Genome-wide identification of CBL family and expression analysis of *CBLs* in response to potassium deficiency in cotton (#17551)

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Genome-wide identification of CBL family and expression analysis of *CBLs* in response to potassium deficiency in cotton

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Calcineurin B-like (CBL) proteins, as calcium sensors, play pivotal roles in plant responses to diverse abiotic stresses and in growth and development through interacting with CBL-interacting protein kinases (CIPKs). However, knowledge about CBLs functioning in upland cotton is scarce at present. Here, we conducted a genome-wide survey and identified 13, 13 and 22 CBL genes in the progenitor diploid *G. arboreum* and *G. raimondii*, and their cultivated allotetraploid *G. hirsutum*, respectively. Analysis of chromosomal location, physical properties, conserved domain and phylogeny indicated rather conserved nature of CBLs among the three cotton species. Moreover, cotton CBLs have closer genetic evolutionary relationship with cocoa than with other plants. Most CBL genes underwent evolution under purifying selection in cotton. Additionally, nearly all cotton CBL genes were expressed in the root, stem, leaf, flower and fiber. Many *CBLs* were preferentially expressed in the flower while several *CBLs* were mainly expressed in roots. Expression patterns of GhCBL genes in response to potassium deficiency were also studied. Most *GhCBLs* were moderately upregulated in roots after treatments with low-potassium stress. Besides, yeast two-hybrid experiments indicated that GhCBL1-2, GhCBL1-3, GhCBL4-4, GhCBL8, GhCBL9 and GhCBL10-3 interacted with GhCIPK23, respectively. Our results provided a comprehensive view of the *CBLs* and valuable information for cotton CBLs, which will help researchers to further investigate the roles and functional mechanisms of the CBLs in cotton.

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ABSTRACT

Calcineurin B-like (CBL) proteins, as calcium sensors, play pivotal roles in plant responses to diverse abiotic stresses and in growth and development through interacting with CBL-interacting protein kinases (CIPKs). However, knowledge about CBLs functioning in upland cotton is scarce at present. Here, we conducted a genome-wide survey and identified 13, 13 and 22 CBL genes in the progenitor diploid *G. arboreum* and *G. raimondii*, and their cultivated allotetraploid *G. hirsutum*, respectively. Analysis of chromosomal location, physical properties, conserved domain and phylogeny indicated rather conserved nature of CBLs among the three cotton species. Moreover, cotton CBLs have closer genetic evolutionary relationship with cocoa than with other plants. Most CBL genes underwent evolution under purifying selection in cotton. Additionally, nearly all cotton CBL genes were expressed in the root, stem, leaf, flower and fiber. Many *CBLs* were preferentially expressed in the flower while several *CBLs* were mainly expressed in roots. Expression patterns of GhCBL genes in response to potassium deficiency were also studied. Most *GhCBLs* were moderately upregulated in roots after treatments with low-potassium stress. Besides, yeast two-hybrid experiments indicated that GhCBL1-2, GhCBL1-3, GhCBL4-4, GhCBL8, GhCBL9 and GhCBL10-3 interacted with GhCIPK23, respectively. Our results provided a comprehensive view of the *CBLs* and valuable information for cotton CBLs, which will help researchers to further investigate the roles and functional mechanisms of the CBLs in cotton.

Keywords Cotton; calcineurin B-like proteins (CBLs); gene family; phylogeny; gene

expression

INTRODUCTION

Calcium ion (Ca^{2+}) is a ubiquitous secondary message in plants. It plays pivotal roles in mediating and regulating many fundamental growth and developmental processes and in response to various environmental stimuli (Luan, 2009; Kudla, Batistič & Hashimoto, 2010; Sarwat et al., 2013). The Ca^{2+} signals are primarily perceived by some Ca^{2+} sensors including Ca^{2+} dependent protein kinases, calmodulins and calcineurin B-like proteins (CBLs), and then are transmitted by these sensors to downstream targets to initiate diverse cellular responses (Luan, 2009; Kudla, Batistič & Hashimoto, 2010; Sarwat et al., 2013).

CBLs are a kind of proteins sharing sequence similarity with the B subunit of calcineurin B in yeast and neuronal calcium sensors in animals (Kudla et al., 1999). Each CBL has at least three EF domains and Ca^{2+}-binding sites (Mohanta et al., 2015; Mao et al., 2016). CBLs have been addressed to relay Ca^{2+} signals through interacting with and activating the CBL-interacting protein kinases (CIPKs). Moreover, CBL-CIPK has been demonstrated to serve as an essential signaling network regulating plant responses to multiple abiotic stresses such as salinity, K^+ deficiency, excess of Mg^{2+} and drought (Sanyal, Pandey & Pandey, 2015; Thoday-Kennedy et al., 2015; Mao et al., 2016). It also modulates growth and development, absorption and/or transport of nitrate, ammonium and iron, sustaining of H^+ homeostasis, and transduction of reactive oxygen species signals in plants (Sanyal, Pandey & Pandey, 2015; Thoday-Kennedy et al., 2015; Mao et al., 2016).

In *Arabidopsis*, 10 genes (*CBL1-10*) encoding CBL proteins have been found (Kolukisaoglu et al., 2004). *CBL1* and/or *CBL9* were reported to positively regulate the uptake and/or transport of K^+, NO_3^-, NH_4^+, aluminum and iron, and the promotion of stomatal opening (Li et al., 2006; Xu et al., 2006; Ho et al., 2009; Mao et al., 2016; Tian et al., 2016; Ligaba-Osena et al., 2017; Straub, Ludewig & Neuhäuser, 2017). CBL1 and CBL9 also affect abscisic acid (ABA)-induced stomatal closure (Pandey et

al., 2004; Cheong et al., 2007), and ROS signaling (Drerup et al., 2013). *CBL2* plays a negative role in the activation of plasma membrane (PM) H^+-ATPase (Fuglsang et al., 2007). Moreover, *CBL2* and *CBL3* are cooperatively implicated in sequestering Mg^{2+} and modulation of pollen germination and tube growth (Steinhorst et al., 2015; Tang et al., 2015). *CBL3* are also engaged in K^+ distribution and translocation (Liu et al., 2013). *CBL4* was proved to be a crucial regulator for excluding Na^+ and translocation of AKT2 (*Arabidopsis* K^+ transporter 2) from endoplasmic reticulum to PM (Held et al., 2011). *CBL10* is involved in enhancing salt tolerance, stimulating K^+ absorption, and modulating GTPase activity (Kim et al., 2007; Ren et al., 2013; Cho et al., 2016). In cotton, *GhCBL2* and *GhCBL3* appear to modulate fiber elongation (Gao et al., 2008). Besides, many *CBLs* in other plant species also play important parts in regulating the responses to various abiotic stress as well as growth and development (Li et al., 2014a; Thoday-Kennedy et al., 2015).

In recent years, multiple CBL gene families have been identified at genome-wide levels in rice, maize, wheat and other plants (Kolukisaoglu et al., 2004; Zhang et al., 2014; Sun et al., 2015; Li et al., 2016; Zhang et al., 2016). Some conserved domains such as EF-hands, myristoylation and palmitoylation sites were discovered in CBLs (Kolukisaoglu et al., 2004; Mohanta et al., 2015). The expression patterns of many *CBL* genes were also investigated in different tissues and in response to various abiotic stresses in plants (Mohanta et al., 2015; Zhang et al., 2016). These findings lay the foundation for people to further explore the functional mechanisms of CBLs in plants. However, to date, knowledge about genomics and evolutionary information of CBLs in upland cotton is limited.

Cotton is an essential fiber crop worldwide. It supplies lint for the textile industry. However, cotton growth and development are severely threatened by diverse abiotic stresses such as drought, salinity and potassium starvation (Allen, 2000). Therefore, enhancing stress tolerance of cotton cultivars is one of most important strategies for us to improve their productivity and quality. Potassium is a vital macronutrient for plants, especially for cotton. Potassium shortage in soil seriously affects the yield and quality of cotton (Oosterhuis et al., 2013). Moreover, it has been demonstrated that K^+ uptake

is controlled by CBLs through interacting with CIPK23 in *Arabidopsis* and rice under potassium deficiency (Li et al., 2014a; Mao et al., 2016). Accordingly, it needs urgently to determine which and how CBLs modulate K^+ absorption in cotton. In this report, genome-wide and comprehensive analyses of the CBL family in *G. arboreum*, *G. Raimondii* and *G. Hirsutum* were conducted. The expression patterns of *CBLs* in tissues and in response to potassium deficiency were monitored. These data will provide a basis for further investigating the functions of CBLs in cotton in the future.

