Genome-wide analysis of the barley non-specific lipid transfer protein gene family

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Abstract
Non-specific lipid transfer proteins (nsLTPs) are small, basic proteins that are characterized by an eight-cysteine motif. The biological functions of these proteins have been reported to involve plant reproduction and biotic or abiotic stress response. With the completion of the barley genome sequence, a genome-wide analysis of nsLTPs in barley (\textit{Hordeum vulgare} L.) (HvLTPs) will be helpful for understanding the function of nsLTPs in plants. We performed a genome-wide analysis of the nsLTP gene family in barley and identified 70 nsLTP genes, which can be divided into five types (1, 2, C, D, and G). Each type of nsLTP shares similar exon and intron gene structures. Expression analysis showed that barley nsLTPs have diverse expression patterns, revealing their various roles. Our results shed light on the phylogenetic relationships and potential functions of barley nsLTPs and will be useful for future studies of barley development and molecular breeding.

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1. Introduction

Lipids are important in plant growth and development. They function in many physiological pathways, especially in stress response, energy storage, and cuticle layer formation [1,2]. Non-specific lipid transfer proteins (nsLTPs) are small, basic proteins, characterized by eight conserved cysteine residues with the general form C-Xn-C-Xn-CC-Xn-CXC-Xn-C-Xn-C. They also have a tunnel-like hydrophobic cavity, capable of transferring lipids between membranes [3]. nsLTPs are abundant in plant species and represent 4% of soluble protein. Investigations of nsLTP classification, structure, gene expression, and chromosome locations will be helpful for revealing their functions in plant development and physiological adaption to environmental changes.

nsLTP has an N-terminal signal peptide, guiding proteins being translocated between intracellular membranes or secreted to the apoplastic space. Each nsLTP structure is

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maintained through disulfide bonding. The hydrophobic cavity binds lipids and other molecules [4]. The secondary structure endows nsLTP with heat and chemical resistance. The tertiary structure of nsLTPs was determined by X-ray crystallography and nuclear magnetic resonance [4]. nsLTPs have a high content of α-helices, with a tertiary fold characterized by an eight-cysteine motif (8CM). A large number of H bonds contributes to protein 3D structure, helping maintain stability to thermal or chemical effects [5]. LTP1 and LTP2 protein structures have been relatively well studied. The two families’ disulfide bridges have different pairings [6]. For LTP1, the cysteine residues 1–6 and 5–8 are paired, differing from the pairing of cysteine residues 1–5 and 6–8 in LTP2. The distinct manners of disulfide bonding lead to different tertiary structures in LTP1 and LTP2, resulting in one long tunnel-like cavity in LTP1 and two adjacent hydrophobic cavities in LTP2 [4]. Many nsLTPs have a glycosylphosphatidylinositol (GPI) anchor [3].

