Genome-wide identification of CBL family and expression analysis of *CBLs* in response to potassium deficiency 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 interaction with CBL-interacting protein kinases (CIPKs). However, knowledge about functions and evolution of CBLs in *Gossypium* plants is scarce. Here, we conducted a genome-wide survey and identified 13, 13 and 22 CBL genes in the progenitor diploid *Gossypium arboreum* and *Gossypium raimondii*, and the cultivated allotetraploid *Gossypium hirsutum*, respectively. Analysis of physical properties, chromosomal locations, conserved domains and phylogeny indicated rather conserved nature of CBLs among the three *Gossypium* species. Moreover, these CBLs have closer genetic evolutionary relationship with the CBLs from cocoa than with those from other plants. Most CBL genes underwent evolution under purifying selection in the three *Gossypium* plants. Additionally, nearly all *G. hirsutum* CBL (GhCBL) genes were expressed in the root, stem, leaf, flower and fiber. Many *GhCBLs* were preferentially expressed in the flower while several *GhCBLs* were mainly expressed in roots. Expression patterns of GhCBL genes in response to potassium deficiency were also studied. The expression of most *GhCBLs* were moderately induced in roots after treatments with low-potassium stress. 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 researchers to further investigate the roles and functional mechanisms of the CBLs in *Gossypium*.

INTRODUCTION

Calcium ion (Ca^{2+}) 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 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 relay Ca^{2+} signals through interaction with and activation of 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, Jacobs & Roy, 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, Jacobs & Roy, 2015*; *Mao et al., 2016*).

In *Arabidopsis*, 10 genes (*CBL1-10*) encoding CBL proteins have been found (*Kolukisaoglu et al., 2004*). *CBL1* and *CBL9* were reported to positively regulate the uptake and 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 and ROS signaling (*Pandey et al., 2004*; *Cheong et al., 2007*; *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 proven 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 (*Gossypium hirsutum*), *GhCBL2* and *GhCBL3* appear to modulate fiber elongation (*Gao et al., 2008*). 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, Jacobs & Roy, 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., 2016a*; *Li et al., 2016b*; *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 *Gossypium* is limited.

Cotton is an essential tetraploid fiber crop that supplies lint for the textile industry worldwide. It is considered to descend from an ancestral combination of two diploid most

similar to modern A (for example *Gossypium arboretum*) and D genome species (*Gossypium raimondii*) (*Wendel, Brubaker & Seelanan, 2010*).

Cotton growth and development are severely threatened by diverse abiotic stresses such as drought, salinity and potassium starvation (*Allen, 2010*). 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, Loka & Raper, 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*). Research is needed 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 *GhCBLs* were monitored in tissues and in response to potassium deficiency in cotton. These analyses will provide a basis for further investigation of the functions of CBLs in *Gossypium*.

MATERIALS AND METHODS

Identification of CBL family in *Gossypium*

The genome sequences 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) were downloaded from the CottonGen database (www.cottongen.org), respectively. The protein sequences of 10 *Arabidopsis* CBLs were applied as queries to search the three genomes using BLAST-2.4.0 software (ftp://ftp.ncbi.nlm.nih.gov/blast/executables/blast+/LATEST) with default parameters (E-value $<$ e^{-10}). EF-hand domains, the typical CBL domains, were analyzed within the candidate CBLs one by one using online software SMART (http://smart.embl-heidelberg.de/). The CBL motifs were also queried against the Pfam databases (*Finn et al., 2010*). The putative CBLs with questionable annotations (i.e., having a typical CBL domain but low E-value or low coverage of a domain) were manually reanalyzed.

Analysis of *Gossypium CBLs* family

The properties of the *Gossypium* CBL proteins were analyzed using online tools ExPaSy (http://web.expasy.org/protparam/). The subcellular localizations of the CBLs were examined in the website http://www.csbio.sjtu.edu.cn/bioinf/Cell-PLoc/. The locations of the *CBLs* in chromosomes were assessed by MapInspect software (http://www.softsea.com/review/MapInspect.html). Structures of the CBLs were determined by GSDS (http://gsds.cbi.pku.edu.cn/). The conserved domains in the CBLs were affirmed by SMART (http://smart.embl-heidelberg.de). The sequence logo of myristoylation motif in the CBLs was generated by MEME program (http://meme-suite.org/tools/meme).

