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Research paper

Genome-wide identification and characterization of the lateral organ boundaries domain gene family in *Brassica rapa* var. *rapa*

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ABSTRACT

The *Lateral Organ Boundaries Domain (LBD)* genes encode highly conserved plant-specific LOB domain proteins which regulate growth and development in various species. However, members of the *LBD* gene family have yet to be identified in *Brassica rapa* var. *rapa*. In the present study, fifty-nine *LBD* genes were identified and distributed on 10 chromosomes. The BrrLBD proteins are predicted to encode hydrophobic polypeptides between 118 and 394 amino acids in length and with molecular weights ranging from 13.31 to 44.24 kDa; the theoretical pI for these proteins varies from 4.83 to 9.68. There were 17 paralogous gene pairs in the BrrLBD family, suggesting that the amplification of the *BrrLBD* gene family involved large-scale gene duplication events. Members of the BrrLBD family were divided into 7 subclades (class I a to e, class II a and b). Analysis of gene structure and conserved domains revealed that most *BrrLBD* genes of the same subclade had similar gene structures and protein motifs. The expression profiles of 59 *BrrLBD* genes were determined through Quantitative Real-time fluorescent PCR (qRT-PCR). Most *BrrLBD* genes in the same subclade had similar gene expression profiles. However, the expression patterns of 7 genes differed from their duplicates, indicating that although the gene function of most BrrLBD genes has been conserved, some BrrLBD genes may have undergone evolutionary change.

Introduction

Genes of the Lateral Organ Boundaries Domain/ASYMMETRIC LEAVES2-like (LBD/AS2) protein family encode plant-specific transcription factors that share a highly conserved LOB domain (Iwakawa et al., 2002; Xu et al., 2016). The conserved lateral organ boundaries domain (LBD) consists of 100 amino acids and contains a conserved $CX_2CX_6CX_3C$ zinc finger-like block for DNA-binding activity, a leucine-zipper-like coiled-coil motif for protein–protein interaction and a GAS (Gly-Ala-Ser) block which may also play a role in DNA-binding (Lee et al., 2013; Majer and Hochholdinger, 2011; Shuai et al., 2002; Xu et al., 2016). The variable C-terminal region of the LBD proteins plays a critical role in controlling the expression of downstream target genes (Liu et al., 2005; Majer et al., 2012). LBD proteins have been divided into two clades (class I and class II) according to their structure. In various species, most members of class I have a complete LOB domain and all members of class II have an incomplete leucine-zipper-like coiled-coil motif (Cao et al., 2016; Lu et al., 2017; Majer et al., 2012; Wang et al., 2013; Yang et al., 2006; Yordanov et al., 2010). In barley, mulberry, rice, grape, *Arabidopsis*, maize and apple, the number of LBD gene family members varies from 24 to 58 (Cao et al., 2016; Guo et al., 2016; Luo et al., 2016; Shuai et al., 2002; Wang et al., 2013; Yang et al., 2006;

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Zhang et al., 2014). The spatio-temporal expression profiles of *LBD* genes vary, implying that during growth and development *LBD* proteins function at specific periods and in specific organs (Cao et al., 2016; Shuai et al., 2002; Wang et al., 2013).

Previous studies have demonstrated that LBD proteins play important roles in plant growth, development, signal transduction, and stress response development (Cabrera et al., 2014; Chen et al., 2012; Cortizo and Laufs, 2012; Fan et al., 2012; Feng et al., 2012; Hu et al., 2014; Kim et al., 2015; Lee et al., 2017; Liu et al., 2014; Mangeon et al., 2011; Oh et al., 2010; Okushima et al., 2007; Thatcher et al., 2012; Yordanov et al., 2010; Zhou et al., 2012). For example, Arabidopsis LBD proteins (AtLBD16, AtLBD18, AtLBD29 and AtLBD33) have been shown to act in the auxin-mediated signaling pathway that regulates lateral root development (Okushima et al., 2007; Xu et al., 2016). More recently, research has demonstrated that the dimerization in transcription factors *LBD16* and *LBD18* plays a crucial role for lateral root formation (Lee et al., 2017). Furthermore, transcription factors from class II *LBD* genes (*AtLBD37*, *AtLBD38* and *AtLBD39*) have been shown to repress the biosynthesis of anthocyanin and regulate the nitrogen metabolism (Rubin et al., 2009).

Turnip (*Brassica rapa* var. *rapa*) is an important vegetable crop in the Tibetan Plateau. However, no report on the turnip LBD gene family exists. The turnip genome has been sequenced and assembled (Lin et al., 2014), providing the basis for characterizing the LBD gene family in turnip.

In this study, 59 *BrrLBD* genes were identified in the turnip genome, their phylogenetic relationship, exon/intron structure, protein motifs and chromosome locations were analyzed. Furthermore, we characterized the expression profiles of *BrrLBD* genes in different tissues.

Material and methods

Plant material and growth conditions

B. rapa var. *rapa* 'KTRG-B36' from Lanping county, Nujiang City, Yunnan Province, China, was used. For root collection, seedlings were grown on a Whatman filter paper and watered with 1/2 MS medium for one week. For other tissues, plants were grown in a greenhouse (22 °C) under long-day conditions (16 h light/day). Leaves and shoot apical meristem were derived from 8-week-old plants; floral buds were derived from 10-week-old plants.

Identification of turnip LBD family genes

The whole genome sequence of *B. rapa* var. *rapa* (Lin et al., 2014) was downloaded from www.bioinformatics.nl/brassica/turnip. Forty-two *Arabidopsis LBD* genes were downloaded from TAIR 12.0 (www.arabidopsis.org). All 43 known *Arabidopsis* LBDs were used as queries to search the turnip genome database at an e-value cut-off of 1e-003 (Du et al., 2017; Lu et al., 2017). The pI (isoelectric points), MW (protein molecular weights) and GRAVY (Grand average of hydropathicity) were obtained by proteomic and sequence analysis tools of the ExPASy proteomics server (http://expasy.org/) (Artimo et al., 2012). Chromosomal locations were found in the turnip database by using a Perl-based program.

