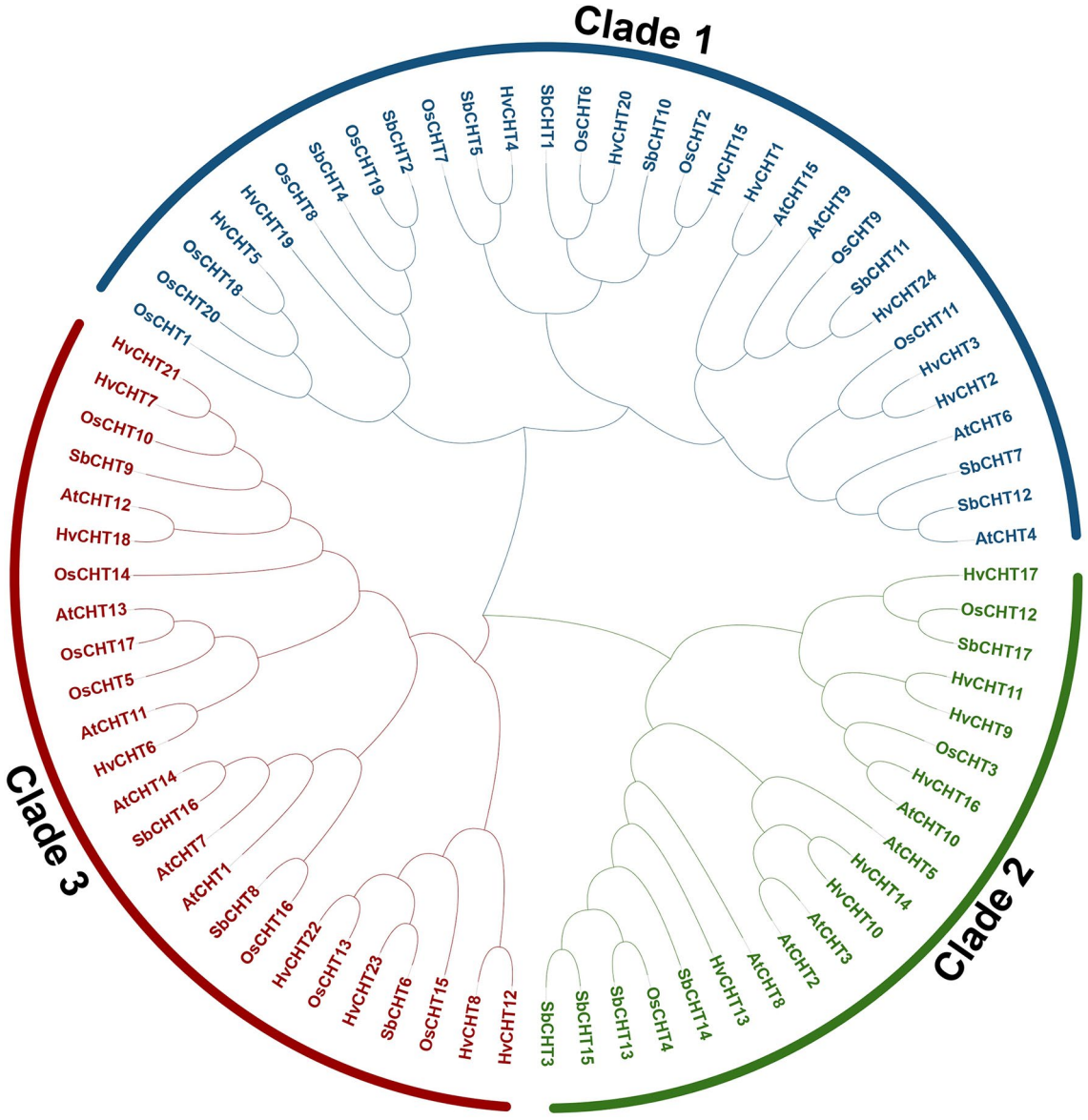

Fig. 1 The phylogenetic tree shows the evolutionary relationship of the chitinase gene family in *Hordeum vulgare* (Hv), *Sorghum bicolor* (Sb), *Oryza sativa* (Os), and *Arabidopsis thaliana* (At), generated using MEGA-11 software. The tree was constructed based on sequence alignment of 76 chitinase gene sequences with bootstrap values

Chromosomes 1 H and 2 H each harbored five HvCHT genes, while chromosomes 3 H and 5 H each contained three genes. The chromosomes "chrUn" contained one gene, visually represented in Figure S3.

The present investigation revealed a diverse array of cis-regulatory elements with distinct physiological and biological functionalities. Notable elements include those responsive to light, ABA, MeJA, salicylic acid, and gibberellins, alongside motifs associated with anaerobic induction, meristem expression, seed-specific regulation, zein metabolism, and defensive regulatory processes (Fig. 2B). The identified HvCHT genes prominently feature *c*is-regulatory elements, including CAAT-box, ABRE, CGTCA-motif, TGACG-motif, as-1, and DRE predominantly located in the promoter region of the genes. These elements are known to regulate gene expression in response to various environmental cues. Notably, the as-1 motif, detected in 19 HvCHT genes, is known to contribute to the plant protective response to xenobiotic stress (Li., 2024), while DRE is present in 21 HvCHT genes, mediating responses to dehydration. Other *cis*-elements may have minimal or negligible contributions, collectively participating in diverse metabolic responses and the induction of various anaerobic processes.

