Genome-Wide Analysis of Soybean LATERAL ORGAN BOUNDARIES Domain-Containing Genes: A Functional Investigation of GmLBD12

Hui Yang, Guixia Shi, Hongyang Du, Hong Wang, Zhenzhen Zhang, Dezhou Hu, Jiao Wang, Fang Huang,* and Deyue Yu*

Abstract
Plant-specific LBD (LATERAL ORGAN BOUNDARIES Domain) genes play critical roles in various plant growth and development processes. However, the number and characteristics of LBD genes in soybean [Glycine max (L.) Merr.] remain unknown. Here, we identified 90 LBD homologous genes in the soybean genome that phylogenetically clustered into two classes (I and II). The majority of the GmLBD genes were evenly distributed across all 20 soybean chromosomes, and 77 (81.1%) of them were detected in segmental duplicated regions. Furthermore, the exon–intron organization and motif composition for each GmLBD were analyzed. A close phylogenetic relationship was identified between the soybean LBD genes and 41 previously reported genes of different plants in the same group, providing insights into their putative functions. Expression analysis indicated that more than half of the LBD genes were expressed, with the two gene classes showing differential tissue expression characteristics; in addition, they were differentially induced by biotic and abiotic stresses. To further explore the functions of LBD genes in soybean, GmLBD12 was selected for functional characterization. GmLBD12 was mainly localized to the nucleus and showed high expression in root and seed tissues. Overexpressing GmLBD12 in Arabidopsis thaliana (L.) Heynh resulted in increases in lateral root (LR) number and plant height. Quantitative real-time polymerase chain reaction (qRT-PCR) analysis demonstrated that GmLBD12 was induced by drought, salt, cold, indole acetic acid (IAA), abscisic acid (ABA), and salicylic acid SA treatments. This study provides the first comprehensive analysis of the soybean LBD gene family and a valuable foundation for future functional studies of GmLBD genes.

Core Ideas
- Ninety LBD homologous genes were identified in soybean genome.
- GmLBD genes had wide tissue expression patterns and were regulated by various stresses.
- GmLBD12 gene was functionally characterized in transgenic Arabidopsis.

The plant-specific LBD (LATERAL ORGAN BOUNDARIES domain) transcription factor family is of great significance for plant growth and development (Shuai et al., 2002; Iwakawa et al., 2007; Matsumura et al., 2009; Majer and Hochholdinger, 2011; Sun et al., 2012). The LBD proteins are defined by a characteristic LOB (LATERAL ORGAN BOUNDARIES) domain that includes a CX2CX6CX3C zinc-finger-like motif; an invariant glycine residue; and a leucine zipper-like coiled-coil motif, which may function in protein–protein interactions (Shuai et al., 2002; Matsumura et al., 2009; Majer and Hochholdinger, 2011). Based on the presence of the leucine zipper-like motif, the LBD gene family can

H. Yang, H. Du, H. Wang, D.Z. Hu, Z. Zhang, J. Wang, F. Huang, and D. Yu, National Key Laboratory of Crop Genetics and Germplasm Enhancement, Nanjing Agricultural University, Nanjing 210095, China; H. Wang, Current Address: Nanjing Decode Genomics Biotechnology Co., Ltd., Nanjing 210019, China; G. Shi, Institute of Industrial Crops, Henan Academy of Agricultural Sciences, Zhengzhou 450002, China. H. Yang and G. Shi contributed equally to this work. Received 17 Mar. 2016. Accepted 13 Oct. 2016. *Corresponding authors (dyu@njau.edu.cn; fhuang@njau.edu.cn).