Categorization of nsLTP based on phylogenetic analysis provides comprehensive information and facilitates nsLTP functional analysis. Based on the length of the mature protein and the molecular weight, nsLTPs have been divided into two major groups: LTP1 (90 amino acids, about 9 kDa) and LTP2 (70 amino acids, about 7 kDa). Boutrot et al. [7] classified nsLTPs in rice (Oryza sativa) and Arabidopsis thaliana into nine types (I–IX). In this classification system, nsLTPs are grouped according to sequence similarity and pattern of spacing between the eight cysteine residues. This classification method has been applied to studies of other species with some modifications [8,9]. Liu et al. [8] clustered Solanaceae nsLTPs in Boutrot’s system, adding a new group, X. Li et al. [9] included a novel XI group in classifying Brassica rapa nsLTPs. The classification methods described above cannot include all identified nsLTPs, especially those from non-flowering plant nsLTPs, owing to low sequence similarity. Edstam et al. [3] developed a new classification system of plant nsLTP families based on intron positions, GPI modification sites, Cys spacing in 8CM, and sequence similarity. In this classification system, the well-established groups based on previous methods, including type 1 and type 2, are retained. Others are classified into several subfamilies: types C-K. Some types overlap with those described in Boutrot’s system, while others comprise new groups in non-flowering species: types F, H, J, and K. Among all the types, D and G occur in early land plant species such as mosses and tracheophytes. Some other types are restricted to a single species: for example, type H is found only in Selaginella moellendorffii. nsLTPs are distributed widely in land plants, but are not present in algae and species outside of the plant kingdom [3]. It is likely that nsLTP genes were acquired when plants colonized land. As land plants evolved, novel nsLTP types arose. Edstam’s classification system gives more information for further functional analysis and a more complete understanding of nsLTPs’ evolutionary history of nsLTPs are expressed in diverse plant organs and tissues. In barley (Hordeum vulgare Linnaeus), nsLTP transcripts were initially observed in the barley aleurone layer. Later, expressions of barley nsLTPs were also detected in vegetative tissues. In other species, nsLTPs have been reported to be expressed in seeds, seedlings, leaves, stems, anthers, microspores, and ovaries [10,11]. The transcription of many nsLTPs is induced by biotic or abiotic stresses [12–14]. Because nsLTPs are targeted to the domain most suitable for conducting their function, nsLTP localization is of great importance in functional studies. One key element determining nsLTP subcellular localization is the signal peptide that induces the secretion of many nsLTPs outside the cell [15] such as those found in barley [16], Arabidopsis (Arabidopsis thaliana) [17], tobacco (Nicotiana tabacum) [18], grapevine (Vitis vinifera) [19], and rice (Oryza sativa) [18]. Using various biological methods such as proteomics [18], cell culture [20], and immunochemical [21] and fusion protein assays [22], nsLTPs have been shown to have various intracellular locations, such as the cell wall [23] or the plasma membrane [22], even though nsLTPs are targeted mainly to the extracellular space. The various locations and diverse expression patterns of nsLTPs suggest their involvement in a wide range of biological functions. In fact, nsLTPs have been shown to be involved in pathogen resistance [24], cutin and wax assembly [25], and plant growth and development [26], as well as being food allergens [27]. DIR1 in Arabidopsis has been shown [28] to be involved in long-distance signaling for pathogen defense. Moreover, the expression of some nsLTP genes is responsive to environmental influences, such as freezing stress [29], salinity [30], and drought [31], suggesting their role in mediating responses to stress during plant growth and development [4]. Overexpression of LTP3 enhances drought and freezing stress tolerance in Arabidopsis [32]. The CALTP1 gene in pepper has been predicted [33] to increase plant tolerance to salinity and drought in developmental stages. It has been suggested [3] that nsLTPs participate in cutin and wax assembly. In Arabidopsis and Brassica oleracea, the expression of nsLTPs is higher in young tissue, where surface waxes are actively synthesized. Previous study [3] has confirmed that nsLTPs are involved in plant pollen development and in recycling endosperm lipids. OsC6 in rice plays a crucial role in regulating postmeiotic anther development [10]. In barley, a major staple food and feed source, nsLTPs play essential roles in plant development and stress response. However, only a small proportion of barley nsLTPs have been well characterized. The lipid transfer protein LTP1 was isolated from the aleurone layer and identified as a novel amylase/protease inhibitor [16]. Two nsLTP genes have been isolated from barley leaves and coleoptile, in which they are specifically expressed [34]. The LTP4 gene promoter from barley responds to ABA treatment and cold treatment [35]. Several barley nsLTP genes are upregulated in response to infection by fungal pathogens [36]. The three-dimensional structure of barley nsLTP has been reported [37]. No genomewide study of the barley nsLTP gene family has yet been reported. The recent release of a high-quality barley reference genome sequence [38–40] makes it feasible to conduct a comprehensive analysis of barley nsLTP genes, which is the basis for further functional characterization. In this study, we identified 70 nsLTP genes in the barley genome. To overcome the difficulty in phylogenetic analysis of nsLTPs due to short and changeable sequences, we grouped these barley nsLTPs into five types based on the sequence identity of mature proteins, GPI modification sites, intron positions, and Cys spacing in 8CM as well as on comparison with nsLTPs of rice and Arabidopsis. We performed a detailed analysis of protein characteristics, chromosome locations, gene expression, and phylogenetic events.
2. Materials and methods

2.1. Retrieval and identification of nsLTPs from the barley genome

Identification of barley nsLTPs from the plant genomics database Phytozone [41] was performed by key word searching in the barley genome. Phytozone v12.1.6 hosts 93 assembled and annotated genomes, including the annotated barley (Hordeum vulgare cv. Morex) genome (International Barley Sequencing Consortium annotation r1 on assembly r1). Repetitive sequences were removed. The downloaded protein sequences were manually examined for an eight-cysteine motif (8CM) and those lacking such a motif were removed. Multiple sequence alignment of the candidate nsLTPs was then performed. Genes showing large sequence differences from other genes were excluded. The remaining candidate proteins were submitted to SMART [42] to confirm the presence of the LTP domains.

