Genome-wide Identification, Evolution and Expression Analysis of LBD Transcription Factors Family in Wheat (*Triticum aestivum*. L)

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Research Article

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Abstract

Background: The LBD (lateral organ boundaries)-domain transcription factor is one unique gene family in plants, playing the crucial role in regulating plant architecture as well as stress tolerance. Although LBD family has been thoroughly characterized in many species, it is not well studied in wheat.

Results: In this study, the *in silico* genome-wide analysis was performed and a total of 90 LBD proteins were identified in wheat (TaLBDs). Phylogenetic analysis classified them into two major groups, of which Class I contained 8 subfamily (IA-IH), and Class II include 2 subfamily (IIA-B). Exon-intron structure, conserved protein motif and cis-regulatory elements analysis showed that the members in the subfamily shared similar gene structure organizations, supporting the classification. Furthermore, these TaLBDs mainly interacted with the genes related to signal transduction cascades and they were also targeted by miRNAs to form the sophisticated regulation network. Then, the tissue-specific and stress-responsive TaLBD genes were identified through RNA-seq analysis. Finally, the expression of 12 TaLBDs were selected to validate by QPCR analysis and the homoeologous genes showed significantly differential expression, suggesting subfunctionalization of them has occurred. In addition, the genetic diversity of TaLBDs in wheat landrace showed slight higher than that of variety while obvious asymmetry at subgenome level.

Conclusions: This study reported the composition, structure, evolution and expression patterns of LBD family in wheat, which will provide potential target genes for further functional analysis and also shed light on the role of LBD gene playing in regulating development and stresses tolerance in wheat and beyond.

Background

LBD (Lateral Organ Boundaries Domain), also called AS2/LOB gene family, is one class of plant specific transcription factor family, which plays an important role in regulating lateral organ development and morphogenesis as well as metabolism in plants[1]. LBDs are expressed in a band of cells at the adaxial base of all lateral organs formed from the shoot apical meristem and at the base of lateral roots. The LBD transcription factors are typically defined by an N-terminal LBD domain, which generally comprises a C-domain containing four highly conserved cysteine (C) residues in a CX₂CX₆CX₃C zinc finger-like motif. Besides, some LBDs also possessed glycine residue and leucine-zipper-like motif LX₆LX₃LX₆L [2, 3]. Generally, the LBD proteins can be divided into Class I and Class II subfamily according to their containing conserved domains. In detail, Class I members usually contain four highly conserved Cys residues in a CX₂CX₆CX₃C motif, together with a leucine zipper-like motif (LX₆LX₃LX₆L) that can form coiled-coiled structure for protein interactions, while Class II members have the conserved Cys residues and zeucine zipper-like domain that cannot form coiled-coil structure [4]. The majority of LBD proteins belong to Class I, and widely particulate in regulating plant development and signal transduction [1, 5].

Up to now, extensive studies have been conduct to investigate the LBD gene family in many plant species, such as 34 putative LBD genes found in barley, 44 in maize, 35 in rice, respectively [6–8]. Meanwhile, the biological function of some LBD genes have been well-studied. In Arabidopsis, AS1 and AS2 gene were found to involve in establishment of a prominent midvein and other veins as well as in formation of the symmetric leaf lamina, which might be related to repression of knotted 1 homeobox genes in leaves [9]. *AtLBD16* and *AtLBD18* functioned in the initiation and emergence of lateral root formation via different pathway, and they could interact with downstream ARF7 and ARF19 [10]. *AtASL 9* was found to be exclusively regulated by the plant hormone cytokinin [11]. In rice, *OsARL 1*, encoded a protein with a LBD domain, was an auxin-responsive factor involved in auxin-mediated cell dedifferentiation [12]. *OsAS2* gene was required for shoot differentiation and leaf development [13]. *OsLBD3-7* might act as the upstream regulatory genes of bulliform cell development to regulate leaf rolling [14]. In soybean, the expression of *GmLBD12* could be induced by drought, salt, cold and hormones [15].

Wheat is one of the most important crops all over the world, occupying around 17% of global cultivated lands [16]. Wheat can provide plenty of proteins, starches and vitamins for human, acting as an essential plant protein resource. Genetically, wheat is an allohexaploid, derived from three diploid donor species through two naturally interspecific hybridization events [17–18].

As a result, wheat has a huge and complicated genome with three sub-genome (A, B and D), making it an ideal model for polyploidization and homoeologous gene interaction and studies in plants [19]. The completion of its reference sequence provides the opportunity to investigate the genomic organization and evolution dynamics of the wheat gene family at the genomic level [20].

At present, the systematical investigation of LBD gene family has not yet been performed in wheat. Here, we conducted an *in silico* genome-wide search of LBD gene family in wheat. Then, the phylogenetic relationship, chromosome localization, gene structure, conserved protein domain, cis-elements and expression profiles as well as regulatory network and genetic variations of the putative wheat LBD genes were systematically analyzed. These results provided the global information on the composition, structure and evolution of wheat LBD gene family, which will facilitate the further functional study of this important transcript factor family in wheat and beyond.

Results And Discussion

Identification of LBD family in wheat

Using the method as described in Method section, a total of 90 genes were remained as putative LBD proteins, which represented the most abundant LBD genes in plant species reported up to now, indicating that LBD gene family expanded significantly in wheat (Tables 1 and 2). Chromosome location analysis found that these 89 TaLBD genes were unevenly distributed on all of the 21 chromosomes in wheat and only one (Ta-U-LBD90) was not mapped on chromosome, of which 4B and 4D had the most abundant PYL genes with each containing 11 TaLBD genes, followed by 4A with 8, 3A with 7, 3B, 3D and 5A with 6 respectively while 6B, 7B and 7D only possessed one TaLBD gene (Fig. 1). Totally, 31, 29 and 29 LBD genes are non-randomly distributed in the wheat A, B and D sub-genome respectively, indicating that no significant variation was occurred on the LBD abundance at subgenome level. Since there is no standard nomenclature, the predicted wheat LBD genes were then named based on their chromosome location and multiple alignment.