Analyses of synteny and Ka/Ks ratio

The homologous gene pairs among the *Gossypium* CBLs were searched by the MCScanx software (http://chibba.pgml.uga.edu/mcscan2/). The gene collinearity results were

obtained by CIRCOS program (http://www.circos.ca/). The ratio of Ka (nonsynonymous substitution rate) to Ks (synonymous substitution rate) of the CBL genes were estimated by PAML program (http://abacus.gene.ucl.ac.uk/software/paml.html).

Phylogenetic analysis of CBLs

The CBL data 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://www.hawaii.edu/microbiology/asgpb/) and castor bean (http://castorbean.jcvi.org). The full-length amino acid sequences of CBL proteins were aligned using Clustal W software through pairwise and multiple alignment with default parameters (*Larkin et al., 2007*). Then, phylogenetic trees were generated based on the alignment results using the neighbor joining method (Neighbor-Joining, NJ) and 1,000 bootstrap trials with the 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 the *GhCBLs* in tissues, samples of roots, stems and leaves were collected from 20-day-old *G. hirsutum* TM-1 plants normally grown in soil containing 1:1 (v:v) peat:vermiculite in a growth chamber (day/night temperature cycle of 28 °C/26 °C, 14 h light/10 h dark, and about 50% relative humidity). Flowers were isolated in the morning at the first day of anthesis from cotton grown in the field. The fibers at elongation stage were obtained from the ovules (23 days post anthesis). 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) (*Murashige & Skoog, 1962*) in the growth chamber described above for 3 weeks. Then, the plants were watered with K^+-lacking 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. Meanwhile, some K^+-starved seedlings for 5 d were resupplied with K^+ (watered with K^+-contained 1/2 MS medium) for 3 h. 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 Biotech, Beijing, China). 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, Madison, WI, USA) following the instructions in the Promega kit (https://tools.thermofisher.com/content/sfs/manuals/superscriptIII_man.pdf).

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	AGTTTGCTCGTGCTCTCTGT	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

Yeast two-hybrid (Y2H) analysis

The full-length CDS sequences of *GhCBLs* and *GhCIPK23* genes were amplified, sequenced and cloned into pGBKT7 and pGADT7 vectors, respectively, using primers listed in Table 2. The plasmids were then transformed into yeast strain AH109 according to the method described in page 18–21 in Yeast Protocols Handbook (Clontech, http://www.clontech.com/xxclt_searchResults.jsp). The cotransformants were plated on non-selective SD/-Leu/-Trp (synthetic dropout medium without Leu and Trp) solid medium and selective SD/-Leu/-Trp/-His/-Ade solid medium. The medium was prepared by ourselves. The concentrations of each component for SD/-Leu/-Trp medium are as follows: L-isoleucine 300 mg/L, L-valine 1.5 g/L, adenine 200 mg/L, L-arginine 200 mg/L, L-lysine 300 mg/L, L-methionine 200 mg/L, L-phenylalanine 500 mg/L, L-threonine 2 g/L, L-tyrosine 300 mg/L, L-histidine 200 mg/L, uracil 200 mg/L, yeast nitrogen base without amino acids 6.7 g/L, glucose 20 g/L. Serial 1:10 dilutions of the cotransformants were made in water, and 2 μl of the dilution was dropped to generate one spot. Plates were incubated at 30 °C for 3-4 d. 5-bromo-4-chloro-3-indoxyl- α-D-galactopyranoside (X- α-Gal) staining assay was carried out following the instruction (the Clontech protocol, page 26).

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	CGCGGATCCATGGGCTGCTTTTGCTTG	GCACTGCAGGTTTTTTCTCAATTCTTCACTGGT
GhCBL8(BD)	Gh_D09G1801	CGCGGATCCATGGGCTGCTTTTGCTTGAAGAA	GCACTGCAGATTCTTCACTGGTTGCTGCAAATCTGAGAC
GhCBL9(BD)	Gh_D08G1764	CCGGAATTCATGGGCTGCTTTCATTCT	CGCGGATCCCGCAGCAACCTCGTCTA
GhCBL10-3(BD)	Gh_A05G0335	CGCGGATCCATGGATTCAACTAGCAAAACC	GCACTGCAGCCGGAGATAGGAAAGGGCCAA
GhCIPK23(AD)	Gh_A06G1219	CCGGAATTCATGGCGAATCGCACTAGT	CGCGGATCCACCATCCTTTTCTTCCAC

Table 3 The CBL family genes in *Gossypium*.