MATERIALS AND METHODS

Identification of CBL family in cotton

The protein sequences of 10 *Arabidopsis* CBLs were applied to search the sequence databases of *G. arboreum* (BGI-CGB v2.0 assembly genome), *G. Raimondii* (JGI assembly v2.0 data.) and *G. Hirsutum* (NAU-NBI v1.1 assembly genome) (www.cottongen.org), respectively. The BLAST program with default parameters (E-value < e^{-10}) was used. The full-length amino acid sequences of CBL proteins were aligned using ClustalW software through pairwise and multiple alignment with default parameters. The cotton CBL domains were identified by Pfam and HMMER software. Genes with questionable CBL annotations (i.e. having a typical CBL domain but low E-value or low coverage of a domain) were manually reanalyzed.

Analysis of *CBLs* family

The properties of CBL proteins were analyzed using online tools ExPaSy (http://web.expasy.org/protparam/). The subcelluler localizations of all cotton CBLs were examined according to the information from the website http://www.csbio.sjtu.edu.cn/bioinf/Cell-PLoc/. The locations of cotton *CBLs* in chromosomes were calculated by MapInspect software. Structures of the CBLs were determined by GSDS (http://gsds.cbi.pku.edu.cn/). The conserved domains in CBLs were affirmed by SMART (smart.embl-heidelberg.de). The motifs in cotton CBLs were analyzed by MEME (meme-suite.org/tools/meme). The nucleotide substitution parameter Ka (non-synonymous) and Ks (synonymous) values data were calculated

by PAML program. The tandem duplications in the gene family were measured by MCscanx soft.

Phylogenetic analysis of CBLs

The databases were downloaded from the websites for various plant species including *Arabidopsis thaliana* (http://www.arabidopsis.org/), *Oryza sativa* (http://rapdb.dna.affrc.go.jp), *Vitis vinifera* (http://www.genoscope.cns.fr/spip/Vitis-vinifera-e.html), *Populus trichocarpa* (http://www.phytozome.net/poplar), *Glycine max* (http://www.phytozome.net/soybean), *Theobroma cacao* (http://cocoagendb.cirad.fr), *Carica papaya* (http://asgpb.mhpcc.hawaii.edu) and castor bean (http://castorbean.jcvi.org). Phylogenetic trees were generated based on the alignment results using the neighbor joining method (Neighbor-Joining, NJ) and 1,000 bootstrap trials with the Clustal W tool and MEGA 5.0 software (http://www.megasoftware.net/).

Expression analysis of *GhCBL* genes in tissues and in response to potassium deficiency

For measuring the expression of *GhCBLs* in tissues, roots, stems and leaves were collected from 20-day-old *Gossypium hirsutum* TM-1 plants normally grown in soil. Flowers were isolated in the morning at the first day of anthesis. The fibers were obtained from the ovules. For monitoring the expression of *GhCBLs* in responding to potassium deprivation, cotton plants grew in clean small pebbles (watered by liquid 1/2 MS medium) in a growth chamber (day/night temperature cycle of 28°C/26°C, 14 h light/10 h dark, and about 50% relative humidity) for 3 weeks. Then, the plants were watered with K^+-lacked liquid 1/2 MS medium (KNO_3 was replaced by NH_4NO_3 and KH_2PO_4 was replaced by $NH_4H_2PO_4$) for 0 h, 6 h, 2 d and 5 d, respectively. The cotton roots were collected, immediately frozen in liquid nitrogen and stored at -70°C. Total RNA of samples was extracted using RNA Pure Plant Kit's protocol (TIANGEN Company). The purity of RNA was examined using a Nanodrop2000 nucleic acid

analyzer. The A260/280 ratio for each RNA sample was about 2.0. Then, total cDNA was synthesized using M-MLV reserve transcriptase synthesis system (Promega, USA).

Quantitative real-time RT-PCR (qRT-PCR) experiments were performed using the cDNA, SYBR Green Master mix, the specific primers of *GhCBL* genes (Table 1), and an ABI 7500 real-time PCR system. *GhUBQ7* was used as the internal control. At least three biological replicates were carried out.

Table 1 Gene primers used for quantitative real-time RT-PCR experiments

Genes	AGI number	Forward primers (5′-3′)	Reverse primers (5′-3′)
GhUBQ7	Gh_A11G0969	GAAGGCATTCCACCTGACCAAC	CTTGACCTTCTTCTTCTTGTGCTTG
GhCBL1-1	Gh_A11G0257	GAGCGTAACGAGGTCAAGCAAA	CTTCCCGTCCTGATTAATGTCC
GhCBL1-2	Gh_D11G0276	TTTTGTTCGAGCACTCAATGTTT	TTGCCTCAATCGTTTCATCAG
GhCBL1-3	Gh_A03G0043	GACATTCTTGGAAGCCGATA	CTGAGGTATGGGAGGGTCAT
GhCBL1-4	Gh_D09G1875	AGAGTAATGACCCTCCCATACCTAA	CGAGCGAGTATTCTCCGACAA
GhCBL1-5	Gh_A09G1766	GGATGCCGACACTAACCAGG	TCCAACAACGTAGCGGCC
GhCBL3-1	Gh_A01G0740	AGTTTGCTCGTGCTCTCTCTGT	ATCATCTGAAAGGTTCATGCCA
GhCBL3-2	Gh_D01G0760	GCAAGAGAGACCGTTTTTAGTG	AATCTTATCGTCAATGGGCG
GhCBL3-3	Gh_A13G1099	GGGCTGATTAACAAGGAGGAGT	ACAGAAAGAGCACGAGCAAACT
GhCBL3-4	Gh_D13G1364	ATGGGCTGATTAACAAGGAGGAG	GACAGAAAGAGCACGAGCGAAC
GhCBL3-5	Gh_A04G0051	GCGGTGATAGATGACGGACT	GACAGAGAGAGCACGAGCAA
GhCBL3-6	Gh_D05G3682	TACACGCTTCCGACCCTATT	ATCAATGAGCCCGTCGTAAC
GhCBL4-1	Gh_A11G0126	ACGGCTAGTGAAGTAGAATCCC	CGAACAAATCAAAAACCCTGTC
GhCBL4-2	Gh_D11G0140	TTCTTGCTGCTGAAACACCT	CGAACAAATCAAAAACCCTG
GhCBL4-3	Gh_A12G2144	TAAGCGTCTTTCATCCCAAC	TGATTCACCAAGCAGAGCCA
GhCBL4-4	Gh_A09G1696	AACTTAGACACAAGGCTGGGTATG	GAGGTTCTGCTTATTGCTGTTTTT
GhCBL4-5	Gh_D12G2320	CCTGAGGAGGTCAAGGAGATG	AAATTGGGTTGCGAGCTACAAA

GhCBL9	Gh_D08G1764	GACATTCTTGGATGCCGACA	ACGCAGCAACCTCGTCTACT
GhCBL10-1	Gh_A06G0800	AGTCTCACAGTGGCGGCA	TTCATTGGCAAGACGGGTAA
GhCBL10-2	Gh_D06G0922	GTCGCGAGAAATGCCGTTAT	ATTCTCGCCGTATGGAGTTTG
GhCBL10-3	Gh_A05G0335	CTGAAATGAATTTGTCCGATGAC	ACTGGAAATAGTAGTTCATCACGGA
GhCBL10-4	Gh_D05G0440	TCTGGAATGAATTTGTCGGATG	CTGGAAATAGGAGTTCTTCACGG

163 **Yeast two-hybrid (Y2H) analysis**

164 The full-length CDS sequences of *GhCBLs* and *CIPK23* genes were amplified,
165 sequencing and cloned into pGBKT7 and pGADT7 vectors, respectively, using
166 primers listed in Table 2. The plasmids were then transformed into yeast strain AH109
167 according to the method described in Yeast Protocols Handbook (Clontech). The
168 cotransformants were plated on non-selective SD/-Leu/-Trp (synthetic dropout
169 medium without Leu and Trp) solid medium and selective SD/-Leu/-Trp/-His/-Ade
170 solid medium. Serial 1:10 dilutions were made in water, and 2 µl of the dilution was
171 dropped to generate one spot. Plates were incubated at 30 °C for 3-4 d. X-α-Gal
172 staining assay was carried out following the instruction

