Comprehensive characterization and gene expression patterns of \textit{LBD} gene family in \textit{Gossypium}

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Abstract

Main conclusion A comprehensive account of the \textit{LBD} gene family of \textit{Gossypium} was provided in this work. Expression analysis and functional characterization revealed that \textit{LBD} genes might play different roles in \textit{G. hirsutum} and \textit{G. barbadense}.

Abstract The Lateral Organ Boundaries Domain (LBD) proteins comprise a plant-specific transcription factor family, which plays crucial roles in physiological processes of plant growth, development, and stress tolerance. In the present work, a systematical analysis of \textit{LBD} gene family from two allotetraploid cotton species, \textit{G. hirsutum} and \textit{G. barbadense}, together with their genomic donor species, \textit{G. arboreum} and \textit{G. raimondii}, was conducted. There were 131, 128, 62, and 68 \textit{LBDs} identified in \textit{G. hirsutum}, \textit{G. barbadense}, \textit{G. arboreum} and \textit{G. raimondii}, respectively. The LBD proteins could be classified into two main classes, class I and class II, based on the structure of their lateral organ boundaries domain and traits of phylogenetic tree, and class I was further divided into five subgroups. The gene structure and motif composition analyses conducted in both \textit{G. hirsutum} and \textit{G. barbadense} revealed that \textit{LBD} genes kept relatively conserved within the subfamilies. Synteny analysis suggested that segmental duplication acted as an important mechanism in expansion of the cotton \textit{LBD} gene family. Cis-element analysis predicated the possible functions of \textit{LBD} genes. Public RNA-seq data were investigated to analyze the expression patterns of cotton \textit{LBD} genes in various tissues as well as gene expression under abiotic stress treatments. Furthermore, RT-qPCR results found that \textit{GhLBDs} had various expression regulation under MeJA treatments. Expression analysis indicated the differential functions of cotton \textit{LBD} genes in response to abiotic stress and hormones.

Keywords Genome-wide analysis · Lateral root · \textit{LBD} gene family · Methyl jasmonate

Abbreviations

LBD Lateral organ boundaries domain
LOB Lateral organ boundary
MeJA Methyl jasmonate

Introduction

The Lateral Organ Boundaries Domain (LBD) proteins are a family of plant-specific transcription factors with a characteristic N-terminal Lateral Organ Boundary (LOB) domain (Iwakawa et al. 2002). The \textit{LOB} gene was first identified in \textit{Arabidopsis thaliana} based on the expression pattern of an enhancer trap insertion and was found to be expressed at the boundaries of lateral organs during vegetative and reproductive plant development (Shuai et al. 2002). Since \textit{LBD} genes are found only in plants, it is implied that this gene family may regulate plant-specific growth and development processes (Shuai et al. 2002). The \textit{LBD} gene family of \textit{A. thaliana} can be divided into two classes according to the structure of the \textit{LOB} domain. Class I has a completely conserved CX2CX6CX3C zinc finger-like motif with a potential DNA-binding ability. \textit{LBD} genes of class I also process an invariant glycine containing GAS block and a
leucine zipper-like structure (LX6LX3LX6L), which allows the formation of coiled-coil protein interactions, whereas class II only contain a conserved zinc finger-like motif (Landschulz et al. 1988; Iwakawa et al. 2002; Shuai et al. 2002; Majer and Hochholdinger 2011). The majority of LBD proteins belongs to class I. Class II LBD proteins have an incomplete, probably not functional, leucine zipper that cannot form a coiled-coil structure (Majer and Hochholdinger 2011). Recently, the crystal structure of the LOB domain in an LBD protein from wheat was crystallized and determined, which reveals that the previously mentioned C-block in fact forms a C4-type zinc finger. Together with GAS motif and the perpendicular conformation between α4 and α5, the zinc finger constitutes an interaction network that precisely determines the spatial configuration of DNA recognition and binding site (Chen et al. 2019).

The lateral LBD gene family was originally characterized as a group of genes typically expressed by these boundary forming cells at the base of emerging lateral organs, indicating a potential role in organ separation and lateral organ development (Iwakawa et al. 2002). Some other studies also revealed additional pivotal roles of the LBD protein family in pollen development and plant regeneration, photomorphogenesis, pathogen response, and nitrogen metabolism (Thatcher et al. 2012b).

LBD genes of class I are mostly involved in plant development such as lateral organ development (Majer and Hochholdinger 2011; Xu et al. 2016), and in auxin signal transduction cascade that leads to the formation of lateral roots (Liu et al. 2005; Lee et al. 2015). In contrast, genes of class II seem to be involved in metabolism, particularly as repressors of anthocyanin synthesis and N availability signals in the plant (Rubin et al. 2009). Expression profiles of LBD family members in Arabidopsis thaliana revealed the induction of almost all LBD genes in class II by multiple pathogens, suggesting functions of LBD genes in plant defense responses (Thatcher et al. 2012a).