Table 1
Comparison of the abundance of LBD genes in different plant species

Species	Class I	Class II	Total
Wheat	60	15	90
Arabidopsis	36	6	42
Rice	29	6	35
Barley	19	5	24
Brachypodium	24	4	28
Maize	37	7	44
Pepper	36	9	45
Tomoto	40	6	46

Table 2
Basic information of the LBD genes identified in wheat

Gene name	EnsemblPlants ID	Gene length (bp)	ORF length (bp)	Deduced Protein				Splice Variants	Subcellular location
				Size (aa)	MW (KDa)	pI	GRAVY		
Ta-1A-LBD1	TraesCS1A02G062200	647	621	206	21.2059	6.7	0.037378	1	Nuclear
Ta-1A-LBD2	TraesCS1A02G111700	1888	714	237	24.7437	8.74	-0.11772	1	Nuclear
Ta-1A-LBD3	TraesCS1A02G222300	808	570	189	20.4461	6.76	-0.18307	1	Nuclear
Ta-1A-LBD4	TraesCS1A02G260000	3986	795	264	27.1022	7.78	-0.02046	1	Nuclear
Ta-1B-LBD5	TraesCS1B02G080600	936	648	215	22.0948	6.61	0.012093	1	Nuclear
Ta-1B-LBD6	TraesCS1B02G131900	1847	711	236	24.6616	8.73	-0.13136	2	Nuclear
Ta-1B-LBD7	TraesCS1B02G235700	1028	570	189	20.4461	7.07	-0.18519	1	Nuclear
Ta-1B-LBD8	TraesCS1B02G270400	4076	792	263	27.0902	7.78	-0.03384	1	Nuclear
Ta-1D-LBD9	TraesCS1D02G110300	744	636	211	22.6064	8.38	-0.50237	1	Nuclear
Ta-1D-LBD10	TraesCS1D02G113100	1930	708	235	24.6746	8.49	-0.18596	1	Nuclear
Ta-1D-LBD11	TraesCS1D02G224000	996	570	189	20.4742	7.07	-0.18836	1	Nuclear
Ta-1D-LBD12	TraesCS1D02G259900	4097	792	263	27.0611	7.78	-0.03726	1	Nuclear
Ta-2A-LBD13	TraesCS2A02G194500	1442	705	234	24.2473	8.41	0.004274	1	Nuclear
Ta-2A-LBD14	TraesCS2A02G271300	1415	924	307	33.541	5.97	-0.64691	1	Nuclear
Ta-2B-LBD15	TraesCS2B02G212400	1205	714	237	24.4175	8.41	0.002954	1	Nuclear
Ta-2B-LBD16	TraesCS2B02G289800	1303	909	302	32.9763	6.29	-0.63576	1	Nuclear
Ta-2D-LBD17	TraesCS2D02G008400	1174	648	215	22.0328	6.61	0.058139	1	Nuclear
Ta-2D-LBD18	TraesCS2D02G193400	1336	732	243	25.7559	12.11	-0.61276	2	Nuclear
Ta-2D-LBD19	TraesCS2D02G270100	1619	912	303	33.0934	5.78	-0.62277	1	Nuclear
Ta-3A-LBD20	TraesCS3A02G093200	1898	774	257	26.492	7.72	0.02179	1	Nuclear

Gene name	EnsemblPlants ID	Gene length (bp)	ORF length (bp)	Deduced Protein				Splice Variants	Subcellular location
				Size (aa)	MW (KDa)	pI	GRAVY		
Ta-3A-LBD21	TraesCS3A02G170500	1200	723	240	25.0127	9.88	-0.54125	1	Nuclear
Ta-3A-LBD22	TraesCS3A02G205500	764	765	254	27.5746	7.09	-0.57126	1	Nuclear
Ta-3A-LBD23	TraesCS3A02G295100	1007	696	231	24.6465	7.07	-0.3316	1	Nuclear
Ta-3A-LBD24	TraesCS3A02G346300	1516	753	250	26.3556	6.51	-0.0492	1	Chloroplast
Ta-3A-LBD25	TraesCS3A02G402300	2718	780	259	26.5147	8.12	-0.02162	1	Nuclear
Ta-3A-LBD26	TraesCS3A02G492000	1346	1146	381	41.3431	4.57	-0.61076	1	Nuclear
Ta-3B-LBD27	TraesCS3B02G106900	1375	888	295	30.8182	6.42	-0.24644	1	Chloroplast
Ta-3B-LBD28	TraesCS3B02G108500	2073	774	257	26.49	7.72	0.046303	1	Nuclear
Ta-3B-LBD29	TraesCS3B02G196100	1152	747	248	25.7215	9.88	-0.56532	1	Nuclear
Ta-3B-LBD30	TraesCS3B02G378100	1559	879	292	30.6535	6.34	0.024657	1	Chloroplast
Ta-3B-LBD31	TraesCS3B02G435700	2926	780	259	26.5588	8.12	-0.00888	1	Nuclear
Ta-3B-LBD32	TraesCS3B02G553000	1455	1155	384	41.5733	4.93	-0.62708	1	Nuclear
Ta-3D-LBD33	TraesCS3D02G091700	1349	894	297	30.9474	7.07	-0.22054	1	Nuclear
Ta-3D-LBD34	TraesCS3D02G093500	2246	774	257	26.46	7.72	0.04786	1	Chloroplast
Ta-3D-LBD35	TraesCS3D02G308000	1127	741	246	26.4426	8	-0.32195	1	Nuclear
Ta-3D-LBD36	TraesCS3D02G340000	1516	747	248	26.0512	6.23	-0.02339	1	Chloroplast
Ta-3D-LBD37	TraesCS3D02G397200	3190	780	259	26.5377	8.12	-0.02317	1	Nuclear
Ta-3D-LBD38	TraesCS3D02G498100	1359	1155	384	41.8898	4.74	-0.61953	1	Nuclear
Ta-4A-LBD39	TraesCS4A02G067300	2149	801	266	27.3337	7.85	-0.22481	1	Nuclear
Ta-4A-LBD40	TraesCS4A02G107300	963	672	223	23.9169	6.88	-0.21794	1	Nuclear
Ta-4A-LBD41	TraesCS4A02G235100	1920	867	288	31.3771	7.36	-0.58854	1	Nuclear