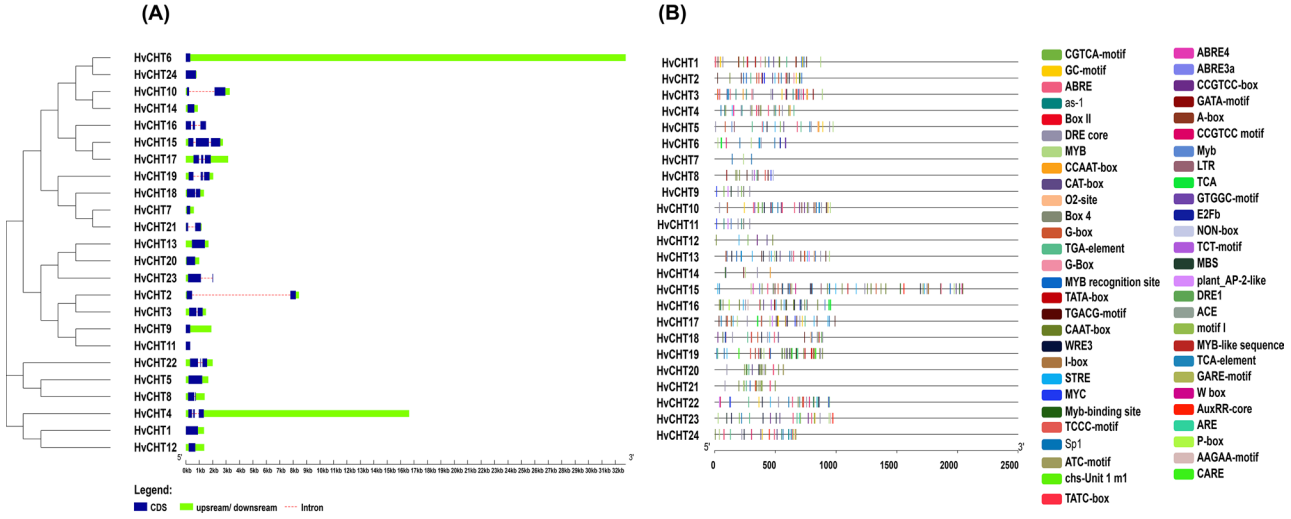

Fig. 2 (**A**) Phylogenetic analysis of the intron-exon structures across various HvCHT genes, illustrating the numbers and arrangement of exons and introns in each gene. (**B**) This diagram shows *cis*-regulatory elements linked to HvCHT genes, revealing their functional roles by investigating the promoter regions of individual genes

Ka/Ks and sub-cellular localization analysis

The analysis of Ka/Ks values (non-synonymous and synonymous substitution rates) within the HvCHT genes revealed a range from 0.129014653 (*HvCHT16_HvCHT17*) to a maximum of 3.383775314 (*HvCHT1_HvCHT7*), the latter representing the highest value across all comparisons. The paralogous gene pair *HvCHT1_HvCHT7* exhibited the lowest divergence at 17.362639 million years ago, whereas the maximum divergence of 445.0775466 million years ago was seen for the gene pair *HvCHT9_HvCHT16*, suggesting segmental duplication events. In the context of evolutionary selection, a ka/ks number below 1 indicates purifying selection, while a value over 1 suggests positive or diversifying selection (Fig. 3). The study's findings indicate that the anticipated subcellular localization of HvCHT genes encompasses various cellular compartments, including the extracellular region, vacuole, chloroplast, nucleus, Golgi apparatus, endoplasmic reticulum (ER), and plastids. The primary localization prediction for the examined genes is predominantly inside the extracellular area, chloroplast, and endoplasmic reticulum (ER) (Figure S4).

Protein-protein interaction and MiRNA

The *HvCHT20* protein showed the highest degree of connectivity within the protein-protein interaction network, signifying a central role in protein interactions. *HvCHT6*, *HvCHT7*, *HvCHT14*, and *HvCHT21* exhibited robust associations with *HvCHT20*, which indicate coordinated biological processes. In contrast, several other genes show limited interactions, such as *HvCHT12* and *HvCHT15* exhibit minimal or no discernible interactions with other genes (Figure S5). The comprehensive analysis of mature micro-RNAs reveals a discernible correlation with the targeting of 24 identified HvCHT proteins, with a specific focus on six micro-RNAs. Notably, the elucidated micro-RNAs, namely hvu-miR1130, hvu-miR6189, hvu-miR6184, hvu-miR6186, hvu-miR5053, and hvu-miR6210, exhibit a pronounced regulatory effect on the HvCHT set. Further investigation demonstrates that hvu-miR6186 and hvu-miR6210 exhibit a targeted interaction with *HvCHT17* genes, thereby intricately modulating cleavage and translation processes. A comprehensive tabulation of detailed information about these micro-RNAs is provided in Table S1 for reference and elucidation (Figure S5).

Gene duplication and syntenic relationship

In the context of the paralogous behavior of HvCHT genes, notable instances of paralogous relationships are observed on barley chromosomes 1 H, 2 H, and 7 H. Specifically, tandem behavior is evident for *HvCHT16*, *HvCHT17*, and *HvCHT18* on chromosome 5 H, while *HvCHT12*, *HvCHT13*, and *HvCHT14* exhibit tandem behavior on chromosome 3 H (Fig. 4). Conducting dual syntenic analysis reveals orthologous behavior between barley genes and those of other plant species. In barley, chromosomes 1 H and 4 H display orthologous relationships with chromosomes 1, 2, and 4 of Arabidopsis. In the case of rice, chromosomes 1 to 6, and 8 exhibit orthologous behavior with chromosomes 1 H, 2 H, 5 H, and 7 H of barley. Similarly, for sorghum, chromosomes 1, 2, 3, 4, 6, 7, 9, and 10 demonstrate orthologous behavior with chromosomes 1 H, 2 H, 5 H, and 7 H of barley (Fig. 4).