Many previous studies have confirmed that LBD proteins play an essential role in the regulation of the growth and development of a variety of plants. AthLBDs are found to be involved in various tissues development such as leaf development (Semiarti et al. 2001) and lateral root initiation (Goh et al. 2019; Cho et al. 2019; Lee et al. 2019a), as well as signaling transduction such as cytokinin (Naito et al. 2007) and gibberellin pathways (Zentella et al. 2007). To be specific, AthLBD16 is involved in instigating the migration of nuclei and in the asymmetric division of lateral root founder cells to promote lateral root initiation (Goh et al. 2012). AthLBD29 is involved in aspects of auxin signaling that inhibits fiber wall thickening in Arabidopsis stems (Lee et al. 2019b). AtLBD20 is a Fusarium oxysporum susceptibility gene that appears to regulate components of jasmonic acid (JA) signalling required for full elicitation of F. oxysporum- and JA-dependent responses (Feng et al. 2012). Besides Arabidopsis, LBD genes have also been studied in many other species, such as fruit, grass, and wood species. OsIG1 is involved in the flower organ number and gametogenesis in rice (Zhang et al. 2015). Additionally, the MdLBD13 protein can inhibit anthocyanin synthesis and nitrogen uptake in apple (Li et al. 2017). Genome-wide analysis of LBD gene family have been studied in plants like Camellia sinensis (Zhang et al. 2019), Solanum tuberosum (Liu et al. 2019), Eucalyptus grandis (Lu et al. 2018), Brassica rapa (Huang et al. 2018), grapevine (Grimplet et al. 2017), and soybean (Yang et al. 2017). However, the function of the LBD genes in Gossypium remains largely unexplored.

Cotton is not only a worldwide leading textile fiber crop, but also a crop that is of significance for edible oil and other production (Zhang et al. 2008). G. hirsutum and G. barbadense are the two most widely cultivated cotton species, both of them are allotetraploids and formed by inter-genomic hybridization of G. raimondii and G. arboreum, which are the putative donor species for the D and A sub-genomic groups of tetraploid cotton species, respectively (Kadir 1976; Grover et al. 2007). In the current study, we performed a genome-wide screening for LBD genes in cotton, based on data gathered from recent whole-genome sequencing results. Here, we used various in silico approaches to identify and characterize LBD genes in cotton, and then focused on the characterization and phylogenetic relationships between G. hirsutum and G. barbadense. The LBD gene structures, conserved domains, synteny, as well as Cis-acting elements were explored in this systematical analysis. Moreover, the tissue-specific expression patterns and the transcriptional responses of GhLBDs to hormone and abiotic stress were studied in this article. These findings may provide inspirations for breeding of cotton with high stress resistance.

Materials and methods

Sequence retrieval and analysis

Two diploid cottons, Gossypium raimondii (Paterson et al. 2012) and G. arboreum (Du et al. 2018), and two allotetraploid cottons, G. hirsutum and G. barbadense (Hu et al. 2019) were analyzed. The genome sequence and annotation information of Gossypium species were obtained from Cottongen (Yu et al. 2014) (https://www.cottongen.org). The LBD genes of Arabidopsis were retrieved from TAIR 10 (https://www.Arabidopsis.org). Both local blast and HMMER were used to identify the LBD sequences in cotton. Putative protein sequences were determined by searching for the LOB domain (PF03195.14) downloaded from Pfam protein family database (https://pfam.xfam.org) using Hmmssearch program (E value as the default).
Besides, local blast was also applied to identify the putative sequences with the retrieved *AthLBDs*. Then, the preliminary sequences to be analyzed in cotton were obtained considering both results above. After that, candidate *LBDs* were further filtered based on their conserved domains using online software SMART (https://smart.embl-heidelberg.de) and Pfam (https://pfam.sanger.ac.uk). All these genes identified were renamed according to their genomic locations. Finally, the acquired sequences of cotton were submitted to ExPASy (https://web.expasy.org/protparam) to calculate the physicochemical parameters like molecular weights (MW) and theoretical isoelectric points (pI).

Phylogenetic tree construction, gene structure and motif composition analysis

Multiple sequence alignment of all these LBD proteins were performed using ClustalX (Larkin et al. 2007) with default parameters. A phylogenetic tree of deduced LBD proteins in the four *Gossypium* species and *A. thaliana* was constructed using the Maximum Likelihood method of MEGA7 (Kumar et al. 2016) with 1000 bootstrap replicates based on the JTT matrix-based model. The phylogenetic tree was subsequently decorated using EvolView (https://www.evolgenius.info/evolview). The exon–intron structure was analyzed based on the full-length genome sequence and the corresponding coding sequences of *LBD* genes in cotton. To identify conserved motifs in the identified sequences, the MEME program (https://meme.ncbi.nlm/meme/intro.html) was used to identify conserved motifs. The parameters were employed as the following descriptions: the maximum number of motifs, 15; and the optimum width of each motif, between 6 and 100 residues. The characteristics of *LBD* gene structure with motif composition were visualized by the TBtools.

Chromosomal mapping and synteny analysis of *LBD* genes in allotetraploid cotton

*LBD* genes were mapped on chromosomes using Mapchart software. The Multiple Collinearity Scan toolkit (MCScanX) was used to determine *LBD* gene synten and collinearity. Then, the Circos (Krzywinski et al. 2009) software was applied to express the syntenic relationship of the duplicated genes. To assess the selection pressure for each gene pair, the nonsynonymous substitutions rate (Ka) and synonymous substitution rate (Ks) were calculated using the KaKs_Calculator (Wang et al. 2010). Generally, Ka/Ks > 1 means positive selection, Ka/Ks = 1 indicates neutral selection, and Ka/Ks < 1 signifies negative or purifying selection (Hurst 2002).