Abbreviations: ABA, abscisic acid; cDNA, complementary DNA; CDS, coding sequences; DAF, days after flowering; EST, expressed sequence tag; GFP, green fluorescent protein; IAA, indole acetic acid; IR, lateral root; PEG, polyethylene glycol; qRT-PCR, quantitative real-time polymerase chain reaction; RNA-Seq, RNA sequencing; SA, salicylic acid; WT, wild-type.
be divided into two classes, Class I and Class II (Semiarti et al., 2001; Iwakawa et al., 2002; Shuai et al., 2002; Majer and Hochholdinger, 2011), and most members belong to Class I (Shuai et al., 2002; Yang et al., 2006b; Zhu et al., 2007; Wang et al., 2010, 2013; Zhang et al., 2014); in addition, rice (*Oryza sativa* L.) encodes a third class (Class III) that contains two LOB domains (Yang et al., 2006b). In *Arabidopsis*, rice, maize (*Zea mays* L.), sorghum (*Sorghum bicolor* (L.) Moench), apple (Malus domestica Borkh.), mulberry (Moraceae: Rosales), and poplar (Populus spp.), 43, 35, 44, 36, 58, 31, and 57 LBD gene family members have been identified, respectively (Shuai et al., 2002; Yang et al., 2006b; Zhu et al., 2007; Wang et al., 2010, 2013; Zhang et al., 2014; Luo et al., 2016). The presence of numerous LBD genes in plants may be the result of genome duplication and potentially indicates functional redundancy among the members of the LBD gene family (Lynch and Conery, 2000; Shuai et al., 2002; Simillion et al., 2002; Yang et al., 2006b; Zhu et al., 2007; Wang et al., 2010, 2013; Zhang et al., 2014). LOB proteins are essential regulators of the development of plant lateral organs, including the embryo, root, leaf, and inflorescence, and are responsive to hormones and stress, suggesting that these proteins function in diverse processes (Iwakawa et al., 2002; Shuai et al., 2002; Chalfun-Junior et al., 2005; Liu et al., 2005; Borghi et al., 2007; Thatcher et al., 2012b). Husbands et al. (2007) determined that the LBD of Class I LBD genes interacts with bHLH (basic helix-loop-helix) transcription factors, resulting in a reduced affinity of the LBD for the consensus DNA motif (Husbands et al., 2007). AS2/*AtLBD6* regulates the establishment of leaf polarity via its interactions with *AtAS1* and *ERECTA*; in addition, *AS2* represses the expression of Class I KNOX genes in the leaf (Semiarti et al., 2001; Lin et al., 2003; Xu et al., 2003; Qi et al., 2004; Belles-Boix et al., 2006; Iwakawa et al., 2007; Guo et al., 2008). The *ELP1* gene barrel clover (*Medicago truncatula* Gaertn.), which encodes a LOB domain transcription factor, regulates motor organ identity in leaflets, and *ELP1* orthologs such as *Apu* in pea (*Pisum sativum* L.) and *SLP* in *Lotus japonicus* (Regel) K. Larsen possess a conserved biological function (Chen et al., 2012). *AtLBD36, AtLBD18, AtLBD29*, and *AtLBD33* control *Arabidopsis* root development but exhibit different regulatory patterns: heterologous *AtLBD18/AtLBD33* dimers can bind the *E2Fa* promoter and drive its expression during LR initiation (Berckmans et al., 2011), whereas *AtLBD8*, in combination with *AtLBD16* and *AtLBD29*, directly regulates root development via an *AtARF7*- and *AtARF9*-dependent pathway (Okushima et al., 2005, 2007; Lee et al., 2009, 2013; Fan et al., 2012; Feng et al., 2012a,b; Goh et al., 2012). *Crl1/Ar1* and *Rtcs*, homologs of *AtLBD29*, regulate crown and LR formation in rice and maize, respectively (Inukai et al., 2005; Liu et al., 2005; Taramino et al., 2007). A chrysanthemum (*Chrysanthemum* spp.) transcription factor gene, designated *CmlLBD1*, responds to auxin and has been shown to participate in the process of adventitious root primordium formation (Zhu et al., 2016). *PtaLBD1* is involved in secondary growth in poplar (Yordanov et al., 2010). *Igl*, an ortholog of *Arabidopsis* AS2, regulates female gametophyte development as well as leaf morphology in maize (Evans, 2007). DH1, a LOB domain-like protein in rice, is required for glume formation, and *dh1* mutants exhibit a degenerated hull as well as naked pistils and stamens (Li et al., 2008). In *Arabidopsis*, the *SCP* gene, which encodes a microspore-specific LOB/AS2 domain protein, is essential for the correct timing and orientation of asymmetric microspore division during male gametophyte patterning (Oh et al., 2010). Overexpression of the apple gene *MdLBD11* in *Arabidopsis* leads to abnormal phenotypes, including upward curling leaves, delayed flowering, downward-pointing flowers, siliques, and other abnormal traits (Wang et al., 2013). *DDA1/LOBD25/ASL3* exhibits possible functions in both auxin signaling and photomorphogenesis pathways (Mangeon et al., 2011). In sorghum, *SbLBD32* is highly induced under IAA, Brassinosteroid, salt, and drought treatments (Wang et al., 2010). Class II LBD proteins lack a leucine zipper-like motif, and thus their functions might differ from those of Class I proteins. *AtLBD37, AtLBD38*, and *AtLBD39* are involved in anthocyanin synthesis and nitrate metabolism and strongly suppress the expression of *PAP1* and *PAP2* as well as N-responsive genes, all of which are key regulators of anthocyanin synthesis (Rubin et al., 2009). *OsLBD37* is associated with nitrogen metabolism (Albinsky et al., 2010), and *AtLBD41* is involved in leaf dorsoventral determination (Meng et al., 2009).

Although *LBD* family members have been identified and functionally characterized in several plant species, their roles in soybean remain unclear. In this study, we investigated soybean *LBD* genes using a genome-wide approach and studied the expression of these genes in various tissues and in response to different stressors. Functional analysis of *GmLBD12* revealed a positive role in LR development. This work provides a foundation for future functional studies of *GmLBD* genes.

**Materials and Methods**

**Identification of LBD Genes in Soybean**

To obtain soybean *LBD* coding sequences (CDS), a total of 300 plant LBD protein sequences deposited in the NCBI database (http://www.ncbi.nlm.nih.gov/gene/) were used as query sequences to perform TBLASTn searches against 88647 soybean CDS (Wm82.a2.v1) downloaded from the soybean genome database (http://phytozome.jgi.doe.gov/pz/portal.html). The hits derived from the soybean CDS were defined by an e-value cutoff of $9.00 \times 10^{-4}$, and redundant sequences were removed. These putative LBD protein sequences were filtered by the presence of a conserved LBD domain using the online domain analysis program Prosite (http://prosite.expasy.org/scanprosite/). To obtain expressed sequence tags (ESTs) supporting *LBD* genes, we used the amino acid sequences of 300 plant *LBD* genes to perform TBLASTn searches against 146,723 soybean ESTs (2015.6 release) sequences in the NCBI
database. After removing vector and redundant sequences, the hits derived from soybean ESTs were assembled using CAP3 software (Huang and Madan, 1999) with a 99% overlap identity cutoff and default settings for other parameters. Because of variations in the EST sequences of different soybean cultivars, the assembled ESTs were mapped to the candidate GmLBD genes without mismatches (our bioinformatics analysis pipeline is shown in Supplemental Fig. S1).

Isoelectric points and protein molecular weights were estimated using the Compute pl/Mw tools on the ExPASy proteomics server (http://expasy.org/).

Chromosomal Locations and Gene Duplication Analysis
GmLBD genes were drawn on the chromosomes with MapDraw (Liu and Meng, 2003). Tandem and segmental duplications are considered to be the two dominant types of duplication (Wang et al., 2015). Tandem duplications are defined as two neighboring GmLBD genes separated by <200 kb (Yang et al., 2006a), whereas segmentally duplicated genes were identified using the Plant Genome Duplication Database (http://chibba.agtec.uga.edu/duplication/).