Fig. 3 The Ka/Ks ratio analysis was used to assess the relationship between non-synonymous (Ka) and synonymous (Ks) substitution rates in HvCHT genes. Gene duplication events and selective pressure acting on paralogous HvCHT gene pairs were evaluated based on these Ks and Ka values

Expression analysis of HvCHT genes

Transcriptional profiling of gene expression in response to salt stress

The transcriptomic gene expression analysis was conducted to investigate the effect of salt stress at 1 h, 24 h, and 10 days after treatment. The gene expression analysis in leaf tissues revealed both upregulation and downregulation of HvCHT genes. *HvCHT17* was upregulated within the first hour, while *HvCHT2*, *HvCHT4*, *HvCHT9*, *HvCHT15*, *HvCHT18*, and *HvCHT22* showed significant upregulation after prolonged salt exposure (Fig. 5). In root tissue, the genes *HvCHT4*, *HvCHT5*, *HvCHT10*, *HvCHT17*, and *HvCHT20* exhibited substantial up-regulation in response to salt stress, detected at several time intervals as shown in Fig. 5.

Transcriptional profiling of gene expression in response to drought stress correlated with Fusarium inoculation

The transcriptomic gene expression analysis was performed to examine the effects of drought stress on barley, in combination with *Fusarium* strain introduction. The plants were inoculated with the *F. avenaceum* strain at 48 and 96 h to assess its impact on drought tolerance. The results show that inoculation with *F. avenaceum* significantly increased barley sensitivity to drought stress, as indicated by the upregulation of *HvCHT5* and *HvCHT9* (Fig. 6). Similarly, the *HvCHT10* gene was upregulated 48 h after the start of drought stress following inoculation with *F. culmorum* strain. Additionally, it shows that *HvCHT2*, *HvCHT18*, and *HvCHT22* exhibited up-regulation when *Fusarium* inoculation occurred at the 96-hour time point.

Transcriptome analysis of barley root meristem under heavy metal (Al) at low pH stress

The comprehensive analysis of the barley transcriptome reveals a noteworthy response to heavy metal stress at low pH. Specifically, twelve HvCHT genes exhibit significant upregulation in both short-term and long-term experiments. This consistent upregulation of HvCHT genes implies their crucial role in conferring heavy metal tolerance to barley under adverse environmental conditions. The expression analysis revealed the highest expression levels in *HvCHT7* and *HvCHT8*, with moderate expression observed in genes *HvCHT 14*, *10*, *11*, and *24*. Conversely, certain genes such as *HvCHT2*, *12*, *13*, and *18* remained silent across all phases. While these silent genes may not be directly associated with