173 Table 2 Gene primers used for yeast two-hybrid experiments

Genes	AGI number	Forward primers (5′-3′)	Reverse primers (5′-3′)
GhCBL1-2(BD)	Gh_D11G0276	CCGGAATTCATGGGCTGCTTTCAATCT	CGCGGATCCTGTGGCAACCTCATCA
GhCBL1-3(BD)	Gh_A03G0043	CCGGAATTCATGGGTTGCTTTCATTCT	CGCGGATCCAGTGGCAACTTCATCTAC
GhCBL1-4(BD)	Gh_D09G1875	CGCGGATCCATGGGCTGCTTGCAATGTAAA	GCACTGCAGTATGCCATTCGCCGAGCGAGT
GhCBL1-5(BD)	Gh_A09G1766	ATAGGATCCATGGGCTGCTTGCAATGTA	GCACTGCAGGTATAACATCGGTATTATGTACCT
GhCBL3-2(BD)	Gh_D01G0760	CGCGGATCCATGTTGCAGTGCATAGAC	GCACTGCAGTGTATCATCAACTTGAGAGTGGAAAA
GhCBL3-4(BD)	Gh_D13G1364	CGCGGATCCATGGGAATTTGTTGTTTT	GCACTGCAGTTTGCCACCCATATTCAACT
GhCBL4-1(BD)	Gh_A11G0126	CGCGGATCCATGAAATGGTGTTTTCAAACT	GCACTGCAGATCTCCATTGACGGAGACGCT
GhCBL4-3(BD)	Gh_A12G2144	CGCGGATCCATGGGTTGTTTTTGCTTG	GCACTGCAGCTTATTCCCAACGATTTCAGCT
GhCBL4-4(BD)	Gh_A09G1696	CGCGGATCCATGGGCTG	GCACTGCAGGTTTTTTCTC

		CTTTTGCTTG	AATTCTTCACTGGT
GhCBL8(BD)	Gh_D09G1801	CGCGGATCCATGGGCTG CTTTTGCTTGAAGAA	GCACTGCAGATTCTTCAC TGGTTGCTGCAAATCTGA GAC
GhCBL9(BD)	Gh_D08G1764	CCGGAATTCATGGGCTG CTTTCATTCT	CGCGGATCCCGCAGCAAC CTCGTCTA
GhCBL10-3(BD)	Gh_A05G0335	CGCGGATCCATGGATTC AACTAGCAAAACC	GCACTGCAGCCGGAGATA GGAAAGGGCCAA
GhCIPK23(AD)	Gh_A06G1219	CCGGAATTCATGGCGAA TCGCACTAGT	CGCGGATCCACCATCCTT TTCTTCCAC

RESULTS

Genome-wide identification of the CBL family in two progenitor diploid and their tetraploid cotton species

The CBL genes in cotton were identified using the homologous alignment method based on the whole genome data of the sequenced *Gossypium* species including diploid *G. Arboreum*, *G. Raimondii* and their tetraploid *G. Hirsutum* as well as of the *Arabidopsis*. A total of 13, 13, and 22 CBL genes were respectively detected in A genome (*G. Arboretum*), D genome (*G. raimondii*) and A_tD_t genome (*Gossypium hirsutum*) using 10 *Arabidopsis* CBL gene coding and protein sequences as queries (Table 3). Further, the CBL candidate genes in *Gossypium* were confirmed by domain analysis programs of Pfam and SMART. The cotton CBL family members were named according to their orthologous similarity to the 10 *Arabidopsis* CBL proteins (Mohanta et al., 2015).

Most cotton CBLs had very similar physical properties (Table 3). The open reading frame (ORF) lengths of the CBL genes in *Gossypium* ranged from 570 bp to 882 bp except that of *GhCBL3-6*, whose ORF length was 3981 bp. The GaCBL and GrCBL proteins contained 199-279 and 209-253 amino acids (AA), respectively, while GhCBLs were composed of 189-293 AA except GhCBL3-6, which consisted of 1326 AA. The molecular weights (MWs) of GaCBLs varied from 23.25 kDa (GaCBL10-1) to 32.43 kDa (GaCBL10-2), and of GrCBLs ranged from 23.25 kDa (GrCBL3-3) to 29.26kDa (GrCBL10-1). The sizes of GhCBLs were 21.64 kDa (GhCBL3-4) to 33.56 kDa (GhCBL10-1) with an exception of GhCBL3-6 (150.21

196 kDa). The theoretical isoelectric point (pI) is small for the overwhelming majority of
197 CBLs, ranging from 4.65 (GaCBL9) to 5.64 (GhCBL4-5). By contrast, PI of
198 GhCBL3-6 was 8.05 (Table 3).
199 Putative subcellular localizations of the cotton CBL proteins were also analyzed.
200 It is predicted that all of CBLs proteins were located in cell membrane except that
201 GhCBL3-6 was in the nucleus (Table 3). The quite different characteristics of
202 GhCBL3-6 from other members suggest that GhCBL3-6 likely play a special role in
203 cotton.
204 Table 3 The CBL family genes in cotton

Gene name	Gene ID	PI	MW (kDa)	Hydro-philicity	Predicted subcellular localization	amino acid residues	coding sequence
GaCBL1-1	Cotton_A_16036	4.74	24.33	-0.163	Cell membrane	213	642
GaCBL1-2	Cotton_A_16034	4.74	24.33	-0.163	Cell membrane	213	642
GaCBL1-3	Cotton_A_16590	5.06	25.39	-0.216	Cell membrane	221	666
GaCBL1-4	Cotton_A_09151	4.72	24.39	-0.142	Cell membrane	213	642
GaCBL2	Cotton_A_07469	4.78	25.94	-0.2	Cell membrane	226	681
GaCBL3-1	Cotton_A_06492	4.77	25.98	-0.189	Cell membrane	226	681
GaCBL3-2	Cotton_A_02147	5.08	27.68	-0.314	Cell membrane	240	723
GaCBL4-1	Cotton_A_02388	4.81	24.88	-0.13	Cell membrane	220	663
GaCBL4-2	Cotton_A_13237	4.97	24.47	-0.173	Cell membrane	215	648
GaCBL8	Cotton_A_08153	4.89	23.48	-0.134	Cell membrane	205	618
GaCBL9	Cotton_A_13238	4. 65	24. 22	-0.141	Cell membrane	210	633
GaCBL10-1	Cotton_A_14000	4.55	23.25	-0.175	Cell membrane	199	600
GaCBL10-2	Cotton_A_34841	4.82	32.43	-0.028	Cell membrane	279	840
GrCBL1-1	Gorai.007G030300	4.72	24.38	-0.143	Cell membrane	213	642
GrCBL1-2	Gorai.003G178700	4.71	24.45	0.075	Cell membrane	214	645
GrCBL1-3	Gorai.004G191400	4.67	23.86	0.016	Cell membrane	209	630
GrCBL1-4D	Gorai.006G214700	4.99	25.39	-0.226	Cell membrane	221	666
GrCBL3-1	Gorai.013G150400	4.79	25.96	-0.208	Cell membrane	226	681
GrCBL3-2	Gorai.002G102900	4.77	25.98	-0.189	Cell membrane	226	681
GrCBL3-3	Gorai.009G450400	4.84	23.25	-0.21	Cell membrane	226	681
GrCBL4-1	Gorai.007G015400	4.78	24.91	-0.193	Cell membrane	233	702
GrCBL4-2	Gorai.006G207100	4.98	25.26	-0.161	Cell membrane	221	666
GrCBL4-3	Gorai.008G255900	5.11	24.02	-0.161	Cell membrane	211	636
GrCBL9	Gorai.008G255800	4.66	24.58	-0.139	Cell membrane	213	642
GrCBL10-1	Gorai.010G101400	4.74	29.26	-0.096	Cell membrane	252	759