Analysis of Cis-acting element in promoters of *GhLBDs* and *GbLBDs*

The upstream sequences (1.5 kb) of the *LBD* sequences were retrieved from the cotton genome sequence based on gene location.

Then, the retrieved promoter sequences were submitted to PlantCARE (https://bioinformatics.psb.ugent.be/webtools/plantcare/html) (Lescot et al. 2002). There were 12 Cis-elements related to phytohormone responses and stress were investigated, such as ethylene-responsive element (ERE), antioxidant response element (ARE), MeJA-responsiveness (CGTCA-motif & TGACG-motif), ABA-responsive element (ABRE), salicylic acid responsiveness (TCA-element), gibberellin-responsive element (P-box), W-box, low-temperature responsiveness (LTR), defense and stress responsiveness (TC-rich repeats), wound-responsive element (WUN-motif), and MYB binding site involved in drought-inducibility (MBS).

Expression patterns in different tissues

The Illumina RNA-seq data were downloaded at the Sequence Read Archive under accession number PRJNA490626 which were provided by Hu et al. (2019). SRA data of 10 tissues (root, stem, leaf, anther, bract, filament, petal, sepal, torus and pistil) as well as expression data of abiotic stress treatments in both *G. hirsutum* and *G. barbadense* were analyzed in this study. The SRA data were converted to fastq data with the SRA Toolkit, the clean data were mapped to the index genomes by TopHat2 program (Kim et al. 2013). The expression levels of the annotated genes were calculated with stringtie program (Pertea et al. 2015). Thereafter, the gene expression levels were visualized with the homogenized method involving log2(TPM + 1) and log2(fold change) using the heatmap program (https://CRAN.R-project.org/package=pheatmap).

Plant materials and treatments

*Gossypium hirsutum* cv. TM-1 was used, which is a standard genetic line of upland cotton (obtained from USDA-ARS, College Station, Texas, USA). Cotton seeds were sown in pots containing the mixed nutrient soil under a 16-h light/8-h dark cycle at 26 °C for 2 weeks. Then, all plants were transferred to Hoagland solution for several days. The seedlings at three-leaf stage were subjected to 100 μM MeJA. The roots of treated plants were harvested at 0, 3, 6, and 12 h. All these samples were immediately frozen in liquid nitrogen and kept at −80 °C for subsequent analysis.
RNA extraction and quantitative real-time (RT-qPCR) analysis

The total RNA of cotton roots were extracted using the RNAprep Pure Plant Kit (Tiangen, Beijing, China). The quantity and quality were determined by a NanoDrop 2000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The cDNA was reverse transcribed with the ReverTra Ace qPCR RT Kit (with gDNA remover) (Toyobo, Osaka, Japan). All the operational procedures followed the manufacturer’s protocols. The qPCR analysis was performed using a LightCycler FastStart DNA Master SYBR Green I kit (Roche, Basel, Switzerland) on a LightCycler96 Real-Time PCR detection system (Roche). For internal reference gene, *UBQ7* was used, which was stably expressed in cotton plants and not affected by treatments and genotypes. All RT-qPCR analyses were performed with three biological replicates and each analysis was carried out in three technical RT-qPCR replicates. Statistical analysis was conducted with biological replicates with mean values of three technique replicates. 2^(-ΔΔCT) method was used to further study the data from real-time PCR amplification (Livak and Schmittgen 2001). The primers were designed using Primer-BLAST (Ye et al. 2012), listed in Supplementary Table S1.

**Results**

**Identification of LBD genes in cotton**

To evaluate the *LBD* gene family in cotton, an HMMER alignment search on the proteome of the *G. hirsutum, G. barbadense, G. raimondii*, and *G. arboreum*, and genome with the hidden Markov model (HMM) profile of the lateral organ boundaries domain (PF3195) was performed. Then, all these retrieved sequences were verified by the Pfam and SMART. The molecular weight was also considered to identify the *LBD* genes. Sequences without a typical LOB domain or with a molecular weight outside of the 11–60-kDa range were excluded. Finally, the confirmed *LBD* genes in *G. raimondii* (GrLBDs), *G. arboreum* (GaLBDs), *G. hirsutum* (GhLBDs), and *G. barbadense* (GbLBDs) amounted to be 68, 62, 131, and 128, respectively (Supplementary Table S2). All these genes were named based on their chromosomal locations. The *LBD* genes in tetraploid cotton (*G. hirsutum* and *G. barbadense*) were almost twice the number of diploid cotton (*G. raimondii* and *G. arboreum*). Therefore, it was proven again that allotetraploid cotton was formed by inter-genomic hybridization of A-genome diploids and D-genome diploids, and followed by chromosome doubling (Paterson et al. 2012).