Gene name	EnsemblPlants ID	Gene length (bp)	ORF length (bp)	Deduced Protein				Splice Variants	Subcellular location
				Size (aa)	MW (KDa)	pI	GRAVY		
Ta-4A-LBD42	TraesCS4A02G236200	2184	702	233	24.0108	7.56	-0.0588	1	Extracellular
Ta-4A-LBD43	TraesCS4A02G297500	3326	522	173	18.4588	8.14	-0.1659	1	Nuclear
Ta-4A-LBD44	TraesCS4A02G312500	993	660	219	24.1458	5.96	-0.43425	1	Nuclear
Ta-4A-LBD45	TraesCS4A02G415300	923	558	185	19.6217	6.98	-0.27838	1	Nuclear
Ta-4A-LBD46	TraesCS4A02G415400	1296	870	289	30.2694	6.74	-0.25813	1	Nuclear
Ta-4B-LBD47	TraesCS4B02G001200	1110	720	239	26.3001	5.97	-0.42134	1	Nuclear
Ta-4B-LBD48	TraesCS4B02G016200	2197	528	175	18.658	8.38	-0.196	1	Nuclear
Ta-4B-LBD49	TraesCS4B02G078800	1904	693	230	23.6684	7.57	-0.04957	1	Extracellular
Ta-4B-LBD50	TraesCS4B02G079900	4020	855	284	30.8945	7.34	-0.58099	1	Nuclear
Ta-4B-LBD51	TraesCS4B02G197100	1167	672	223	24.021	5.64	-0.19776	1	Nuclear
Ta-4B-LBD52	TraesCS4B02G224600	2045	789	262	27.1386	7.86	-0.1813	1	Nuclear
Ta-4B-LBD53	TraesCS4B02G316100	741	564	187	19.7849	6.7	-0.21177	1	Nuclear
Ta-4B-LBD54	TraesCS4B02G316200	1319	867	288	30.3994	7.29	-0.38854	1	Nuclear
Ta-4B-LBD55	TraesCS4B02G346500	578	507	168	18.3305	5.52	-0.42738	1	Nuclear
Ta-4B-LBD56	TraesCS4B02G361100	1179	882	293	30.5339	7.77	-0.19898	1	Chloroplast
Ta-4B-LBD57	TraesCS4B02G361200	1155	834	277	28.6916	7.79	-0.23357	1	Chloroplast
Ta-4D-LBD58	TraesCS4D02G001700	1042	654	217	23.8554	6.27	-0.44608	1	Nuclear
Ta-4D-LBD59	TraesCS4D02G014600	2210	885	294	31.8152	8.94	-0.23027	1	Nuclear
Ta-4D-LBD60	TraesCS4D02G077600	1454	693	230	23.7696	7.56	-0.03783	1	Extracellular
Ta-4D-LBD61	TraesCS4D02G078800	3868	855	284	30.8425	7.36	-0.55669	1	Nuclear
Ta-4D-LBD62	TraesCS4D02G197400	1112	672	223	23.9208	5.64	-0.21525	1	Nuclear

Gene name	EnsemblPlants ID	Gene length (bp)	ORF length (bp)	Deduced Protein				Splice Variants	Subcellular location
				Size (aa)	MW (KDa)	pI	GRAVY		
Ta-4D-LBD63	TraesCS4D02G225200	2152	783	260	26.9293	7.85	-0.20692	1	Nuclear
Ta-4D-LBD64	TraesCS4D02G312700	879	564	187	19.7428	6.7	-0.2246	1	Nuclear
Ta-4D-LBD65	TraesCS4D02G312800	1393	861	286	30.3505	7.21	-0.35699	1	Nuclear
Ta-4D-LBD66	TraesCS4D02G341500	621	522	173	19.1583	5.51	-0.49422	1	Nuclear
Ta-4D-LBD67	TraesCS4D02G354100	1203	897	298	31.1127	6.92	-0.17282	1	Chloroplast
Ta-4D-LBD68	TraesCS4D02G354200	1107	828	275	28.8449	7.77	-0.29891	1	Chloroplast
Ta-5A-LBD69	TraesCS5A02G152200	1695	534	177	19.7023	7.37	-0.29831	1	Nuclear
Ta-5A-LBD70	TraesCS5A02G191900	1574	1092	363	38.5621	5.72	-0.38843	1	Nuclear
Ta-5A-LBD71	TraesCS5A02G284000	652	567	188	20.1495	5.3	-0.42447	1	Nuclear
Ta-5A-LBD72	TraesCS5A02G515300	846	540	179	19.719	4.89	-0.42123	1	Nuclear
Ta-5A-LBD73	TraesCS5A02G529300	1168	870	289	30.2026	7.77	-0.23114	1	Chloroplast
Ta-5A-LBD74	TraesCS5A02G529400	806	807	268	27.9669	7.09	-0.23769	1	Chloroplast
Ta-5B-LBD75	TraesCS5B02G150800	1044	534	177	19.7163	7.37	-0.29774	1	Nuclear
Ta-5B-LBD76	TraesCS5B02G191200	1415	1140	379	40.1759	5.3	-0.39077	1	Nuclear
Ta-5B-LBD77	TraesCS5B02G282700	658	573	190	20.7041	4.94	-0.50684	1	Nuclear
Ta-5D-LBD78	TraesCS5D02G157400	1151	534	177	19.7183	7.37	-0.32542	1	Nuclear
Ta-5D-LBD79	TraesCS5D02G199000	1593	1155	384	40.6975	5.44	-0.36406	1	Nuclear
Ta-5D-LBD80	TraesCS5D02G291300	655	570	189	20.2766	5.28	-0.40106	1	Nuclear
Ta-6A-LBD81	TraesCS6A02G053700	1078	714	237	26.0791	6.49	-0.31814	1	Nuclear
Ta-6A-LBD82	TraesCS6A02G398400	1043	621	206	21.2541	7.99	0.151942	1	Nuclear
Ta-6B-LBD83	TraesCS6B02G072200	978	714	237	26.105	6.42	-0.36245	1	Nuclear