Gene structure and conserved motif analyses

BrrLBD gene exon and intron structures were identified with Gene Structure Display Server 2.0 (GSDS, http://gsds.cbi.pku.edu.cn/) (Hu et al., 2015). Conserved motifs of the BrrLBD proteins were analyzed using the Multiple Em for Motif Elucidation (MEME) program (http://meme-suite.org/index.html) (Bailey and Elkan, 1994) with the following parameters: (1) the optimum motif width was set from 6 to 200, and (2) the maximum number of motifs was set to identify 20 motifs.

Phylogenetic analysis

All phylogenetic trees of protein sequences were constructed with the neighbor-joining method using the MEGA 7.0 program. The reliability of the trees was tested by the bootstrapping method with 1000 replicates. All images of phylogenetic trees were drawn in MEGA 7.0 (Kumar et al., 2016).

Chromosome distribution, and divergence time estimation of BrrLBD genes

BrrLBD genes were mapped on chromosomes by confirming their detailed chromosomal positions supplied by the Turnip Genome Database. To determine their physical location, the starting positions of all *BrrLBD* genes on each chromosome were confirmed based on a local database of the complete sequence of the turnip genome by searching BLASTn. Segmental and tandem duplication regions were obtained from MCscanX. Analysis of synteny blocks in turnip genes was visualized using Circos (http://circos.ca/). Synonymous (Ks) and nonsynonymous (Ka) substitution rates were estimated using the codeml program of the PAML4 package (Yang, 2007). The divergence time (T) of turnip gene pairs was calculated using formula $T = Ks/2\lambda$, where λ represents the divergence rate of 1.5×10^{-8} for *Arabidopsis* (Gaut et al., 1996).

Quantitative RT-PCR

Total RNA was extracted from KTRG-B36 leaves and shoot apical meristem of 8-week-old plants, floral buds of 10-week-old plants using Eastep™ Universal RNA Extraction Kit (Promega, Shanghai, China). All quantitative real-time PCR primers were designed by the online NCBI program Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) applying the following parameters: 150–200 bp of polymerase chain reaction (PCR) product size, 58 °C–62 °C primer melting temperatures (Tm), and organism (taxid:3711) for *B. rapa*. At least one of two primers was derived from differential sequences of highly similar genes. qRT-PCR was performed in three technical repetitions with cDNAs synthesized from three biological replicates of KTRG-B36 different tissues. *BrrTUB2* was used as reference gene.

Subcellular localization and confocal laser scanning microscopy

The coding region sequence (CDS) of *BrrLBD* genes were subcloned into *pRI101-GFP* using the EZ-cloning method. *35S:GFP-BrrLBDs* were transferred into *Agrobacterium tumefaciens* EHA105 using electroporation. Positive transformants were prepared for leaf infiltration according to Du et al. (2017), and laser confocal microscopy was performed.

Results

Identification and annotation of LBD genes in turnips

The release of the complete turnip genome sequences allowed the genome-wide identification of turnip genes (Lin et al., 2014). In the present study, BLAST was used to search *BrrLBDs* in the turnip genome. The obtained sequences were further verified through the Hidden Markov Model of the SMART and pfam 31.0. A total of 59

LBD-like sequences were identified from the turnip genome, all of which possessed conserved LOB motifs. The *BrrLBD* genes were annotated following the nomenclature of *Arabidopsis thaliana* depending on protein sequence similarities (Fig. 1).

BrrLBD proteins were classified into seven subclades (subclades a to e within class I, and subclades a and b within class II). Among them, most *BrrLBDs* were clustered into class I. Thirteen *BrrLBDs* were classified as class Ia; 6 *BrrLBDs* fell into class Ib; 16 *BrrLBDs* were included in class Ic, 10 *BrrLBDs* were clustered into class Id, 4 *BrrLBDs* were classified as class Ie. Sequence analysis revealed that *AtLBD5*, *8*, *9*, *26*, *32, 34* and 36 had no orthologs in turnip. *AtLOB*, *AtLBD3*, *11*, *12*, *13*, *14*, *15*, *16, 19*, *24*, *25*, *29*, *30*, *37*, *38*, *39* and 41 had more than one ortholog in the turnip genome. The standard annotations of 59 *BrrLBD* genes are shown in Table 1; accession numbers for sequences were submitted to GenBank.

The BrrLBD proteins were predicted to encode polypeptides of 118–394 amino acids with molecular weights ranging from 13.31 to 44.24 kDa. The theoretical pI ranged from 4.83 to 9.68. All values of GRAVY were less than zero, indicating that all polypeptides of BrrLBD protein are hydrophilic.

BrrLBD genes in turnip are distributed in all 10 turnip chromosomes (Fig. 2). The maximum number of *BrrLBD* genes per chromosome was found for chromosome A04 with 11 *BrrLBD* genes. Ten genes were found at chromosome 9, eight genes each were located at chromosome 3 and 5, and six genes each were located at chromosome 2 and 6, respectively. Chromosome 8 and 10 contain the minimum numbers of *BrrLBD* genes, with only one each. Turnip chromosome 7 has five *LBD* genes, and the chromosome 1 contains three *BrrLBD* genes.

In this study, a total of 17 pairs of putative LBD paralog proteins were found in the segmental duplication blocks distributed in different chromosomes. These results demonstrate that the expansion of *BrrLBD* gene family in turnip was involved in large-scale segmental duplication events.