Gene characteristics, including the size of the protein sequence (amino acid), the protein molecular weight (MW), isoelectric point (pI), and the subcellular localization were listed in Supplementary Table S2. It was observed that the length of the GbLBD proteins ranged from 106 to 521 amino acids, and GhLBD was from 137 to 524 amino acids. The average length of GhLBDs was much longer than that of GbLBDs. The average MW for GhLBDs was 23,591.72 Da, and that of GbLBDs, GaLBDs, and GrLBDs were 23,710.96 Da, 23,407.13 Da, and 23,056.92 Da, respectively. The PI value of LBDs in four cotton species above showed similar statistical results (about 7.3). The predicted subcellular location of LBD proteins suggested that nuclear was the only area where cotton LBDs implements their biological functions as most of the LBDs in cotton were located in the nuclear region except for the none results.

**Phylogenetic analysis of the cotton LBD gene family**

To detect the evolutionary relationships of LBD proteins among four cotton species, an unrooted phylogenetic Maximum Likelihood (ML) tree was constructed using full-length amino acid sequences of these identified genes. The phylogenetic analysis (Fig. 1) revealed that the LBDs could be divided into two main clades corresponding to class I and II of *Arabidopsis* as defined by Mangeon et al. (2012). There were 330 and 59 LBD genes involved in class I and II of the fore mentioned cotton species. Therefore, the quantity of LBD genes in class I was fifth as big as that in class II of each cotton species, approximately. Class II consisted of 20, 19, 10, and 10 LBD genes separately in *G. hirsutum, G. barbadense, G. raimondii*, and *G. arboreum*. According to previous studies of traditional classification and distribution of LBDs (Coudert et al. 2013), class I could be further grouped into five sub-classes (1A–1E) based on tree topology and branch lengths. Each of the subgroups contained proteins from both diploid and allotetraploid cotton species. Subgroup 1B was the smallest in class I with 8, 7, 4, and, 4 genes in *G. hirsutum, G. barbadense, G. raimondii*, and *G. arboreum*, respectively. While the subgroup 1C was the largest with 31, 31, 16, and 15 LBD genes in the same cotton species. The LBD genes in tetraploid cotton were almost twice as many as diploid cotton in each subgroup. Each clade comprised members of all the species analyzed, which suggested that LBDs were homologous with those in *Arabidopsis*. According to previous studies (Majer and Hochholdinger 2011), many LBD genes in *Arabidopsis* (class I) were involved in lateral organ development, including AthLBD13, AthLBD3, AthLBD12, AthLBD6, AthLBD36 and so on. Genes such as AthLBD16, AthLBD18, and AthLBD29 participated in the auxin signal transduction cascade that lead to the formation of lateral roots in *Arabidopsis*. However, class II LBD genes, AthLBD37, AthLBD38 and AthLBD39 were
involved in metabolism. \textit{AtLBD37} was down-regulated by gibberellin and up-regulated by DELLA proteins (Majer and Hochholdinger 2011). The phylogenetic analysis indicated that the homologs in cotton might have similar biological functions as \textit{AthLBDs}.

**Gene structure and motif composition analysis**

To further explore the possible evolutionary relationship of LBD proteins in allotetraploid cotton (\textit{G. hirsutum} and \textit{G. barbadense}), phylogenetic trees of both GhLBDs and GbLBDs were represented by stars in red, green, yellow, black, and white, respectively.
were constructed (Figs. 2a, 3a). Conserved motifs in these proteins were identified using MEME software. Here, 15 conserved motifs (named motif 1 to motif 15) were identified in both GhLBDs and GbLBDs, and the results were represented in schematic diagrams (Fig. 2b, Fig. 3b). The sequence information for each motif of G. hirsutum and G. barbadense was provided in Supplementary Table S3. LBD members within the same clade were usually found to share a similar motif composition, which was conspicuous in class II. All the proteins belonged to class II only contained motif 3 in G. barbadense. While in G. hirsutum, motif 3, motif 13 and motif 15 were existed in LBD proteins of class II. As for the LBD proteins of class I, the number of motifs was two to four in G. hirsutum and two to five in G. barbadense. The different compositions of the motifs may indicate functional diversity. The subgroups of GbLBDs in class I showed similar motif arrangements, revealing that the protein architecture was conserved within a specific subfamily. The functions of most of these conserved motifs remain to be elucidated. Then, comparative analysis of intron–exon structures was performed by the alignment of genomic DNA with full-length cDNA (Figs. 2c, 3c). The gene structure patterns were similar in the two cotton species. GhLBDs and GbLBDs possessed 1 – 13 exons and could be divided into four categories based on exon number. Therefore, in G. hirsutum, 27 (20.6%) LBD genes had 1 single exons, 93 (71%) LBD genes possessed 2 exons, 10 (7.6%) LBD genes owned 3 exons and only 1 (0.8%) LBD gene had more than 3 exons. In G. barbadense, 93 genes (73%) only had 1 exon, 25 genes (20%) had 2 exons, 9 genes (7%) possessed 3 exons, and none had more than 3 exons. Notably, the intron–exon structures were consistent with the alignment clusters of GbLBDs, indicating that exon–intron structure and the phylogenetic relationship among GhLBDs were highly correlated.