Gene name	EnsemblPlants ID	Gene length (bp)	ORF length (bp)	Deduced Protein				Splice Variants	Subcellular location
				Size (aa)	MW (KDa)	pI	GRAVY		
Ta-6B-LBD84	TraesCS6B02G438700	1033	636	211	21.7136	8	0.120379	1	Nuclear
Ta-6D-LBD85	TraesCS6D02G382600	1109	630	209	21.4853	7.99	0.190909	1	Nuclear
Ta-7A-LBD86	TraesCS7A02G066100	350	351	116	12.542	4.73	-0.17759	1	Nuclear
Ta-7A-LBD87	TraesCS7A02G228900	1559	690	229	24.6203	6.65	-0.38079	1	Nuclear
Ta-7B-LBD88	TraesCS7B02G195100	1394	690	229	24.5704	6.49	-0.27773	1	Nuclear
Ta-7D-LBD89	TraesCS7D02G229900	1392	690	229	24.5012	6.66	-0.35808	1	Nuclear
Ta-U-LBD90	TraesCSU02G132900	*	708	235	26.0411	6.73	0.102128	1	Nuclear

As reported in rice and Arabidopsis, the LBD gene family could be divided into two major groups, class I and class II according to the specific conserved domain [3, 8]. We further investigated the conserved signature motif in these TaLBDs. Results showed that all the putative wheat LBDs possessed conserved cysteine rich C-motif (CX₂CX₆CX₃C) signature motifs (Figure S1). Among them, 73 TaLBD genes shared cysteine rich C-motif, GAS-block and leucine zipper-like structure basically, which could categorized into class I, while the remaining 17 TaLBD genes had a reminiscent of these motifs, belonging to the class II. Furthermore, the length of putative TaLBD proteins ranged from 116 to 384 amino acids, with the putative molecular weight (Mw) ranging from 12.5 to 41.9 KDa and theoretical isoelectric point (PI) ranging from 4.57 to 12.11, respectively. Meanwhile, the subcellular localization prediction found that most of the TaLBDs (76) localized in nuclear, despite of 11 in chloroplast and 3 in extracellular (Table 2)

Phylogenetic relationship, conserved motif and gene structure analysis

To evaluate the evolutionary relationships of TaLBDs, phylogenetic analysis was further conducted based on multiple protein sequence alignment of all of the TaLBD together with rice and Arabidopsis LBD proteins. Phylogenetic tree clustered these LBD genes into two major clades (class I, class II), which was consistent with the categorization depending on their domain composition as described above (Figure 2). The class I clades could further divide into 8 groups (IA-IH), and class II divided into two group according to the phylogenetic relationship. It is obvious that the phylogenetic tree was monophyletic and the TaLBDs clustered together with their orthologous counterpart in rice and Arabidopsis in each subgroup, respectively (Figure 3A). Furthermore, a clear paralogous expansion by gene tandem duplication was occurred in wheat LBD gene family and each orthologous loci had two or three homoecologous copies, indicating that the allohexaploidization together with tandem duplication contributed to the expansion of wheat LBD gene family [21, 22]. However, compared to rice and Arabidopsis, no significant paralogous expansion was happened on wheat LBD family. Interestingly, the IE subgroup only contained AtLBDs without rice and wheat orthologous genes, suggesting this subgroup might be lost in rice and wheat.

Furthermore, all of the deduced TaLBD proteins were submitted to MEME web server and 15 conserved motifs were identified. Result showed that motif 1 and motif 3 were most conserved among 15 motifs which inserted in LBD domain and shared by all of LBD proteins (Figure 3B). Further analysis found that the class I and class II LBD proteins possessed the specific motif composition. All of class I proteins harbored motif 1, motif 2 and motif 3, however, class II proteins contained motif 1, motif 3 and motif 5 (Figure 3B and Figure S2). Interestingly, we found that motif 5 was uniquely in class II and could be used as the

reference to divide the two class. It is noteworthy that proteins from the same subgroup are likely to share one or more motifs that outside the LBD domain region. For example, motif 9 and 10 were shared by 3 members in the 1E subfamily while motif 13 was specifically shared by 9 members in the class II subfamily. These results confirmed our phylogeny-based groupings.