The divergence time (T) of seventeen pairs of BrrLBD paralog proteins was estimated by measuring the synonymous (Ks) and nonsynonymous (Ka) mutation rates (Table 2). The estimated divergence time (T) for *BrrLBD* duplicated gene pairs was approximately from 3.9 to 24.30 million years ago (MYA), with an average duplication time of ~10.4 MYA. The ω-values (Ka/Ks) for the BrrLBD paralogs were less than one. However, the duplicated pair BrrLBD19/BrrLBD19a ($\omega = 0.5624$) had a relatively large ω value, which implies that this pair may have evolved rapidly from the last common ancestor.

Gene structure and conserved motifs

To mine information concerning exon/intron organization of *BrrLBDs* and conserved motifs of BrrLBDs, a new neighbor-joining (NJ) tree was constructed using the protein sequences of BrrLBDs (Fig. 3A). Gene structure analysis revealed that most *BrrLBD* genes in the same subclade have similar gene structures (Fig. 3B). The exon numbers of *BrrLBD* gene family members vary from one to three. Fourteen *BrrLBD* genes have only one exon (24%), most members of *BrrLBD* gene family have two exons (71%), and only three *BrrLBD* genes have three exons. The MEME online tool was performed to predict the conserved motif composition of turnip LBD proteins (Fig. 3C). The numbers of BrrLBD protein motifs range from 2 to 8. The motifs of BrrLBDs were annotated using InterProScan. The conserved $CX_2CX_6CX_3C$ zinc finger-like domain sequence was detected in motif 2, which exists in various species in all known LBD proteins. Furthermore, the leucine zipper-like coiled-coil was found in motif 1 and 3, which exists only in majority of the class I LBD proteins (Majer and Hochholdinger, 2011; Shuai et al., 2002).

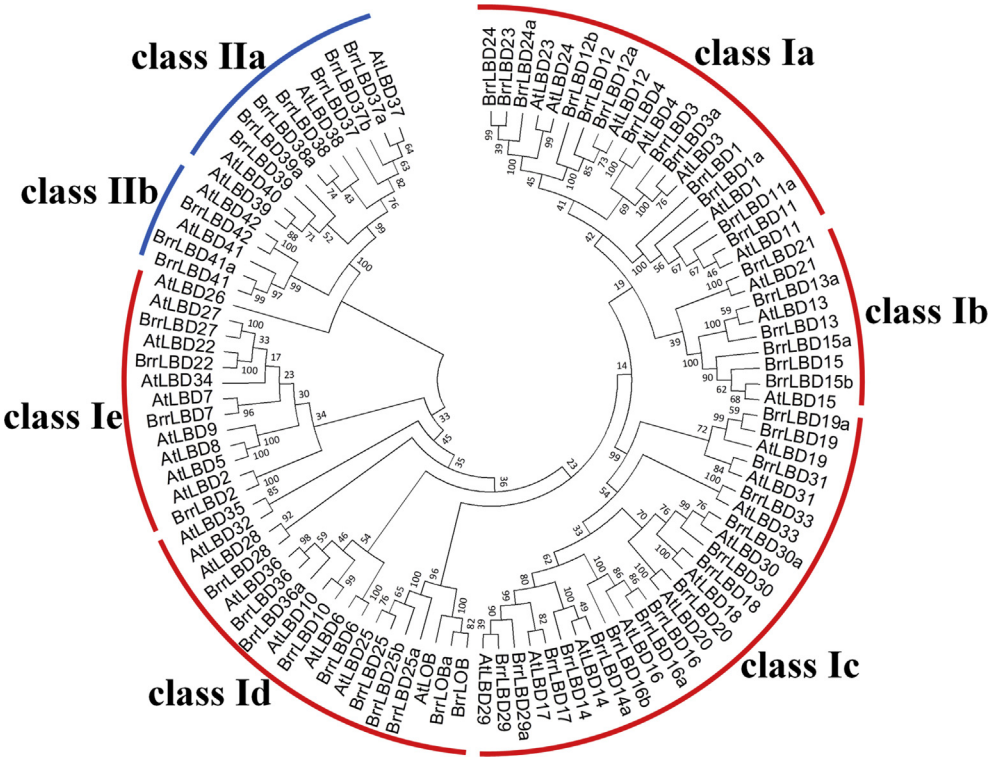

Fig. 1. Phylogenetic tree of LBD proteins from turnip and *Arabidopsis*. The evolutionary history of LBD proteins was inferred by using 59 BrrLBD protein and 43 AtLBD protein sequences to construct a Neighbor-Joining (NJ) cluster tree. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. Evolutionary analyses were conducted in MEGA7.

Table 1
Identification and characteristics of *LBD* genes in turnip.

