Genome-Wide Identification and Expression Profile under Abiotic Stress of the barley Non-Specific Lipid Transfer Protein Gene Family and its Qingke Orthologs

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Abstract

Background

Plant non-specific lipid transfer proteins (nsLTPs), a group of small, basic ubiquitous proteins to participate in lipid transfer, cuticle formation and stress response, involved in the regulation of plant growth and development. To date, although the nsLTP gene family of barley (*Hordeum vulgare* L.) has been preliminarily identified, it is still unclear in the recently completed genome database of barley and Qingke and its transcriptional profiling under abiotic stress has not been elucidated as well.

Results

We identified 40 barley nsLTP (*HvLTP)* genes through strict screening strategy based on the latest barley genome and 35 Qingke nsLTP (*HtLTP*) Orthologs used blastp, and these *LTP* genes were divided into four types (1, 2, D and G). At the same time, the comprehensive analysis of physical and chemical characteristics, homology alignment, conservative motifs, gene structure and evolution of HvLTPs and HtLTPs were further supported their similar nsLTPs' characteristics and classification. The genomic location of *HvLTPs* and *HtLTPs* showed that these genes were unevenly distributed, and obvious *HvLTP* and *HtLTP* gene clusters were found on the 7 chromosomes including six pairs of tandem repeats and one pair of segment repeats in barley genome, indicating that these genes may be co-evolution and co-regulated. Spatial expression analysis showed that most *HvLTPs* and *HtLTPs* were differently tissue-specific expression patterns. Moreover, the upstream cis-elements analysis of *HvLTPs* and *HtLTPs* showed that there had many different stress-related transcriptional regulatory elements, and the expression pattern of *HvLTPs* and *HtLTPs* under abiotic stress also indicated that numerous *HvLTP* and *HtLTP* genes were found to be related to abiotic stress response. Taken together, it may be due to the differences in promoter, rather than by genes themselves resulting in different expression pattern under abiotic stress.

Conclusion

Due to a stringent screening and comprehensive analysis of *nsLTP* gene family in barley and Qingke, and its expression profile under abiotic stress, this study can be considered as a useful source for the future studies of *nsLTP* genes in either barley and Qingke or comparison of different plant species.

Background

Plant lipid transfer protein (LTPs), named for their function that transfer phospholipids and fatty acids between cell membranes in vitro, were also named non-specific LTPs (nsLTPs) because of the characteristic of non-specific binding to different lipids[1, 2]. Plant nsLTPs are usually 6.5 to 10.5 kDa, but some nsLTPs that are approximately 15 kDa, and their isoelectric points (pI) is usually between 8.5 and12 (occasionally less than 5). Therefore, nsLTPs were a group of small, basic and ubiquitous proteins, which have eight cysteine residues motifs (8CM) in a highly conserved backbone sequence, C-Xn-C-Xn-CC-Xn-CXCXn-C-Xn-C, and a high content of α-helices with a central hydrophobic cavity to binding lipid. And almost all nsLTPs carry an N-terminal signal peptide in their nascent polypeptides[3–5]. Plant nsLTPs are involved in multiple physiological functions, such as cuticular lipids transport, cutin synthesis, cell wall extension, pollen development, pollen tube growth and guidance, stigma and pollen adhesion, plant signaling, seed maturation, and so on [1, 6–8]. Besides, some plant nsLTPs have been identified as related allergens in plant food and pollen [9–11]. The expression of some nsLTPs can be induced by biotic and abiotic stresses, including low or high temperature, drought, heavy metal exposure and disease[12–18]. In particular, many studies have shown that nsLTP genes are closely related to abiotic stress resistance in plant [9, 12, 13, 19, 20].

Barley (*Hordeum vulgare* L.) is the fourth largest crop in the world after wheat, rice, and maize, and is one of the oldest food and feed crop in the world (http://faostat.fao.org). It is an important crop in China, which has high economic and food value, especially in the Qinghai-Tibet Plateau. Tibetan hulless barley (highland barley), called as "Qingke" in Chinese and "nas" in Tibetan, is the principal cereal cultivated on the Qinghai-Tibetan Plateau for at least 3,500 years. Due to its high soluble dietary *β*-glucans and arabinoxylan content, it is beneficial to human health, which has attracted considerable interest [21–23]. Karen Skriver cloned and analysed the gene structure of the LTP1 gene in barley[24]. The crystal structure of this gene, specifically in ligand binding preferences, was also studied[25]. Then, some other barley genes (*LTP2*, *LTP3*, and *LTP4*) have been cloned in succession [26]. Interestingly, barley nsLTP gene (*blt4*) was also found in the cold stress response [27, 28]. The latest physical, genetic and functional sequence assembly of the barley and Qingke genome were completed in 2012, 2016[29, 30] and 2015, 2018, 2020[22, 31, 32], respectively, which provided important reference for future crop breeding, improvement, gene function and evolution research.

Barley has strong drought resistance, low-temperature tolerance and salt and alkali capacity, and its environmental adaptation mechanism is complex[19, 20]. Mainly cultivated in the Tibetan Plateau (> 4,000 m above sea level) where is high UV-B radiation, low temperatures and low barometric pressure, Qingke may therefore have greater extreme environmental adaptability compared with the cultivated barley from other regions [21, 31]. Now the nsLTP genes and its multi-gene family have been studied from many plants, including Arabidopsis, carrot, rape(*Brassica napus*), broccoli(*Brassica oleracea*), tobacco(*Nicotiana tabacum*), sugar beet (*Beta vulgaris*), sesame(*Sesamum indicum*), tomato(*Solanum lycopersicum*), maize(*Zea mays*), sorghum (*Sorghum vulgare)*, cotton(*Gossypium hirsutum*), rice(*Oryza sativa*) and wheat(*Triticum aestivum*) [33]. For barley, although the 70 barley nsLTP genes have been identified in 2012 barley genome [34], there are still many deficiencies, which need further comprehensive research by using the genome database of barley recently completed in 2016. At the same time, in order to further understand the molecular mechanism that Qingke has stronger ability to adapt to extreme environment than barley. Therefore, this study carried out the identification of nsLTP gene family members and bioinformatics analysis further in barley and Qingke through strict screening based on the latest barley and Qingke genome. Using qRT-PCR technology, the expression profile of this gene family under

abiotic stress was also discussed. The results of this study lay a foundation for further study on the biological and molecular functions of nsLTP genes in barley and Qingke.

Results

3.1 Identification, sequence analysis and classification of HvLTPs and HtLTPs

To identify the entire collection of putative non-redundant nsLTP genes in barley genome, an accurate search workflow of nsLTP identification and data mining was performed (Fig. 1). Initially, three search methods were used to identify barley nsLTP family members. Firstly, 109 amino acid sequences were obtained after conducting BLASTP analysis in IPK Barley BALST Server using previously reported nsLtp protein sequences of Arabidopsis (79), maize (63), cabbage (63) and rice (77) as the queries (Table S1), and the redundancy was checked. Secondly, a total of 265 proteins with the conserved Tryp alpha amyl domain (Pfam domain PF00234) were retrieved by HMM search method. Thirdly, the relevant 39 barley nsLTP genes were downloaded by keyword searches from the NCBI, IPK and Phytozome databases. Then, the above results were merged, and the redundant sequences were examined and removed. Finally, all the barley deduced nsLTPs protein sequences were downloaded from the NCBI and IPK database and the presence of LTP domains cl07890 was verified by Batch Web CD-Search and Pfam validation using the domain PF00234. After that initial identification step, a total of 160 putative nsLTP protein sequences were identified. Then each of the deduced protein sequences was manually assessed through the analysis of the cysteine residue motifs (8CM), and 107 proteins lacking the Cys residues were omitted from the remaining set. In addition, 11 proteins lacking N-terminal signal sequences (NSS) were also excluded by NSS prediction, and 8 proteins with C-terminal glycosylphosphatidylinositol (GPI) anchors were remained by GPI anchor signal prediction. Subsequently, 2 proline-rich proteins were also excluded, and no proteins similar to At2S1- At2S4 and RATI were found. Finally, we identified 40 nsLTPs in the whole barley genome and named as HvLTPs, and 35 nsLTPs were also identified in Qingke genome by local blast with HvLTPs and named as HtLTPs (Table 1). Furthermore, to better understand the characteristics of HvLTP and HtLTP proteins, we analyzed the theoretical isoelectric point (pI) and molecular weight (MW) for all putative HvLTP and HtLTP proteins and summarized in Table 1. As shown in Table 1, considering the mature form of nsLTPs, the average length is 132 aa (91–200 aa) with a molecular mass ranging from 9206.81 to 19981.15 Da. And average Mw of HvLTPs is 13,344 Da and average theoretical pI of HvLTPs is 8.07, while average Mw of HtLTPs is 13,238 Da and average theoretical pI of HtLTPs is 7.94, which shows its small, basic properties with subtle differences. We also analysed Instability index, Aliphatic index and Grand average of hydropathicity (GRAVY) of HvLTPs and HtLTPs. The results showed that the most instability index of HvLTPs and HtLTPs were greater than 40, the most Aliphatic index was greater than 75.5 and the GRAVY value was greater than zero, which indicated that most HvLTPs and HtLTPs were unstable, aliphatic and Hydrophobic proteins.