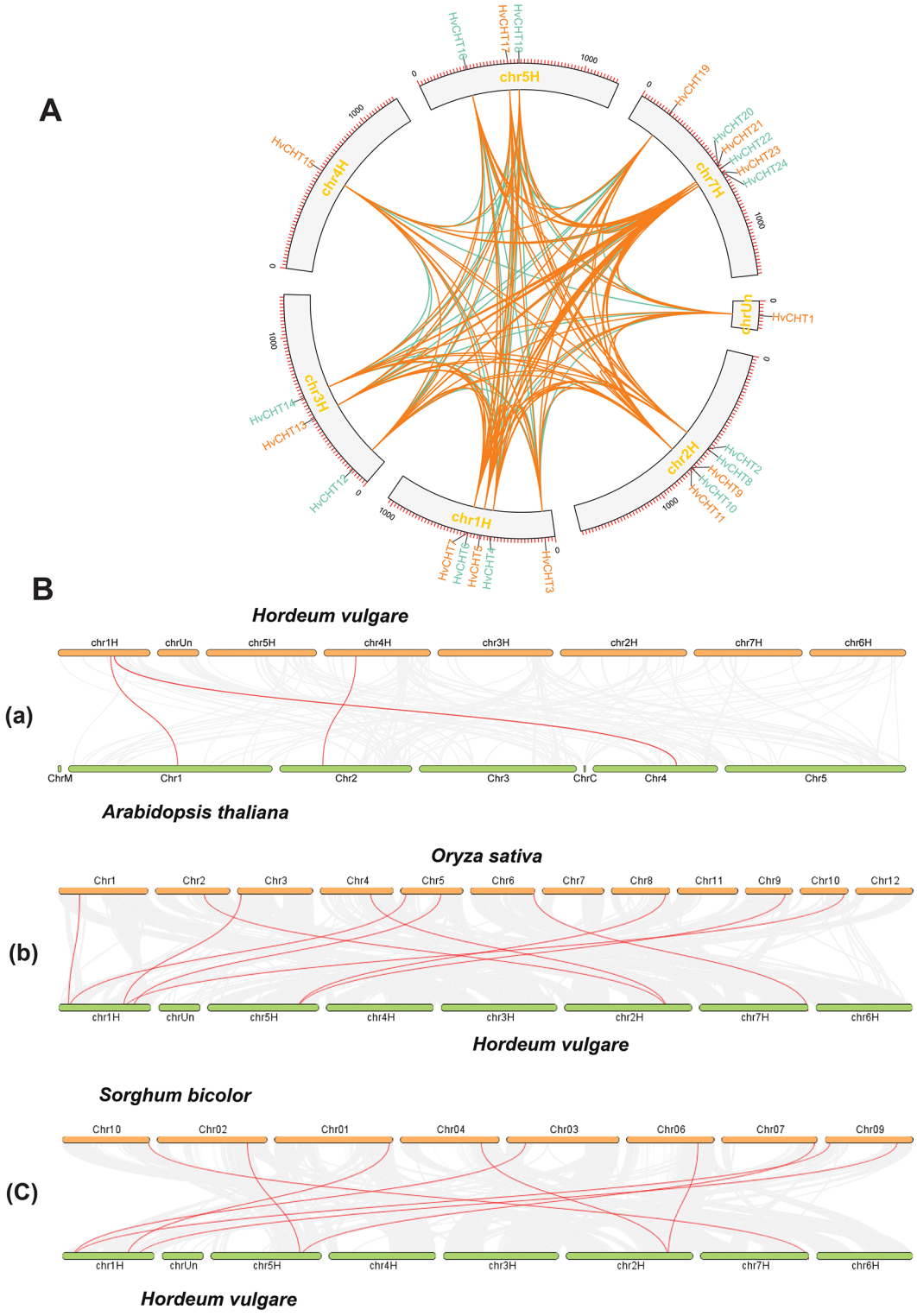

Fig. 4 The synteny analysis of identified genes across different plant species. (**A**) Circos plot showing paralogous gene relationships within *Hordeum vulgare* (barley), underlining gene duplication events. (**B**) Collinearity analysis between *Hordeum vulgare* and other species: (**a**) *Arabidopsis thaliana* and (**b**) *Oryza sativa* and their orthologous gene relationships. The Red lines indicate orthologous gene pairs, while gray lines represent additional syntenic relationships

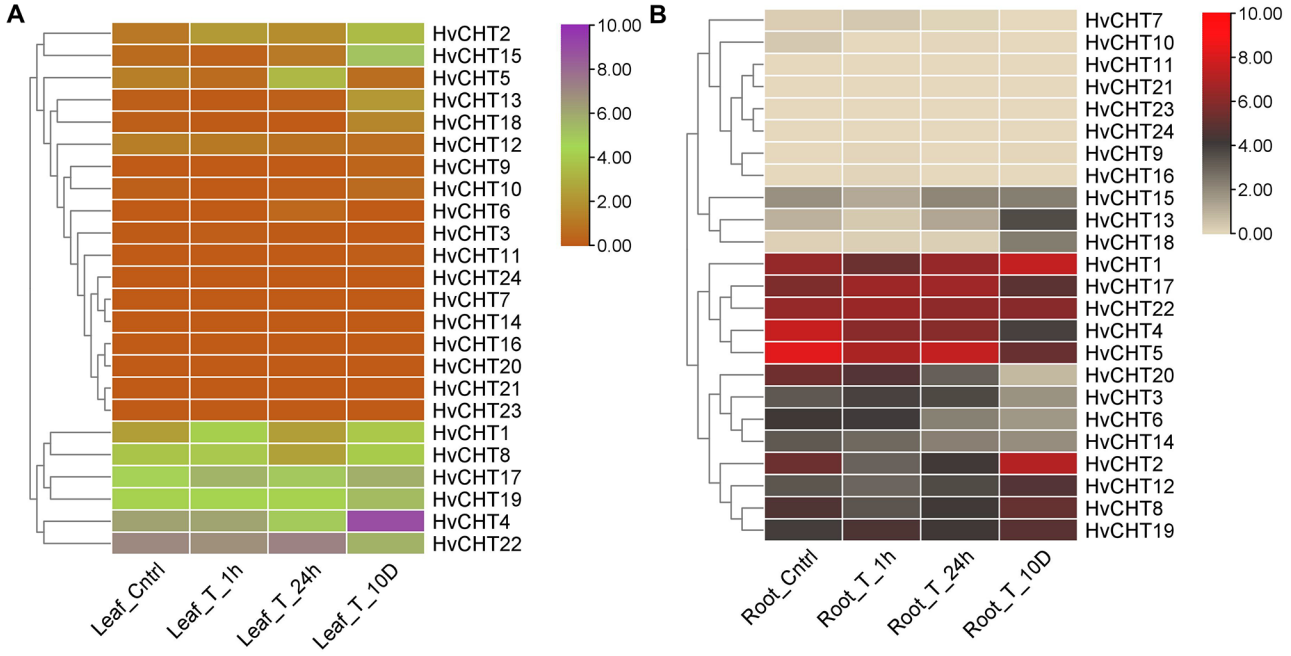

Fig. 5 Heatmap depicting the transcriptional response of HvCHT gene expression in leaf (**A**) and root (**B**) tissues under salt stress at 1 h, 24 h, and 10 days