Phylogenetic and Structural Analyses
The LOB sequences of 90 soybean LBD proteins and 41 previously reported LBD genes from other plant species (Supplemental Table S6) were used to perform multiple sequence alignments with ClustalW (Thompson et al., 1994) using the default options in MEGA Version 6.0 (Tamura et al., 2013). Secondary structures were predicted using COILS (http://www.ch.embnet.org/software/COILS_form.html) with the following parameters: MTIDK matrix and 2.5-fold weighting of positions a and d. Phylogenetic trees were constructed based on bootstrap neighbor-joining algorithms with the Kimura two-parameter model of MEGA6 v6.0. The stability of the internal nodes was measured by bootstrap analysis of 1,000 replicates. Gene structures were generated using GSDS (http://gsds.cbi.pku.edu.cn/). Motif analysis was performed using the MEME program (http://meme-suite.org/tools/meme).

Digital Expression Data Analysis
Two databases were used to identify the tissue and stress-response expression patterns of GmLBD genes. RNA sequencing (RNA-Seq) data from 14 tissues, including three vegetative tissues (leaves, root, and nodules) and the whole seeds from 11 stages of reproductive tissue development (flower, pod, and seeds), were downloaded from SoyBase (http://www.soybase.org/soyseq/), and soybean microarray expression data obtained from different experiments were downloaded from Plant Expression Database (http://www.plexdb.org). The expression data for GmLBD genes were formatted as heat maps using Cluster 3.0 and Java TreeView 1.1.6 software in red and green to indicate up- and downregulation, respectively, on the basis of the log ratio.

Plant Materials and Plant Growth Conditions
The soybean cultivar Williams82 was used for gene sequencing and gene expression analysis. Tissue expression of GmLBD genes in root and shoot (leaf and stem), flower, pod, and seed was examined at the seedling stage, flowering stage, and 20 d postanthesis. The soybean cultivar Nannong 94-16 and the cco mutant were used to measure the tissue expression of GmLBD12. Root, stem, leaf, flower, and seed (at 7, 10, 15, 30 and 40 d postflowering) were collected from Nannong 94-16 and cco mutant plants for qRT-PCR analysis. All seeds were grown under natural conditions in the field at Jiangpu Experimental Station, Nanjing Agricultural University.

Arabidopsis (Col-0) was used as wild type. Seeds were sterilized and sown on Murashige and Skoog (MS) medium [MS, salt, 30 g L⁻¹ sucrose, 3.5 g L⁻¹ phytagel, and 3 mM 2-(N-morpholino) ethanesulfonic acid] after incubation for 48 to 72 h at 4°C. The medium plates were placed vertically in a growth room under conditions of 16:8 h light/dark, at 23 and 22°C, respectively, and 70% relative humidity. The length of the primary roots and the number of LRs were measured using 7- and 15-d-old seedlings; 30 plants of each genotype were analyzed. Leaf samples were collected from 2-wk-old wild-type (WT; Col-0) and GmLBD12 transgenic Arabidopsis plants for genomic DNA and total RNA isolation. Transgenic Arabidopsis was identified by both PCR and semiquantitative RT-PCR methods (primers listed in Supplemental Table S5).

Hormone and Abiotic Treatments
Seeds of the soybean cultivar Williams82 were germinated in plugs containing a mixture of peat and vermiculite (3:1, v/v) and placed in growth chambers at 25°C with 60% relative humidity under a 16:8 h light/dark cycle. Once the cotyledons had fully expanded, the soybean seedlings were selected and precultured in one-half-strength Hoagland’s nutrient solution for 3 d. The uniformly grown seedlings were washed and transferred to different abiotic conditions. For drought treatment, the seedlings were transferred to water supplemented with 15% polyethylene glycol (PEG), and leaves were sampled after 0.5 and 2 h. For salt and cold treatments, the seedlings were transferred to water supplemented with 250 mM NaCl and a 4°C growth chamber, respectively, and leaves were sampled after 2 and 4 h. Control seedlings were submerged in water, and the leaves were sampled at 0, 0.5, 2, and 4 h. For the hormone treatments, 20 μM IAA, 100 μM ABA, and 100 μM SA was sprayed on 15-d-old soybean seedlings using a handheld mist sprayer, and untreated seedlings were used as a control. Leaf samples (treated and control) were collected at 0, 1, 3, 6, and 12 h. All samples were immediately frozen in liquid nitrogen and stored at −80°C until RNA extraction.

Cloning and Sequencing of GmLBD Genes
Ten randomly selected GmLBD genes (primers listed in Supplemental Table S4) were PCR amplified from the mixed complementary DNA (cDNA) samples including
root, stem, leaf, flower, pod, and seed tissues of Williams82 for direct sequencing after gel purification (HuaDa).

GmLBD12 was cloned from the leaf cDNA of Nannong 94-16 (primers listed in Supplemental Table S4). The PCR products were gel-purified, cloned into the pMD19-T vector, and sequenced (HuaDa, Shenzhen, China).

**Nucleic Acid Extraction, Complementary DNA Synthesis, and Gene Expression Analysis**

*Arabidopsis* genomic DNA was extracted according to the CTAB method. Total RNA was extracted from soybean and *Arabidopsis* using a Plant RNA Extract Kit (TianGen) following the manufacturer’s instructions. Complementary DNA was synthesized from 2 μg of mRNA in a 20-μl volume using a HiScript II Q RT Super Mix kit (Vazyme). Semiquantitative RT-PCR was performed in a 25-μl reaction volume. Quantitative RT-PCR was performed in a 20-μl volume containing 5 μl of diluted (1:10 v/v) cDNA, 10 μl 2× SuperReal PreMix Plus (TianGen) and 0.3 μM of each primer on a Bio-Rad CFX96 Real-Time PCR instrument. Three biological and technical replicates were used in each experiment. The endogenous soybean *tubulin* gene (GenBank accession no. AY907703.1) was used as a control, and the relative expression was estimated using the 2−ΔΔCt method as follows (Livak and Schmittgen, 2001): fold change = 2−ΔΔCt, for tissue expression, \( \Delta \Delta Ct = (Ct_{target \, gene} - Ct_{Tubulin \, stem}) - (Ct_{target \, gene} - Ct_{Tubulin \, root}) \); for abiotic stress experiments, \( \Delta \Delta Ct = (Ct_{target \, gene} - Ct_{Tubulin \, PEG}) - (Ct_{target \, gene} - Ct_{Tubulin \, Water}) \); and for hormone experiments, \( \Delta \Delta Ct = (Ct_{target \, gene} - Ct_{Tubulin \, IAA}) - (Ct_{target \, gene} - Ct_{control}) \).