Table 1
Characteristics for the different types of non-specific lipid transfer proteins found in barley (HvLTPs) and Qingke

Gene name	barley gene ID	Qingke gene ID	Chromosome	Amino acids		pI		Mass (Da)		Ins
				Barley	Qingke	Barley	Qingke	Barley	Qingke	Ba
Type1										
HvLTP1.1	HORVU2Hr1G107470.2		2	118		8.89		11560.52		23
HvLTP1.2/HtLTP1.2	HORVU2Hr1G107480.1	KAE8807755.1	2	120	120	9.03	9.03	11856.79	11856.79	31
HvLTP1.3/HtLTP1.3	HORVU2Hr1G107460.1	KAE8775919.1	2	125	125	9.82	9.82	12233.34	12233.34	38
HvLTP1.4	HORVU3Hr1G009560.1		3	115		9.46		11221.08		25
HvLTP1.5/HtLTP1.5	HORVU3Hr1G009510.1	KAE8776966.1	3	126	126	4.35	4.35	12730.43	12730.43	61
HvLTP1.6/HtLTP1.6	HORVU3Hr1G009370.5	KAE8788659.1	3	115	115	9.32	9.32	11099.06	11099.06	33
HvLTP1.7/HtLTP1.7	HORVU3Hr1G009490.4	KAE8788658.1	3	115	115	9.32	9.32	11175.17	11175.17	34
HvLTP1.8/HtLTP1.8	HORVU3Hr1G009520.2	KAE8788661.1	3	115	115	9.88	9.88	11410.5	11410.5	40
HvLTP1.9	HORVU3Hr1G009360.8		3	168	168	8.97		16215.03		34
HvLTP1.10/HtLTP1.10	HORVU3Hr1G029430.1	KAE8792517.1	3	155	155	8.65	8.65	16185	16185	28
HvLTP1.11/HtLTP1.11	HORVU4Hr1G022780.2	KAE8800812.1	4	111	111	8.7	8.7	10982.81	10982.81	49
HvLTP1.12/HtLTP1.12	HORVU4Hr1G022770.1	KAE8800813.1	4	120	120	9.41	9.41	11746.77	11746.77	42
HvLTP1.13/HtLTP1.13	HORVU5Hr1G046550.1	KAE8791291.1	5	117	117	8.7	8.7	12297.24	12297.24	35
HvLTP1.14/HtLTP1.14	HORVU5Hr1G046520.1	KAE8791289.1	5	149	149	9.58	9.58	15377.09	15377.09	45
HvLTP1.15/HtLTP1.15	HORVU7Hr1G080360.2	KAE8796122.1	7	120	120	3.74	3.74	12209.7	12209.7	63
HvLTP1.16/HtLTP1.16	HORVU5Hr1G111050.1	KAE8788807.1	5	128	128	8.51	8.51	12881.01	12881.01	49
Type2										
HvLTP2.1/HtLTP2.1	HORVU1Hr1G083160.1	KAE8821628.1	1	91	91	9.27	9.27	9206.81	9206.81	27
HvLTP2.2/HtLTP2.2	HORVU1Hr1G083170.1	KAE8802268.1	1	94	94	8.93	8.93	9370.08	9370.08	41
HvLTP2.3/HtLTP2.3	HORVU2Hr1G108660.1	KAE8821633.1	2	95	95	7.48	7.48	9602.32	9602.32	47
HvLTP2.4/HtLTP2.4	HORVU4Hr1G089490.2	KAE8784379.1	4	91	91	8.72	8.72	9439.32	9439.32	37
HvLTP2.5/HtLTP2.5	HORVU4Hr1G089500.1	KAE8777687.1	4	97	97	9.72	9.72	10393.17	10393.17	66
Type D										
HvLTPd1	HORVU1Hr1G043430.1		1	139		9		14388.36		56
HvLTPd2/HtLTPd2	HORVU2Hr1G102110.1	KAE8788006.1	2	105	105	8.15	8.15	10966.95	10966.95	41
HvLTPd3/HtLTPd3	HORVU2Hr1G102170.1	KAE8781084.1	2	113	113	4.85	4.85	11439.15	11439.15	45
HvLTPd4/HtLTPd4	HORVU2Hr1G073730.1	KAE8786049.1	2	125	125	8.67	8.67	13033.35	13033.35	55
HvLTPd5/HtLTPd5	HORVU2Hr1G102050.1	KAE8787999.1	2	105	105	8.47	8.47	10985.01	10985.01	43
HvLTPd6/HtLTPd6	HORVU2Hr1G102100.1	KAE8788000.1	2	105	105	8.47	8.47	10994.02	10994.02	41
HvLTPd7/HtLTPd7	HORVU2Hr1G104430.1	KAE8799817.1	2	132	132	7.53	7.53	14042.31	14042.31	55
HvLTPd8/HtLTPd8	HORVU4Hr1G082600.1	KAE8799709.1	4	98	98	4.85	4.85	10347.06	10347.06	55
HvLTPd9/HtLTPd9	HORVU7Hr1G102030.1	KAE8770127.1	7	114	114	8.14	8.14	11569.65	11569.65	54
HvLTPd10/HtLTPd10	HORVU5Hr1G109100.1	KAE8808658.1	5	183	183	8.11	8.11	18559.7	18559.7	29
HvLTPd11/HtLTPd11	HORVU7Hr1G026570.1	KAE8795354.1	7	103	103	9.22	9.22	10461.46	10461.46	37
Type G										
HvLTPg1/HtLTPg1	HORVU1Hr1G009490.1	KAE8777605.1	1	177	177	7.48	7.48	16847.26	16847.26	44
HvLTPg2/HtLTPg2	HORVU2Hr1G098500.2	KAE8795728.1	2	189	189	6.06	6.06	19086.3	19086.3	65
HvLTPg3/HtLTPg3	HORVU4Hr1G071790.1	KAE8805373.1	4	194	194	5.52	5.52	18793.44	18793.44	67

Gene name	barley gene ID	Qingke gene ID	Chromosome	Amino acids		pI		Mass (Da)		Ins
HvLTPg4/HtLTPg4	HORVU5Hr1G109040.1	KAE8808654.1	5	197	197	8.66	8.66	19981.15	19981.15	44
HvLTPg5/HtLTPg5	HORVU5Hr1G076000.1	KAE8769277.1	5	190	190	4.41	4.41	19076.74	19076.74	68
HvLTPg6	MLOC_57612.1		5	176		8.47		17033.39		52
HvLTPg7/HtLTPg7	HORVU0Hr1G016430.1	KAE8793545.1	0	174	174	7.5	7.5	17574.28	17574.28	46
HvLTPg8/HtLTPg8	HORVU6Hr1G082080.1	KAE8808175.1	6	200	200	8.76	8.76	19385.55	19385.55	85

Based on the Mw of the mature proteins, plant nsLTPs can be classified into two main types: nsLTP1 (9 kDa) and nsLTP2 (7 kDa). Then, according to sequence similarity, Boutrot divided nsLTPs into nine types (I, II, III, IV, V, VI, VII, VIII and IX) [4]. Recently, plant nsLTPs have been categorized into four major and several minor types (1, 2, C, D, E, F, G, H, J, K, X) by intron position, sequence identity and spacing between the cysteine residues in the 8CM, as well as post-translational modifications [35, 36]. Compared with the classification proposed by Edstam, 40 HvLTPs and 35 HtLTPs could be divided into four types, including 16/13(type 1), 5/5(type 2), 11/10(type D) and 8/7(type G) nsLTP genes, respectively (Table 1). As shown in Table 1, the molecular weight of HvLTPs and HtLTPs are usually between 9206.81 Da and 19981.15 Da, where the molecular weight of Type 2 is smaller than other types, with an average of 9 kDa. The molecular weight of type G nsLTPs is relatively large, mostly between 16–19 kDa, with an average of 18 kDa. In type G, the transcripts encode not only a C-terminal signal sequence, but also the N-terminal one, leading to a post-translational modification which a glycosylphosphatidylinositol (GPI)-anchor is added to the protein to attach the protein to the exterior side of the plasma membrane[37], leading to the molecular weight of type G nsLTP being far higher than that of other types.