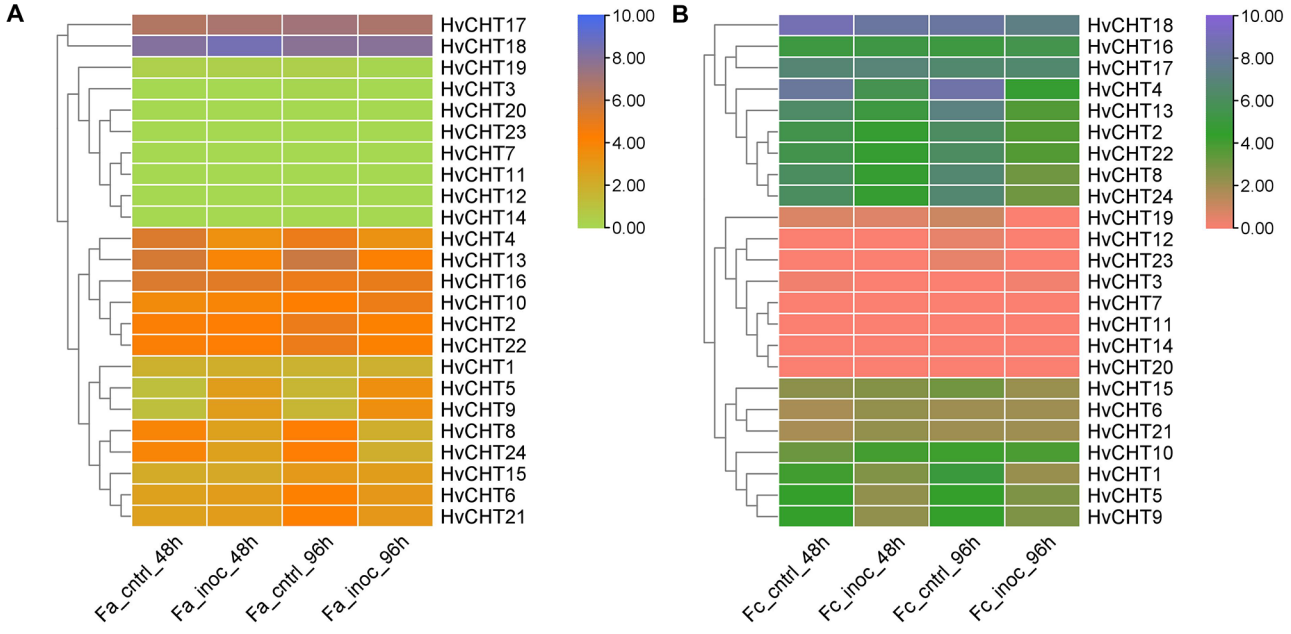

Fig. 6 Heatmap illustrating the transcriptional response of HvCHT gene expression under biotic stress conditions. The response of HvCHT genes to drought stress, including Fusarium avenaceum (**A**) and Fusarium culmorum (**B**), inoculation at 48 and 96 h

stress-related functions, they could potentially serve specialized roles. Overall, these findings highlight the diverse functionalities of the chitinase gene family, which are crucial for plant adaptation and survival in challenging environments (Fig. 7).

Expression pattern of HvCHT genes under salt and hormone treatment

Investigates the expression dynamics of the *HvCHT* gene family in response to salt stress and the phytohormones abscisic acid (ABA) using quantitative real-time PCR (qRT-PCR). Experiments were conducted using ABA at a concentration of 110 µmol and NaCl at 160 mmol. The gene expression was assessed at three time

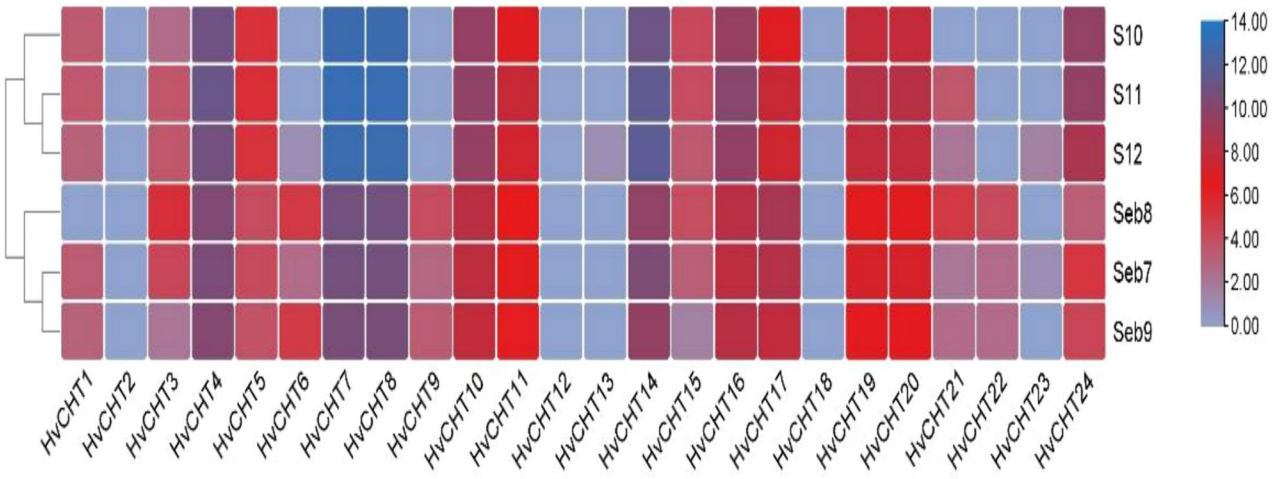

Fig. 7 The expression pattern of HvCHT genes in barley root meristems under heavy metal stress and low pH level. The S10, S11, and S12 represent short-term (24 h) treatments, while Seb7, Seb8, and Seb9 correspond to long-term (7 days) treatments under pH 6, pH 4, and aluminum stress. The heatmap shows relative gene expression levels

points, including 0 h, 6 h, and 12 h (Fig. 8). In response to ABA treatment, significant upregulation was observed for *HvCHT4*, *HvCHT5*, *HvCHT17*, and *HvCHT18* at the 6-hour time point. Conversely, *HvCHT1*, *HvCHT7*, *HvCHT20*, and *HvCHT23* showed initial upregulation but exhibited minimal expression changes throughout the experiment. Under salt stress conditions (160 mmol NaCl), *HvCHT1*, *HvCHT4*, and *HvCHT17* exhibited enhanced expression at all-time points, with HvCHT4 reaching peak expression at 6 h. In contrast, *HvCHT18* and *HvCHT20* demonstrated slight initial upregulation followed by downregulation as the stress response progressed (Fig. 8; Table S2).