**Subcellular Localization of GmLBD12**

The CDS of GmLBD12 was PCR amplified from the leaves of Nannong 94-16 and cloned into a modified pAN580 vector that contains the soybean ubiquitin gene promoter GmUbi3pro. The construct Gmubi3pro:GmLBD12-GFP was transformed into *Arabidopsis* (Col-0) protoplast cells as previously described (Yoo et al., 2007). The images were captured by confocal laser-scanning microscopy (Leica TCS SP2).

**Transformation of Arabidopsis**

A pBI121 vector with a termination codon at the end of GmLBD12 was used for transformation. The gene construct was introduced into Agrobacterium tumefaciens strain EHA105. Transgenic *Arabidopsis* (Col-0) were generated by the floral dipping method (Mara et al., 2010).

**Results**

**Genome-Wide Identification of Soybean LBD Genes**

A TBLASTn search of soybean cDNA sequences (88,647; Wm82.a2.v1) and soybean ESTs (146,723; 2015.6 release; NCBI) using 300 known LBD proteins as the query yielded 125 CDS and 346 ESTs, respectively. A total of 90 genes (98 transcripts) containing conserved LOB domains were identified (Supplemental Fig. S1). By mapping the assembled ESTs to the GmLBD genes, we found 41 genes supported by 329 ESTs (Table 1). The 41 genes included 22 genes with EST (290) coverage from the 5′ end, 5′ and/or 3′ end support (290) coverage from the 3′ end, 5′ end, or both. Expressed sequence tags (2) that only supported internal sequences corresponded to one gene (Table 1). In addition, 12 genes (supported by 17 ESTs) were found to contain novel alternative splicing sites that differed from existing genome annotations. However, these new transcripts were not predicted to encode proteins, indicating that these genes might be alternatively spliced during some specific biological processes based on the previous studies showing that alternative splicing plays an important role in the post-transcriptional regulation in higher eukaryotes (Unoki et al., 2006; Lira et al., 2008). To verify the accuracy of the predicted GmLBD gene sequences, we selected 10 genes (GmLBD12, GmLBD20, GmLBD23, GmLBD35, GmLBD37, GmLBD45, GmLBD51, GmLBD70, GmLBD77, and GmLBD79) and sequenced their CDS. Our experimental results demonstrated that the obtained CDS of all 10 genes agreed with the annotated sequences (data...
not shown). Furthermore, detailed information for each GmLBD gene, including gene identifier, genomic position, open reading frame length, and characteristics of the encoded protein (size, molecular weight, and isoelectric point), was annotated (Supplemental Table S1).

**Chromosomal Locations and Duplication Patterns of Soybean LBD Genes**

Of the identified GmLBD genes, 88 were nearly evenly distributed throughout the 20 soybean chromosomes (Fig. 1), while the remaining two genes (GmLBD89 and GmLBD90) were mapped to unassembled genomic sequence scaffolds. The number of GmLBD genes on each chromosome ranged from two to seven. Sixteen chromosomes carried four to six genes. Chromosome 7 contained three genes, and chromosomes 9, 12, and 16 contained two genes each. Most GmLBD genes were located in clusters on chromosome arms (Fig. 1), which are associated with high rates of recombination (See et al., 2006).

Compared with the number of LBD genes in Arabidopsis, rice, maize, sorghum, apple, mulberry, and poplar, which contain 43, 35, 44, 36, 58, 31, and 57 LBD genes, respectively, the soybean LBD family is remarkably large at 90 members, suggesting that the expansion of LBD genes in soybean occurred more rapidly, possibly the result of two rounds of whole-genome duplication events in soybean (Schlueter et al., 2004; Schmutz et al., 2010; Van neste et al., 2014). Thus, we investigated the duplication patterns of soybean LBD family, and the results showed that 77 out of 90 GmLBD genes (85.56%) were present in duplications (Fig. 1; Supplemental Table S2), indicating that duplications largely contributed to the amplification of LBD family in the soybean genome. Among the 77 duplicated genes, we identified four GmLBD genes with tandem duplications (on chromosomes 1, 3, 18, and 19), 61 GmLBD genes with segmental duplications (on 19 soybean chromosomes), and 12 GmLBD genes with both types of duplications (on chromosomes 1, 3, 10, 13, 14, 18, and 19) (Fig. 1; Supplemental Table S2), suggesting that segmental duplication appears to be the main driver of the expansion of LBD genes in soybean.
Domain Structures of LBD Proteins in Soybean

Arabidopsis LBD genes encode a conserved, N-terminal LOB domain, and the LOB domains of the Class I proteins contain two conserved blocks: the C block and the GAS block. To identify conserved domains within GmLBD proteins, all the LOB domain sequences were examined by alignment analysis (Supplemental Fig. S2). All GmLBD proteins contain four conserved Cys residues in the C block except for the following: GmLBD11 lacks the first three Cys residues, the first Cys residue of GmLBD68 is substituted with a Tyr residue, and the third Cys residue of GmLBD34 is substituted with a His residue (Supplemental Fig. S2). As with AtLBD proteins, the spacing between these Cys residues is essentially conserved in soybean, but the intervening amino acids differ. In the GAS block, only GmLBD68 has no corresponding sequences, whereas the remaining GmLBD proteins contain two completely conserved residues (P, G) in the motif (D/N) PX2G (Supplemental Fig. S2). Secondary structure prediction revealed the presence of a leucine zipper-like coiled-coil motif in most members (89%) of Class I LBD proteins. However, unlike most GmLBD proteins having one coiled-coil, GmLBD proteins (4, 17, 25, 40, and 46) are predicted to encode two coiled-coil structures (Supplemental Fig. S3); this phenomenon has also been observed in rice (Yang et al., 2006b). As with other species (Shuai et al., 2002; Yang et al., 2006b; Wang et al., 2010, 2013; Zhang et al., 2014), no Class II GmLBD proteins are predicted to form coiled-coil structures.