The main characteristic of plant nsLTPs is the presence of 8CM motifs. To establish a specific 8CM consensus for each nsLTP type obtained, we conducted a multiple sequence alignment using the 8CMs from 40 HvLTPs and 35 HtLTPs. The amino acid sequence alignment of the 8CMs of HvLTPs and HtLTPs revealed a variable number of inter-cysteine amino acid residues. We also found that the alignment results are consistent with the classification results. For the CXC motif, most of the residues at the X position in type 1 nsLTP are only hydrophilic, while in Types 2, D and G, the X position is usually occupied by hydrophobic residues (Fig. 2). These conserved hydrophobic or hydrophilic residues may play an important role in the biological function of HvLTPs and HtLTPs and are also consistent with their classification.

3.2 Phylogenetic analysis, conservative motif and gene structure of the HvLTPs and HtLTPs families

To analyze the evolutionary relationship, a phylogenetic tree of 293 nsLTPs from four species, including the HvLTPs and HtLTPs with maize, rice and Arabidopsis, was constructed. Comparing the previous classification data with phylogenetic analysis, it was found that the previous classification and phylogenetic analysis have the same type of nsLTPs; that is, the four groups of the HvLTPs and HtLTPs classification were consistent with the 1, 2, D and G types of the nsLTPs of the other three species, except for sporadic interlaces in types D and G (Fig. 3). The members of type 1 and 2 formed specific clades, indicating that these genes share a common ancestor in major nsLTP types.

Based on the distribution of predicted motifs, 40 HvLTPs and 35 HtLTPs were categorized into four distinct subfamilies, which was consistent with the classification from the phylogenetic analysis (Fig. 4). Both the HvLTPs and HtLTPs have four different subfamilies. Type 1 has a similar motif, and the other types of motif structure are completely different. Common Motif 2 were presented in all HvLTPs and HtLTPs, except for six genes in type 2 (HvLTP2.1/HtLTP2.1, HvLTP2.2/HtLTP2.2, HvLTP2.4/HtLTP2.4 and HvLTP2.5/HtLTP2.5) and type D (HvLTPd8/HtLTPd8 and HvLTPd11/HtLTPd11). Special motifs appear in special types, for instance, Motifs 1 and 15 are only present in Type 1, Motifs 14 and 16 are only present in Type D, and Motif 13 only exists in Type G HvLTPs and HtLTPs.

As a type of evolutionary relic, the intron-exon arrangement carries the imprint of gene family evolution. The gene structures of the HvLTPs and HtLTPs were also investigated (Fig. 5). Investigation of HvLTPs and HtLTPs gene structure revealed a low diverse distribution of intronic regions amid the exonic sequences. 40 HvLTP and 35 HtLTPs genes were predicted to be interrupted by 0–2 introns positioned − 9 to 104 bp downstream of the codon encoding the eighth cysteine in 8CM (Table 1). Additionally, it is interesting to find a similar exon/intron pattern in each group. For instance, the HvLTP and HtLTPs genes in type 2 lack introns, while type G contains 2 introns. Except for HvLTPd1, HvLTPd7/HtLTPd7, and HvLTPd9/HtLTPd9, no intron was detected in the coding regions of type D genes.

3.3 Chromosomal localization and gene duplication of HvLTPs and HtLTPs

After genomic location information of 40 HvLTP and 35 HtLTPs genes were determined, one of them (MLOC_57612.1) genomic location information belonging to the 2012 IPK genes was not found. The results of Chromosomal localization showed that 39 HvLTP and 35 HtLTPs genes were unevenly distributed on 7 chromosomes of barley and Qingke, and there were obvious HvLTP and HtLTPs gene clusters. The maximum number of HvLTP and HtLTPs genes were contained on chromosome 2 (11), and the minimum number (1) was shown on chromosome 6 (Fig. 6).

Gene duplication is generally considered to be a major driving force in evolutionary innovation, giving rise to genomic complexity. In this study, six tandem repeats were identified in barley genome (Table S2), which was consistent with obvious *HvLTP* gene clusters in barley chromosome. Two significant clusters were found on chromosomes 2 and 3. Besides, one sister pair appeared to be generated from segmental duplication (Table S2, Figure S1A). Furthermore, we

analysed the collinearity of *HvLTPs* with the nsLTP genes in rice and wheat. Eleven out of 39 *HvLTP* genes had collinear genes with rice, while 22 *HvLTP* genes had syntenic members between barley and wheat (Figure S1B).

3.4 Promoter and stress expression analysis of HvLTPs and HtLTPs

Plant nsLTPs display a complex tissue-specific and developmental expression pattern, mainly expressed in the tapetum, pericarp and epidermal cells of embryo, stem, leaf and root [36–38]. According to on the RPKM values of each *HvLTP* gene published on the IPK website, an expression heatmap of 40 *HvLTP* genes in the 16 different tissues were mapped (Fig. 7A). The results showed that the *HvLTPs* had distinctly different expression patterns during different developmental stages as well as in different plant tissues. All *HvLTPs* were not expressed in INF1, and the expression level of *HvLTPs* in INF2 was also extremely low. Among different *HvLTPs* types, the relative expression levels of type 1 and type 2 genes were higher and more specific than those of type D and type G, except that the expression of HvLTP1.10 was lower in all tissues. Type 1 and type 2 *HvLTP* genes showed obvious tissue-specific expression patterns. For example, *HvLTP1.2* is mainly expressed in LEA. *HvLTP1.13* is specifically expressed in CAR15, and *HvLTP2.4* and *HvLTP2.5* are specifically highly expressed in CAR5 and CAR15, which indicates that these three genes might be involved in grain development. In view of the high expression level of barley type 1 *HvLTPs* in the above transcriptome data, we simultaneously detected the tissue expression patterns of 10 type 1 barley and Qingke ns*LTP* genes in five different tissues by qRT-PCR, respectively (Fig. 7B). The results showed that the expression level of type 1 *LTP* genes of barley and Qingke were very low in roots, and the expression pattern of barley type 1 *HvLTPs* was basically consistent with that of transcriptome data. However, the expression patterns of 10 *nsLTP*s genes were different in barley and Qingke tissues. For example, the expression level of barley *LTP*1.4 was higher in leaves, while that of Qingke *LTP*1.4 was lower in leaves; The expression level of barley *LTP*1.11 was higher only in seeds, and that of Qingke *LTP*1.11 was higher in stems and flowers.

In silico analysis of the 1.5 kb upstream region (starting from the translation initiation site) of 35 *HvLTP* and *HtLTP* genes revealed the existence of various regulatory elements associated with development, abiotic or biotic stress signaling (Fig. 8.). In addition to TATA-box and CAAT-box, the A-box is the most common type of cis-elements in *HvLTP* and *HtLTP* genes. In this study, we found that the cis-elements of the *HvLTP* and *HtLTP* genes included stress response elements (ARE, MBS, MYB, LTR, TC-rich motif, DRE), hormone-related elements (ABRE, TGACG-element, CGTCA-motif, TGA-element, TCA-element, GARE-motif, P-box, TATC-box, AuxRR-core, ERE, Wbox), indicating that *HvLTP* and *HtLTP* are involved in stress response and hormone signaling. Also, the regulatory element involving light responsiveness also appears to be enriched in *HvLTP* and *HtLTP* genes, including G-Box, ACE, Box 4, GT1-motif, Sp1, TCT-motif, GATA-motif, I-box, AE-box, Box II, TCCC-motif. And some *HvLTP* and *HtLTP* genes have specific developmental response elements such as zein metabolism regulation (O_2-site), meristem expression (CAT-box), seed-specific regulatory elements (RY-elements). Comparison of 35 gene promoters of *HvLTPs* and *HtLTPs*, only 6 genes have the same cis-elements organization, indicating that there are more stress response and hormone related elements in Qingke. And there are both similarities and differences between *HvLTPs* and *HtLTPs*. Similarly, all have a similar number of stress response elements, most of which have ARE (essential for the anaerobic induction), LTR (involved in low-temperature responsiveness) and MBS (MYB binding site involved in drought-inducibility). For nsLTP, MBS has been previously reported as a target for MYB transcription factor to modulate plant tolerance to freezing and drought stress[39], suggesting that these *HvLTPs* and *HtLTPs* containing MBS or MYB elements may participate in some abiotic stress signaling of MYB. The difference is that the number of hormone response elements and light responsive elements in Qingke was higher than in barley, and ABRE and G-box are the most important and different hormone and light responsive regulatory elements in *HvLTP* and *HtLTP* genes, which may mean that Qingke may rely on abscisic acid hormone pathway and G-box related light response pathway to adapt to more severe plateau environment [40].