Gene Name	Accession Number	CDS length (bp)	Protein size (aa)	MV(kD)	PI	GRAVY	Chr. Location
BrrLOB	MF953601	546	181	19.91	8.29	−0.442	Chr06:14089874..14090419
BrrLOBa	MF953602	531	176	19.27	8.56	−0.453	Chr09:3249530..3250060
BrrLBD1	MF953603	624	207	23.13	4.87	−0.267	Chr09:35566092..35567209
BrrLBD1a	MF953604	723	240	26.61	5.36	−0.215	Chr06:2669916..2671082
BrrLBD2	MF953605	594	197	22.49	9.68	−0.909	Chr08:20861229..20861822
BrrLBD3	MF953606	492	163	18.08	8.53	−0.187	Chr06:6348336..6349858
BrrLBD3a	MF953607	495	164	18.32	9.22	−0.200	Chr09:33285226..33286439
BrrLBD4	MF953608	519	172	18.67	7.61	−0.361	Chr07:5298495..5299499
BrrLBD6	MF953609	609	202	22.09	7.10	−0.375	Chr02:9571227..9571835
BrrLBD7	MF953610	558	185	21.10	8.30	−0.350	Chr02:12903480..12904037
BrrLBD10	MF953611	927	308	34.41	4.83	−0.640	Chr04:10470622..10471548
BrrLBD11	MF953612	699	232	25.53	4.92	−0.308	Chr04:12704785..12706200
BrrLBD11a	MF953613	696	231	25.40	4.90	−0.376	Chr03:11340842..11342322
BrrLBD12	MF953614	573	190	21.13	5.91	−0.354	Chr04:13390300..13391118
BrrLBD12a	MF953615	570	189	21.08	6.03	−0.320	Chr05:7475733..7476562
BrrLBD12b	MF953616	750	249	28.02	7.09	−0.368	Chr03:6995346..6998651
BrrLBD13	MF953617	807	268	29.28	8.98	−0.523	Chr05:7375325..7377428
BrrLBD13a	MF953618	801	266	28.87	9.04	−0.411	Chr04:13502689..13505830
BrrLBD14	MF953619	543	180	20.20	5.48	−0.216	Chr05:6926370..6927053
BrrLBD14a	MF953620	543	180	20.27	5.00	−0.248	Chr04:14150149..14150839
BrrLBD15	MF953621	681	226	24.79	7.69	−0.419	Chr04:17194524..17195851
BrrLBD15a	MF953622	672	223	24.46	8.73	−0.432	Chr05:830241..831235
BrrLBD15b	MF953623	579	192	20.67	8.23	−0.255	Chr03:9761557..9762501
BrrLBD16	MF953624	741	246	26.62	8.11	−0.468	Chr04:17743892..17745306
BrrLBD16a	MF953625	735	244	26.27	8.15	−0.403	Chr05:1354391..1355425
BrrLBD16b	MF953626	717	238	25.95	6.88	−0.372	Chr03:10167241..10169052
BrrLBD17	MF953627	600	199	22.59	6.23	−0.524	Chr04:17751076..17751838
BrrLBD18	MF953628	780	259	27.10	8.51	−0.224	Chr04:18509449..18511269
BrrLBD19	MF953629	567	188	20.74	6.29	−0.256	Chr05:2384192..2384849
BrrLBD19a	MF953630	507	168	18.60	7.68	−0.179	Chr04:18502804..18503399
BrrLBD20	MF953631	828	275	29.14	7.27	−0.300	Chr07:496401..497739
BrrLBD21	MF953632	618	205	22.77	8.28	−0.135	Chr05:21715551..21716168
BrrLBD22	MF953633	774	257	29.51	5.62	−0.755	Chr05:20593305..20594078
BrrLBD23	MF953634	357	118	13.31	9.17	−0.305	Chr03:16849276..16849721
BrrLBD24	MF953635	366	121	13.59	8.90	−0.267	Chr06:22637883..22638342
BrrLBD24a	MF953636	366	121	13.84	9.03	−0.347	Chr02:22273191..22273670
BrrLBD25	MF953637	396	131	14.58	7.60	−0.344	Chr09:1251553..1251948
BrrLBD25a	MF953638	399	132	14.56	7.58	−0.314	Chr06:22268459..22268857
BrrLBD25b	MF953639	399	132	14.68	6.89	−0.391	Chr02:21758022..21758420
BrrLBD27	MF953640	1041	346	39.36	5.15	−0.605	Chr06:10206064..10207198
BrrLBD28	MF953641	537	178	20.08	6.58	−0.553	Chr01:15707079..15707615
BrrLBD29	MF953642	678	225	24.80	6.21	−0.478	Chr09:28873054..28873853
BrrLBD29a	MF953643	660	219	24.32	6.28	−0.481	Chr04:1562942..1563718
BrrLBD30	MF953644	675	224	23.73	6.53	−0.164	Chr02:16017746..16020434
BrrLBD30a	MF953645	675	224	23.59	6.69	−0.056	Chr09:1062280..1064275
BrrLBD31	MF953646	669	222	24.23	6.16	−0.378	Chr09:1068061..1069446
BrrLBD33	MF953647	552	183	20.62	5.72	−0.183	Chr10:15419817..15420448
BrrLBD36	MF953648	1011	336	37.36	6.56	−0.645	Chr07:9891804..9892814
BrrLBD36a	MF953649	1185	394	44.24	5.34	−0.726	Chr09:4268544..4269728
BrrLBD37	MF953650	711	236	25.72	9.03	−0.333	Chr09:3854563..3855367
BrrLBD37a	MF953651	726	241	25.99	8.46	−0.337	Chr02:27506908..27507701
BrrLBD37b	MF953652	771	256	27.87	8.40	−0.398	Chr07:9589107..9589970
BrrLBD38	MF953653	738	245	26.70	6.82	−0.249	Chr09:24819925..24820971
BrrLBD38a	MF953654	711	236	25.99	6.34	−0.320	Chr03:21473516..21474319
BrrLBD39	MF953655	714	237	26.02	7.55	−0.312	Chr01:627066..627939
BrrLBD39a	MF953656	699	232	25.30	9.10	−0.119	Chr03:30663003..30663878
BrrLBD41	MF953657	795	264	28.18	8.77	−0.308	Chr01:25183419..25184297
BrrLBD41a	MF953658	780	259	27.76	8.51	−0.214	Chr03:14495479..14496347
BrrLBD42	MF953659	714	237	25.81	8.05	−0.355	Chr07:17868701..17869608

MW, molecular weight; PI, isoelectric point; GRAVY, Grand average of hydropathicity.