Phylogeny, Gene Structure, and Motif Analysis of GmLBD Genes

To evaluate the evolutionary relationships among GmLBD genes, a phylogenetic tree was constructed based on the LOB domain sequences. As shown in Fig. 2, all 90 GmLBD genes were divided into two subclasses (Class I and Class II). As Class I contained a large number of genes (74 members), it was further separated into 10 subgroups (a through j). Exon–intron structure divergences play an important role during the evolution of duplicate genes (Xu et al., 2012); thus, we analyzed the exon–intron organization of individual GmLBD genes (Fig. 2). In soybean, 27 genes contained one exon and six genes contained three exons; the remainder of the genes contained two exons. The exon numbers and lengths were relatively conserved within each subgroup, whereas the gene size varied, indicating that the total intron length and number mainly contributed to the wide-ranging sizes of the GmLBD genes. To further understand the characteristics of the soybean LBD proteins, we predicted 10 motifs in GmLBD proteins using MEME (Fig. 3; Supplemental Table S3). Motifs 1, 2, 3, 4, 5, and 6 were identified in the LOB domains (motifs 2, 3, and 6 belong to the C-block; Motif 1 belongs to the GAS block; Motifs 4 and 5 belong to the coiled-coil region) (Supplemental Fig. S4). The remaining motifs are located outside the LOB domain in the C-termini of the proteins. As shown in Fig. 3, Motifs 1 and 2 are present in the majority of GmLBD proteins, thus supporting their conservation in the domain structure. Motifs 3 and 4 are detected in most members of Class I, whereas Motifs 5 and 6 are present in all Class II proteins; these different motif compositions indicate their classification on the phylogenetic tree. By analyzing the subgroups, we found that Motif 7 is present in Class I-f, Motif 8 was identified in Class I (c and e), Motif 9 in Class I-b, and Motif 10 in Class I; these results suggest that the C-terminal motifs are highly divergent compared with those in the N-termini of GmLBD proteins.

To explore the functions of soybean LBD genes, we built another phylogenetic tree (Fig. 4) using the LOB domain sequences of 131 LBD genes including the 41 previously reported plant LBD proteins (Supplemental Table S6) and 90 soybean LBD proteins. As with Fig. 2, these various LBD proteins from different species were also classified into two primary clades comprising 10 smaller subgroups (Fig. 4) including Subclass I (Subgroups a-j) and Subclass II. Based on their phylogenetic relationships, the functions of soybean LBD proteins could be inferred from known LBD proteins. Except for Class I-h and Class I-j, having no orthologs with identified functions, all the other subgroups have at least one LBD gene functionally characterized. For example, Class I-e contains five LBD genes that are involved in the regulation of leaf and flower development (Semiarti et al., 2001; Chalfun-Junior et al., 2005; Evans, 2007; Ma et al., 2009; Kim et al., 2015). Class I-g includes six LBD genes related to LR formation and development (Inukai et al., 2005; Liu et al., 2005; Taramino et al., 2007; Berckmans et al., 2011; Feng et al., 2012a,b). And Class II members were clustered with LBD genes involved in nitrate metabolism and biotic and abiotic stresses (Rubin et al., 2009; Albinsky et al., 2010; Wang et al., 2010; Thatcher et al., 2012b).

Tissue Expression Patterns of GmLBD Genes

To characterize the tissue expression patterns of GmLBD genes, two expression datasets were analyzed. A total of 59 (65.6%) GmLBD genes had corresponding digital expression data. Fifty-eight and 15 genes were identified in the RNA-Seq and microarray data, respectively, with 14 genes shared between them. These results suggest that the remaining 31 LBD genes (34.5%) in soybean may be weakly expressed. RNA sequencing analysis indicated that Class II GmLBD genes were nearly expressed in all tissues and relatively highly expressed compared with the Class I (Fig. 5A). By contrast, the Class I genes were predominantly expressed in some specific tissues. For instance, GmLBD33, 21.2, 69, 47, and 80.2, were predominantly expressed in one tissue; GmLBD43, 10.2, 63, 59, 66, 49, and 90 were expressed in several tissues but not seeds; GmLBD6, 81, 39, and 76 were mainly expressed in seed tissue (Fig. 5A). In the microarray tissue expression experiments, 10 tissues in three different embryo microcosmic stages (globular stage, heart stage, and cotyledon stage) during seed development were specifically analyzed. As shown in Fig. 5B, four genes (GmLBD23, 45, 51, and 37) were highly expressed in three stages compared with...
Fig. 2. The phylogenetic tree and gene structure analysis of GmLBDs. The LOB domain sequences of LBD proteins were aligned using ClustalW, and the phylogenetic tree was constructed using the neighbor-joining method in MEGA 6.0 software. Each node is represented by a number that indicates the bootstrap value for 1000 replicates. Classification within the phylogenetic tree was based on bootstrap values >50%. The right side illustrates the exon–intron structures of the corresponding LBD genes. Exons and introns are designated by green boxes and black lines, respectively.
Fig. 3. Protein motifs in the soybean LBD family. Motifs present in soybean LBD proteins are shown as colored boxes. Motifs 1, 2, 3, 4, 5, and 6 are in the LOB domains, and Motifs 7, 8, 9, and 10 are located outside the LOB domain in the C-termini of the LBD proteins. The scale on the bottom can be used to estimate the length of a motif (unit: amino acid). The GmLBD proteins are listed according from Subclass I to Subclass II based on the phylogenetic tree shown in Fig. 2, and different subgroups are highlighted in different colors.
other genes; however, they exhibited different expression patterns: GmLBD23 expression continuously decreased in the embryo proper and suspensor during all three stages; GmLBD45 showed lower expression in the embryo proper and endosperm at the cotyledon stage; and although GmLBD51 and GmLBD77 were both weakly expressed in young trifoliate leaves during the globular and heart stages, their expression patterns varied in other tissues.