qRT-PCR was performed further to explore the expression patterns of 16 *HvLTP* and 7 *HtLTP* genes in root and leaf tissue under abiotic stress, means that after barley seedlings were treated for 2 days with drought, cold and salt stress, and then recovered for 2 days(Fig. 9, Figure S2). Similar to Spatio-temporal tissue expression of *HvLTPs*, the expression level of Type 1 genes is higher than that of other types in *HvLTPs* under abiotic stress, so the expression of type 1 genes of *HtLTP* genes were also select to perform qPCR. The seven Type 1 *HvLTP* and *HtLTP* genes had significantly responses in leaf under cold, drought and salt stress, but no or low responses in root, which was consistent with the low expression in root in IPK database (Fig. 9). The expression level of all barley Type 1 *HvLTP* genes were decreased under cold stress, while four *HtLTP* genes were up-regulated in Qingke. The 4 Type 1 *HvLTP* and *HtLTP* genes were up-regulated in barley and Qingke under drought stress, but two of them were different. For example, the expression of *HvLTP* 1.7 was down-regulated in barley, but *HtLTP* 1.7 was up-regulated in Qingke; the expression of *HvLTP* 1.3 was up-regulated in barley, but *HtLTP* 1.3 was down-regulated in Qingke. Only one Type 1 *HvLTP* gene was up-regulated in barley under salt stress, while six Type 1 *HtLTP* genes were up-regulated in Qingke. In general, the expression patterns of seven Type 1 *HvLTP* and *HtLTP* genes in barley and Qingke under abiotic stress were different. After abiotic stress was removed, the change trend of type 1 gene also was different between barley and Qingke. For example, there were 3 genes in barley, while there were 6 genes with opposite change trend in Qingke under cold stress; there were 5 genes in barley, but 4 genes with the same change trend in Qingke under drought stress; there were 6 genes in barley, but only 3 genes with the same change trend in Qingke under salt stress. At the same time, there also have differences in genes with opposite trends, such as, *HvLTP* 1.4 and *HvLTP* 1.6 genes were down-regulated first and then up-regulated under cold stress in barley, while *HtLTP* 1.4 and *HtLTP* 1.6 were up-regulated first and then down-regulated in Qingke.

Unlike type 1, two genes (*HvLTP2.1/HvLTP2.2*) of type 2 responded significantly to drought, cold and salt stresses in root and *HvLTP2.1* responded significantly to salt stress in leaf. The two genes showed same change trends in root under removal of abiotic stresses basically. For example, the two genes were up-regulated continuously under cold stress and after removal of cold stress, but they were down-regulated under drought stress and up-regulated under removal of drought stress, and there were slightly different under salt stress between them. *HvLTP2.1* was up-regulated continuously under and removal salt stress, while *HvLTP2.2* gene changes in opposite directions, it was down regulated first and then up regulated. The type D genes were down-regulated under cold and drought stress basically, while most type D genes were up-regulated under salt stress in root. *HvLTPd2* and *HvLTPd5* gene also showed significant effects and were up-regulated in leaf under drought and salt stress. After removal of abiotic stress, the overall fluctuation of type D genes was the opposite, but the *HvLTPd7* gene was the same trend. In type G, the response of *HvLTPg1* in root was up-regulated significantly under cold stress, while the response in leaf was up-regulated significantly under drought and salt stress. *HvLTPg2* was down-regulated significantly in root and leaf under three abiotic stresses. Only *HvLTPg5* was up-regulated significantly in leaf under cold stress. In general,, the type G genes were not affected due to low expression after removal of abiotic

stress, only some genes have the opposite changed trends, including the *HvLTPg1* genes in root and *HvLTPg2* genes in root and leaf under drought stress; and *HvLTPg*5 gene in leaf under three abiotic stresses (Figure S2).

Discussion

Plant nsLTPs are a large transporter family composed of 79 members in Arabidopsis, 77 in rice, 63 in maize, 58 in sorghum, 63 in cabbage and 156 in wheat, all of which are classified as different types [4, 17, 40–42]. In 2018, the wheat nsLTP family was comprehensively analysed again and 461 putative TaLTPs have been identified from the whole wheat genome [43]. Edstam provided comprehensive information about the categorization of nsLTPs gene family based on phylogenetic clustering and facilitated further functional analysis [35]. In this study, the 40 and 38nsLTPs were identified in the barley and Qingke genome and classified into 4 subfamilies (Type 1, 2, D and G) following Edstam's classification (Table 1, Fig. 2). Besides, the number and classification of HvLTPs were also compared with those in Arabidopsis, rice, maize and Cabbage (Table 2). The result showed that the total number of nsLTP gene families in barley and Qingke was less than that in other species. It was also found that type E is unique to dicotyledons, while HvLTPs and HtLTPs are also deficient in type C and type X (Table 2). Meanwhile, the proportion of nsLTPs in each subfamily indicated that type G seemed to have contracted in barley, but no expansion was observed. Therefore, the decreased of nsLTP gene number in barley may be due to the loss of individual type and the reduction of individual populations. In addition, this number may be due to the inhibition of gene expansion. Zhang (2019) showed that 70 barley nsLTP genes were identified by keyword searching in Phytozome v12.1.6 and divided into five types (1, 2, C, D, and G) [34], which indicates that the research results of this barley nsLTP gene family expansion are different from ours. Compared with Zhang's research, our research collected more comprehensive data through three methods of blast P, HMM search and keyword search from the barley genomes, including IPK, Ensembl, NCBI and Phytozome, and obtained 160 potential HvLTP genes. Referring to the previous screening methods [17], the screening method is also more stringent, and finally identified 40 barley HvLTP genes, so our results are more accurate, comprehensive and reliable.

Table 2
Numbers of nsLTP genes in different species

species	Total number of members	Type 1	Type 2	Type C	Type D	Type E	Type G	Type X
Hordeum vulgare	40	16	5	0	11	0	8	0
Qingke	35	13	5	0	10	0	7	0
Arabidopsis thaliana	79	13	13	3	12	2	33	3
Oryza sativa	77	18	13	2	14	0	27	3
Zea mays	63	8	9	2	15	0	26	3
Brassica oleracea var. capitata	89	19	12	1	18	2	28	9

The role of gene duplication in the origin of evolutionary novelty and complexity has long been recognized [44]. In our study, we found one segmental duplication and six tandem duplication events in the barley nsLTP gene family (Table S2; Figure S1). These results suggest that overall gene expansion is inhibited, and the evolution of the barley nsLTP gene family not only involves gene retention, but also gene loss and mutation. The retention and loss of genes may be associated with the related functions during plant evolution [45]. Besides, these paralogs duplicated genes may retain some essential functions in the subsequent evolution. For example, five pairs of tandem duplication genes (HvLTP1.1/ HvLTP1.2/ HvLTP1.3, HvLTP1.5/HvLTP1.7/HvLTP1.8, HvLTP1.6/ HvLTP1.9, HvLTP2.1/ HvLTP2.2, HvLTPd2/ HvLTPd5/ HvLTPd6) shared similar expression profiles. However, one pair of duplication gene (HvLTP2.4/HvLTP2.5) showed significant divergence in expression. At the same time, another pair of segmental duplication gene (HvLTP1.12/HvLTP1.13) also showed different expression patterns. The differential expression patterns of these duplicated genes in barley indicated that these genes may be functionalizated after duplication events during the evolutionary process, leading to significant variation in gene regulation [46, 47].

There are sufficient evidences that nsLTPs are involved in various types of stress resistance, including resistance to phytopathogens, freezing, drought and salt [36, 48]. In our study, the stress dependent cis-elements in the promoter region of the HvLTP and HtLTP genes were analysed. The results showed that the promoters of HvLTP and HtLTP genes contained stress response elements (STRE, DRE, MBS, MYB, TC-rich repeats), hormone-related elements (ARE, LTR, ABRE, ERE, TCA-element, TGA-element, TGACG-element, CGTCA-motif, W box), indicating that the HvLTPs and HtLTPs are involved in stress response. Meanwhile, the expression patterns of 17 HvLTP genes in response to abiotic stresses also demonstrated their correlation with abiotic stresses. In general, the response of these genes in root is weaker than that in leaf. Under different abiotic stresses, the response levels of different type members were inconsistent. For example, the response of HvLTP type1 was significantly higher than that of other subfamilies. The *blt4* gene of barley is a low-temperature response gene with different response to drought, pathogen attack, as well as abscisic acid (ABA) [28]. Previous studies have shown that *blt4* belongs to the barley nsLTPs, located on chromosome 3, and can act as a regulatory protein to stabilize plasma membrane activity and resist low-temperature injury [12]. Interestingly, blt4 genes belongs to barley nsLTP type 1, and some of them appear in tandem duplication events, such as blt4.3 (HvLTP1.8)/ blt4.9 (HvLTP1.7) and blt4.1 (HvLTP1.6) (Table S2, Fig. 6). The expression patterns of these tandem duplication genes are similar, indicating that these genes may retain the same function and coordinate the regulation of stress response through tandem repeat of gene cluster.