GrCBL10-2	Gorai.009G045600	4.83	29.23	-0.095	Cell membrane	253	762
GhCBL1-1	Gh_A11G0257	4.72	24.44	-0.148	Cell membrane	213	642
GhCBL1-2	Gh_D11G0276	4.79	24.38	-0.145	Cell membrane	213	642
GhCBL1-3	Gh_A03G0043	4.98	22.76	-0.163	Cell membrane	199	600
GhCBL1-4	Gh_D09G1875	5.06	25.69	-0.194	Cell membrane	224	675
GhCBL1-5	Gh_A09G1766	5.51	23.23	-0.165	Cell membrane	200	603
GhCBL3-1	Gh_A01G0740	4.77	25.98	-0.189	Cell membrane	226	681
GhCBL3-2	Gh_D01G0760	4.77	25.99	-0.189	Cell membrane	226	681
GhCBL3-3	Gh_A13G1099	4.84	23.25	-0.21	Cell membrane	202	609
GhCBL3-4	Gh_D13G1364	4.98	21.64	-0.205	Cell membrane	189	570
GhCBL3-5	Gh_A04G0051	5.14	21.76	-0.274	Cell membrane	189	570
GhCBL3-6	Gh_D05G3682	8.05	150.21	-0.284	Nucleus	1326	3981
GhCBL4-1	Gh_A11G0126	4.77	23.01	-0.059	Cell membrane	201	606
GhCBL4-2	Gh_D11G0140	4.82	24.97	-0.185	Cell membrane	220	663
GhCBL4-3	Gh_A12G2144	4.97	24.5	-0.175	Cell membrane	215	648
GhCBL4-4	Gh_A09G1696	5.27	28.4	-0.184	Cell membrane	248	747
GhCBL4-5	Gh_D12G2320	5.64	25.06	0.023	Cell membrane	218	657
GhCBL8	Gh_D09G1801	4.85	24.74	-0.177	Cell membrane	217	654
GhCBL9	Gh_D08G1764	4.74	23.8	-0.032	Cell membrane	209	630
GhCBL10-1	Gh_A06G0800	5.18	33.56	-0.143	Cell membrane	293	882
GhCBL10-2	Gh_D06G0922	4.95	30.41	-0.159	Cell membrane	265	798
GhCBL10-3	Gh_A05G0335	5.16	30.48	-0.114	Cell membrane	262	789
GhCBL10-4	Gh_D05G0440	5.01	30.25	-0.08	Cell membrane	262	789

Distribution of cotton CBL family members in the whole genome

Chromosomal distributions of the cotton *CBL* genes were examined. In general., *CBL* members were unevenly distributed among the *Gossypium* chromosomes. Thirteen *GaCBLs* were distributed on 7 chromosomes. Among them, three *GaCBLs* were located on each of Gachr07 and Gachr11 chromosomes. Two *GaCBLs* harbored each of Gachr06 and Gachr13, and one *GaCBL* was on Gachr01, Gachr08 and Gachr09, respectively (Fig.1). Thirteen *GrCBL* genes were identified on 9 chromosomes. Each of the four chromosomes Grchr06, Grchr07, Grchr08 and Grchr09 owned 2 genes, and other chromosomes (Grchr02, Grchr03, Grchr04, Grchr10, Grchr13) individually contained 1 gene (Fig.1). Likewise, 22 *GhCBL* family members were mapped onto 17 chromosomes dispersedly. Each of the five chromosomes Ghchr09, Ghchr11, Ghchr19, Ghchr21 and Ghchr23 had 2 members, and other chromosomes individually

carried one member (Fig.1). We observed the phenomena of two CBL genes jointing together in a chromosome. For instance, GaCBL4-2 and GaCBL9 were mapped within 16.0 Mb in Gachr06, and GrCBL4-3 and GrCBL9 were mapped within 53.8 Mb in Grchr08. These findings suggest that tandem duplication play a role in generating these genes during evolution.

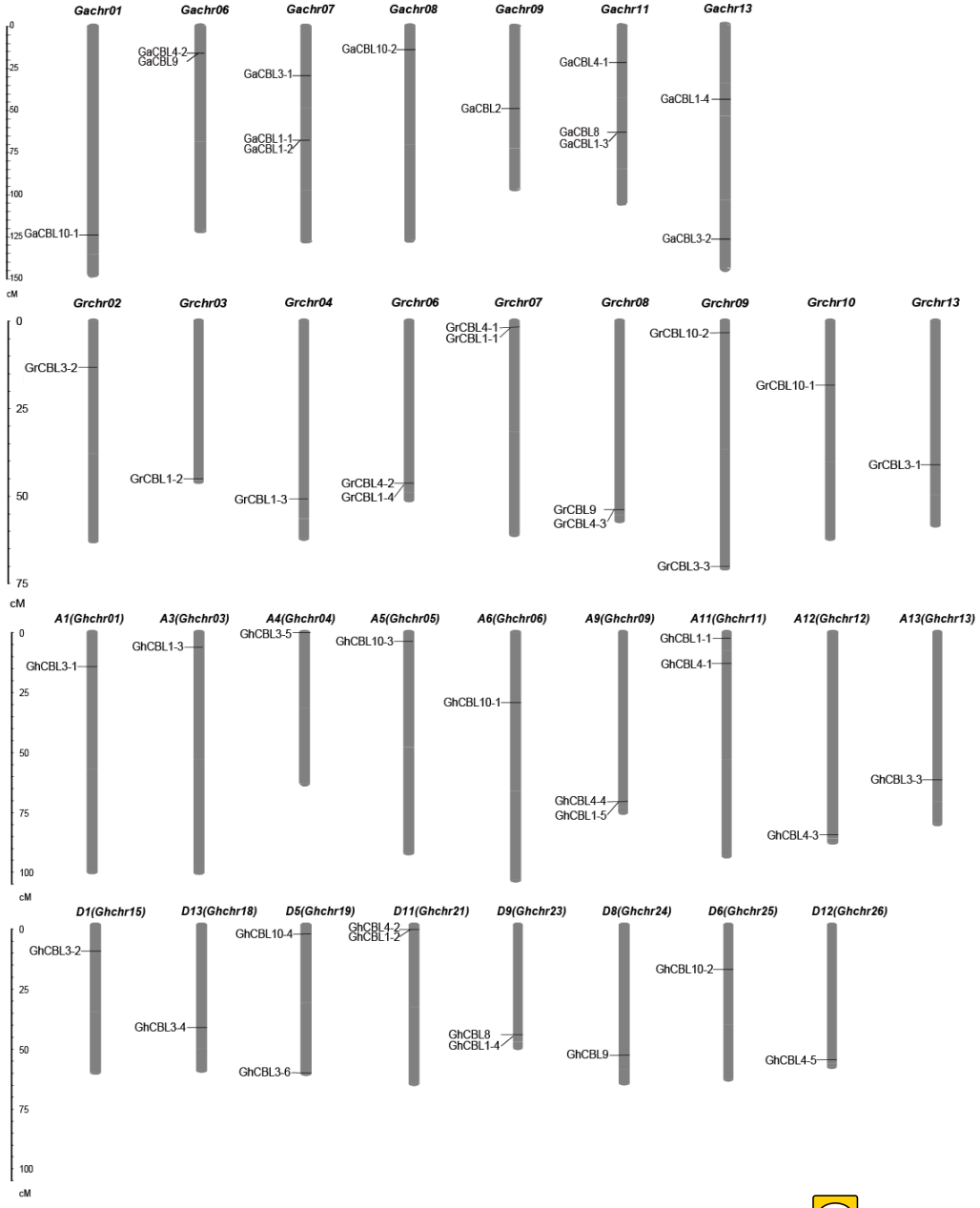


Fig.1 Distributions of the *CBL* family genes on chromosomes in cotton

GaCBLs are from *G. Arboreum*, *GrCBLs* are from *G. Raimondii* and *GhCBLs* are from *G. Hirsutum.*