Gene structure can provide important clues for analyzing evolution feature and phylogenetic relationships of gene family [23]. Therefore, we analyzed the intron and exon structure of TaLBD genes. Result showed that, most of the genes were intron-less and the number of exons ranged from 1 to 4 (Figure 3C). Totally, 25 TaLBD genes had no exon, 42 had one exon and 23 genes had multiple exons. The subfamily A has more sophisticated structure than other subfamily because of the various number of intron. Furthermore, the members within the same subfamily shared the similar intron-exon structure and gene size, which supported their close evolutionary relationship and the stated classification of subfamilies. Through comparing the LBD gene structure between wheat with barley and Arabidopsis [3, 6], we found that they shared similar intron/exon structures, proving that LBD gene family was rather conserved. Based on phylogenetic tree and gene structure, the homoeologous groups of these 90 TaLBDs were identified and a total of 22 group with each containing A, B and D homoeologous copies were identified in wheat LBD gene family, and 8 gene pairs with a copy on only 2 of the 3 homoeologous chromosomes were also found, while the remaining 8 genes were not found homologous copy in wheat genome. This result demonstrated homoeologous copy loss event might occurred in LBD gene family during wheat polyploidization. The specific retention and dispersion patterns of TaLBDs in homoeologous chromosomes provided the important insight on the mechanism of wheat chromosome evolution and interaction [24]. Interestingly, although the chromosome location of Ta-U-LBD90 was unknown, its location could be deduced to on 6D based on the homoeologous grouping.

Cis-elements and miRNA targets analysis

The Cis-elements are the important regulatory factor that involved in the transcriptional regulation of genes during plant growth and development as well as stress response [25]. The 1.5kb genomic sequences upstream from 5'-UTR of the 90 TaLBD genes were extracted from wheat genome sequences and used to identify cis-elements. A total of 36 cis-elements were identified belonging to the different functional categories (Table S1 and S2). Obviously, a lot of phytohormone-responsive elements were widely found in the promoters of the wheat LBD genes. In detail, 79 TaLBDs contained ABA-responsive element (ABRE) in their promoters, suggesting these TaLBD genes were probably induced by ABA. And 62 TaLBDs contained MeJA-responsive element (TGACG-motif and CGTCA-motif) (Figure S2). In addition, three gibberellin-responsive elements, including GARE-motif, P-box motif and TATC-box were also found, which was consistent with previous study that LBD genes may involve in signaling transduction such as gibberellin pathway [10]. Furthermore, it is also shown that TaLBD promoters were highly enriched in light related responsive elements, for example, sp1 and G-box. We found that 68 out 90 TaLBDs had the G-box motif. According to previous study, light-responsive element combined with other cis-element such as ABA-related, may provide a link to the light and stress mediated signaling in plant defense responses [26].

The microRNAs (miRNAs) are a class of small non-coding regulatory RNAs that involved in gene expression through guiding target mRNA cleavage or translational inhibition [27]. Recently, some miRNA was found to involve in response to abiotic stress in different plants through targeting on transcription factors [28-30]. In order to investigate the potential regulatory association between LBD transcription factor and miRNA, the putative miRNA-TaLBDs relationship were predicted. Results showed that a total of 30 TaLBDs were predicted to be targeted by 21 miRNAs (Figure 4), of which tae-miR5384-3p could targeted on 11 TaLBDs, tae-miR444, tae-miR9677b and tae-miR9659-3P could target on 3 TaLBDs. Most of TaLBDs were targeted by one miRNA, but Ta-4A-LBD39, Ta-3A-LBD25 and Ta-3B-LBD31 as well as Ta-3D-LBD37 were targeted by 3 miRNAs. What's more, Ta-3A-LBD25, Ta-3B-LBD31 and Ta-3D-LBD37 belonged to the same homoeologous group but targeted by 3 different miRNAs. The specific miRNA-LBD pairs provide the crucial insight for precisely manipulating these LBD genes' function through miRNA-based method. Although miRNA inhibition mostly involved the transcript cleavage, some genes such as Ta-4B-LBD55 was predicted to be inhibited by translation. In addition, we found that the homoeologous group genes Ta-6A-LBD82, Ta-6B-LBD84 and Ta-6D-LBD85 were regulated by tae-miR444a and tae-miR444b. According to previous study,

miR444 family played important role in root development, tiller formation and stress response[31], indicating that they might involve in wheat development and stress tolerance through miRNA regulation. The miRNAs-LBD complex identified in this study would be useful in interpreting the post-transcriptional control of gene expression during various stress-induced physiological and cellular processes in wheat as well as other cereal crops.

Regulatory network between LBDs and other genes in wheat

LBDs transcription factor superfamily plays the crucial role in regulating the complex processes of plant growth, development and stresses response through interacting with other functional genes [10, 13]. To get the preliminary information about the interaction relationship between LBD and other genes in wheat, we constructed the interaction network they involved in based on the orthology-based prediction followed the network in Arabidopsis (Figure 5). Results showed that these TaLBDs widely interacted with the functional genes associated with organ development and morphogenesis (KNAT, EXPA17 and PRR1) and also interacted with other transcription factors to form signal transduction cascades (ARF19, NAC075 and WOX). Among them, Ta-4A-LBD40 was found to interact with KAN and KAN2 gene, which has been reported to involve in the molecular mechanism of lateral axis-dependent development of lateral organs in seed plants [32], suggesting that Ta-4A-LBD40 might act on regulator in wheat lateral axis-dependent development. Previous study found that LBD18/ASL20 could up-regulated EXPA17 to promote lateral root formation through auxin signaling in Arabidopsis [33, 34]. Here, we found that Ta-3A-LBD21, the orthology of AtLBD18, could interacted with EXPA17, indicating that it might regulate root development in wheat. In conclusion, the co-expression network analysis of LBD genes provided the vital information for the better understanding LBD transduction pathways in wheat.