It has been reported that different promoter cis-elements and their epigenetic changes may affect gene regulation, resulting in different gene expression levels, further affecting adaptation to environment, including altitude changes[49–51].We also compared the nsLTP gene promoter and stress expression pattern between barley and Qingke, and found that the nsLTP gene sequences between barley and Qingke were basically same, but the promoter and stress expression pattern were different. Moreover, the expression of nsLTP gene was directly related to its promoter, which indicated that the stress expression pattern of nsLTP gene was changed by its promoter. Combined with the analysis of upstream cis-elements of *HvLTPs* and *HtLTP*s and their expression

patterns under abiotic stress, it was found that a large number of *HvLTP* and *HtLTP* genes may change their regulatory modes due to different upstream cis-elements, and causing different abiotic stress responses.

Conclusions

In summary, 40 HvLTPs and 35 Qingke nsLTPs were identified in barley and Qingke in this study. A comprehensive study of the HvLTPs and HtLTPs will provide some important features of the nsLTP gene family, such as gene structure, evolution, chromosome distribution, conservative motifs, segmental and tandem duplication, upstream cis-element, and stress expression pattern. The study results could be considered as a useful source for future nsLTP gene research in either barley and Qingke or comparison of different plant species. And it will help to provide the foundation for future research on the molecular mechanisms of barley and Qingke stress adaption.

Materials And Methods

2.1 Plant materials, growth conditions and abiotic stress treatment

Barley variety 'Morex' was used as plant material in this study. The seeds were surface sterilised with 10% H_2O_2(v/v) for 10 min and rinsed with deionized water for several times. And then soaked seeds were kept at 25°C for 48hr in darkness to germinate in a light growth chamber. The germinating seeds were planted in 1.5L pot and filled with Hoagland's nutrient solution and were grown in a greenhouse with 22 ℃, photoperiod of 16-h (12000 lux) and dark period of 8-h. Seedling were grown to maturity(14 days of germination) under normal condition then treated under different abiotic stress conditions including drought, cold and salt. For salt stress, seedling were grown application of 200 mM NaCl to the nutrient solution for 48 hours, the same as for drought stress, seedling were grown application 18% polyethylene glycol to the nutrient solution for 48 hours, and were treated with 4℃ to represent the cold stress for 48 hours. Then tissue samples composed leaf, root from every stress treatment were taken once. After 48 hours, the seedlings were transferred to the nutrient solution without stress and continued to culture for 48 hours in the growth chamber. Then, the tissue samples were taken again. Then, the plant materials were collected and immediately frozen in liquid nitrogen for RNA extraction. All samples were replicated three times.

2.2 Identification of putative nsLTP genes in the barley and Qingke genome

All known nsLTP amino acid sequences from Arabidopsis (*A. thaliana*), maize (*Zea mays* L), cabbage (*Brassica rapa* L) and rice (*O. sativa*) were used as queries (Table S1) by searching against the barley database using the BLASTP (IPK) program with the default parameters (http://webblast.ipk-gatersleben.de/barley_ibsc/). Simultaneously, an HMM search was performed on the barley genome release-41 from Ensembl Genomes (http://ensemblgenomes.org/), and amino acid sequences containing the domain PF00234 (Tryp alpha amyl domain, plant lipid transfer/seed storage/trypsin-alpha amylase inhibitor) were obtained. Besides, the protein associated with nsLTP was searched by keyword from NCBI (https://www.ncbi.nlm.nih.gov/), IPK (http://apex.ipk-gatersleben.de/apex) and Phytozome((https://phytozome.jgi.doe.gov/pz/portal.html) database. The results from BLASTP, HMM search and keyword search were combined to remove redundant sequences. To increase the probability of detecting putative nsLTPs in barley, all barley protein sequences were downloaded from the NCBI and IPK databases and were submitted to the Batch Web CD-Search Tool (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) to verify the presence of nsLTP domains cl07890(PF00234 belong to cl07890). Pfam(http://pfam.sanger.uk/) validation was then performed using the domain PF00234. Then, the deduced protein sequences of candidate nsLTPs were manually examined to harbor the ECMs (C…C…CC…CXC…C…C), and proteins lacking the essential cysteine residues were removed. Subsequently, the proteins without NSSs (N-terminal signal sequence prediction, http://www.cbs.dtu.dk/services/SignalP, checked by the PROTTER http://wlab.ethz.ch/protter/start/) were also removed and remained C-terminal glycosylphosphatidylinositol (GPI) anchors (GPI anchor signal prediction, http://mendel.imp.ac.at/gpi/plant_server.html and http://psort.hgc.jp/form.html). After that step, the putative proline-rich or hybrid proline-rich proteins, which are characterized by a high proportion of proline, histidine and glycine residues in the sequence located between the NSS and the ECM, were excluded from further analyses. The protein sequences of At2S1-At2S4 and RAT1 were then Blast-searched against the rest of the candidate nsLTP proteins to exclude the possible inhibitors and cereal storage proteins. The proteins with more than 120 amino acids at maturity were also discarded, and the final remaining amino acid was identified as the target amino acid sequence, named HvLTPs (the nsLTPs in barley). The entire screening process is undertaken strictly according Fig. 1.

The Qingke (Tibetan hulless barlay) genome were downloaded from NCBI (https://ftp.ncbi.nlm.nih.gov/genomes/genbank/plant/Hordeum_vulgare/all_assembly_versions/GCA_004114815.1_Hulless_Barley_ass.V2/), and the putative Qingke nsLTPs Orthologs were identified using HvLTPs sequences by local blast, named HtLTPs (the nsLTPs in Qingke).

2.3 Multiple sequence alignment and classification

The amino acid sequences of the putative 40 HvLTPs and 35 HtLTPs were downloaded and the multiple alignment of the ECM part of these sequences was then conducted and manually edited using the DNAMAN program. Additionally, the amino acid sequences were submited to the online site Compute pI/Mw tool (http://web.expasy.org/compute_pi/) to calculate the isoelectric point and molecular weight. Sub-cellular localization of these genes was predicted by Plant-mPLoc online service (http://www.csbio.sjtu.edu.cn/bioinf/plant-multi/). The three-dimensional structures of all putative HvLTPs and HtLTPs were also predicted by SWISS-ODEL (https://swissmodel.expasy.org).

The HvLTPs and HtLTPs can be divided into four major and several minor types according to sequence identity, spacing between the Cys residues and intron position in the 8CM, as well as the post-translational modifications based on the presence of a GPI modification site. In the second round of classification, the HvLTPs and HtLTPs were sorted based on the identity matrix calculated from the multiple sequence alignments [35].

2.4 Phylogenetic construction

The nsLTP amino acid sequences of Arabidopsis, maize, cabbage and rice were downloaded from the TAIR (http://www.Arabidopsis.org/), gramene (http://ensembl.gramene.org/Zea_mays/Info/Index), BRAD (http://brassicadb.org/brad/index.php) and RGAP (http://rice.plantbiology.msu.edu/) databases, respectively. Multiple alignments of the mature proteins were carried out and phylogenetic tree was built using MEGA5.0, with the neighbour-joining (NJ) method and 1000 bootstrap replications. After that, the results were imported to iTOL (https://itol.embl.de/) online service output the picture.

2.5 Protein motif and gene structure analysis

The predicted barley and Qingke nsLTP protein sequence were submitted to the online MEME (http://meme-suite.org/tools/meme) to identify 20 distinct conserved motifs in the nsLTPs. The following parameters are used: repetitions are arbitrary, the maximum number of bases is 10, and the optimal base widths are limited to between 6 and 50 residues.

The prediction analysis of gene structure was carried out using GSDS (http://gsds.cbi.pku.edu.cn/) using the DNA and cDNA sequences of each predictive HvLTP and HtLTP gene from the barley and Qingke genome.

2.6 Chromosomal mapping and gene duplications

The chromosome location information of the nsLTPs was searched in the barley and Qingke genome database and MapInspect software was used to generate chromosomal distribution images.

Gene duplication was investigated following the method described by Kong et al[52]. The MCScanx and Circos programs were used to retrieve and map collinearity between different plant genomes.