**Chromosomal distribution and synteny analysis of GbLBD genes**

Based on the public information of G. hirsutum and G. barbadense genome, the location of genes and the length of
Chromosomes were available to analyze the chromosomal distribution (Fig. S1, Fig. S2). High similarity was found in the chromosomal distribution patterns of these two cotton species. The \( \text{LBD} \) genes were unevenly arranged on chromosomes. However, no \( \text{GbLBDs} \) were found on chromosome A02 and A04 in both \( \text{G. hirsutum} \) and \( \text{G. barbadense} \). There were 131 \( \text{GhLBDs} \) distributed throughout the 24 chromosomes comprising 66 located on the At subgenome and 65 located on the Dt subgenome. For \( \text{G. barbadense} \), 63 \( \text{LBD} \) genes were located on the At subgenome and 65 located on the Dt subgenome. The majority of \( \text{GhLBDs} \) and \( \text{GbLBDs} \) was located on the proximate or the distal ends of the chromosomes. In the two allotetraploid cotton species, the homologous chromosome pair A11 and D11 separately contained the largest gene clusters in At and Dt sub-genome. The chromosome A01 and D01 had the minimal \( \text{LBD} \) gene numbers in its sub-genomes. There were no positive correlations between the length of chromosome and the \( \text{LBD} \) gene number.

According to previous reports, gene duplications are considered to be one of the primary driving forces in the evolution of genomes and genetic systems (Moore and Purugganan 2003), and segmental and tandem duplications have been suggested to represent two of the main causes of gene family expansion in plants (Cannon et al. 2004). Segmental duplications multiple genes through polyploidy followed by

**Fig. 3** Phylogenetic relationships, architecture of conserved protein motifs and gene structure in \( \text{LBD} \) genes of \( \text{G. barbadense} \). a The phylogenetic tree was constructed based on the full-length sequences of GbLBD using MEGA 7 software. b The motif composition of LBD proteins. The 15 motifs were displayed in different colored boxes. The sequence information for each motif was provided in Supplementary Table S3. c Exon–intron structure of \( \text{GbLBDs} \). Green boxes indicated exons; black lines indicated introns.
chromosome rearrangements (Yu et al. 2005). It occurs most frequently in plants, because most plants are diploidized polyploids and retain numerous duplicated chromosomal blocks within their genomes (Cannon et al. 2004). Tandem duplications were characterized as multiple members of one family occurring within the same intergenic region or in neighboring intergenic regions (Ramamoorthy et al. 2008).

According to a previous study, a chromosome region containing two or more genes within 200 kb could be defined as a gene cluster (Holub 2001). In the present study, blast and MCScanX were used to further investigate the expanded mechanism of LBD gene family by conducting genome synteny analysis (Fig. 4). Eight GhLBDs, GhLBD22/23, GhLBD29/30, GhLBD88/89, and GhLBD95/96 were clustered into three tandem duplication event regions on chromosome A06, A08, D06, and D08, respectively. However, for GbLBDs, there were three tandem duplication events, GbLBD28/29, GbLBD85/86, and GbLBD92/93, on chromosome A08, D06, and D08, respectively. While segmental duplication accounted for a large part of the GhLBDs and GbLBDs, indicating that compared to tandem duplication, the segmental duplication played a predominant driving force in LBD gene family evolution. In addition, the segmental duplication events were mainly occurred among different subgenomes. To decipher the evolutionary relationships of LBD genes, intergenomic synteny analysis was further performed among different cotton species (Fig. 5). There were 60, 58, 49, and 63 collinear gene pairs between G. hirsutum and G. arboreum, G. barbadense and G. arboreum, G. hirsutum and G. raimondii, and G. barbadense and G. raimondii, respectively. Therefore, it is illustrated that LBD genes in G. raimondii and G. barbadense possess closer evolutionary relationships. Thus, the current results provided evidences that LBD genes may have experienced some genomic rearrangements during polyploidization. To better understand the evolutionary constraints acting on LBD genes functional divergence after gene duplication, non-synonymous to synonymous substitution ratio (Ka/Ks) for each pair of duplicated LBD genes were calculated (Supplementary Table S4). There were 15 duplicated GhLBDs pairs predicted to undergo positive selection with the Ka/Ks < 1. As for the GbLBDs, a majority of Ka/Ks ratio was more than 1 except for 7 pairs of genes (Ka/Ks < 1), suggesting that the LBD gene family in G. hirsutum and G. barbadense might have experienced strong purifying selective pressure during evolution.