Expression profiles of TaLBD genes

The spatio-temporal expression specificity of genes will provide the helpful information to understand their function in growth and development [35]. In this study, the tissue-specific expression profiles of the 90TaLBD genes in different developmental organs (grain, leaf, root, shoot and spike) were investigated using RNA-Seq data based on the FPKM analysis (Figure 6). Based on the log10-transformed (FPKM + 1) values, we found that the expression levels of the TaLBD genes varied significantly in different tissues and showed obvious tissue specificity. In detail, a total of 72 TaLBD genes were detected to express in at least one of the tested tissues, while 18 showed no expression in all these tissues. . We deduced that during long time evolution, these genes may undergone functional differentiation and redundancy. Ta-1A-LBD2, Ta-6B-LBD84 and Ta-4B-LBD47 displayed specially high expression in root. Ta-5A-LBD73, Ta-4B-LBD56 and Ta-4D-LBD67 highly expressed in spikes, but little expression in other tissues, suggesting they might involve in seed development and mature. Ta-3A-LBD25, Ta-3D-LBD35 and Ta-4D-LBD61 showed specific expression in spike. Ta-3B-LBD28, Ta-4A-LBD46, Ta-4B-LBD54 specially expressed in stem with relative low level, and Ta-2B-LBD18 displayed high expression in leave but lower expression in other tissues. By comparison, there is much less genes showing specific expression in stem and leave and most of TaLBD genes were much more highly expressed in the root and grain. Furthermore, most of the homologous genes shared similar expression pattern among different tissues, nevertheless, we also found that some homologous genes showed different express pattern, such as Ta-4D-LBD61 showed special expression in spike, while Ta-4A-LBD41, Ta-4B-LBD50 did not detected any expression in spike.

Previous studies have showed that the LBD genes especially Class Ⅱ genes were generally considered to be also involved in stress tolerance [36, 37]. To gain insight into the expression and putative functions of LBD genes in response to stress, the expression patterns of TaLBDs in four abiotic (cold, heat, drought and salt) stresses were investigated using RNA-seq (Figure 7). Results revealed that most of the TaLBDs showed differential expression patterns under these abiotic stress, particularly under salt stress. Under salt stress, 54 TaLBDs were found to show differential expression at different time points. Ta-6B-LBD81, Ta-4B-LBD51 and Ta-U-LBD90 displayed high expression level at all 4 time points, while Ta-2B-LBD16, Ta-2D-LBD19 and Ta-1B-LBD5 showed specifically up-regulated expression at 6h. Ta-5A-LBD69, Ta-4B-LBD54, Ta-4D-LBD65 expressed

specifically at 24h after salt stress. Furthermore, the expression levels of Ta-1A-LBD1 and Ta-4D-LBD62 increased continually from 6h to 24h, while Ta-1A-LBD2, Ta-1D-LBD10 and Ta-4B-LBD47 possessed high expression at 6h, 12h and 24h and then declined to little expression at 48h. These results suggested that LBD widely involved in salt stress induction in wheat and the different members played the differential roles in regulating salt stress response. Under cold stress, Ta-4A-LBD40 and Ta-4D-LBD62 were induced to highly express at 4 while Ta-4B-LBD49 specially expressed at 23. In addition, Ta-2A-13, Ta-2B-LBD15 and , Ta-2D-LBD18 showed up-regulated expression under drought stress, while Ta-5A-LBD72, Ta-5D-LBD79, Ta-5B-LBD76 and Ta-3D-LBD37 showed up-regulated firstly by heat stress induction at 1h and then were detected to be down-regulated after expose to heat stress 6h..The spatio-temporal expression profiles of these TaLBDs provided the useful information to better understand the roles of TaLBD playing in the growth and development as well as stress tolerance.

Validation of the expression of wheat LBD genes using qRT-PCR

Based on the expression patterns, 12 TaLBDs belonging to 4 homoeologous groups with significantly differential expression based on RNA-seq analysis were selected to investigate their expression levels under salt stress conditions by qPCR analysis (Figure 8). The expression divergence among homoeologous genes was also analyzed. The group A (comprising of Ta-1A-LBD1, Ta-1B-LBD5 and Ta-2D-LBD17) and the group B (comprising Ta-2A-LBD14, Ta-2B-LBD16 and Ta-2D-LBD19) showed up-regulated expression under salt stress, while the group A showed highest expression at 6h and then decreased expression continually but group B showed highest expression at 24h after salt stress. Comparing to their A and D homoeologous genes, Ta-1B-LBD5 and Ta-2B-LBD16 showed the higher expression levels at all of 4 tested time point under salt stress (Figure 8A and 8B). At the same time, the 6 genes in group C (Ta-4A-LBD44, Ta-4B-LBD47 and Ta-4D-LBD58) and group D (Ta-6A-LBD81, Ta-6B-LBD83, Ta-U-LBD90) were induced to down-regulated under salt stress except for Ta-4A-LBD44 at 6h (Figure 8C and 8D). The homoeologous genes also showed differential expressions. For group C, Ta-4A-LBD44 had highest expression level at 6h and 12h while its B homoeologous copy Ta-4B-LBD47 showed highest expression level at 24h and 48h (Figure 8C). In group D, Ta-6B-LBD83 had a higher expression level compared to its homoeologous gene Ta-6A-LBD81 and Ta-U-LBD90 at all of the 4 time points (Figure 8D). These results demonstrated the overall expression trend of these genes obtained by qRT-PCR analysis was basically consistent with that of RNA-seq analysis. Furthermore, the expression levels of homoeologous genes showed significantly divergent, suggested that subfunctionalization has occurred in these homoeologous genes when responded to salt stress. Further studies on the underlying effect of subfunctionalization will contribute to better understand the function of LBD genes and the mechanism of salt tolerance in wheat.