Subcellular localization analyses of HvCHT

To study the subcellular localization of *HvCHT1* and *HvCHT4*, which encode CHT proteins, their full CDS were combined with GFP to construct 35 S- HvCHT -GFP vectors. The subcellular localization analysis showed that HvCHT proteins are mainly located in the extracellular space, chloroplasts, and the endoplasmic reticulum (ER), consistent with the predicted nuclear localization of CHT proteins (Fig. 9). In contrast, the GFP signal from the positive control was detected in both the cytoplasm and nucleus. These results indicate that *HvCHT1* and *HvCHT4* are primarily nuclear proteins.

Discussion

The current study provides a comprehensive genome-wide analysis of the HvCHT gene family in barley, offering valuable insights into their role in stress response and evolutionary dynamics [46]. Chitinases are found in a wide range of organisms, including bacteria, fungi, plants, insects, animals, and humans [4]. They have been widely studied in various plant species, such as apples [47], cucumber, tomato, and mustard greens [48–50] as well as crops like sugarcane [51], cotton [52], and trees like *Eucalyptus grandis* [53] and *Populus trichocarpa* [54]. However, the exploration of this gene family in barley remains relatively unexplored. A total of 24 HvCHT genes were identified, revealing their complex roles in biotic and abiotic stress responses. This investigation sheds light on the molecular and functional diversity of these genes, highlighting their crucial role in enhancing barley defense mechanisms.

A genome-wide examination of the chitinase gene family in barley under the influence of the inoculation of *Fusarium* strains was assumed to exhibit the intricate defensive mechanisms activated in barley in response to drought, salt, and heavy metals stress conditions [55]. The findings of this investigation unveil a sophisticated interplay of molecular mechanisms that play a pivotal role in barley defense response. A systematic exploration of available databases, including Phytozome and TAIR [56], was conducted to identify and analyze the distribution of Chitinase gene clusters across barley chromosomes. This scrutiny revealed a diverse spatial arrangement of these gene clusters. The distribution pattern of chitinases in barley bears resemblance to that observed in other tree species, such as Eucalyptus grandis [53]. Phylogenetic analyses were conducted to demonstrate the evolutionary relationships and duplication events within the chitinase gene family across various crops [57]. The analysis of paralogous and orthologous gene behavior provides valuable insights into the evolution and functional roles of HvCHT genes in barley. Paralogous genes, resulting from gene duplication, may exhibit functional diversification, while orthologous genes maintain conserved functions across species, such as *Arabidopsis thaliana*, rice, and *sorghum* [58], which may play a crucial role