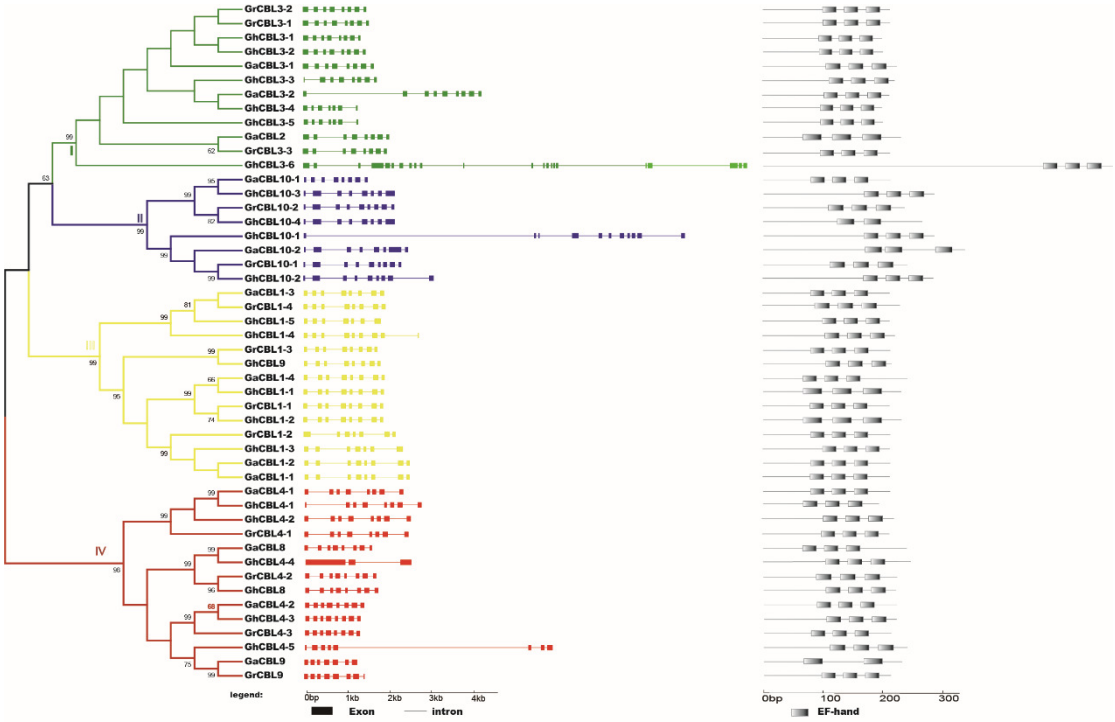
Phylogenetic analysis and structural properties of *CBL* genes in different cotton species

To determine the evolutionary relationship of the CBLs among *G. arboreum*, *G. raimondii*, and *G. hirsutum*, the phylogenetic tree for the cotton CBLs was constructed. The cotton CBLs can be classified into four families (I to IV) (Fig.2a). Family I consisted of 12 CBLs (3 GaCBLs, 3 GrCBLs and 6 GhCBLs). The members in family II were 8 CBLs (2 GaCBLs, 2 GrCBLs and 4 GhCBLs). Family III contained 14 CBLs (4 GaCBLs, 4 GrCBLs and 6 GhCBLs). Family IV had 14 CBLs (4 GaCBLs, 4 GrCBLs and 6 GhCBLs) (Fig.2a).

The structure of a protein is closely related to its functions in cells. We therefore identified the intron-exon structures of *CBL* genes in *Gossypium* by mapping the cDNA sequences onto their genomic sequences. Most of *GaCBLs* and *GrCBLs* owned 8 exons except that *GaCBL3-2*, *GrCBL10-1*, *GrCBL10-2* had nine and *GaCBL9*, *GrCBL1-2* had seven. The majority of *GhCBLs* carried 7-11 exons, but *GhCBL4-4* had 3 exons and *GhCBL3-6* had 22 exons (Fig. 2a).

The putative domains in the cotton CBL proteins were also investigated. EF-hand motifs, which bind to Ca^{2+} ions to transfer calcium signals, were observed in all cotton CBL members. Each CBL proteins had 3 EF-hand motifs except for GaCBL9, which contained 2 such motifs (Fig. 2a). Furthermore, a conserved myristoylation motif (MGCXXS/T) was detected in the N-terminal regions of 11 cotton CBL proteins. These proteins included 4 GaCBLs, 2 GrCBLs and 5 GhCBLs (Fig. 3b, c). A conserved palmitoylation site with N-terminal Cys residue at third, fourth, fifth or sixth position in AA sequence also existed in many cotton CBL members. The two sites are important in the attachment of a protein to membrane (Mohanta et al., 2015).

a



b

c

Name	Start	Myristoylation site
GaCBL1-1	1	MGCFHSKVTRQ HPGYEDPVTL
GaCBL1-2	1	MGCFHSKVTRQ HPGYEDPVTL
GaCBL1-4	1	MGCFQSKVTRQ YPGHEDPIIL
GaCBL4-1	1	MGCASSKRSKQ LHGYEDPSVL
GrCBL1-1	1	MGCFQSKVTRQ YPGHEDPIIL
GrCBL4-1	1	MGCGSSKRSKQ LHGYEDPSVL
GhCBL1-3	1	MGCFHSKVTRQ HPGYEDPVTL
GhCBL1-1	1	MGCFQSKVTRQ YPGHEDPIIL
GhCBL1-2	1	MGCFQSKVTRQ YPGHEDPIIL
GhCBL4-2	1	MGCASSKRSKQ LHGYEDPSVL
GhCBL9	1	MGCFHSKVTRH EDPIELASQT

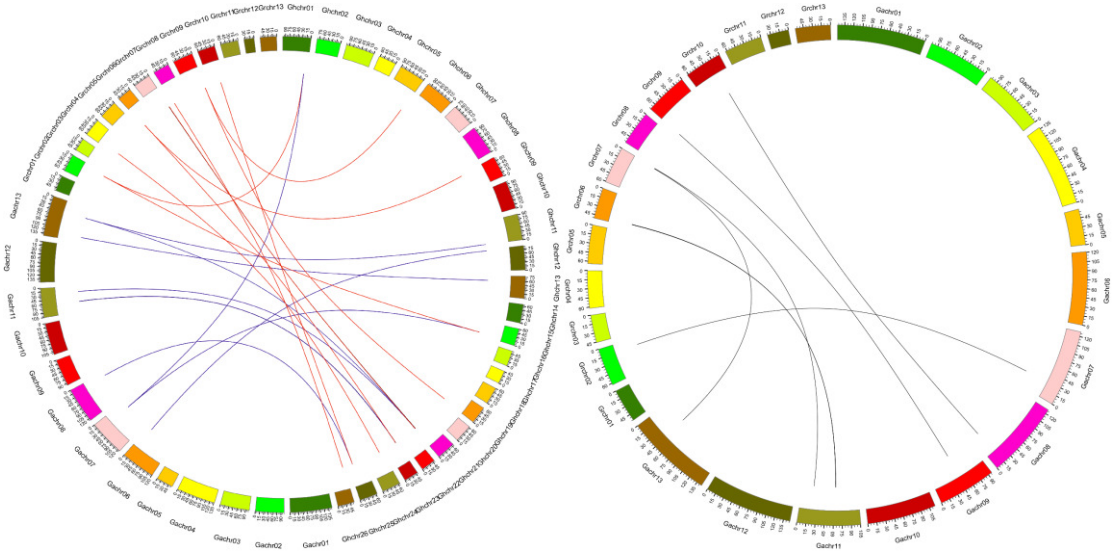
Fig. 2 Analysis of phylogenetic relationship, gene architecture and conserved domains of cotton CBLs

(a) The phylogenetic tree, exon-intron architecture and EF-hand domains of CBLs in *G. Arboreum*, *G. Raimondii*and and *G. hirsutum*. The four major subfamilies are numbered I to IV. The color boxes indicate exons, and the color lines indicate introns; (b) The Logo of the myristoylation motif; (c) Multiple sequence containing the myristoylation motif in cotton CBLs.