2.2. Primary sequence analysis

All identified nsLTPs were submitted to SignalP 4.1 [43] for signal peptide prediction using the default cutoff. All full length proteins were submitted to ProtParam [44] for calculating number of amino acids, molecular weight, and theoretical pI. Intronic position was obtained from sequence information provided by Phytozone, which was determined from publicly available barley full-length cDNAs and RNA-seq data generated in the International Barley Genome Sequencing project (the genome annotation workflow is described in [38,40]). GSDS software [46] was used to illustrate gene features. For better visualization and comparison, 5’ UTR sequences were removed. The prediction tool GPI Modification Site Prediction [45] was used to check or the presence of GPI anchor sites. To predict subcellular targeting, the TargetP 1.1 [45] was used. Gene distribution on chromosomes was drawn manually based on data from Gramene [46].

2.3. Sequence alignment and phylogenetic reconstruction

Multiple alignment of the 8CM motifs of nsLTP proteins was performed with MUSCLE [47], using default settings. Based on these alignments, a maximum-likelihood tree was generated with Phyml 3.0 in SeaView [48] using the following parameters: LG model; aLRT (SH-like) branch support; amino acid model equilibrium frequencies: model-given; optimized invariable sites and across-site rate variation; tree searching operations: NNI; and starting tree: BioNJ with optimized tree topology. The rice and Arabidopsis homologs used were those reported by Edstam et al. [3]. The rice and Arabidopsis nsLTP protein sequences were downloaded from Phytozone and TAIR [49], respectively.

2.4. Gene expression analysis

The expression profiles of nsLTPs genes were compared. RNA-seq data of different barley tissues or the same tissues at different development stages were obtained from BARLEX (Barley Genome Explorer) [50]. A heat map showing expression differences on the log2 scale was generated with the gplots package in R [51]. A hierarchical clustering algorithm was used to identify similar patterns in expression profiles.

3. Results

3.1. The barley nsLTP gene family is composed of 70 members

A total of 70 unique nsLTPs were found in the barley genome [14]. To obtain a full and nonredundant set of nsLTPs in barley, barley protein-encoding genes were extracted from Phytozone. Initially, 90 potential HvLTPs were identified in the barley genome sequence by keyword searching, following the removal of sequences with incomplete 8CM domains. Each of the candidate protein sequences was then manually assessed to identify an eight-cysteine pattern. Among the 90 candidates, two containing no 8CM domain, 13 with incomplete patterns, and two redundant sequences were removed. Three others with large sequence differences from the others were also removed on the basis of the sequence alignment, leaving 70 proteins selected as barley nsLTPs.

Classification of the identified barley nsLTPs was performed based on sequence identity of mature proteins, GPI modification sites, intron positions, Cys spacing in 8CM, and comparison with nsLTPs of rice and Arabidopsis. The 70 HvLTPs were classified into five types: 1, 2, C, D, and G. Type G proteins accounted for the largest proportion of barley nsLTP proteins: 31 nsLTPs, comparable to the 27 (of 78) in rice and Arabidopsis. The 70 HvLTPs were classified into five types: 1, 2, C, D, and G. Type G proteins accounted for the largest proportion of barley nsLTP proteins: 31 nsLTPs, comparable to the 27 (of 78) in rice and Arabidopsis. The 70 barley sequences, 27 were assigned to type D, more than the corresponding numbers in the other two species. The numbers of type C sequences was low in all three species. The barley nsLTPs were named according to their types. The gene encoding a barley type 1 nsLTP was named HvLTP1, with corresponding numbers being assigned for the other types.