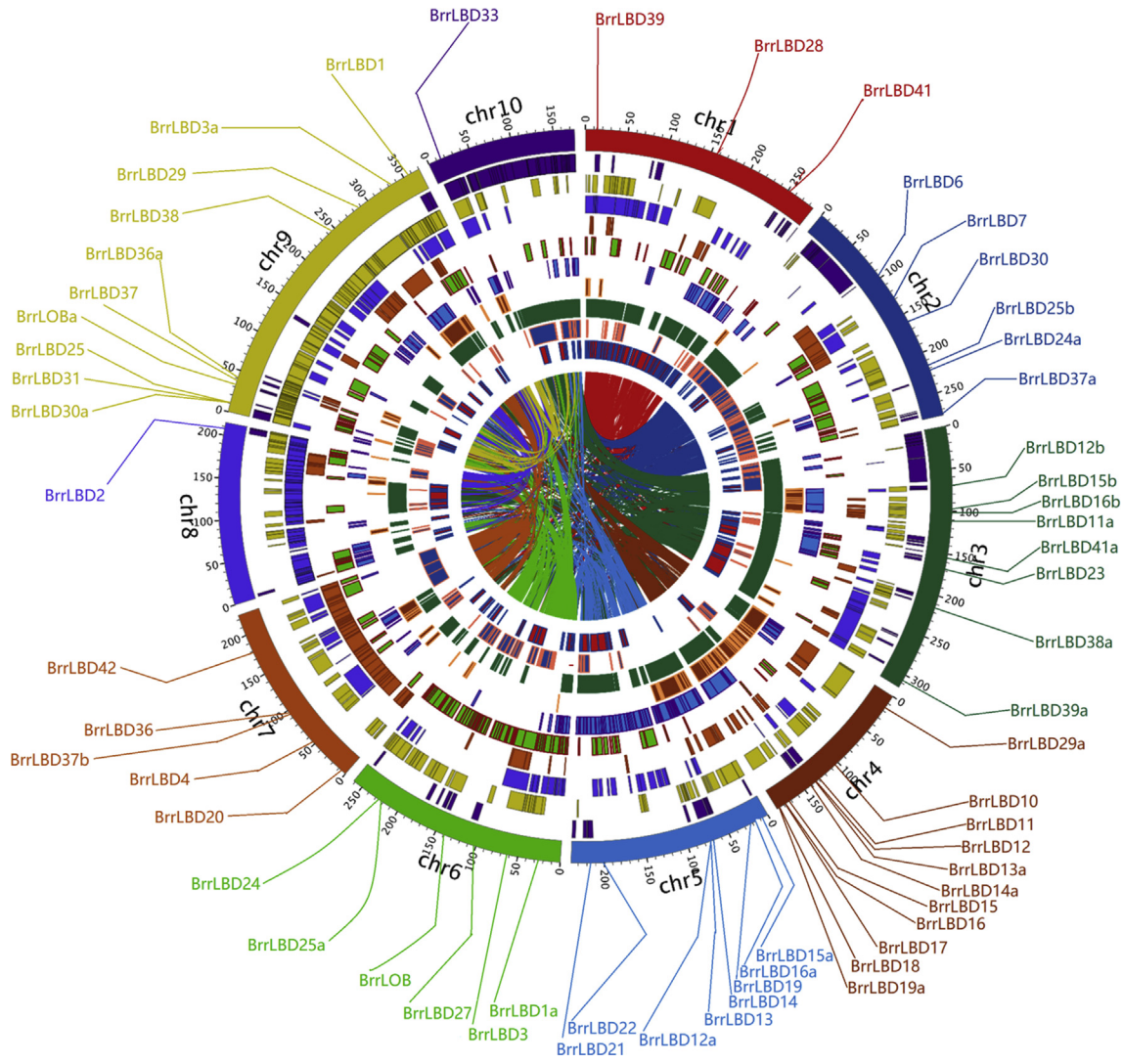

Fig. 2. Chromosome distribution of *BrrLBD* genes. Turnip Chromosomes are shown in different colors in the outer circle, where the numbers represent the chromosome length in 100 Kb. The *BrrLBD* genes are marked at the approximate positions with specific color lines on the circle. Filled blocks in different colors represent the syntenic relationships of *BrrLBD* genes.

Table 2
Estimated divergence time of paralogous pairs of *BrrLBD* genes.

Seq1	Seq2	Identity (%)	Ks	Ka	ω	T (MYA)
BrrLBD12	*BrrLBD12a*	0.9319	0.1616	0.0202	0.1250	5.3867
BrrLBD23	*BrrLBD24*	0.9008	0.1784	0.0536	0.3004	5.9467
BrrLBD3	*BrrLBD3a*	0.8354	0.3331	0.0585	0.1756	11.1033
BrrLBD11	*BrrLBD11a*	0.8397	0.3155	0.0245	0.0777	10.5167
BrrLOB	*BrrLOBa*	0.8798	0.2346	0.0205	0.0874	7.8200
BrrLBD25	*BrrLBD25a*	0.9167	0.4132	0.0334	0.0808	13.7733
BrrLBD13	*BrrLBD13a*	0.8963	0.3768	0.0102	0.0271	12.5600
BrrLBD15	*BrrLBD15b*	0.7832	0.3670	0.0288	0.0785	12.2333
BrrLBD19	*BrrLBD19a*	0.6974	0.1161	0.0653	0.5624	3.8700
BrrLBD30	*BrrLBD30a*	0.8705	0.3602	0.0332	0.0922	12.0067
BrrLBD16	*BrrLBD16a*	0.9024	0.2881	0.0081	0.0281	9.6033
BrrLBD14	*BrrLBD14a*	0.8556	0.2311	0.0535	0.2315	7.7033
BrrLBD29	*BrrLBD29a*	0.8451	0.2743	0.0323	0.1178	9.1433
BrrLBD41	*BrrLBD41a*	0.8491	0.2845	0.0041	0.0144	9.4833
BrrLBD39	*BrrLBD39a*	0.7890	0.2530	0.0484	0.1913	8.4333
BrrLBD38	*BrrLBD38a*	0.8462	0.7290	0.0165	0.0226	24.3000
BrrLBD37	*BrrLBD37a*	0.8272	0.3750	0.0206	0.0549	12.5000

Ks, synonymous substitution rates; Ka, Nonsynonymous substitution rates; MYA, million years ago.