Based on the RNA-Seq data, qRT-PCR was used to examine the expression patterns of 13 GmLBD genes (11 of which had digital expression data) in seven tissues including root, stem, leaf, flower, pod, and seed (at 20 d postflowering). As shown in the Fig. 6, the relative expression levels of these 10 genes were generally in agreement with the digital expression data (Fig. 5A) except for one gene (GmLBD46), which showed higher expression in flower and seed via qRT-PCR (Fig. 6I) but showed higher expression in leaf, flower, and pod in the RNA-Seq data (Fig. 5A). This discrepancy possibly is due to the differences in the RNA samples collected from two different cultivars (G. max A81-356022 for RNA-Seq and Williams 82 for qRT-PCR). Moreover, two genes without related RNA-Seq data appeared in the qRT-PCR analysis: GmLBD15 was predominately expressed in root.
tissue (Fig. 6E) and GmLBD35 was mainly expressed in seed tissue (Fig. 6G). This difference might have been caused by low sequencing depth. Furthermore, we found that duplicated GmLBDs exhibited similar expression patterns but different expression levels (Fig. 6A–C). For example, GmLBD23 and GmLBD70 had higher expression in root tissue, and GmLBD37, GmLBD51, and GmLBD77 were expressed higher in seed tissue, indicating functional redundancy.

Response of GmLBD Genes to Stress and Hormone Treatment

We investigated the differential expression of GmLBD genes in tissues under biotic and abiotic stresses based on EST and microarray data. As shown in Table 2, 27 genes, accounting for 30% of the identified soybean LBD genes, were induced by the different treatments including 17 of 74 Class I genes (23%) and 10 of 16 Class II genes (62.5%). Moreover, 15 of 27 genes responded to both biotic and abiotic stresses. GmLBD9, GmLBD23, GmLBD30, GmLBD37, and GmLBD51 were induced in the root and hypocotyl after Bradyrhizobium japonicum and Phytophthora sojae mycelia infection and were also responsive to salt, Mg, and Al abiotic stresses. GmLBD48 was induced by Bradyrhizobium japonicum and ozone. GmLBD59 was induced by both biotic and abiotic stresses and was also responsive to SA treatment. Of the 12 genes only responsive to biotic or abiotic stresses, GmLBD69 was induced by Fusarium solani, whereas GmLB15 and GmLBD20.2 were induced by drought.

To verify these results, the expression of eight GmLBD genes (seven genes with digital data) following abiotic stresses, including PEG, salt, cold, and hormone treatments, such as IAA, ABA, and SA, was examined. Four genes (GmLBD23, 45, 51, and 70) responsive to the abiotic stresses and hormone treatments in the digital datasets (Table 2) were induced by PEG, salt, cold (4°C), and the three hormone treatments (Fig. 7, 8). Another three genes (GmLBD5, 74, and 77) that did not respond to abiotic stresses and hormone treatments in the digital data were induced by the examined treatments (Fig. 7, 8). Moreover, GmLBD12, which lacked digital data, was also induced by the examined treatments (Fig. 7, 8). These inconsistent induction results of certain GmLBD genes from the two methods might be due to differences in the experimental conditions and methods or in the RNA samples (various cultivars for EST and microarray data and Williams 82 for qRT-PCR). From the results, we
found that these eight genes were differentially expressed under abiotic stresses (Fig. 7) and hormone treatments (Fig. 8). Interestingly, GmLBD74 was downregulated following the three abiotic stresses (Fig. 7) and GmLBD12 was upregulated by all three hormone treatments. Furthermore, various differential expression patterns were observed in duplicated GmLBD genes under abiotic stresses and hormone conditions (Fig. 7, 8), such as GmLBD23/GmLBD70 and GmLBD51/GmLBD77, indicating that specific genes may function under certain environmental conditions.

Functional Analysis of GmLBD12

We previously demonstrated that a soybean curled-cotyledon mutant (cco) derived from sodium azide (NaN₃) and 60Coγ ray-mutagenized seeds of the Nannong 94-16 cultivar (hereafter designated CK) exhibits defective cotyledon morphology and other aberrant agronomic characteristics such as reduced root systems (Supplemental Fig. S5A), longer growth periods, and smaller plants (Supplemental Fig. S5B) (Yu et al., 2012; Shi et al., 2014). Analysis of RNA-Seq data (Shi et al., 2014) revealed three GmLBD members (GmLBD12, GmLBD55, and GmLBD59) were differentially expressed between CK and the cco mutant. In particular, the transcript levels of GmLBD12 were greatly reduced in the cco mutant compared with CK. Thus, GmLBD12 was selected for functional characterization.