3.2. Characteristics of lipid transfer proteins in barley

The characteristics of the 70 HvLTPs are summarized in Table 1. Of the 70, 47 were predicted to have a signal peptide, with length ranging from 19 to 49 amino acids. Most of the proteins were predicted to lie in the secretory pathway, whereas some were predicted by signal peptide analysis to be localized in the mitochondria. Barley nsLTPs are small and of low molecular weights ranging from 9439.32 to 46,722.68 Da. Types 1, 2, and D were mostly 10 kDa proteins whereas types G and C were mostly 20-kDa proteins. The molecular weight of type G was much higher than that of the other types because of the C-terminal extra amino acid residues. Judging from the pI value,
Table 1 – Occurrence and features of nsLTPs in barley.

<table>
<thead>
<tr>
<th>Name</th>
<th>Signal peptide</th>
<th>Mature protein</th>
<th>GPI</th>
<th>Number of introns</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amino acid</td>
<td>Target</td>
<td>Amino acid</td>
<td>Mass (Da)</td>
</tr>
<tr>
<td>Type 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HvLTP1.1</td>
<td>28 s</td>
<td>122</td>
<td>12,306.42</td>
<td>8.98</td>
</tr>
<tr>
<td>Type 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HvLTP2.1</td>
<td>m</td>
<td>133</td>
<td>13,933.36</td>
<td>8.96</td>
</tr>
<tr>
<td>HvLTP2.2</td>
<td>28 s</td>
<td>96</td>
<td>9995.91</td>
<td>8.68</td>
</tr>
<tr>
<td>Type C</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HvLTPc1</td>
<td>44 s</td>
<td>230</td>
<td>22,988.88</td>
<td>8.33</td>
</tr>
<tr>
<td>Type D</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HvLTPd1</td>
<td>24 s</td>
<td>127</td>
<td>13,983.17</td>
<td>8.02</td>
</tr>
<tr>
<td>Type G</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HvLPg1</td>
<td>21 s</td>
<td>184</td>
<td>18,612.6</td>
<td>8.14</td>
</tr>
<tr>
<td>HvLPg2</td>
<td>29 s</td>
<td>177</td>
<td>16,873.3</td>
<td>7.63</td>
</tr>
</tbody>
</table>

**Note:**
- a: Target indicates signal peptide presence.
- b: Target indicates GPI presence.
- c: Number of introns indicates presence or absence.
- d: No GPI presence.
types 1, 2, C, and D are mostly alkaline proteins, except for some type D proteins. Some of the type G nsLTPs are alkaline and some are acidic (Table 1). The average molecular weight is 16,408.21 Da and the theoretical pI is 7.76 pI.

Most type G proteins contained GPI modifications whereas few putative GPI modification residues were observed in other types. Among the nsLTPs, 11 type G but only two type D HvLTPs contained putative GPI modification residues.

3.3. Sequence analysis of the barley nsLTPs

The main characteristic of plant nsLTPs is the presence of a highly conserved eight-cysteine motif (C-Xn-C-Xn-CC-Xn-CXn-C-Xn-C). The conserved residues form four disulfide bonds that stabilize the tertiary structure of the hydrophobic cavity. In an attempt to build a specific 8CM consensus for each nsLTP type found, the eight-Cys spacing was examined in all of the classified HvLTPs (Table 2). Because all the cysteine residues were located at a conserved position, BioEdit was used to perform the multiple alignment (Fig. 1).

The numbers of inter-cysteine residues in different types of HvLTPs varied widely (Tables 2, 3). Three HvLTP types could be identified according to typical spacings of the motif. Type 2 and type C HvLTPs contained respectively 7 and 11 residues between the conserved Cys1 and Cys2 residues. Similarly, type 1 HvLTPs contained 19 residues between the conserved Cys4 and Cys5 residues. The resulting features of type 1 and 2 proteins were consistent with those described in previous reports [3,7]. In contrast, types D and G could not be distinguished based on the cysteine spacing features. Some HvLTPs harbor an additional cysteine, such as HvLTPd14, which has an extra cysteine between Cys6 and Cys7.