Expression profiles of turnip LBD genes

The gene expression pattern is always relative to its function (Xu et al., 2015). To investigate possible functions of *BrrLBD* genes in turnip, quantitative real-time fluorescence PCR (qRT-PCR) was performed to examine expression levels of 59 *BrrLBD* genes in different turnip tissues (Fig. 4). Except for *BrrLBD30a*, which is expressed specifically in leaves, 14 of 49 class I *BrrLBD* genes are highly expressed in roots, 34 are strongly expressed in flower buds, and all class I *BrrLBD* genes are weakly expressed in leaves.

Tissue-specific expression patterns are similar for genes within each phylogenetic subclade. For instance, all BrrLBD genes in class Ib and class Ie, 12 of 16 BrrLBD genes in class Ic, and 7 of 10 class Id genes are expressed highly in flower-buds. However, class Ia genes showed more widespread but less tissue-specific expression patterns; for example, *BrrLBD1*, *12*, *12a*, *23*, *24* and *24a* are highly expressed in flower-buds, and *BrrLBD1a*, *3*, *3a*, *4*, *11*, *11a* and *12b* are expressed highly in roots. Seventeen duplicated BrrLBD pairs (*BrrLBD11/11a*, *BrrLBD12/12a*, *BrrLBD13/13a*, *BrrLBD14/14a*, *BrrLBD15/15b*, *BrrLBD23/24*, *BrrLBD25/25a*, *BrrLBD38/38a*, *BrrLBD39/39a* and *BrrLBD41/41a*) have similar tissue-specific expression

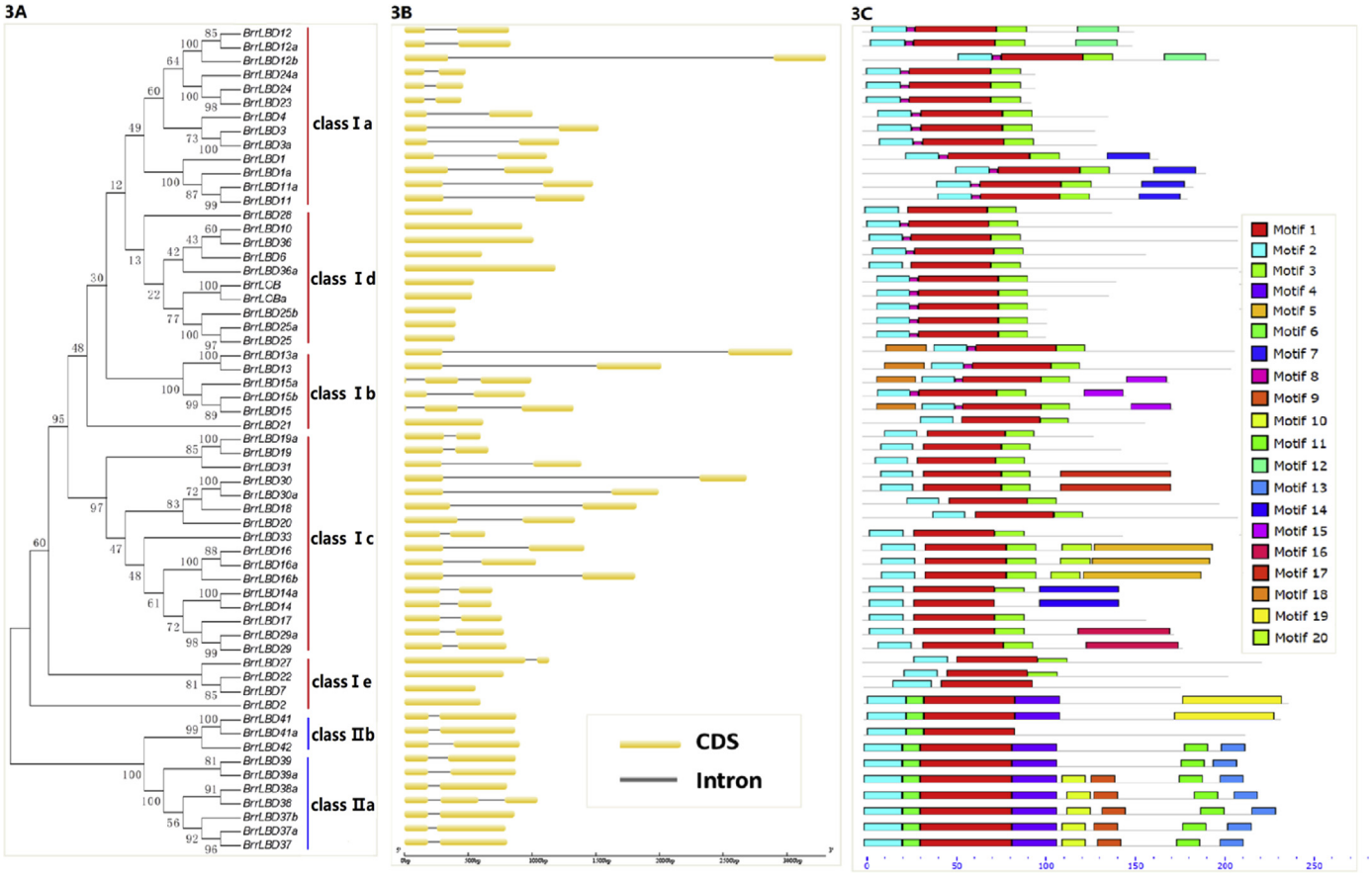

Fig. 3. The gene structure and motif composition of *BrrLBD* genes. (3A) Phylogenetic tree and classification of BrrLBD proteins was constructed by MEGA 7.0.21. (3B) Gene structure of *BrrLBD* genes. The yellow boxes and black lines represent the CDS and intron. (3C) Conserved motif compositions of BrrLBD proteins were identified using MEME. Each color represents a specific motif.

patterns, respectively. In contrast, *BrrLOB/BrrLOBa*, *BrrLBD16/16a*, *BrrLBD19/19a*, *BrrLBD29/29a*, *BrrLBD30/30a* and *BrrLBD37/37a* have different tissue expression characteristics. These results imply that most turnip LBD gene functions have been conserved, although some gene functions may have been altered through gene duplication events.