Synteny analysis of cotton CBL genes

To better know the genetic origins and evolution of the tetraploid cotton species and the two diploid cotton species, we chose the most similar homologous gene pairs for

analysis. Collinear analysis results revealed that 10 homologous gene pairs existed between *G. arboreum* and *G. hirsutum* (Fig. 3a). Moreover, 11 homologous gene pairs were found between *G. raimondii* and *G. Hirsutum* (Fig. 3a). Using the same method, 7 homologous gene pairs were observed between *G. Arboreum* and *G. Raimondii*. They distributed on 5 chromosomes in *G. Arboreum* and 5 chromosomes in *G. Raimondii*, respectively (Fig. 3b). Moreover, 215 homologous gene pairs (both orthology and paralogy) were found among the CBLs from the 3 cotton species of cotton (Supplementary 1). These results imply that many cotton CBL genes may evolve through segmental duplication.



a b

Fig. 3 Genome-wide synteny analysis of cotton CBL genes

(a) Synteny analysis between *G. hirsutum* and two diploid species *G. Arboreum* and *G. Raimondii.* Blue lines link gene pairs between *G. Arboreum* and *G. Hirsutum*, and red lines link gene pairs between *G. Raimondii* and *G. Hirsutum*; (b) Synteny analysis between *G. Arboreum* and *G. Raimondi*.

Analysis of Ka/Ks values of cotton CBLs

To better understand the divergence of the *CBL* genes after polyploidization, the value Ka and Ks and their ratio (Ka/Ks) were evaluated for the homologous gene pairs among *G. arboreum*, *G. raimondii* and *G. hirsutum* (Fig. 4). Results showed that the

263 Ka/Ks values among most of the homologous genes were less than 1, indicating they
264 evolved under the purifying selection effect. Only GhCBL10-2/GrCBL10-1 has a
265 Ka/Ks ratio more than 1, hinting that the gene pair may generate via the directional
266 selection.

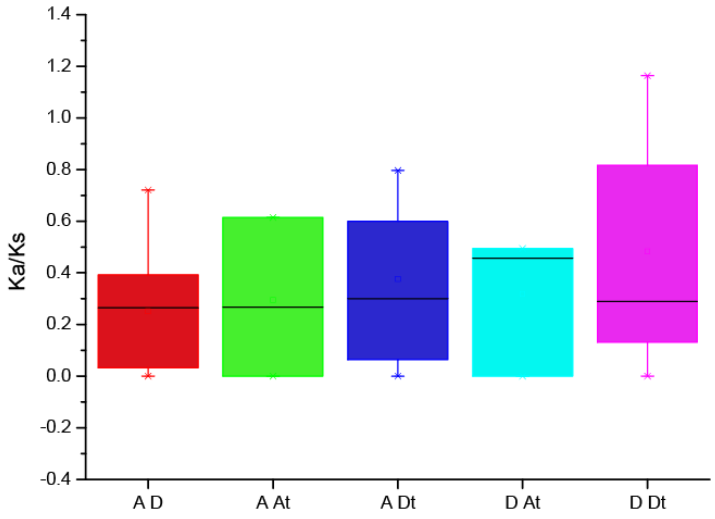


Fig 4. The Ka/Ks values of the homologous genes between the A genome, D genome and subgenomes of *G. Hirsutum* (A_tD_t)

267 **Phylogenetic relationship of CBLs in cotton and other plant species**

268 To gain insight into the evolutionary relationships among GaCBLs, GrCBLs, GhCBLs
269 and those of other plant species, we constructed a phylogenetic tree. Full-length
270 amino acid sequences of 126 predicted CBL proteins were obtained from *G.*
271 *arboretum*, *G. raimondii*, *G. Hirsutum*, *A. thaliana*, *C. papaya*, *G. max*, *V. vinifera*, *T.*
272 *cacao*, *P. trichocarpa*, *R. Communis* and *O. sativa*. Phylogenetic trees were generated
273 using the neighbor-joining method and MEGA 5.0 software. The CBLs family was
274 divided into thirteen subfamilies according to the topology of the phylogenetic tree
275 (Fig.5). As expected, the three cotton CBLs commonly clustered closely in a
276 subfamily. Most of them belonged to subfamily two, eight and thirteen. We found that
277 the CBL members from different dicotyledon species and rice always clustered in a
278 subfamily, indicating the CBLs emerged before the divergence from eudicots and
279 monocots. Moreover, the CBLs from cotton often clustered together with those from *T.*

Cacao (Fig.5). The results imply that cotton CBLs have closer relationship with those from *T. Cacao* relative to other plants.

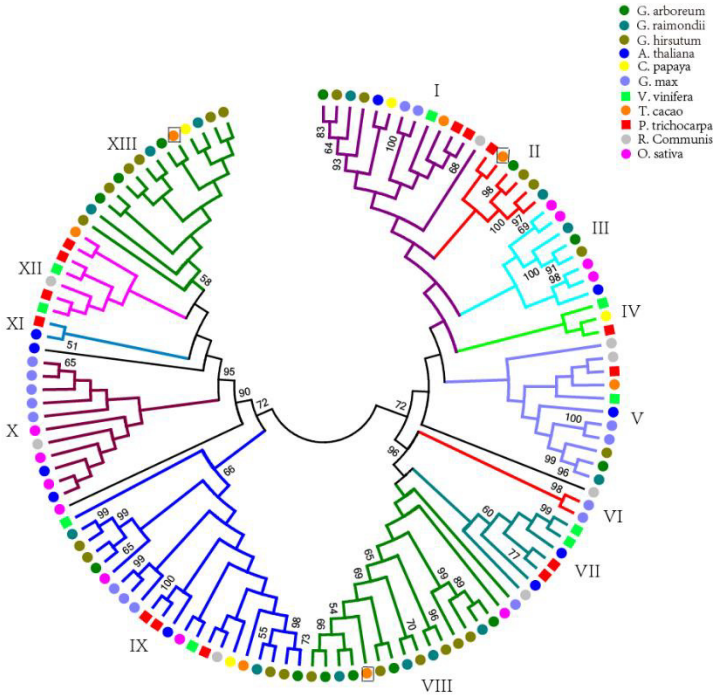


Fig. 5 Phylogenetic tree of CBLs in cotton and other plant species

The plants in the square frame indicated that the CBL genes have closer evolutionary relationship with cotton *CBLs*.

Annotation analysis of GhCBLs

Putative functions of GhCBLs were analyzed using KOG database. Only GhCBL3-6 was blasted out. It was predicted that GhCBL3-6 played roles in modulation of RNA processing and modification, signal transduction, and coenzyme transport and metabolism. Gene ontology (GO) database for these GhCBLs was also assessed. The results showed that all of the 22 GhCBL members were capable of binding calcium ion, like those of other plant species. These data indicate that GhCBLs and other CBLs are of great importance in Ca^{2+} signal transduction in plants.

Expression analysis of *GhCBL* genes in tissues

The expression patterns of all the 22 *GhCBL* genes in tissues were monitored by qRT-PCR. We found that most genes were highly expressed in flowers except that *GhCBL4-3*, *GhCBL4-4*, and *GhCBL8* were dominantly expressed in roots. Moreover, the transcripts of *GhCBL1-5*, *GhCBL1-1*, *GhCBL1-4* and *GhCBL9* were relatively

abundant in fiber, and those of *GhCBL4-3* were also numerous in flowers (Fig. 6). These results suggest that *GhCBL4-3*, *GhCBL4-4* and *GhCBL8* may mainly function in roots and other genes may chiefly act in flowers. *GhCBL1-1*, *GhCBL1-4*, *GhCBL1-5* and *GhCBL9* probably play a part in fiber development in cotton.