Genetic diversity of wheat LBD genes

To obtain the information on genetic variations of wheat LBD genes, we further investigated their genetic diversities using the available wheat resequencing data [38]. A total of 404 (A:183, B:172 and D:49) and 550 (A:231, B: 218 and D: 101) SNPs were found in LBD genes of wheat landrace and variety populations, respectively (Table S3). The average genetic diversity (Pi value) of landrace population was 0.889E-04, compared to that of variety population with 0.886E-04 (Figure 9). No significant genetic variation was observed between them, suggesting no severe bottleneck was occurred on LBD gene family during wheat improvement. Furthermore, at subgenome level, the average genetic diversity of A, B and D subgenome in landrace population was 1.31E-04, 1.15E-04 and 2.08E-05 respectively, while in variety population was 1.06 E-04, 1.31E-04 and 2.87E-05 (Table S4). The D subgenomes of varieties showed higher diversity than that of the landraces, which was consistent with the previous study using the whole genome resequencing data [38]. It demonstrated that during modern wheat breeding processes, introgressive hybridization with its wild D relatives had the genetic effect on LBD gene family to enrich its diversity [39]. At the same time, the B subgenomes of varieties also had higher diversity than that of the landraces, which was not consistent with the general finding landrace with higher diversity on B subgenome [38-40]. It suggested that some specific alien introgression events might occur in the wheat LBD genes on B subgenome. Further association the variations with the agronomic traits will not only contribute to the function of TaLBDs and also to better understand the evolutionary mechanism of this family.

Conclusion

This study systematically identified and characterized the LBD gene family in wheat. Using the latest available wheat genome information, a total of 90 putative TaLBD genes were obtained, which could be classified into two subfamilies based on the conserved motif signatures and phylogenetic relationship. Furthermore, the gene structure and conserved protein domain analysis supported the prediction and the same subfamily showed similar gene structures. The interaction network and miRNA-TaLBDs pairs provided the useful clues for revealing LBD mediated regulation pathway. The tissue-specific or stress-responsive TaLBDs were identified based on RNA-seq data, of which 12 were validated by QPCR, proving subfunctionalization of homoeologous genes has occurred in wheat. Finally, the asymmetry genetic variation and diversity of TaLBDs was also observed at subgenome level. This study not only provides the candidates for further functional analysis of the TaLBDs, but also contribute to better understanding the regulatory mechanism of LBD genes involving in regulating growth and development as well as stress tolerance in wheat.

Methods

Databases search and sequences analysis

The reference sequence (release-49) of Chinese Spring genome and its annotated proteins was downloaded from Ensemble plants database (<http://plants.ensembl.org/index.html>). Then, two methods were adopted to identify putative wheat LBDs. Firstly, the LBD proteins of rice and Arabidopsis were retrieved and downloaded from RGAP (<http://rice.plantbiology.msu.edu/index.shtml>) and TAIR (<https://www.arabidopsis.org/>) database respectively, and then used as the queries to perform a BLASTP search against the local protein database of wheat (IWGSC_v1.1) with an e-value of $1e-5$ and identity of 50 % as the threshold. Meantime, the LBD domain (PF03195) obtained from PFAM database (<http://pfam.xfam.org/>) was used as the query for Hidden Markov Model (HMM) search using HMMER 3.0 program (<http://hmmer.org/>). The protein sequences identified by both above methods were integrated and parsed by manual editing to remove the redundant. The remaining proteins were considered as the putative TaLBDs. The chromosome locations of these genes were obtained through searching TaLBD proteins against the wheat genome using TBLASTN tool as well as referred from the annotation information. The diagrams were visualized using the program Circos v0.67 [41]. Finally, the NCBI-CDD web server (<http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>) and SMART database (<http://smart.embl-heidelberg.de/webcite>) were used to further confirm the predicted TaLBD genes. The theoretical isoelectric point (PI) and molecular weight (MW) of the obtained proteins were calculated by ExPASy server (<http://www.expasy.org/>). The subcellular localization prediction of each gene was predicted using the cello software [42].

Phylogenetic relationship, gene structure analysis and conserved motif prediction

All of the identified TaLBD proteins together with LBD proteins of rice and Arabidopsis were used to perform a multiple sequence alignment using DNAMAN tools [43] with the default parameters. An un-rooted neighbor joining (NJ) tree with 1000 bootstrap replications was constructed using MEGA 8.0 [44] based on the full-length protein alignment. The exon-intron organizations and splicing phase of these predicted LBD genes were retrieved from the annotation file of wheat genome and then graphically displayed by the Gene Structure Display Server (<http://gsds.cbi.pku.edu.cn/>). Conserved motifs or domains were predicted using the MEME tool (<http://meme-suite.org/>), with the following parameters, maximum number of motifs set at 15 and optimum width of motifs set from 5 to 200 amino acids.

Promoter analysis and identification of miRNAs targets

The upstream 1.5 kb genomic DNA sequences of each predicted TaLBDs were extracted from the wheat genome, and then submitted to Plant Care database (<http://bioinformatics.psb.ugent.be/webtools/plantcare/>) to identify the putative cis-regulatory elements in the promoter regions. Furthermore, all the identified LBD transcripts were searched against the published wheat miRNAs in the miRBase using psRNATarget tool ([http:// plantgrn.noble.org/psRNATarget/](http://plantgrn.noble.org/psRNATarget/)) to predict the LBD targeted by miRNA.