A closer analysis of the sequences indicates that aside from the eight Cys residues, Type 1, 2, and C HvLTPs were characterized by a leucine residue present three aa 5′ to Cys2. In type 1, the number of amino acids between Cys4 and Cys5 was greater than that in other types. Leucine is a hydrophobic residue and the most frequent residue appearing in the X position in the CXC motif of all the HvLTPs. The conserved residues may play important roles in the biological function of nsLTPs. There were seven different residues (Arg, Leu, Phe, Met, Val, Ile, and Ala) at the X position of the CXC motif (Fig. 1). Five (Leu, Phe, Val, Ile, and Ala) are hydrophobic and two (Arg and Met) hydrophilic.

3.4. Intron–exon structures of barley nsLTPs

Intron–exon structure predicts the evolution of a gene family. Forty-two HvLTP genes were predicted to be interrupted by 1–6 introns (Fig. 2). Similar intron-exon patterns were found

<table>
<thead>
<tr>
<th>Name a</th>
<th>Signal peptide</th>
<th>Mature protein</th>
<th>GPI c</th>
<th>Number of introns</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amino acid</td>
<td>Target b</td>
<td>Amino acid</td>
<td>Mass (Da)</td>
</tr>
<tr>
<td>HvLTPg22</td>
<td>27</td>
<td>s</td>
<td>185</td>
<td>18,855.91</td>
</tr>
<tr>
<td>HvLTPg23</td>
<td>30</td>
<td>s</td>
<td>181</td>
<td>19,065.2</td>
</tr>
<tr>
<td>HvLTPg24</td>
<td>Other</td>
<td></td>
<td>164</td>
<td>16,891.56</td>
</tr>
<tr>
<td>HvLTPg25</td>
<td>24</td>
<td>s</td>
<td>150</td>
<td>14,952.49</td>
</tr>
<tr>
<td>HvLTPg26</td>
<td>Other</td>
<td></td>
<td>122</td>
<td>12,357.29</td>
</tr>
<tr>
<td>HvLTPg27</td>
<td>25</td>
<td>s</td>
<td>176</td>
<td>18,103.08</td>
</tr>
<tr>
<td>HvLTPg28</td>
<td>m</td>
<td></td>
<td>157</td>
<td>16,204.77</td>
</tr>
<tr>
<td>HvLTPg29</td>
<td>25</td>
<td>s</td>
<td>173</td>
<td>18,233.81</td>
</tr>
<tr>
<td>HvLTPg30</td>
<td>31</td>
<td>s</td>
<td>183</td>
<td>17,750.42</td>
</tr>
<tr>
<td>Single</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HvLTPx1</td>
<td>21</td>
<td>s</td>
<td>230</td>
<td>22,483.59</td>
</tr>
<tr>
<td>HvLTPx2</td>
<td>49</td>
<td>m</td>
<td>220</td>
<td>21,468.48</td>
</tr>
</tbody>
</table>

Table 2 – The spacing patterns of eight-cysteine motifs of HvLTPs.

<table>
<thead>
<tr>
<th>Type</th>
<th>Spacing pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>C 9 C 15 C 19* C-1-C 21 C 13 C</td>
</tr>
<tr>
<td>2</td>
<td>C 7* C 13 C 8 C-1-C 23 C 6 C</td>
</tr>
<tr>
<td>C</td>
<td>C 11* C 16 C 12 C-1-C 28 C 2 C</td>
</tr>
<tr>
<td>D</td>
<td>C 6, 9, 10, 14 C 12, 14, 16–18 C 9–12 C-1-C 21–24, 26, 27 C 6–10 C</td>
</tr>
<tr>
<td>G</td>
<td>C 6, 9, 10, 12 C 8, 10, 14–18, 21 C 11, 12, 14 C-1-C 22–27, 29 C 8, 9, 11–13 C</td>
</tr>
</tbody>
</table>

The consensus motif of each LTP type was deduced from analysis of the mature sequences of 70 putative HvLTPs. * Direct identification of the LTP type.
within groups. For instance, the nsLTP genes in type 2 lacked an intron, the genes in types 1, C, and D contained one intron, and the genes in type G contained variable numbers of introns from none to six. HvLTP9 had the highest number of introns in all barley nsLTP genes. The exact number of introns in some proteins could not be determined, owing to incomplete sequencing. In types I and C, the intron was positioned respectively 5 and 4 bp downstream of the codon encoding the eighth cysteine in the SCM. In nine of 11 type D nsLTPs carrying one intron, the intron was positioned 4 bp downstream of the eighth cysteine codon. In HvLTP22 and HvLTP23, the intron was 16 bp downstream of the eighth cysteine codon. HvLTP gene structures were similar to those of nsLTPs in Arabidopsis, rice and maize [14].