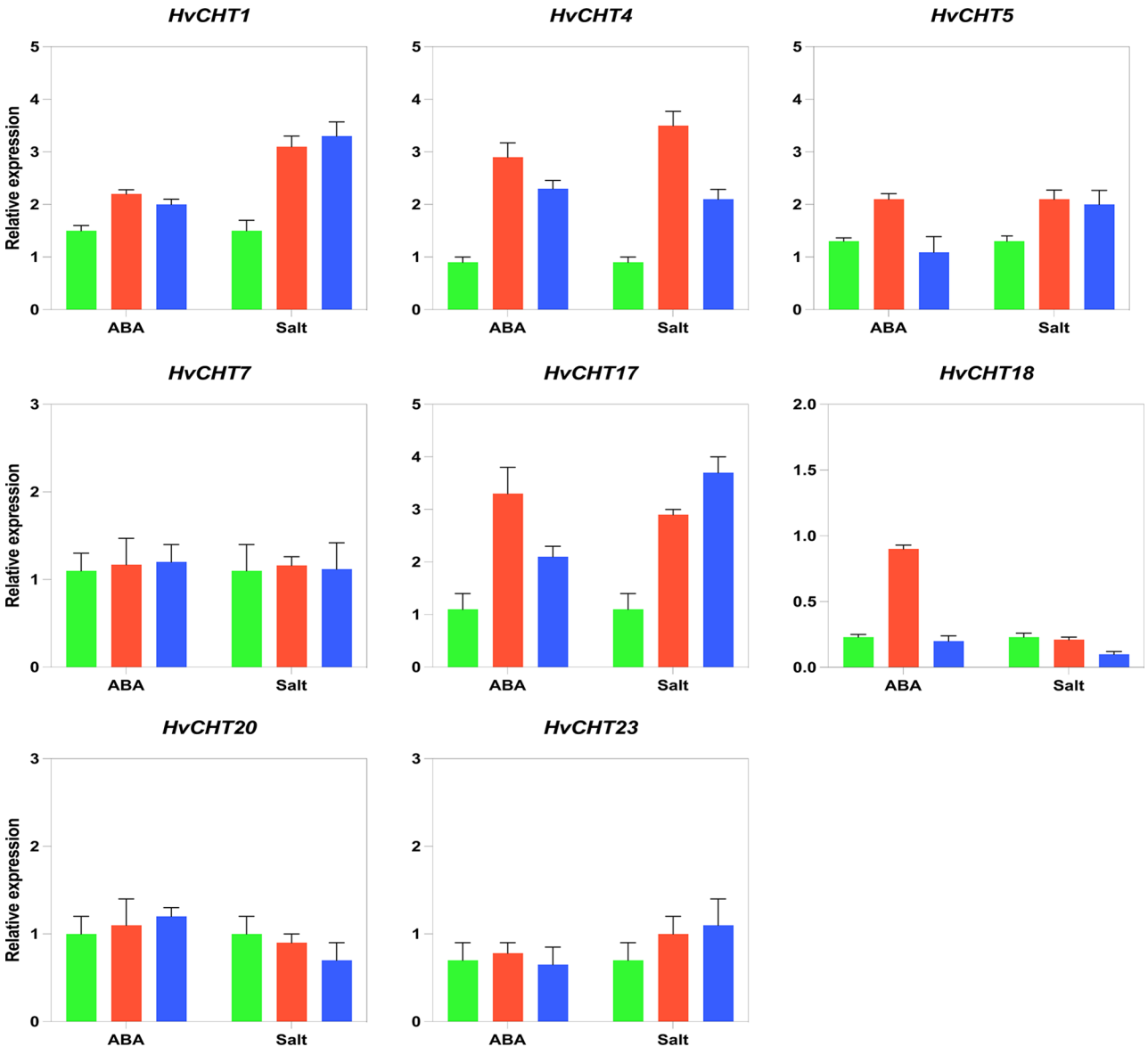

Fig. 8 The qRT-PCR heatmap shows the relative expression of HvCHT genes in response to ABA and salt stress. The colors correspond to specific time intervals: green bars represent the 0-hour, orange bars indicate the 6-hour, and blue bars represent the 12-hour interval

in stress response and their evolutionary adaptation. The conserved genetic mechanisms involved in barley's defense response, provide insights into potential evolutionary constraints shaping the functions of these genes [59, 60]. Moreover, the analysis of conserved motifs and *cis*-regulatory elements [61] within these genes provided insights into their functional diversity and the regulatory intricacies governing their expression. Cis-regulatory elements exploration provides perception into the transcriptional regulation of *HvCHT* genes. The presence of phytohormones responsive elements, such as ABRE, CGTCA-motif, and TGACG-motif, indicates that these genes are tightly regulated by ABA, ethylene and MeJA signaling pathways. This regulation correlates with light stress, cold stress, heavy metal, drought and biotic stress responses, providing insights into the regulatory mechanisms enhancing the HvCHT gene family's responses to abiotic and biotic stress [62].

Protein-protein interaction networks were investigated to shed light on the complex molecular interplay among Chitinase genes. The significant interconnectedness among these genes emphasized their coordinated response to biotic and abiotic challenges. Most of the chitinase proteins examined in the current study possessed a signal peptide, although it was absent in some cases. It's worth noting that plant chitinase proteins undergo maturation through the removal of the N-terminal signal peptide [63], contributing to increased plant resistance

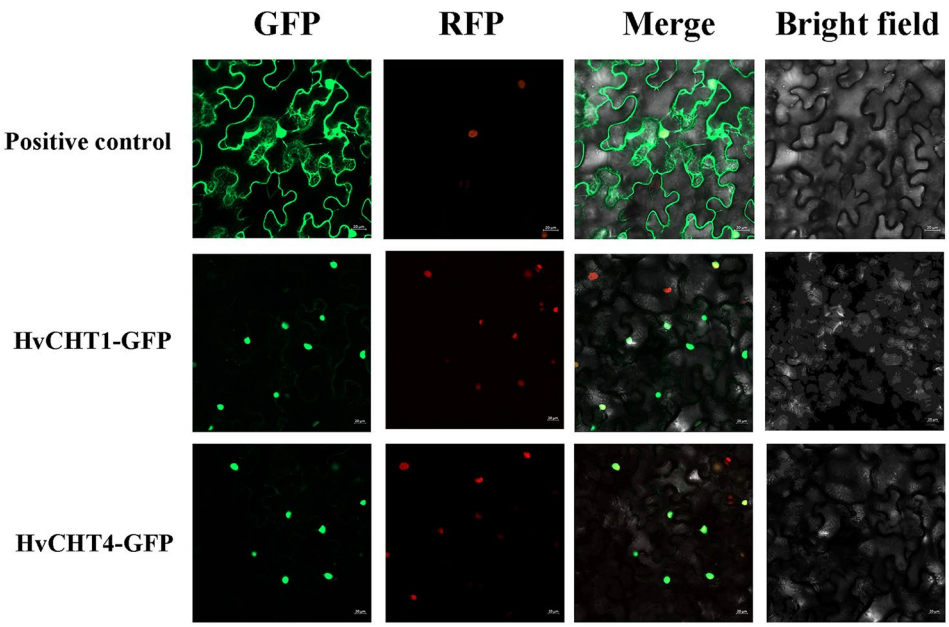

Fig. 9 The subcellular localization of HvCHT genes in *N. benthamiana* leaves. The bars represent 20 µm

against fungal pathogens [64]. Additionally, miRNA analysis uncovered specific regulatory networks associated with distinct HvCHT genes such as hvu-miR1130, hvu-miR5053, hvu-miR6184, hvu-miR6186, hvu-miR6189 and hvu-miR6210, shedding light on their post-transcriptional regulation. Previous studies demonstrated that miRNAs like those identified in barley are crucial for regulating genes involved in defense response, supporting the role of these HvCHT-related miRNAs in modulating the plant defense mechanisms against pathogens [65].