Gene name	Gene ID	pI	MW (kDa)	Hydrophilicity	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
GrCBL1-4	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	1,326	3,981
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

Table 3 (*continued*)

Gene name	Gene ID	pI	MW (kDa)	Hydrophilicity	Predicted subcellular localization	Amino acid residues	Coding sequence
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

RESULTS

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

The CBL genes in *Gossypium* were identified using the homologous alignment method. 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 (*G. hirsutum*) using 10 *Arabidopsis* CBL protein sequences as queries (Table 3). Further, the CBL candidate genes in *Gossypium* were confirmed by domain analysis programs of Pfam and SMART. The CBL family members were named according to their orthologous similarity to the 10 *Arabidopsis* CBL proteins (*Mohanta et al., 2015*). In general, the *CBLs* in *G. arboretum*, *G. raimondii* and *G. hirsutum* were named *GaCBLs, GrCBLs* and *GhCBLs*, respectively.

Most CBLs had very similar physical properties in the 3 *Gossypium* plants (Table 3). The open reading frame (ORF) lengths of the CBL genes 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 29.26 kDa (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 kDa). The theoretical isoelectric point (pI) is small for overwhelming majority of the CBLs, ranging from 4.65 (GaCBL9) to 5.64 (GhCBL4-5). By contrast, pI of GhCBL3-6 was 8.05 (Table 3).

Putative subcellular localizations of the *Gossypium* CBL proteins were also analyzed. It was predicted that all of CBLs were located in cell membrane except that GhCBL3-6 was in the nucleus (Table 3). The quite different characteristics of GhCBL3-6 from other members suggest that GhCBL3-6 likely plays a special role in cotton.

Distribution of the *Gossypium* CBL family members in the whole genome

Chromosomal distributions of the *CBL* genes were examined in *Gossypium*. In general, the *CBLs* were unevenly distributed among the *Gossypium* chromosomes. Thirteen *GaCBLs* were distributed on seven chromosomes. Among them, three *GaCBLs* were located on each

of Gachr07 and Gachr11 chromosomes. Two *GaCBLs* were situated in each of Gachr06 and Gachr13, and 1 *GaCBL* was on Gachr01, Gachr08 and Gachr09, respectively (Fig. 1). Thirteen *GrCBL* genes were identified on nine chromosomes. Each of the four chromosomes Grchr06, Grchr07, Grchr08 and Grchr09 owned 2 genes, and other chromosomes (Grchr02, Grchr03, Grchr04, Grchr10, Grchr13) individually contained one gene (Fig. 1). Likewise, 22 *GhCBL* family members were mapped onto 17 chromosomes. Each of the five chromosomes Ghchr09, Ghchr11, Ghchr19, Ghchr21 and Ghchr23 had two *CBL* members, and other chromosomes individually carried one *CBL* member (Fig. 1). We observed the phenomena of 2 CBL genes joining 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 plays a role in generating these genes during evolution.

Phylogenetic analysis and structural properties of *CBL* genes in *Gossypium*

To determine the sequence similarity relationship of the CBLs among *G. arboreum*, *G. raimondii*, and *G. hirsutum*, the phylogenetic tree for the 48 CBLs was constructed. The CBLs can be classified into four families (I to IV) (Fig. 2A). Family I consisted of 12 CBLs (three GaCBLs, three GrCBLs and six GhCBLs). The members in family II were 8 CBLs (two GaCBLs, two GrCBLs and four GhCBLs). Family III contained 14 CBLs (four GaCBLs, four GrCBLs and six GhCBLs). Family IV had 14 CBLs (four GaCBLs, four GrCBLs and six GhCBLs) (Fig. 2A).

The structure of a protein is closely related to its functions in cells. We therefore identified the intron-exon structures of the *CBL* genes in *Gossypium* by mapping the cDNA sequences onto their genomic sequences. Most of *GaCBLs* and *GrCBLs* owned eight 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 three exons and *GhCBL3-6* had 22 exons (Fig. 2A).

The putative domains in the *Gossypium* CBL proteins were also investigated. EF-hand motifs, which bind to Ca^{2+} ions to transfer calcium signals, were observed in all CBL members. Each CBL proteins had three EF-hand motifs except for GaCBL9, which contained two such motifs (Fig. 2A). Furthermore, a conserved myristoylation motif (MGCXXS/T) was detected in the N-terminal regions of 11 CBL proteins. These proteins included four GaCBLs, two GrCBLs and five GhCBLs (Fig. 2B, 2C). A conserved palmitoylation site with N-terminal Cys residue at third, fourth, fifth or sixth position in amino acid 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*).