3.5. Chromosomal locations of HvLTP genes

The 70 HvLTPs were unevenly located on the seven barley chromosomes (Fig. 3). The maximum number of HvLTPs (14) was located on chromosome 1. A further 12 were located on chromosome 4, 11 on chromosome 2, and nine and eight on chromosomes 3 and 5, respectively. Seven genes were located on chromosome 7 and six on chromosome 6. Most of the HvLTPs were located close to the ends of chromosomes, but absent from some regions on several chromosomes, such as the long arm of chromosome 5 and the short arm of chromosome 3. HvLTP41, HvLTP42, and HvLTP21 were not assigned to any chromosomes but were included in Chr.Un. Chr.Un is composed of sequence fragments from BAC overlap clusters not placed in the Hi-C (high-throughput/resolution chromosome conformation capture) map, gene-bearing fragments of BAC sequences and Morex WGS contigs selected in addition to the nonredundant sequence [40]. The positions of HvLTPs on barley chromosomes are shown in Table S2.

Analysis of physical chromosome localization revealed that 36 of the 70 HvLTPs were arranged in 15 direct tandem duplicate repeats (Fig. 3). Two tandem repeats of type 2 were identified on chromosome 1 and one on chromosome 3. Eighteen type D HvLTPs were clustered into six tandem repeats, located on chromosomes 1, 2, 3, 4, and 7. Seven tandem repeats of type G were found on chromosomes 2, 4, 5, and 6. Genes in the same tandem repeat were closely related. For instance, HvLTP26 and HvLTP27 shared 95% identity.

3.6. RNA-seq-based expression profile of HvLTPs

Gene expression pattern analysis gives useful clues to gene function. Hierarchical clustering was used to investigate the
spatial and temporal expression patterns of the nsLTP genes in the barley life cycle. Given that HvLTPg25 was not expressed in any tissues based on RNA-seq data, it was removed for expression profiling. Visual global transcription profiles of the remaining 69 HvLTP genes across 15 different developmental stages were constructed based on expression data provided by BARLEX database (https://apex.ipk-gatersleben.de/apex/?p=284:10). As shown in Fig. 4, the heat map can be divided into four clusters. Clusters I–IV contained respectively 23, 15, 13, and 18 members. Genes in cluster I showed relatively high expression levels and those in cluster IV relatively low expression levels. In cluster II, genes were expressed at a moderate level. In cluster III, genes were expressed specifically in some tissues. Compared with those in other tissues, the expressions of most HvLTPs were diminished in senescing leaves (SEN). HvLTP transcripts were also less abundant in developing inflorescences (INF) and lodicules (IOD). Genes in the same tandem repeat showed generally similar expression patterns. HvLTP genes in cluster I showed the most ubiquitous and stable expression in root, embryo, grain, seedling, tillers, internode, inflorescence, and floral organs (Fig. 4). HvLTPg30 is the closest homolog of Arabidopsis AtLTPG1, which has been shown [25] to be required for normal export of wax to the cuticle. HvLTPg30 is widely expressed and may also be involved in cuticular lipid deposition. Genes in cluster II showed dynamic expression throughout the entire life cycle. For example, the expressions of HvLTPd9, HvLTPd10, and HvLTPd11 were strong in the epidermal strip (EPI), but much lower in developing grain. Genes in cluster III were markedly more specific and were high in developing grain (5 DAP and 15 DAP). The 13 members occur almost exclusively in kernel tissues. In cluster IV, gene expressions were low and in root, embryo and epidermal strips, gene expressions were relatively high.