**Cis-element analysis of LBD genes in allotetraploid cotton**

In this current study, Cis-elements are responsive to corresponding elicitors to regulate the expression of genes. In this study, a 1.5-kb region upstream from the start codon of each LBD gene was selected to investigate putative...
Cis-elements involved in the mediation of gene expression using the PlantCARE server. Some cis-elements predicted to be involved in phytohormone responses and stress were found in *GhLBDs* and *GbLBDs* (Fig. S3). In *G. hirsutum*, 95 *GhLBD* gene promoters processed the ethylene-responsive element (ERE), and 90 *GhLBD* genes had at least 1 antioxidant response element (ARE). There were 87 *GhLBDs* processed the elements involved in the MeJA-responsiveness (CGTCA-motif and TGACG-motif), 71 *GhLBDs* had ABA-responsive element (ABRE), 53 *GhLBDs* had the cis-acting element involved in salicylic acid responsiveness (TCA-element), and 35 *GhLBDs* had gibberellin-responsive element (P-box). The putative Cis-elements exposed to stress were less as compared to hormones. W-box is a cis-acting element recognized by WRKY transcription factors, involved in pathogen related responses (Eulgem et al. 1999). Some of these W-boxes were also responsive to ethylene (Miao et al. 2004). 73 *GhLBDs* processed the W-box. The remaining elements related to stress, like cis-acting element involved in low-temperature responsiveness (LTR), defense and stress responsiveness (TC-rich repeats), wound-responsive element (WUN-motif), and MYB-binding site involved in drought inducibility (MBS), were existed in about 40 *GhLBD* genes. However, as compared to *G. hirsutum*, fewer cis-elements were found in the promoter regions of *GbLBDs*. That was 90 contained ARE, 88 possessed ERE, 67 had ABRE, 57 had W-box, and 55 possessed TCA-element. Many of the *GhLBDs* also harbored cis-elements such as TGACG-motif (41 *GhLBDs*), CGTCA-motif (40 *GhLBDs*), TC-rich repeats (38 *GhLBDs*), WUN-motif (37 *GhLBDs*), MBS (37 *GhLBDs*), and LTR and P-box (35 *GhLBDs*).

These results suggested that *LBD* genes might play an important role in the phytohormone signal transduction and stress response in both *G. hirsutum* and *G. barbadense*.
Tissue-specific expression patterns of \textit{GhLBDs} and \textit{GbLBDs}

To gain further insights into the potential developmental roles of \textit{LBD} genes in allotetraploid cotton, the published transcriptomic data (Hu et al. 2019) were investigated to analyze the expression of cotton \textit{LBD} genes in various tissues, including vegetative (root, stem, and leaf) and reproductive (anther, bract, filament, petal, sepal, torus, and pistil) tissues. According to the heat map (Fig. 6), those expression characteristics of \textit{GhLBDs} and \textit{GbLBDs} in different tissues were quite various. In \textit{G. hirsutum}, a large part of \textit{GhLBDs} witnessed low expression levels in leaf, while the majority showed high transcriptional levels in stem and root, indicating that they had a conserved functional role in root and stem development. Genes ranging from \textit{GhLBD87} to \textit{GhLBD106} were highly expressed in both root and stem, and among them only one gene, \textit{GhLBD47}, was in class II. The situation was more harmonious in anther, bract, filament, petal, sepal, torus, and pistil, where the expression level of \textit{GhLBDs} was similar among these reproductive tissues, except for the anther and filament with a higher expression level. It was confirmed with the previous finding that \textit{LBD} protein family played pivotal roles in pollen development (Thatcher et al. 2012b). Moreover, a majority of \textit{GhLBDs} predominantly expressed in the vegetative tissues might express with a relatively low level in those reproductive tissues and vice versa. For \textit{LBD} genes in \textit{G. barbadense}, the situation was quite similar. \textit{GbLBDs} were also most abundant in root and stem, followed by these reproductive tissues and leaf.

Expression profiles of cotton \textit{LBD} genes under abiotic stress and hormone treatment

The \textit{LBD} gene expression profiles under abiotic stress were checked to investigate their potential functions in \textit{G. hirsutum} and \textit{G. barbadense} using transcriptome data (Hu et al. 2019). According to the results of \textit{GhLBDs} expression patterns (Fig. 7a), more than half of them were induced by PEG treatment and a small part of them were down-regulated by PEG. These genes responding to PEG also suggested that the influences may happen immediately, and 12 h and 24 h past treatment may play a more important role in this process. Treatments such as hot, cold and salt played insignificant effects on \textit{GhLBDs} expression with a small amount of genes differently expressed. Among these genes, \textit{GhLBD123} was
up-regulated by heat, and reached a peak at 24 h past treatment, \textit{GhLBD125} was induced by cold and the expression level reached a peak at 12 h past treatment. Nevertheless, the situation was quite different in \textit{G. barbadense} (Fig. 7b). Great majority of \textit{GbLBDs} did not seem to express differently under the abiotic stresses, even under PEG treatment. 

MeJA, belonging to a class of cyclopentanone compounds, is a naturally and ubiquitously occurring phytohormone involved in signal transduction pathway and plant response to environmental stressors (Santino et al. 2013), and \textit{LBD} genes played roles in response to MeJA treatment (Thatcher et al. 2012b). The present results showed that a majority of \textit{GhLBDs} processed the cis-elements involved in the MeJA-responsiveness, so these \textit{GhLBD} genes might have functions in response to MeJA treatment. To verify these predictions, the expression changes of \textit{GhLBDs} were monitored by RT-qPCR at different time (0, 3, 6, and 12 h) under MeJA treatment. Finally, 22 genes covering almost all the subfamilies were picked up to be analyzed through RT-qPCR. Nine genes, \textit{GhLBD01}, \textit{GhLBD07}, \textit{GhLBD12}, \textit{GhLBD14}, \textit{GhLBD38}, \textit{GhLBD54}, \textit{GhLBD85}, \textit{GhLBD91}, \textit{GhLBD106}, and \textit{GhLBD123}, with higher relative expression level were analyzed (Fig. 8). Almost all these nine genes reached the highest expression level at 12 h except for \textit{GhLBD91}, which increased to the maximum level at 6 h. Additionally, \textit{GhLBD123} showed strongest up-regulation compared to the other eight \textit{GhLBDs}.