Subcellular localization

The known members of the *LBD* gene family function as transcription factors that regulate plant-specific process. The *GFP* gene was fused with *BrrLBDs* as a reporter. The GFP signals of *BrrLBD-GFP* were detected in the nucleus of tobacco leaf epidermal cells (Fig. 5), which suggests that *BrrLBD16*, *BrrLBD18*, *BrrLBD29*, and *BrrLBD33* might function as transcription factors.

Discussion

LBD proteins belong to a novel family of plant-specific transcription factors involved in the regulation of processes during plant development and growth in higher plants, such as pollen development, plant regeneration, pathogen response, secondary growth, photomorphogenesis, pulvinus identity and petiole development (Xu et al., 2016). Turnip underwent polyploidization events, such as γ triplication (135 MYA) and β (90–100 MYA) and α (24–40 MYA) duplications. Following these polyploidization events, the Brassica genome underwent chromosome reduction and rearrangement and numerous gene losses took place, resulting in highly complex gene families (Du et al., 2017). Phylogenetic analysis of LBD proteins in apple, grape, eucalyptus, rice and Arabidopsis have led to the division of LBD families into two classes (class I and class II) (Cao et al., 2016; Lu et al., 2017; Wang et al., 2013; Yang et al., 2006). In this study, 59 *BrrLBD* genes were identified from turnip genome sequences, which were distributed on 10 chromosomes. Some BrrLBD proteins were detected in the nucleus. An unrooted phylogenetic tree divided BrrLBDs into 7 subclades (subclade a to e within class I, and subclade a and b within class II). Alignment analysis revealed that in turnip genome 7 *AtLBD* genes had no orthologs and 17 *AtLBDs* had more than one ortholog. These results demonstrate that the expanded of *BrrLBD* gene family in turnip may have been involved in chromosomal duplication events, containing multiple segmental duplication, tandem duplications, transposition events and genome-wide duplications.

Structural analysis is another effective method to mine the evolutionary history of the gene duplication events and phylogenetic relationship within a gene family. In this investigation, most *BrrLBD* genes had a similar exon/intron structure within the same subclade, which is consistent with LBD genes in *A. thaliana*, *Oryza sativa*, *Eucalyptus grandis*, *Vitis vinifera* and *Malus domestica* (Cao et al., 2016; Lu et al., 2017; Shuai et al., 2002; Wang et al., 2013; Yang et al., 2006). Motif analysis demonstrated that most BrrLBD proteins possessed similar motif distributions within the same subclade. The characteristic LOB domain was discovered in BrrLBD proteins, which consists of a $CX_2CX_6CX_3C$ zinc finger-like motif required for the DNA-binding activity within motif 2, a Gly-Ala-Ser (GAS) Block within motif 1, and a leucine-zipper-like coiled-coil motif