Gene expression profiling from the NCBI GEO database was employed to examine the temporal dynamics of barley's response to salt, heavy metals, and pathogenic fungi. The differential expression patterns of chitinase genes during various stages of *F. avenaceum* strain inoculation, with up-regulation of *HvCHT5* and *HvCHT9*, and *F. culmorum* strain inoculation, with up-regulation of *HvCHT2*, *HvCHT10*, *HvCHT18*, and *HvCHT22*, highlighted the critical roles played by these genes in barley's defense mechanism against pathogen infestation [66]. These findings contribute to a comprehensive understanding of the molecular mechanisms underlying barley's response to biotic stress and its evolutionary adaptation in the context of chitinase gene family dynamics. The exhaustive analysis delineated in this study provides an enhanced comprehension of the pivotal role played by the chitinase gene family in barley molecular and defensive mechanisms against pathogenic inoculation. The present findings establish a fundamental framework for targeted genetic engineering interventions to augment barley's resilience to both biotic and abiotic stresses.

These signaling are known for their role in stress situations and pathogen resistance, showing that HvCHT genes are significant performers in these processes. The mechanism of HvCHT gene expression in response to salt stress and phytohormones (ABA) was evaluated in this study through qRT-PCR. The ABA treatment led to the upregulation of *HvCHT4*, *HvCHT5*, *HvCHT17*, and *HvCHT18*, suggesting their potential involvement in ABA-mediated stress response. *HvCHT1*, *HHvCHT4*, and *HvCHT17* also showed the highest relative expression in the 6 h interval after treatment of salt stress (NaCl), indicating that these genes help the plant survive under salt stress. *HvCHT18* and *HvCHT20* genes show little expression but are later downregulated in response to salt stress. These findings highlight the importance of the chitinase gene family under abiotic stress [62, 67]. These investigations provide a possible link for understanding the role of chitinase gene expression and several stresses on barley. Subcellular localization analysis confirmed that *HvCHT1* and *HvCHT4* are localized in the nucleus, suggesting that these genes may have more regulatory features beyond their enzymatic functions in the cell wall. This nuclear localization indicates an important connection in transcriptional regulation or signaling that controls stress responses at the cellular level. The identification of key regulators intricate in drought, salt, and pathogen response offers valuable targets for further functional investigations and probable crop improvement strategies. The upcoming research concentrating on gene-editing technology might be used to employ the HvCHT gene to boost barley resistance to many stress

dynamics, accordingly increasing production and resilience in the appearance of environmental challenges.

Conclusions

This study provides a comprehensive investigation of the HvCHT gene family in barley under biotic and abiotic stress conditions, including heavy metal stress, salt and drought stresses, *Fusarium* strains, and pH variations. Our findings highlight the significant roles of HvCHT genes in barley defense mechanisms, with conserved gene sequences and motifs distributed across multiple chromosomes. Subcellular localization and *c*is-regulatory element analyses suggest that HvCHT genes play pivotal roles in both intracellular and extracellular stress responses, particularly in response to light, Abscisic acid, and salicylic acid signaling. The study suggests that *HvCHT20* plays a central role in protein-protein interaction networks based on its extensive connectivity in the present analysis. Additionally, microRNA regulatory pathways are identified as potential modulators of HvCHT expression. The evolutionary analysis includes a phylogenetic tree, non-synonymous, and substitution rates, revealing the relationship of HvCHT genes in different crops, such as *Oryza sativa*, *Sorghum bicolor*, and *Arabidopsis thaliana*. Gene expression profiling demonstrates the dynamic behavior of HvCHT genes in response to both biotic and abiotic stressors, further supporting their key role in barley stress tolerance mechanisms.

Abbreviations

PR	Pathogenesis-related
Al	Aluminium
ABA	Abscisic acid
CHT	Chitinase
ER	Endoplasmic reticulum
MeJA	Methyl jasmonate
SA	Salicylic acid
miRNA	microRNA
Mw	Molecular weight
pI	Isoelectric points
GRAVY	Grand Average of Hydropathy
Ks	Synonymous substitution rates
Ka	Nonsynonymous substitution rates

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

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Author contributions

IAS and MQ conceived and designed the experiments; FU, MAM and MS contributed reagents/materials/analysis tools; IAS, FN, and MQ wrote the manuscript; AH, EFA and JA writing, reviewing, and editing. AH and EFA funding acquisition. All authors read and approved the final manuscript.

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Data availability

The transcriptomic data of Hordeum vulgare were obtained from the NCBI GEO database under accession numbers GSE230751, GSE223521, and GSE167271. The amino acid sequences of the HvCHT genes were retrieved from the Phytozome database (https://phytozome-next.jgi.doe.gov/.).

Declarations

Ethics approval and consent to participate

The plant materials used in this study were grown in South China Agricultural University (SCAU), Guangzhou, Guangdong. All methods were carried out in accordance with relevant guidelines and regulations.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

Author details

[1]South China Agricultural University (SCAU), Guangzhou, Guangdong 510642, China
[2]Department of Botany, University of Swabi, Swabi, Pakistan
[3]Department of Botany and Microbiology, College of Science, King Saud University, P.O. Box. 2455, Riyadh 11451, Saudi Arabia
[4]Department of Plant Production, College of Food and Agricultural Sciences, King Saud University, P.O. Box. 2460, Riyadh 11451, Saudi Arabia

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