**Discussion**

\textit{LBD} genes comprise a plant-specific transcription factor family, which had been proven to have essential roles in the regulation of lateral organ development and metabolic processes, such as anthocyanin and nitrogen metabolism in higher plants (Iwakawa et al. 2002; Shuai et al. 2002; Majer and Hochholdinger 2011). Comprehensive analyses of this gene family had been conducted in many species such as \textit{Arabidopsis} (Liu et al. 2005), \textit{Eucalyptus grandis} (Lu et al. 2018), \textit{Brassica rapa} (Huang et al. 2018), grapevine (Grimplet et al. 2017), soybean (Yang et al. 2017), and \textit{Camellia sinensis} (Teng et al. 2018). However, no systematic study of \textit{LBD} genes has been conducted in allotetraploid cotton. The \textit{de-novo} assembly of the genomes by integrating data from Illumina PCR-free short-read sequencing, 10×Genomics sequencing, Hi-C, and optical and super-dense genetic maps have resulted in substantial improvements to the contiguity and accuracy of assembly, with a notable improvement.
in the assembly of centromeres (Hu et al. 2019). In recent years, higher quality de-novo-assembled genomes of the two allotetraploid species (Hu et al. 2019) and G. arboreum (Du et al. 2018) were published, which will enable a new era of genome-wide analyses of cotton gene families. In the current study, 68, 62, 131, and 128 candidate LBD genes were systematically identified in G. raimondii, G. arboreum, G. hirsutum, and G. barbadense, respectively. The number of LBD genes in the two tetraploid species was almost the sum of those in the two ancestral diploid progenitors, which provided evidence that allotetraploid cottons may have appeared through hybridization and subsequent polyploidization events between the A- and D-subgenome progenitors (Lynch and Conery 2000). The characteristic LOB domain comprises a C-block containing four cysteine with spacing (CX2CX6CX3C) required for DNA-binding activity, a Gly-Ala-Ser (GAS) block and a leucine zipper-like coiled-coil motif (LX6LX3LX6L) responsible for protein dimerization (Shuai et al. 2002; Majer and Hochholdinger 2011). The LBD genes in Gossypium could be divided into two main subfamilies, class I and class II, according to the structure of LOB domain. Most of LBD genes belong to class I. 20 (15.3%), 19 (14.8%), 10 (16.1%), and 10 (14.7%) LBD genes were classified as class II in G. hirsutum, G. barbadense, G. arboreum, and G. raimondii, respectively. Members of class I could be grouped into five sub-classes (1A–1E) based on tree topology and branch lengths. The intron–exon structures and motif compositions of GhLBDs

Fig. 8 Relative expressions of 9 GhLBD genes under MeJA treatment based on RT-qPCR. Data were normalized to UBQ7 gene. The values are means ± standard deviation (SD) of three replicates. The details of the primer sequences were listed in Supplementary Table S1
and GbLBDs were consistent with the alignment clusters of these LBD genes. Finally, many similarities could be found between GhLBDs and GbLBDs in the gene structure, which indicated that LBD genes in the two allotetraploid cotton species might serve close functions, and they might have the same origin.

Gene duplication generates functional divergence, which is essential for environmental adaptability and speciation (Conant and Wolfe 2008). During evolution, duplicated gene pairs can experience functional divergence resulting in neo-functionalization, sub-functionalization, or non-functionalization (Prince and Pickett 2002). Gene duplication is the most commonly evaluated mechanism for gene family expansion (Cannon et al. 2004). It is widely accepted that the homologous genes created by a duplication inside a genome might have occurred through various mechanisms, including (i) single gene duplications (tandem repeats, clustering, short range duplications, and transposition); (ii) reverse transcript insertion, (iii) duplications of chromosomal blocks (translocations); (iv) duplications of single chromosomes (aneuploidy); or (v) duplication of the whole genome (polyploidy) (Prince and Pickett 2002). To reveal the expansion mechanism of the LBD gene family, intragenomic and intergenomic duplication data of these cotton species were filtered by MCScanX. According to the current intragenomic analysis results, only several tandem duplication events existed in both G. hirsutum and G. barbadense, which revealed that segmental duplication played a predominant driving force in LBD gene family evolution. In both G. hirsutum and G. barbadense, the number of homologous LBD genes in At and Dt subgenomes was identical, thus we can infer that the translocations and reverse transcript insertion rarely occurred in the generation of cotton LBD gene family. The fates of duplicated genes are diverse (Charon et al. 2012). First, when the original function is maintained in both copies, this leads to functional redundancy. Another possibility is that both copies of a gene share the ancestral function, gradually developing partially different functions, the so-called sub-functionalization. In addition there is also a possibility, although rare, that one of the two copies evolves, acquiring a new function. Finally, intermediate evolutionary stages between sub- and neo-functionalization in which only genes critical for plant growth and development are retained. The expression level of many duplicated GhLBDs and GbLBDs was quite similar (Fig. 6), which suggested that the duplicated LBD genes in G. hirsutum and G. barbadense may lead to functional redundancy mainly.