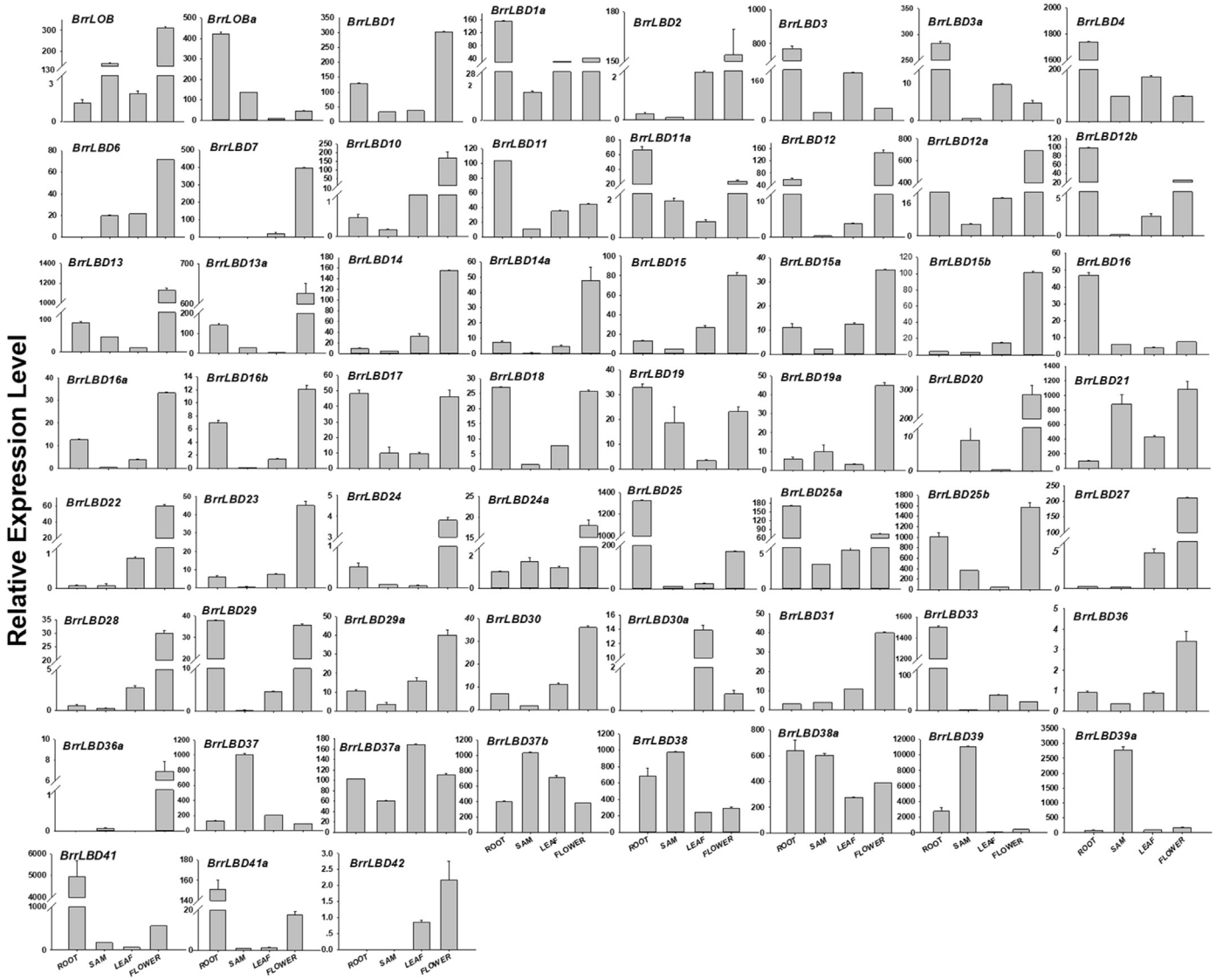

Fig. 4. Expression patterns of *BrrLBD* genes in root, shoot apical meristem (SAM), leaf, and flower buds (Flower). All data obtained from quantitative real-time fluorescence PCR. Constitutive β-tubulin gene served as an internal control. The error bar represents standard deviations for three technical duplications.

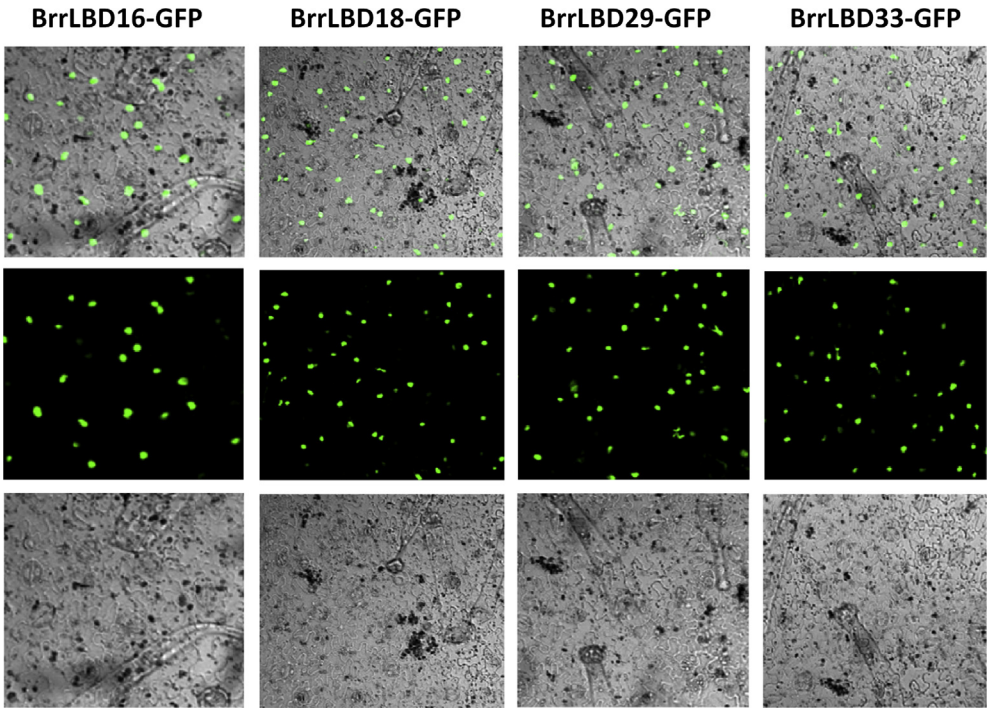

Fig. 5. Subcellular localization of *35S:BrrLBD-GFP* in *Nicotiana benthamiana* leaves. BrrLBD16-GFP, BrrLBD18-GFP, BrrLBD29-GFP and BrrLBD33-GFP were localized in the nucleus.

responsible for protein interactions within motif 1 and 3. The zinc finger-like motif and GAS block were discovered in all 59 BrrLBDs. However, the leucine-zipper-like motif was discovered in 47 of 49 BrrLBDs within class I and all remaining BrrLBD proteins contained an incomplete structure, which is similar to other species (Lu et al., 2017; Majer and Hochholdinger, 2011; Shuai et al., 2002; Xu et al., 2016). These results suggest that the gene structure and protein motif compositions of *LBD* genes are relatively conserved in different angiosperms.

In *Arabidopsis*, previous studies have demonstrated that *LBD* genes participate in plant growth and development as response factors and regulate nitrogen metabolism and anthocyanin synthesis as repressors (Kim and Lee, 2013; Mangeon et al., 2011; Okushima et al., 2007; Rubin et al., 2009). One important indicator of gene function is its expression profile. In this study, we detected the expression profiles of 59 *BrrLBD* genes in different tissues using qRT-PCR. These genes displayed distinct expression profiles in different turnip organs. Meanwhile, most genes exhibited similar expression profiles within each subclade. Most (10 of 17) duplicated BrrLBD protein pairs demonstrated similar tissue-specific expression patterns. In contrast, the remaining 7 gene duplicated pairs showed different tissue expression characteristics. These results imply that most gene functions have been conserved, although some gene functions may have changed during gene duplication events. Furthermore, the expression of *BrrLBD33* in roots suggests that this gene may play a role in the development of the lateral root in turnip.

Declaration of Competing Interest

The authors declare no conflict of interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pld.2019.11.004.

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