2.7 Promoter analysis of *HvLTPs* and *HtLTPs*

To investigate cis-elements in promoter sequences of barley and Qingke nsLTP genes, the upstream sequences (~ 1500 bp) of each identified *HvLTP* and *HtLTP* were retrieved from the barley and Qingke genome using a Perl script. The PlantCARE website (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) was used to identify cis-elements in the promoters.

2.8 Tissue expression profile analysis of *HvLTPs*

The publicly available Barley RNA-Seq datasets were downloaded from the IPK database, and the value of fragments per kilobase of transcript per million fragments mapped (FPKM) of these genes were used for visualizing the heat map using the HEMI software and then used to analyze the barley tissue expression profiles of the identified *HvLTP* genes.

2.9 RNA isolation and quantitative real-time PCR

Total RNA was extracted using the Plant RNA isolation kit (Takara, Shiga-ken, Japan) following the manufacturer's instructions. The RNA quality was checked using 1.0% (w/v) agarose gel stained with ethidium bromide (EB), and the RNA samples were inspected for quality and quantity using a NanoDrop® spectrophotometer and gel imager analysis. First-strand cDNA was synthesized from DNase-treated RNA with a PrimerScript 1st Strand cDNA synthesis kit (TIANGEN, Beijing, China). HvLTPs and HtLTPs gene-specific primers were designed based on their coding sequences (CDSs) using an online tool in NCBI (https: //www.ncbi.nlm.nih.gov/tools/primer-blast) and then synthesized commercially (shenggong, Shanghai, China) (Table S3). qRT-PCR was performed with SYBR GREENI and the CFX96 Real-time System (Bio-Rad, France) by strictly following the manufacturer's instructions. The thermal cycling conditions were as follows: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 sec, and the relative transcription levels were calculated using the $2^{-\Delta\Delta}$ CT method. Three technical replicates were performed for each sample.

Abbreviations

nsLTP: Plant non-specific lipid transfer proteins; 8CM(ECM): eight cysteine residues motifs; IPK: Leibniz Institute of Plant Genetics and Crop Plant Research; NSS: N-terminal signal sequences; GPI: C-terminal glycosylphosphatidylinositol anchors; qRT-PCR: quantitative real-time PCR

Declarations

Acknowledgements

Not applicable.

Authors' contributions

J.D., H.X. and R.D. conceived and designed the experiments. J.D., X.W. and H.X. performed the experiments and participated to the data analysis. Y.L. and C.Z. performed the qRT-PCR experiments. R.D. and J.S. revised the manuscript and improved the English. All authors reviewed and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this article and its additional files. The genome data of *Hordeum vulgare* was downloaded from

https://webblast.ipk-gatersleben.de/downloads/barley_pangenome/Morex/ and

http://plants.ensembl.org/Hordeum_vulgare/Info/Index [29, 30], the genome data of *Qingke* was downloaded from

https://ftp.ncbi.nlm.nih.gov/genomes/genbank/plant/Hordeum_vulgare/all_assembly_versions/GCA_004114815.1_Hulless_Barley_ass.V2/ and http://www.ibgs.zju.edu.cn/ZJU_barleygenome.htm [31],[22], All the RNA-Seq data are available at IPK from https://apex.ipk-gatersleben.de/apex/f?p=284:57.

Ethics approval and consent to participate

Plant materials (barley and Qingke) used in this article were obtained from Academy of Agriculture and Forestry of Qinghai University. All plant materials were provided free of charge and maintained in accordance with the international guidelines. This article did not contain any studies with human participants or animals and did not involve any endangered or protected species.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

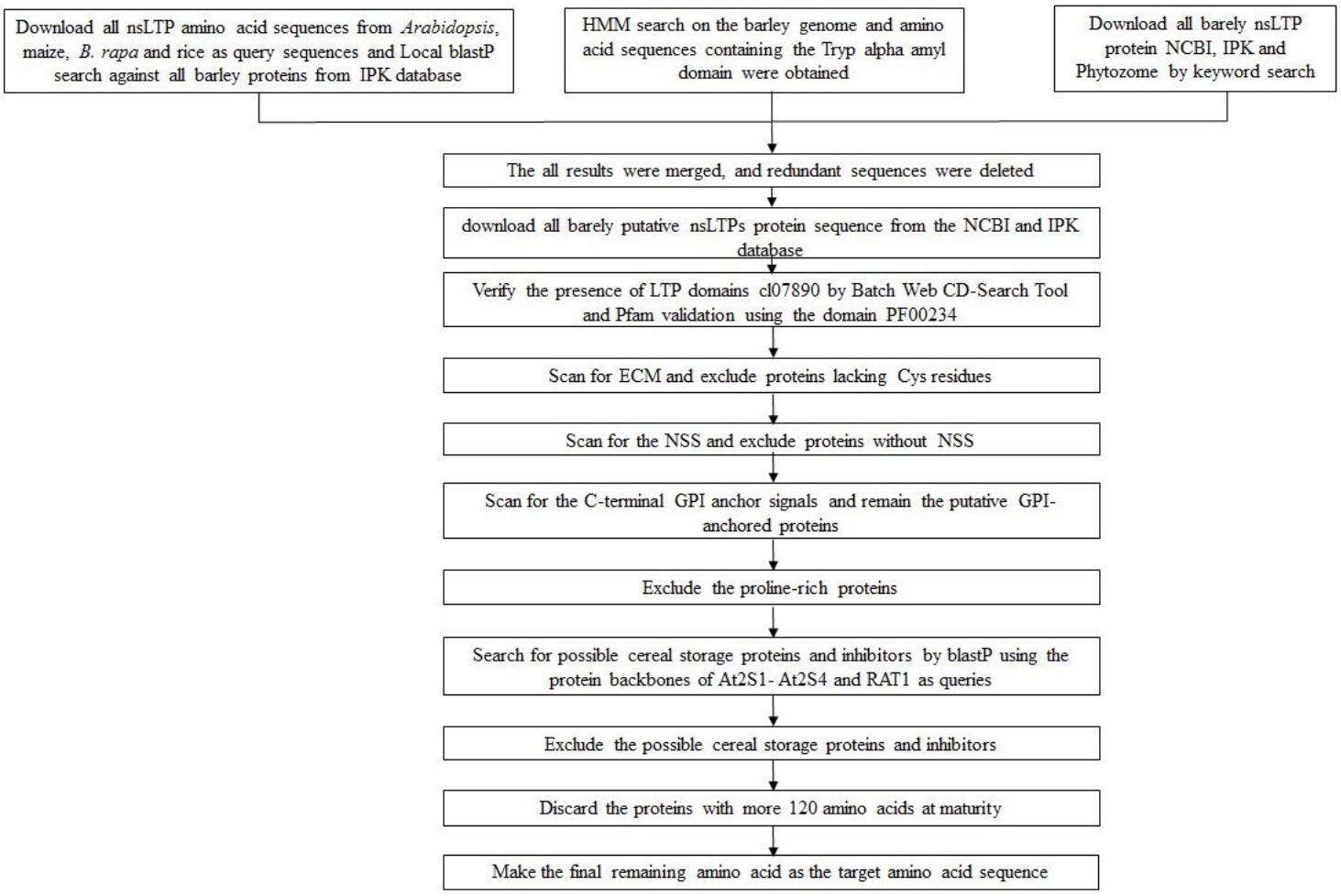

Figure 1

Workflow of HvLTPs identification and data mining.

Figure 2

Multiple sequence alignment of the HvLTPs and HtLTPs 8CM domain sequences Note: * is eight cyc (ECM).