Synteny analysis in different species is a way to provide insights to their evolution and relationship. In our study, the synteny analysis of LBD genes between diploid and allotetraploid cotton was conducted (Fig. 5). Only two pairs of GhLBD and GbLBD genes were within the intraspecific rearrangement blocks between G. hirsutum and G. raimondii, as well as G. barbadense and G. raimondii, which were far less than that located in rearrangement blocks between G. hirsutum and G. arboream, or G. barbadense and G. arboream. The result was consistent with the conclusion that the A-derived subgenomes have apparently been more active than D-derived subgenomes during evolution (Yang et al. 2019). To illuminate the divergence after gene duplication, the synonymous substitution rate (Ks) and nonsynonymous substitution rate (Ka) of duplicated LBD genes from G. hirsutum and G. barbadense were calculated. The current results suggested that GhLBDs and GbLBDs were under strong purifying selection, and the LBD genes from G. hirsutum had experienced stronger positive selection than that from GbLBDs.

LBD proteins orchestrate a lot of plant developmental processes and respond to environmental stimuli, especially play the essential roles in the regulation of lateral organ development and metabolic processes. Several LBD genes, such as LBD16, LBD13, LBD18, and LBD29 have been proven to play important roles in regulating lateral root development in Arabidopsis (Cannon et al. 2004; Goh et al. 2019; Cho et al. 2019; Lee et al. 2019a). LBD18 controls lateral root development via inhibition of LBD18 DNA-binding activity (Lee et al. 2013). Thus, it is reasonable to predicate that the LBD genes may play roles in plant resistance as the root architecture plasticity is an important feature in both abiotic and biotic responses. Generally speaking, the main root may be affected by the overgrowth of lateral roots, which may reduce the ability of plant to extract water and nutrients. Too many lateral roots spread in the soil will increase the possibility of being attacked by pathogens. Some previous work has proven these predications. LBD1 in Medicago controls root architecture under salt stress via the inhibition of primary root growth and maintenance of lateral root emergence (Ariel et al. 2010). AtLBD20, a root-specific LBD gene in Arabidopsis, is a negative regulator of susceptibility to the root-infecting fungal pathogen Fusarium oxysporum (Thatcher et al. 2012b). LBD genes are crucial molecular targets for plant pathogen invasion due to their transcriptional regulation of a range of downstream genes (Xu et al. 2016).

It has been demonstrated that LBD proteins make differences in response to phytohormone signaling. In Arabidopsis, LBD40 was reported to be down-regulated by gibberellin (Zentella et al. 2007), LBD20 was proposed playing a role in JA-response (Thatcher et al. 2012a). In Camellia sinensis, LBD genes had differential functions in response to the treatments with gibberellic acid (GA3), salicylic acid (SA), MeJA, ABA, and ethephon (ETH) (Teng et al. 2018). Considering that LBD genes are conservative in structure and function in different species (Wang et al. 2013; Yang et al. 2016), and the results of Cis-acting
element in promoters of GhLBDs and GbLBDs led us to draw the predication that the LBDs in cotton were involved in phytohormone responses and stress resistance. In this study, expression profiles of GhLBDs and GbLBDs responding to abiotic stress were studied using the published transcriptome data. More LBD genes in upland cotton expressed differently after the hot, cold, salt, and PEG treatments. Furthermore, expression levels of GhLBD were widely induced under PEG, which was hard to find in G. barbadense. Therefore, it indicated that GhLBD genes might be involved in cotton adapting to drought especially, while GbLBDs might play an unimportant role in improving cotton abiotic stress tolerance. Several GhLBDs were selected by the expression level in different tissues (Fig. 6) as well as the results of Cis-element. According to the current study, GhLBDs in response to MeJA showed different expression regulation, seven genes witnessed up-regulated at 12 h. Especially, for the GhLBD123, the expression level owned huge increase (Fig. 8). These results demonstrated that GhLBD genes had differential functions in response to hormone like MeJA and water-deficit stress. The present work not only established a foundation for the further functional analyses of LBD genes in cotton, but can also provide valuable information for potential candidate genes involved in plant growth, development, and stress tolerance.

In conclusion, comparisons of gene structure, chromosomal distribution, phylogenetic relationship, synteny characteristic as well as cis-element traits between GhLBDs and GbLBDs were performed in the present study, which largely enriched our knowledge of the cotton LBD gene family. In addition, expression and functional characterization indicated the involvement of GhLBDs in abiotic stresses responses, especially in drought condition. It also indicated the differential functions of GhLBD genes in response to hormones like MeJA. These findings could provide valuable information for subsequent elucidation roles of LBD genes.

**Author contribution statement** SZ and JY designed the research. JY and QX conducted experiments. CL and JY performed the data analyses. JY wrote the manuscript. All authors read and approved the manuscript. SZ and JC contributed substantially to revisions.

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