GmLBD12 is located in a region of chromosome 3 that is derived from both segmental and tandem duplications (Fig. 1). Gene-structure analysis showed that GmLBD12 contains two exons and one intron (Fig. 2). The deduced GmLBD12 protein contains 251 amino acids with a molecular mass of 25.97 kDa and an isoelectric point of 8.12 (Supplemental Table S1). Phylogenetic analysis suggested that GmLBD12 belongs to Class I-g and is clustered closely to Arabidopsis AtLBD18 (87.38% similarity), AtLBD30 (83.50% similarity), AtLBD20 (79.61% similarity), and L. japonicas LjLOB4 (50.49% similarity) (Fig. 4). To assess the subcellular
localization of GmLBD12, we fused the full-length open reading frame of GmLBD12 to the green fluorescent protein (GFP) reporter gene and obtained the construct Gmubi3pro:GmLBD12-GFP. Confocal images suggested that the GmLBD12-GFP fusion protein was mainly distributed in the nucleus in Arabidopsis (Col-0) protoplasts (Fig. 9). We further investigated the expression pattern of GmLBD12 in different tissues in both cco mutant and CK plants. The results showed that GmLBD12 was highly expressed in roots and seeds compared with other tissues and was significantly downregulated in all tissues except for seeds in the cco mutant compared with CK (Fig. 10) with the greatest reduction in roots. In seed tissue, RNA levels of GmLBD12 were reduced at 7 and 10 d after flowering (DAF), and were higher at 15 and 40 DAF (Fig. 10). Considering the low transformation efficiency in soybean (Atif et al., 2013), we used the model plant Arabidopsis (Col-0) to analyze the biological function of GmLBD12 and obtained six independent transgenic lines as confirmed by RT-PCR analysis (Supplemental Fig. S6). The effect of GmLBD12 on root development was first observed using 35S:LBD12 (T4 generation) and WT Arabidopsis plants grown on MS medium. After 7 d of growth, 35S:LBD12 plants produced three or more visible LRs, whereas WT plants had just begun to grow the first LR (Fig. 11A, C). At 15 d, 35S:LBD12 plants continued to produce the most LRs (Fig. 11B, C). We also observed other phenotypes during subsequent growth: compared with WT plants, 35S:LBD12 plants grew faster and exhibited increased plant height (Fig. 11D–F). These results suggest that GmLBD12 may promote the growth and development of Arabidopsis by increasing its number of LRs. Since GmLBD12 was showed high expression in seed tissue, we weighed 1000 seeds from transgenic and WT Arabidopsis plants and determined that the seed weight of GmLBD12-overexpressing plants was comparable to that of WT (Supplemental Fig. S7), suggesting that GmLBD12 overexpression has no effect

Table 2. LBD genes in soybean exhibiting responses to biotic and abiotic stresses and hormone treatment in specific tissues.

<table>
<thead>
<tr>
<th>Genes</th>
<th>ESTs†</th>
<th>Microarray Types‡</th>
<th>Tissues</th>
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<th>Microarray Types§</th>
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† EST, expressed sequence tag.
‡ H, B, P, and F, infection with Heterodera glycines, Bradyrhizobium japonicum, Phytophthora sojae mycelia, and Fusarium solani, respectively.
§ S, D, O3, M, A, and I, stress caused by salt, drought, ozone, Mg, Al, and Fe, respectively.
¶ SA, salicylic acid.
Fig. 7. Expression profiles of GmLBDs following abiotic stress treatments measured by quantitative real-time polymerase chain reaction. The selected GmLBD genes were examined in leaves after polyethylene glycol, NaCl, and 4°C treatments. The numbers in parentheses indicate hours after treatment. GmLBD expression at 0 h was used as a control (expression value=1). Statistical analysis was performed using paired-samples t-test (one-tail). A single asterisk (*) indicates significance at 0.01<P<0.05 probability level; A double asterisk (**) indicates significance at 0.01 probability level.
The selected GmLBD genes were examined in leaves after indole acetic acid, abscisic acid, and salicylic acid treatments. The numbers in parentheses indicate hours after treatment. GmLBD expression at 0 h was used as a control (expression value = 1). Statistical analysis was performed using paired-samples t-test (one-tail). A single asterisk (*) indicates significance at 0.01 < \( P \) < 0.05 probability level; A double asterisk (**) indicates significance at 0.01 probability level.

Fig. 8. Expression profiles of GmLBDs following hormone treatments measured by quantitative real-time polymerase chain reaction. The selected GmLBD genes were examined in leaves after indole acetic acid, abscisic acid, and salicylic acid treatments. The numbers in parentheses indicate hours after treatment. GmLBD expression at 0 h was used as a control (expression value = 1). Statistical analysis was performed using paired-samples t-test (one-tail). A single asterisk (*) indicates significance at 0.01 < \( P \) < 0.05 probability level; A double asterisk (**) indicates significance at 0.01 probability level.

Discussion

The LBD Gene Family in Soybean

LBD genes encoding LOB domains are widely distributed among higher plants and may regulate plant-specific functions in Arabidopsis yield. In addition, induction experiments revealed that GmLBD12 responded to drought, salt, cold, IAA, ABA, and SA treatments (Fig. 7, 8), which implies that GmLBD12 is involved in responses to abiotic stresses and in different signaling pathways.
In this study, we performed a systematic investigation of the soybean family of LBD genes and their expression patterns. A total of 90 LBD genes (98 transcripts) were identified in soybean via genome-wide analysis. Soybean is known to be an ancient tetraploid and has undergone two rounds of large-scale duplication events (Schmutz et al., 2010), which were important for the rapid expansion and evolution of the plant’s gene families. Some gene families in soybean contain more members than those in corresponding families in other species, and segmental and chromosome duplication has been posited as the major factor leading to this gene expansion (Liu et al., 2016; Zhou et al., 2016), which is possibly because segmental duplicated genes have more chances to be retained (Wang et al., 2005). Tandem duplication plays an important role in generating new duplicated genes (Cannon et al., 2004), whereas segmental duplication drives genomic distribution of duplicated genes (Baumgarten et al., 2003) and results in many functional redundant genes to avoid fitness cost (Song et al., 2014).

**Domain Structure and Phylogenetic Analysis**

All known LBD proteins in different species encode a conserved zinc finger-like domain (Shuai et al., 2002; Yang et al., 2006b; Wang et al., 2013; Zhang et al., 2014). In our study, we identified three soybean LBD genes (GmLBD11, GmLBD34, and GmLBD68) that exhibited variations in the conserved amino acids (Supplemental Fig. S2). Similar results were found in L. japonicus: LjLOB1 lacks the first Cys residue, LjLOB4 lacks the all four conserved Cys residues, and the first Cys residue of LjLOB3 is substituted with a Gln residue (Luo et al., 2006). However, LBD proteins in other plants, such as *Arabidopsis*, rice, maize, sorghum, apple and mulberry, feature invariant residues in their C and GAS blocks, suggesting that there is more variation within the legume LBD family.