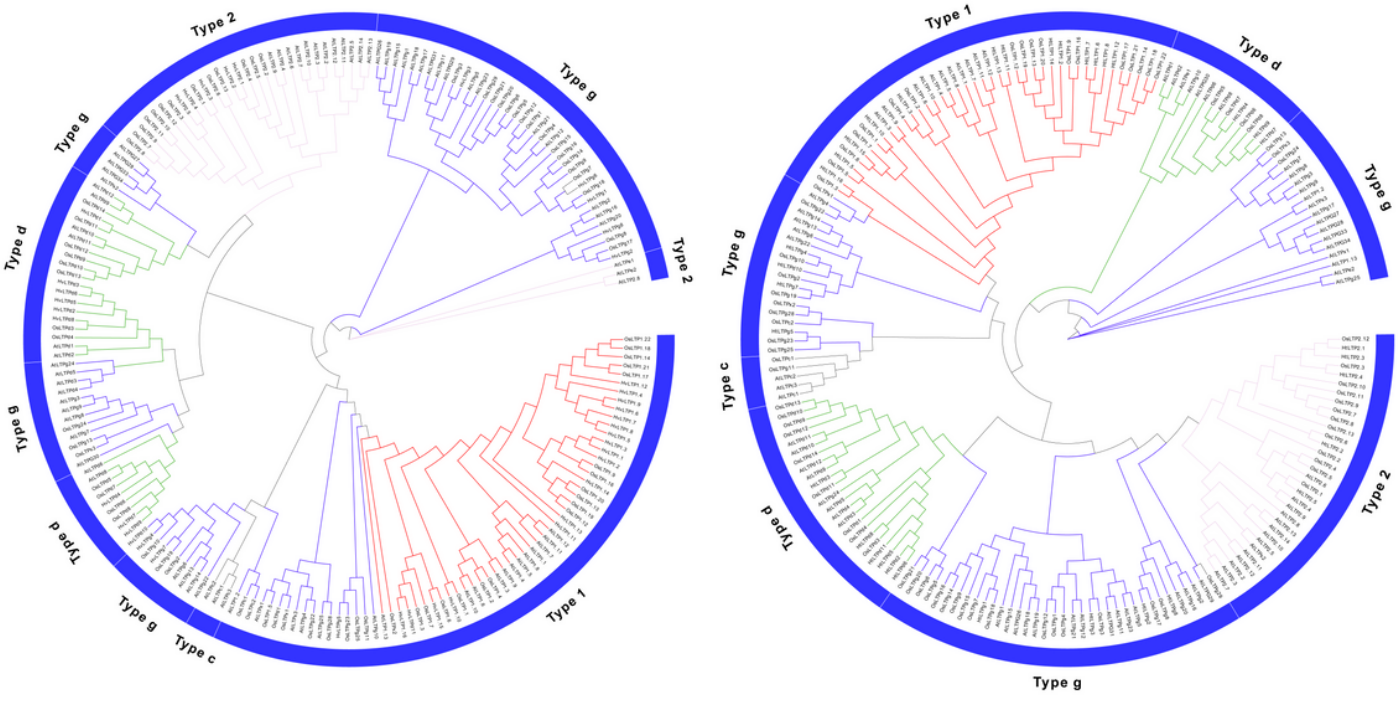

Figure 3

Phylogenetic relationships of the nsLTP family from barley/Qingke, mazie, rice and Arabidopsis

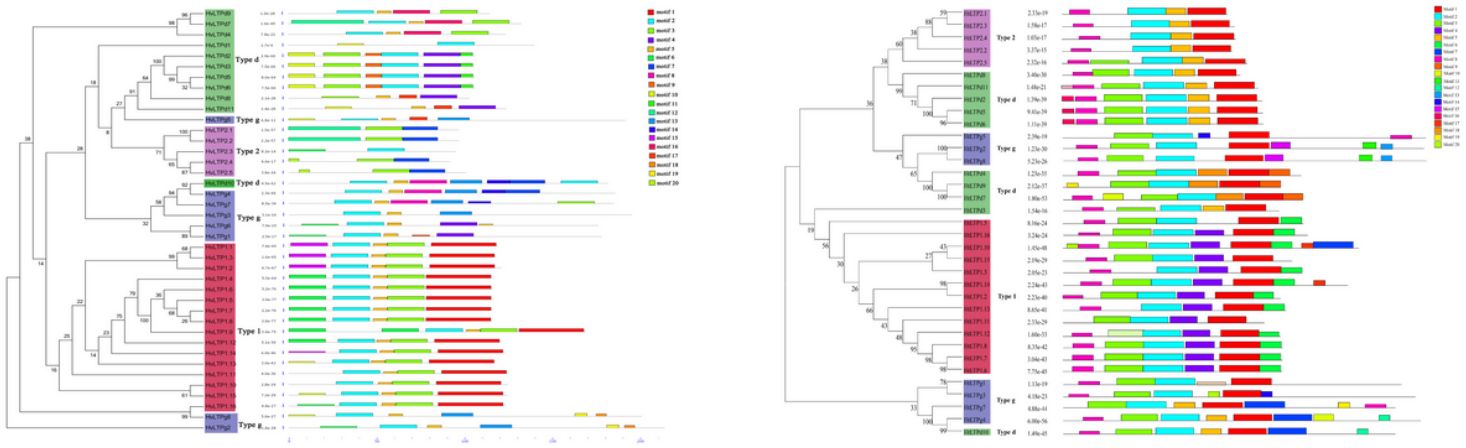

Figure 4

Motif analysis of the HvLTP and HtLTP gene family

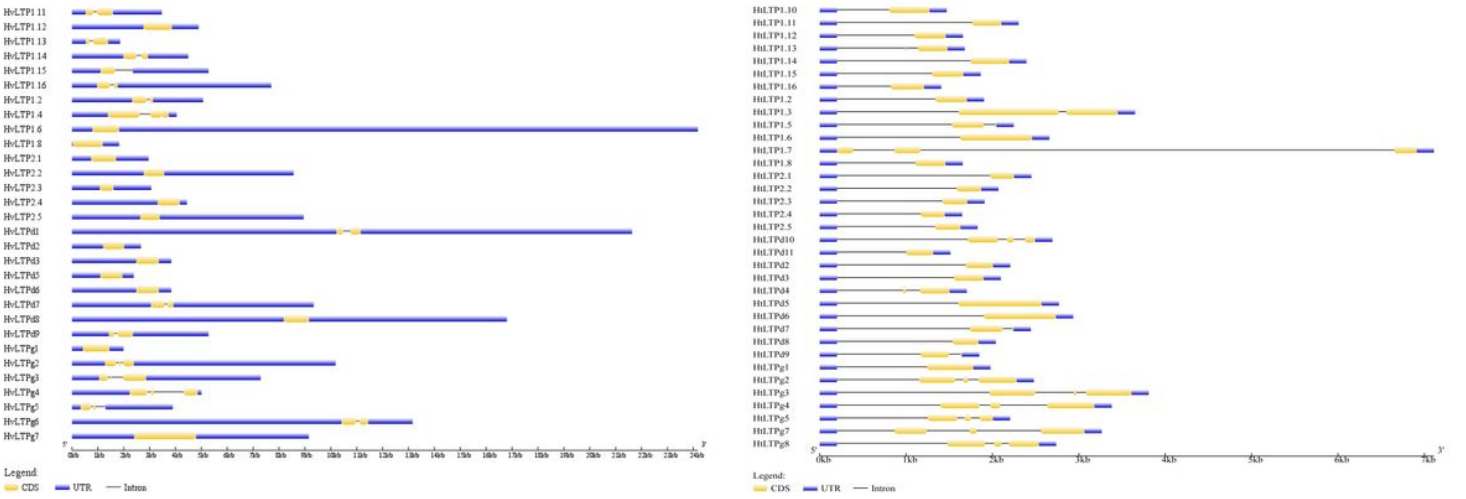

Figure 5

Gene structure of the HvLTPs and HtLTPs

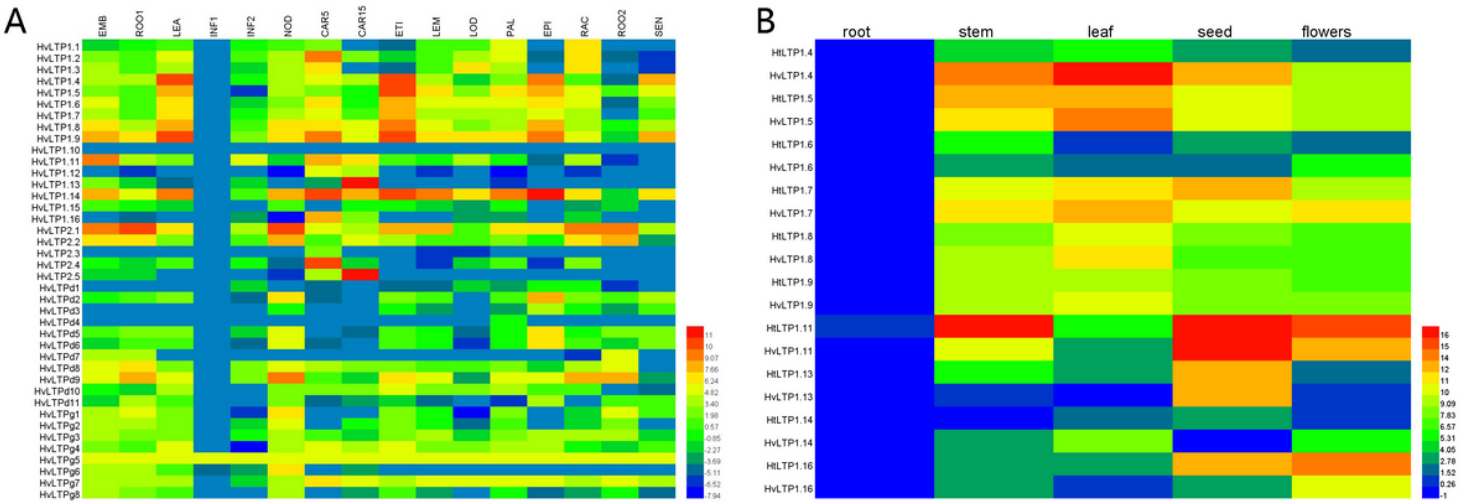

Figure 6

Genomic localization of the HvLTP and HtLTP genes on the barley and Qingke chromosomes