3.7. Phylogenetic analysis of barley, rice and Arabidopsis nsLTPs

To characterize the phylogenetic relationship of the nsLTPs among barley, rice and Arabidopsis, 219 nsLTPs from these three species were analyzed (Fig. S1). A multiple sequence alignment of the eight-cysteine domain sequences
from barley, rice and Arabidopsis was performed and a phylogenetic tree was generated with PhyML using the maximum-likelihood method. nsLTPs from the three species were classified into five types: types 1, 2, C, D, and G, in agreement with the previous report [3]. As shown in Fig. S1, type 1 and type 2 members from the three species formed a single clade in the tree, indicating that genes in these two types originated from a common ancestor. Type D and G sequences are placed in specific clusters. The sequences from the minor nsLTP type C form a clade close to type D. But none of these types forms a distinct monophyletic clade supported with high bootstrap values.

### 4. Discussion

Barley is well known for its tolerance to salinity, alkali, drought, and cold. nsLTPs have been reported to be involved in abiotic stress tolerance and may play roles in the adaptation of barley to diverse environmental conditions. However, the barley nsLTP family has not been comprehensively characterized. In this study, 70 nsLTP genes were identified in the barley genome and divided into five types (1, 2, C, D, and G) supported by phylogeny, protein characteristics and gene structures.

The finding that most of the nsLTP genes are located at the distal region of chromosomes is consistent with the observation [38] that both ends of barley chromosomes are especially gene-rich. Higgins et al. [51] reported that in barley, meiotic homologous chromosome recombination is confined predominantly to distal regions on all chromosomes. Biased distribution to recombination-rich regions ensures flexibility for generating sequence diversity required to cope with dynamic environmental changes and stressful conditions.

Gene structure and chromosome locations suggest that numerous gene birth events have occurred in the barley genome during evolution. Among the 70 barley nsLTP genes, 36 are clustered into 15 tandem duplication repeats. Similarly, large numbers of gene duplication events are also observed in several other angiosperms, including Arabidopsis, rice, sorghum, maize, and wheat [7,14,52]. By contrast, many fewer gene duplication events occur in lower plants, such as liverworts, mosses, and lycopods [3]. Sequence variation and differential expression profiles suggest the occurrence of neofunctionalization or subfunctionalization following gene duplication. For instance, HvLTPg20 (HORVU7Hr1G104750) and HvLTPg21 (HORVU7Hr1G104760), two tandem nsLTP genes in a distal region of barley chromosome 5 share only 38% identity at the nucleic acid level and 19% identity at the protein level. HvLTPg20 is broadly expressed in several tissues, whereas HvLTPg20 is weakly expressed in roots, embryos, and developing tillers. In another example, HvLTPd26 (HORVU7Hr1G105960) and HvLTPd27 (HORVU7Hr1G106020) exhibit distinct expression profiles although they share more than 95% identity at both DNA and protein levels. HvLTPd26 is highly expressed in the embryo and carpel, but only very low levels of HvLTPd27 transcripts are detected in the carpel. These differential expression patterns suggest that they may diverge in the regulatory regions of their gene promoters. These observations support the hypothesis that the nsLTP gene family has expanded considerably during flowering plant evolution, allowing both conservation and divergence of gene function.

The expression profiles of HvLTPs in different tissues across developmental stages indicate that nsLTP genes

![Fig. 3 – Genome distribution of LTP genes from barley. Distribution of 70 HvLTP genes on seven barley chromosomes and Chr. Un. Chromosomal distances are given in Mb. The name of each chromosome is shown at the top of the corresponding bars. The approximate locations of LTPs are marked on each chromosome and gene names are shown to right of the bar.](image-url)

![Fig. 2 – Intron-exon arrangement of barley LTP genes. Exons and introns are drawn based on the scale of the respective encoding regions. Exons are depicted as yellow boxes and introns as connecting thin lines. Non-encoding regions are depicted in blue boxes.](image-url)
perform a variety of functions in different tissues at multiple developmental stages. But to date, only a few HvLTPs have been functionally characterized. Barley LTP1 is involved in membrane biogenesis, responses to stresses, and transport of cutin [53,54]. Overexpression of barley LTP2 in tobacco and Arabidopsis enhances plant tolerance to bacterial pathogens [55]. However, the biological functions of most barley nsLTPs remain enigmatic. Further investigations, using molecular genetic, biochemical, physiological, and developmental approaches will be required to clarify these functions.

In summary, our analysis has established a foundation for further understanding the functional significance of this dynamic and fascinating gene family in barley development and stress resistance, which will contribute to barley molecular breeding in the future.

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