Gene expression and regulatory network analysis

To study the expression profiles of TaLBDs, a total of 49 public available RNA-seq samples from 5 tissues (root, stem, leaf, spike, and grain) and 4 stressed conditions (cold, salt, heat, drought) were downloaded from WHEAT URGI (<https://urgi.versailles.inra.fr/files/RNASeqWheat/>) and NCBI Sequence Read Archive (SRA) database. The accession numbers and sample information were listed in Table S5. The FPKM value (fragments per kilobase of transcript per million fragments mapped) was calculated for each LBD gene by Hisat2 and Stringtie software [45] and the spatial-temporal expression patterns of them were obtained. The heatmap was drawn based on the log10-transformed (FPKM + 1) values through pheatmap package in R software.

To obtain the regulation relationship between LBD gene and other wheat genes, the interaction network which these putative TaLBDs involved in were investigated based on the orthologous genes between wheat and Arabidopsis using the STRING tool (<http://string-db.org/cgi/>) and AraNet V2 tool (<http://www.inetbio.org/aranet/>). The predicted interaction network was displayed through Cytoscape software.

Genetic variations of TaLBDs

The genetic diversity of LBD family in wheat landrace and variety populations was investigated based on the resequencing data [38]. VCF file was downloaded from Genome Variation Map (<https://bigd.big.ac.cn/gvm>) and the SNPs in TaLBD genes were extracted from VCF file. Then, the nucleotide diversity (π) in landrace and variety populations was calculated using vcftools (version 0.1.16).

qPCR validation of TaLBDs under salt stress

The seeds of cv. Chinese Spring were germinated in petri dishes and grown in water at a growth chamber with the controlled conditions (23 ± 1 °C, 16-h light/8-h dark cycle). The three-leaf seedlings were used for salt stress treatments. The healthy plants were incubated in 150 mM NaCl solution for 6h, 12h, and 24h with seedlings under normal condition used as the control. Leaves of all these samples were collected with three biological replications. Total RNA was isolated by Plant RNA Kit reagent (Omega Bio-Tek, USA) according to the manufacturer's instructions. A total of 12 TaLBDs belonging to 4 homoeologous groups were selected to investigate their expression levels under salt condition by qPCR analysis using the primers as listed in Table S6. qPCR were performed on the QuantStudio™ 7 Flex System (Thermo Fisher Scientific, USA) with SYBR® Premix Ex Taq™ II (TaKaRa, Dalian, China) with the thermal cycling condition was 95°C for 30 s followed by 40 cycles of 95°C for 3s, 60°C for 30 s. Three technological replications were applied and the expression level was calculated using the $2^{-\Delta\Delta CT}$ method.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

All of the datasets supporting the results of this article are included within the article and its Additional files. The phylogenetic data generated and/or analysed during the current study are available in the TreeBASE repository, <http://purl.org/phylo/treebase/phyloids/study/TB2:S27654>.

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

NX and SW conceived and designed the study. WZ and GY prepared the materials, performed the analysis and also drafted the manuscript. HF and SY contributed to plant material collection. PW and LP conducted the qRT-PCR analysis. NX revised the manuscript. All authors read and approved the final manuscript.

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Competent interest

All authors have declared that there no competent interest existed.

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Figures

Figure 1

Distribution of the identified 90 TaLBD genes across the seven chromosome groups and A(red), B(blue) and D(green) subgenomes of wheat. All wheat chromosomes are drawn to scale based on their actual physical lengths.

Figure 2

Phylogenetic analysis of LBD gene family in wheat. Tree was constructed by MEGA8.0 using neighbor-joining method with 1000 bootstraps. Purple, green, Orange colors represent LBD protein sequences from *Triticum aestivum* (Ta), *Oryza sativa* (Os), *Arabidopsis thaliana* (At) respectively. The branch length represents the magnitude of genetic change. Different clade colors represent subfamilies respectively.

Figure 3

Phylogenetic relationship(A), conserved motifs (B) and gene structure (C) of LBD genes in wheat. The conserved motifs analysis of the LBDs based on their phylogenetic relationship were identified using MEME software. The different colors represent the 15 identified motifs. The yellow and blue rectangles represent the CDSs and UTRs respectively, and the black lines represents introns. The lengths of the CDSs, UTRs and introns for each LBD gene are shown proportionally.

Figure 4

A schematic representation of the regulatory network relationships between the putative miRNAs and their targeted TaLBD genes. The miRNAs that regulate one TaLBD gene are shown in yellow circles, and miRNAs that regulate two TaLBDs are shown in orange circles as well as miRNAs that regulate more than two TaLBDs are shown in red circles. The miRNAs and TaLBDs linked by the blue line indicate a putative regulatory relationship.

Figure 5

Predicted protein-protein interaction networks of TaLBD proteins with other wheat proteins using STRING tool. The blue circles represent wheat LBD proteins, and the circles on the outside represent proteins that interact with TaLBDS. The two circles connected by the red line represent the interaction between the proteins.

Figure 6

The expression profiles of 90 TaLBDS in different tissues including grain, leaves, spike, root, and stem. The X axis represents the samples of different tissues. The Y axis represents the 90 different TaLBDS. The color scale represents Log2 expression values. The expression level is equal to the mean values and transform log 2 values.

Figure 7

The expression profiles of 90 TaLBDs under different stresses including drought, salinity, cold and heat stresses. The X axis represents the samples of diverse stresses. The Y axis represents the 90 different TaLBDs. The color scale represents Log2 expression values. The expression level is equal to the mean values and transform log 2 values.

Figure 8

The expressions of the 12 TaLBDs belonging to 4 homologous groups under salt stress was investigated using qRT-PCR method. The relative expression levels were calculated and compared. Error bars represent standard errors of three independent replicates.

Figure 9

The Pi values of 90 TaLBDs in wheat landrace and variety populations. The X axis represents different populations. The Y axis represents the Pi values.

Supplementary Files

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

- [SupplementaryFigures.docx](#)
- [SupplementaryTables.xlsx](#)