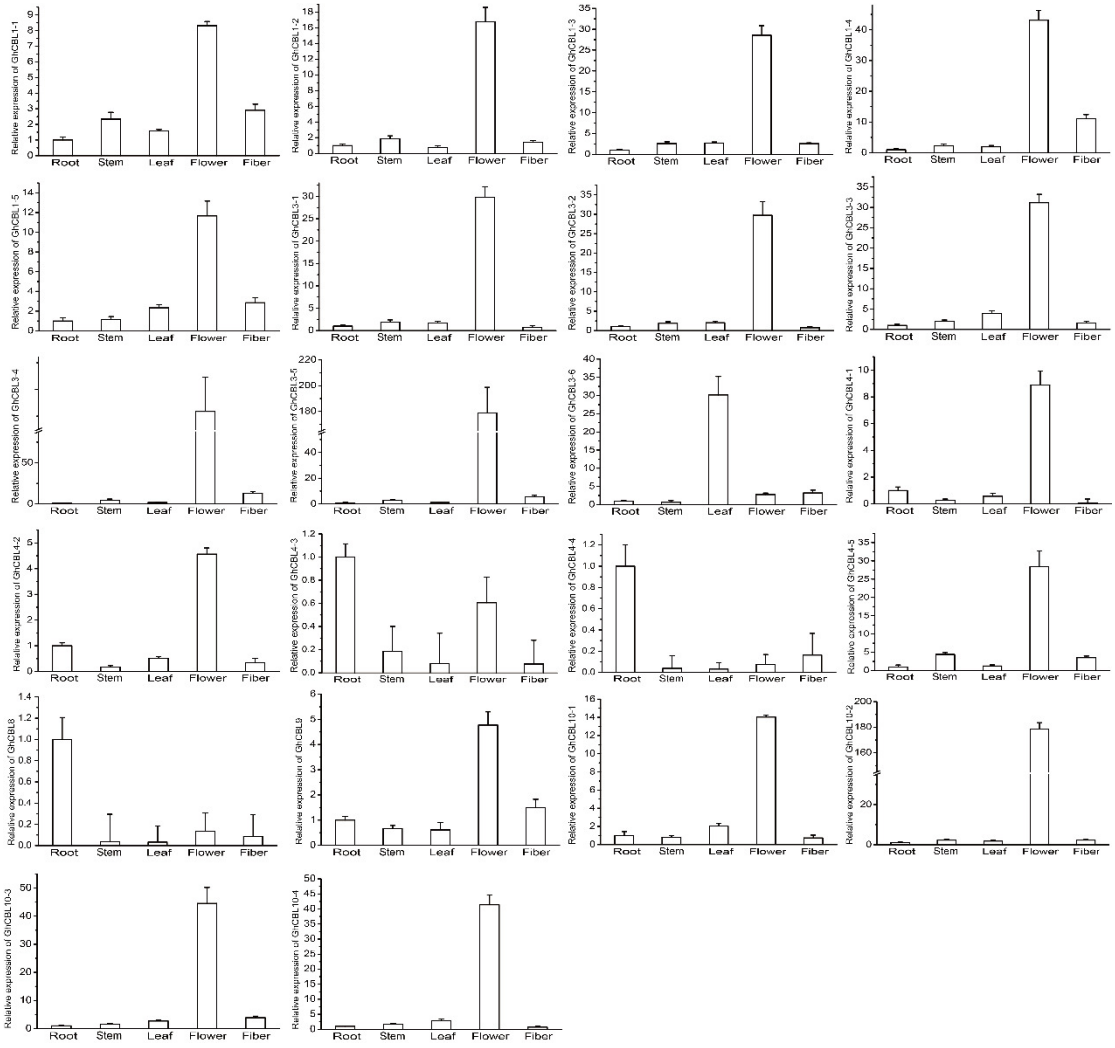


Fig. 6 Expression of 22 *GhCBL* genes in tissues of cotton

The relative expression of genes was calculated from 3 independent replicates. The expression value of a gene in roots was set as 1. The vertical bars represent the standard error.

Expression patterns of *GhCBLs* in responding to potassium deficiency

CBLs have been addressed to play key roles in response to K^+ deprivation in *Arabidopsis* and rice (Li et al., 2014a; Mao et al., 2016). Accordingly, we measured the expression patterns of the 22 *GhCBL* genes in response to potassium deficiency. As a whole, potassium deficiency moderately altered the expression levels of *GhCBL*

genes (Fig. 7). Under potassium deficiency, many genes were downregulated at 6 h, but upregulated at 2 d and/or 5 d. These gene included *GhCBL1-1*, *GhCBL1-2*, *GhCBL1-3*, *GhCBL1-5*, *GhCBL3-3*, *GhCBL3-4*, *GhCBL4-4*, *GhCBL10-1*, *GhCBL10-3* and *GhCBL10-4*. The expression levels of *GhCBL3-6*, *GhCBL4-2*, *GhCBL4-3*, *GhCBL8* and *GhCBL9* were decreased while those of *GhCBL1-4*, *GhCBL3-5*, and *GhCBL10-2* were unchanged after shortage of potassium (Fig. 7). These results suggest that a number of GhCBLs may play roles in response to potassium starvation in cotton

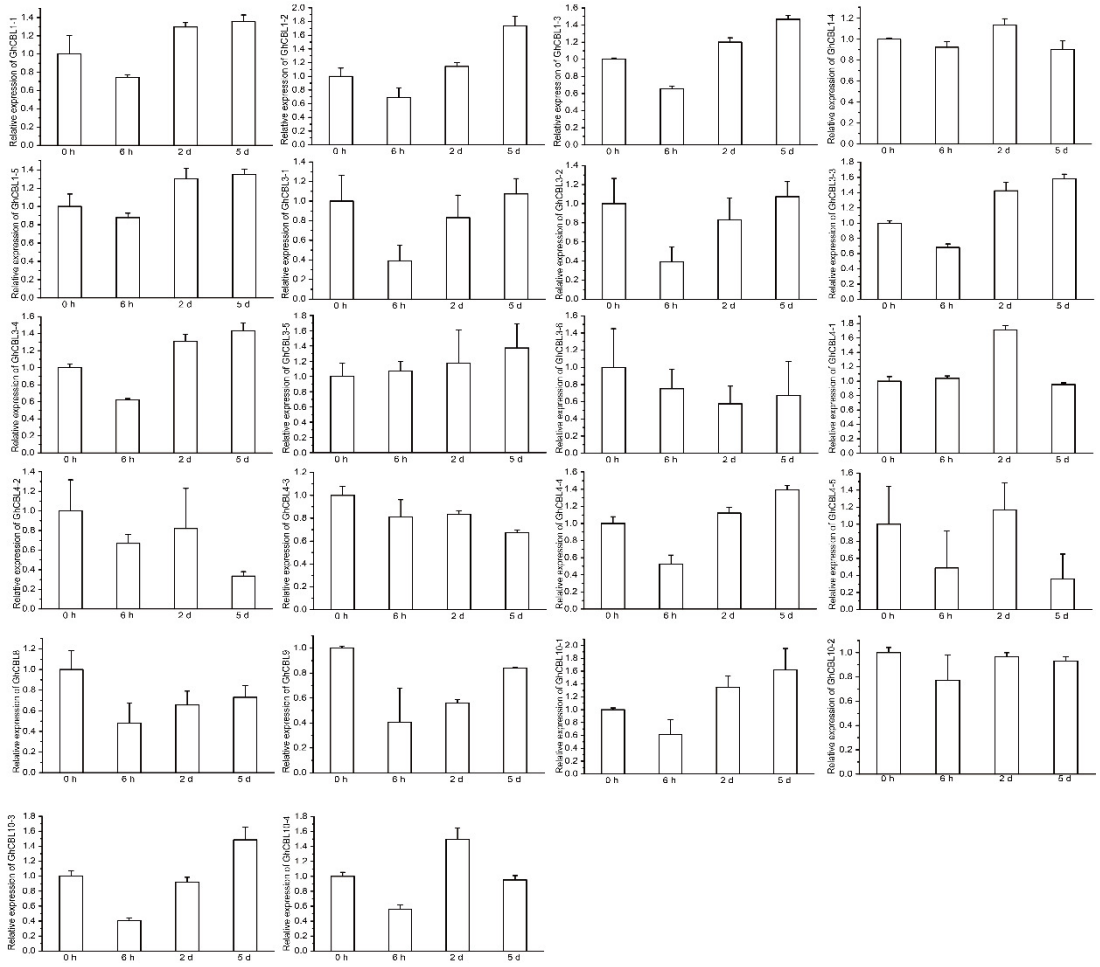


Fig. 7 Expression of 22 *GhCBL* genes under potassium deprivation

The relative expression of *GhCBLs* was examined under potassium deficiency for indicated period of time. The expression value of the gene at 0 h was set as 1. The vertical bars mean the standard error.

Several GhCBLs can interact with GhCIPK23 *in vitro*

To examine whether GhCBLs interact with GhCIPK23, yeast two-hybrid experiments

were performed and total of 12 GhCBLs were measured. Among them, GhCBL1-2, GhCBL1-3, GhCBL4-4, GhCBL8, GhCBL9 and GhCBL10-3 were observed to interact with GhCIPK23. Furthermore, GhCBL1-2 and GhCBL9, the respective homologues of Arabidopsis CBL1 and CBL9, displayed more strong interactive signals with GhCIPK23 in yeast, suggesting that GhCBL1-2 and GhCBL9 may direct regulate GhCIPK23 in cotton.