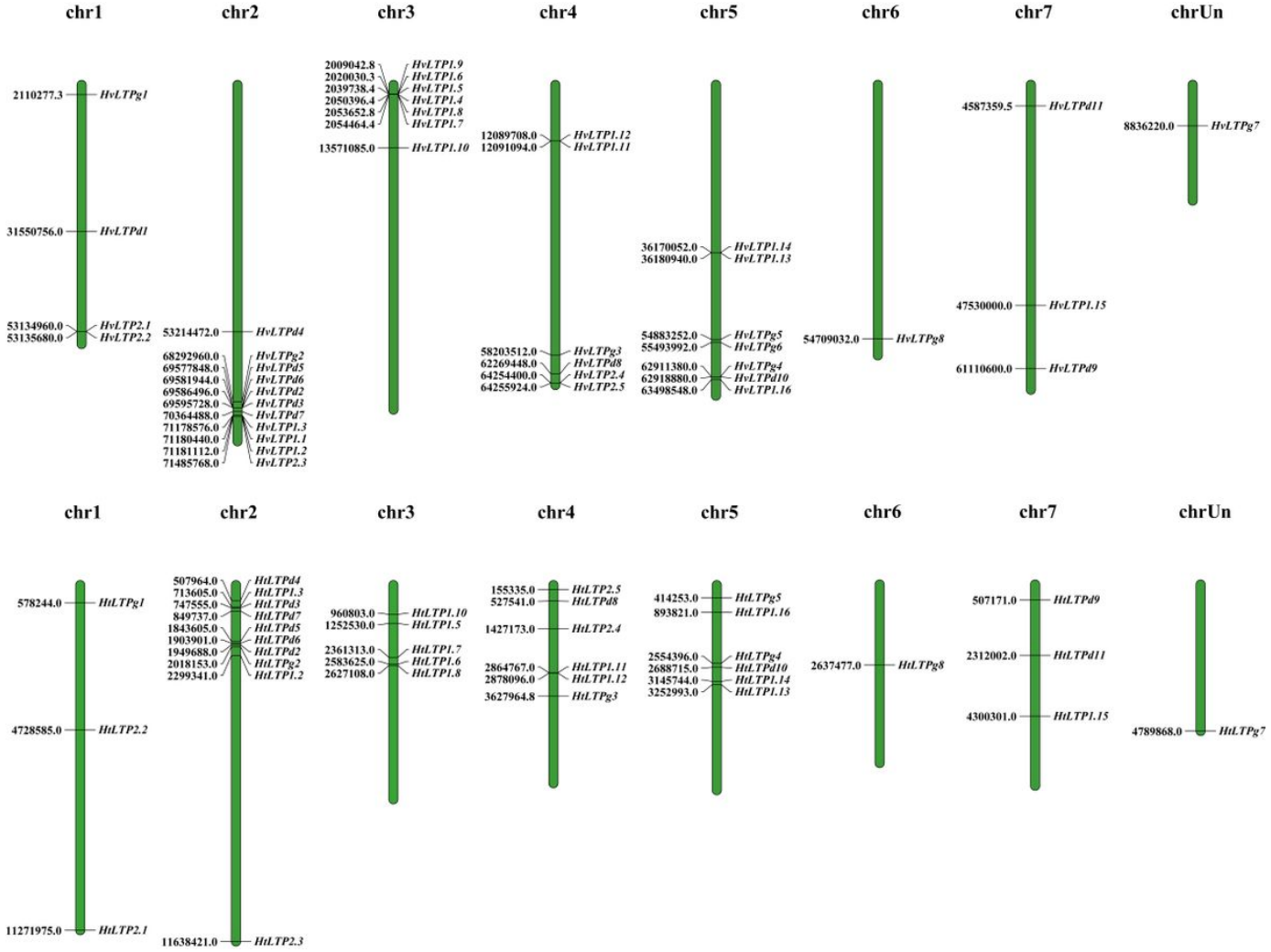

Figure 7

Tissue expression pattern in the HvLTP and HtLTP genes Note：EMB: 4-day embryos; ROO1: Roots from seedlings (10 cm shoot stage); LEA: Shoots from seedlings (10 cm shoot stage); INF1: Young developing inflorescences (5 mm); INF2: Developing inflorescences (1-1.5 cm); NOD: Developing tillers, 3rd internode (42 DAP); CAR5: Developing grain (5 DAP); CAR15: Developing grain (15 DAP); ETI: Etiolated seedling, dark cond. (10 DAP);LEM: Inflorescences, lemma (42 DAP); LOD: Inflorescences, lodicule (42 DAP); PAL: Dissected inflorescences, palea (42 DAP); EPI: Epidermal strips (28 DAP); RAC: Inflorescences, rachis (35 DAP); ROO2: Roots (28 DAP); SEN: Senescing leaves (56 DAP).

Figure 8

Cis-element analysis of the HvLTP and HtLTP genes

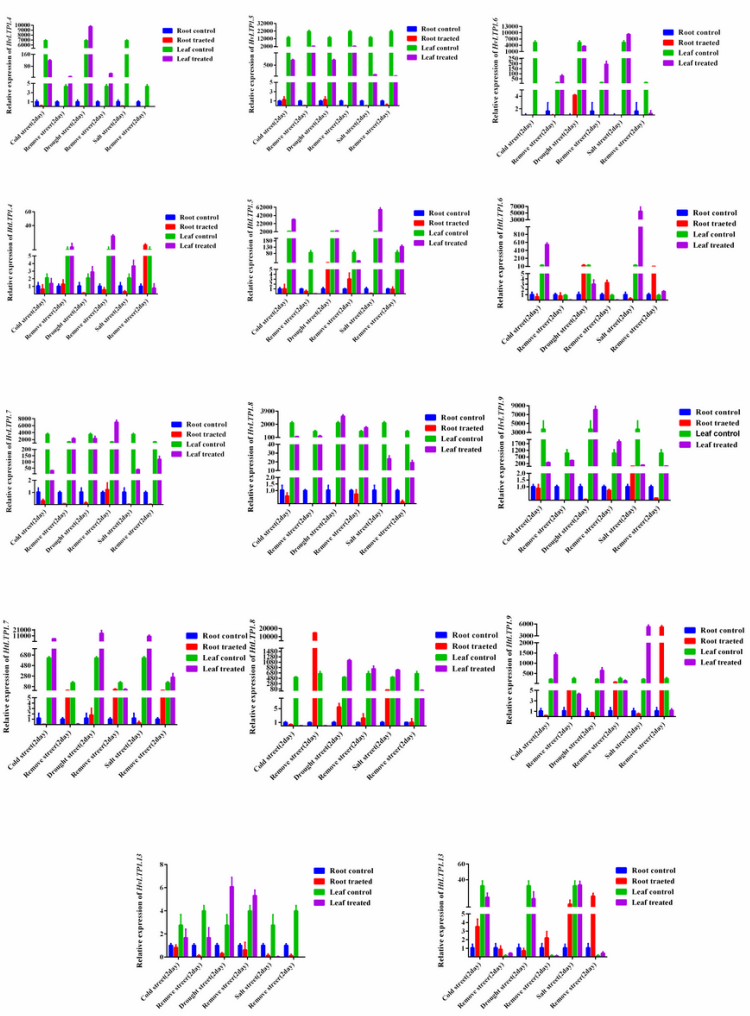

Figure 9

Expression analysis of HvLTP1 and HtLTP1 genes

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Table1CharacteristicsforthedifferenttypesofnonspecificlipidtransferproteinsfoundinbarleyHvLTPsandQingkeHtLTPs.xlsx
- Table2NumbersofnsLTPgenesindifferentspecies.xlsx
- TableS2GeneduplicationeventofHvLTPgenesineachbarleychromosome.xlsx
- TableS1QuerygenesforthebarleynsLTPgenefamilyscreening.xlsx
- TableS3QuantitativePCRprimersforHvLTPandHtLTPgenes.xlsx
- FigureS1CollinearanalysisofinterspeciesandintraspeciescomparisoninHvLTPgenes.tif
- FigureS2ExpressionanalysisofType2DandGHvLTPgenes.tif