As with other plants (Shuai et al., 2002; Yang et al., 2006b; Wang et al., 2013; Zhang et al., 2014; Luo et al., 2016), the soybean LBD family can be divided into two phylogenetic classes. Furthermore, GmLBDs were aligned with 41 previously reported plant LBD proteins. Since most of the genes with similar functions have a tendency to cluster together, the soybean LBD genes in the same group or subgroup may have similar functions with their homologs. The phylogenetic tree, to some extent, indicates that most Class I LBD members tend to be involved in plant tissue and organ developments, whereas Class II genes are more likely to participate in metabolism or stress responses (Fig. 4; Supplemental Fig. S6). However, not all Class I members are specific to development regulation, such as Class I-f, which contains a banana (*Musa acuminata* Colla) gene MaLBD5 regulating cold tolerance through MeJA-mediated pathway (Ba et al., 2016). Likewise, Class II proteins may also function in regulating plant development; for example, *AtLBD41* is not only responsive to pathogens but is also involved in leaf dorsoventral determination (Meng et al., 2009). Our current results provide an important foundation for further functional analysis of GmLBDs.

**Expression Pattern of GmLBDs**

To date, many *Arabidopsis* LBD genes have been assigned functions in regulating plant root, shoot, leaf, flower,
Fig. 10. Expression analysis of GmLBD12 in the soybean mutant cco (MU) and Nannong 94-16 (CK) plants. GmLBD12 was examined in root, stem, leaf, flower, and seed (at different developmental stages) tissues and expression in roots was used as a control (expression values = 1). Statistical analysis was performed in cco vs. CK using paired-samples t-test (one-tail). A single asterisk (*) indicates significance at 0.01 < P < 0.05 probability level; A double asterisk (**) indicates significance at 0.01 probability level. DAF, days after flowering.

Fig. 11. Phenotypic comparison of T4 35S:GmLBD12 and wild-type (WT) Arabidopsis plants. (A) The roots of 7-d-old seedlings. (B) The roots of 14-d-old seedlings. (C) Lateral root (LR) density (i.e., the number of LRs/total primary root length) in WT and 35S:GmLBD12 plants at 7 and 15 d. (D) Phenotypes of 3-wk-old GmLBD12 transgenic and WT plants. (E) Sizes of GmLBD12 transgenic and WT plants close to maturity. (F) Heights of 35S:GmLBD12 and WT plants close to maturity. Statistical analysis was performed by comparisons of each parameter in 35S:LBD12 vs. WT using paired-samples t-test (one-tail). A single asterisk (*) indicates significance at 0.01 < P < 0.05 probability level; A double asterisk (**) indicates significance at 0.01 probability level.
and callus tissues (Semiarti et al., 2001; Fan et al., 2012; Feng et al., 2012b; Sun et al., 2013; Kim et al., 2015). Accordingly, we examined the expression patterns of soybean LBD genes in various tissues (Fig. 5, 6). The different expression characteristics between two classes may indicate their different functions as shown in the phylogenetic tree (Fig. 4). GmLBD15 was mainly expressed in root tissue by qRT-PCR analysis and closely clustered with CmLBD1 (LR formation) in Class I-g (Fig. 4; 6; Supplemental Table S6). GmLBD46, with higher expression in flower, was clustered with AtLBD10 (pollen development) and AtLBD36 (flower development) in Class I-e (Fig. 4; 6; Supplemental Table S6). And GmLBD48 and GmLBD74, predominately expressed in leaf and stem, were clustered with LjLOB1 (leaf development) in Class I-d (Fig. 4; 6; Supplemental Table S6).

Digital (EST and microarray) and experimental (qRT-PCR) results showed that soybean LBD genes could be induced by stress and hormone treatments similar to the proportions of apple and sorghum LBD genes known to be regulated by abiotic stresses and hormone treatments (Wang et al., 2010, 2013). Among the soybean LBD family, a total of 27 genes (30%) were induced by the different treatments, and these genes included 17 of 74 Class I genes (23%) and 10 of 16 Class II genes (62.5%), suggesting that Class II genes have a greater role in plant stress responses than Class I genes. Previous work in Arabidopsis demonstrated that LBD genes whose expression is responsive to multiple pathogens belong to the smaller Class II of LBD proteins (subgroups formed between AtLBD37, 38, and 39 and AtLBD40, 41, and 42) and that Class II LBD genes are the most responsive (Thatcher et al., 2012a). Consistent with these findings, our investigation of soybean biotic stress responses revealed that Class II LBD genes in soybean, particularly GmLBD23, 30, 37, 45, 51, and 70, are induced by more varieties of pathogens than Class I genes and are maintained at a higher expression level after infection (supported by microarray data). However, some Class I genes, such as AtLBD20 in Arabidopsis and CsLOB1 in citrus, were found to be involved in fusarium wilt (Thatcher et al., 2012b) and citrus canker (Hu et al., 2014), respectively.

GmLBD Genes May Have a Conserved Function Similar to Arabidopsis Homologs

GmLBD12, which shows a close phylogenetic relationship and high degree of sequence similarity to Arabidopsis AtLBD18, may possess similar functions to AtLBD18 in LR development, but its regulatory network remains unclear. AtLBD18 is known to be regulated by IAA1-ARF7/ARF19 in auxin signaling to directly stimulate LR formation (Lee et al., 2009, 2013). Our experiments showed that GmLBD12 responds to IAA, indicating an association between its roles in regulating LR development and auxin signaling. A protein’s subcellular localization pattern can provide significant clues to its function. Previous studies have demonstrated that LBD proteins in different plant species are localized to the nucleus (Liu et al., 2005; Lee et al., 2009; Chen et al., 2012; Zhu et al., 2016). In our study, GmLBD12 was mainly localized at nucleus (Fig. 9), implying that GmLBD12, like other LBDs, functions as a transcription factor. However, there were some low GFP signals in other cellular regions except nuclei, which might be the result of potential artifact and requires more experimental verification.

Overall, our work indicates that LBD genes are important for regulating plant growth and development and exhibit conserved structures and functions across different species. Bioinformatic and experimental approaches were used to comprehensively identify and analyze the structures and functions of soybean LBD genes, providing a foundation for further in-depth functional analysis of the biological roles of GmLBD genes.

Supplemental Information

Supplemental information for this article is available online.

Acknowledgments

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References