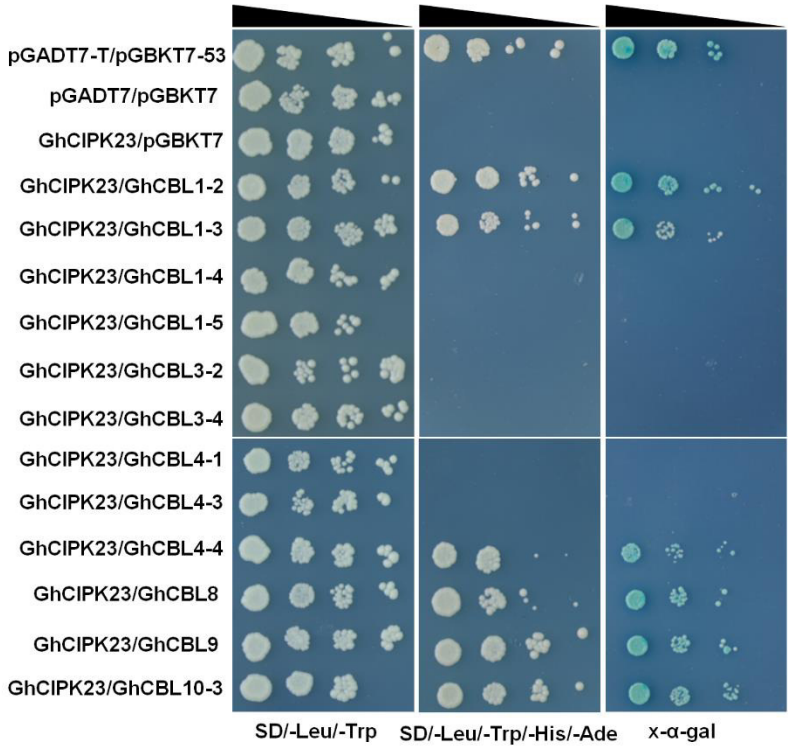


Fig. 8 Yeast two-hybrid analysis of interactions between GhCBLs and GhCIPK23

The yeast cells containing the indicated plasmids were grown on the non-selective SD/-Leu/-Trp solid medium and selective SD/-Leu/-Trp/-His/-Ade solid medium, followed by X-α-Gal staining. The reduced cell densities in the dilution series are shown by narrowing triangles. The first row represents a positive control, the 2th and 3th rows represent two negative controls.

DISCUSSION

In the present study, we identified 13, 13 and 22 *CBL* genes in *G. Arboreum*, *G. Raimondii* and *G. Hirsutum* genomes, respectively (Table 3). Among the 22 *GhCBL* genes, 11 and 11 were assigned to the A_t and D_t subgenome, respectively. They were similar to the number of *CBL*s found in *G. Arboreum* and *G. Raimondii*, respectively.

We detected that 8 *GaCBLs* and 9 *GrCBLs* were homologous genes of *GhCBLs*. However, homologues of 5 GaCBLs and 4 GrCBLs were not discovered in genome of *G. hirsutum*. These findings indicate that the 8 *GaCBLs* and 9 *GrCBLs* have been maintained in *G. hirsutum* after polyploidization event, while the 5 *GaCBLs* and 4 *GrCBLs* are lost or regenerated after the event. Moreover, we observed 5 *GhCBLs* (*GhCBL1-3*, *GhCBL3-5*, *GhCBL4-1*, *GhCBL4-4*, *GhCBL10-1*) in A_t subgenome and 2 *GhCBLs* (*GhCBL3-4*, *GhCBL3-6*) in D_t had no homologues in A genome of *G. Arboreum* and D genome of *G. Raimondii*, implying that they are new evolved genes.

The physical properties of most GaCBLs and GrCBLs were similar to those of GhCBLs (Table 3), suggesting that the functions of the CBLs from the three cotton species remained to be conservative during evolutionary. The majority of cotton CBLs was predicted to localize in the membrane, just like many CBLs in *Arabidopsis* and rice. In *Arabidopsis*, CBL1 and CBL9 were described to localize in the PM. CBL2, CBL3 and CBL6 localize in tonoplast whereas CBL10 is in both PM and tonoplast (Mao et al., 2016). Rice CBL1 is also present in PM. The localizations of the CBLs should be consistent with their primary roles of sensing and transferring Ca^{2+} signals in cotton. However, GhCBL3-6 was predicted to be nuclear. Its roles are unknown at present. Experimental characterization of GhCBL3-6 might shed light on some novel functions of it.

Analysis of gene distributions on chromosomes showed that most homologues of *GaCBLs* and *GrCBLs* in *G. hirsutum* were present in their corresponding A_t and D_t homologous chromosomes, respectively. These findings indicate that *GhCBLs* originate from DNA polyploidization. However, some *GhCBLs* homologues of *GaCBLs* and *GrCBLs* did not distribute on their corresponding A_t or D_t chromosomes, indicating gene conversion events occurred during the evolution of GhCBLs. Additionally, separated and jointed distributions of cotton CBLs genes in chromosomes in combination with the colinearity results of these genes imply that both segmental duplication and tandem duplication are essential for the generation of cotton CBLs during genetic evolution.

The number of introns in coding region of most cotton CBL genes is six or seven,

very similar to that in CBLs genes in *Arabidopsis*, rice, maize, wheat, canola and eggplant (Kolukisaoglu et al., 2004; zhang et al., 2014; Sun et al., 2015; Li et al., 2016; Zhang et al., 2016), reflecting the rather conserved structure of CBL genes in different species. Moreover, nearly all cotton CBLs shared three conserved EF hand domains with other higher plants (Fig. 2). In addition, many *CBLs* from cotton contained the myristoylated and palmitoylated sites, which may facilitate the targeting of CBL-CIPK complex to membrane. These features are also similar to those in *Arabidopsis*, rice and other plants (Kolukisaoglu et al., 2004; Mohanta et al., 2015). The conserved structure of these CBL family members in different plants might reflect a very similar mode of action and/or conserved interaction with their target protein CIPKs (Mohanta et al., 2015).

Results from phylogenetic analysis of CBLs from cotton and many other plants revealed that CBLs in cotton have closer relationship with those in cocoa than in other plants tested (Fig. 5). These findings hint that cotton may have a more recent common ancestor with cacao relative to other plant species, in line with the results of other gene families in cotton (Li et al., 2014; Li et al., 2016).

Expression analysis results showed that almost all of the GhCBL genes were expressed in various tissues including the root, stem, leaf, flower and fiber. Of note, most genes were dominantly expressed in the flower (Fig. 6), suggesting that these genes may play important parts in the reproductive development in cotton. Besides, the expression levels of *GhCBL4-3* and *GhCBL4-4* in roots were clearly higher than those of other genes. These data imply that the two genes may function in modulation of ion transport or acclimation to diverse abiotic stresses in roots.

The expression of 22 *GhCBLs* in responding to potassium starvation was determined. Most genes were moderately upregulated at 2 d and/or 5 d post low-potassium treatments (Fig. 7), indicating multiple GhCBL genes likely regulate cotton response to potassium deprivation. Strikingly, in *Arabidopsis*, the expression of *CBL1* and *CBL9* was reported to be stable, and the transcripts of *CBL10* in roots were moderately decreased under low-potassium conditions (Cheong et al., 2007; Ren et al., 2013). These results imply that constitutive expression of some *CBL* genes may be

enough for transmitting Ca^{2+} signals to downstream targets in response to potassium deficiency in plants. Thus, those *GhCBLs* that were not upregulated by low-potassium stress likely participate in modulation of potassium absorption and/or transport. However, which and how GhCBLs regulate potassium uptake remained to be investigated in the future.

CIPK23 has been addressed to function in diverse cellular processes in *Arabidopsis* (Mao et al., 2016). In this study, 6 out of 12 GhCBLs could interact with GhCIPK23 in yeast (Fig. 8), indicating that different GhCBL members may interplay with and modulate GhCIPK23 in various growth and/or stress responses in cotton. The cotton homologues of *Arabidopsis* CBL1 and CBL9 suggest that GhCBL1 and GhCBL9 probably play similar roles to CBL1 and CBL9 in cotton.

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