Synteny analysis of CBL genes in *Gossypium*

To investigate the genetic origins and evolution of the CBLs in *Gossypium*, the homologous gene pairs among the *CBLs* from *G. arboretum*, *G. raimondii* and *G. hirsutum* were monitored, and the collinear analysis was carried out. The results revealed that 10 homologous gene pairs existed between *G. arboreum* and *G. hirsutum*, and 11 homologous
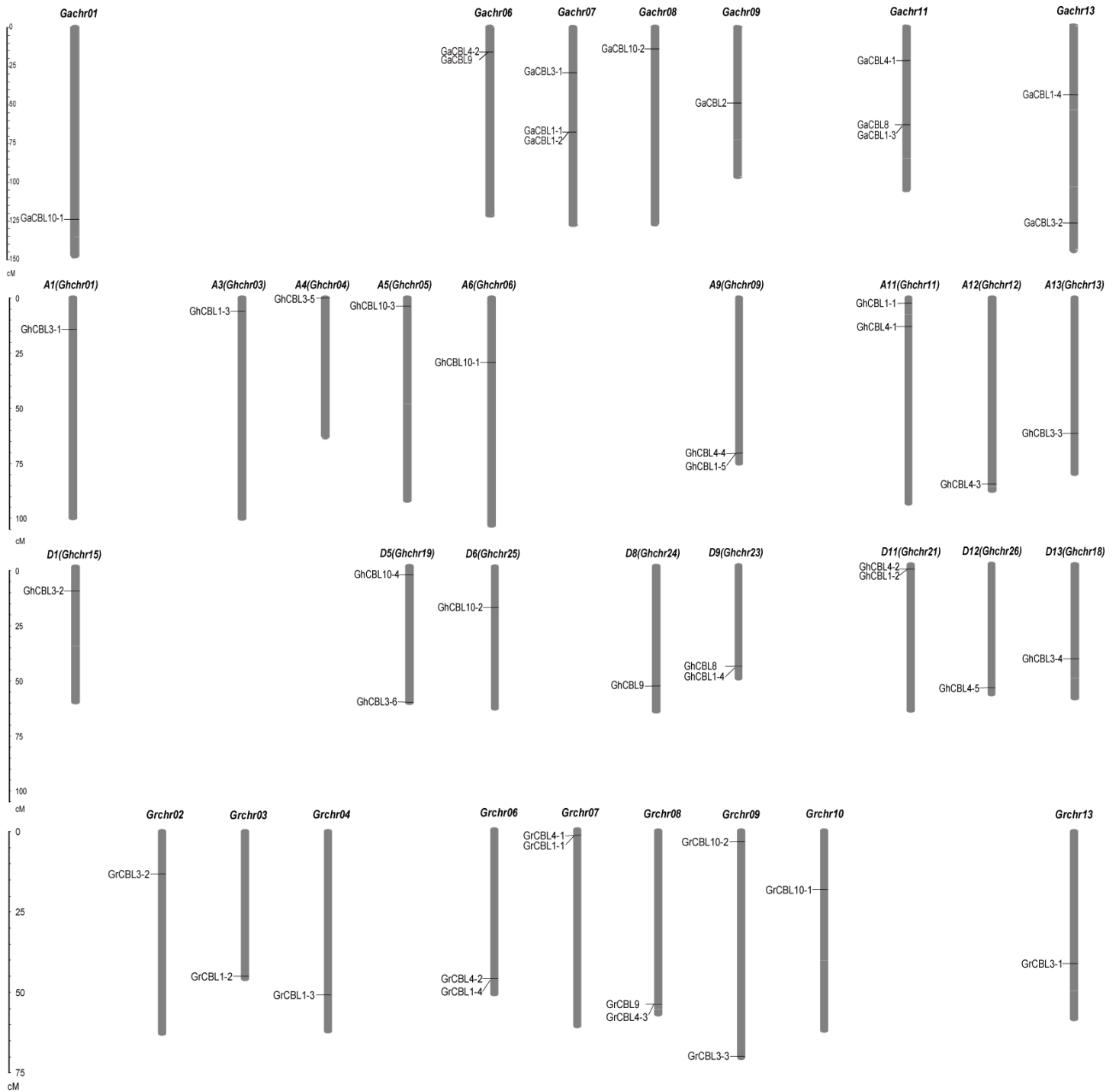
Figure 1 **Distributions of the *CBL* family genes on chromosomes in *Gossypium*.** The *GaCBLs*, *GrCBLs* and *GhCBLs* are from *G. arboreum*, *G. rai-mondii* and *G. hirsutum*, respectively.
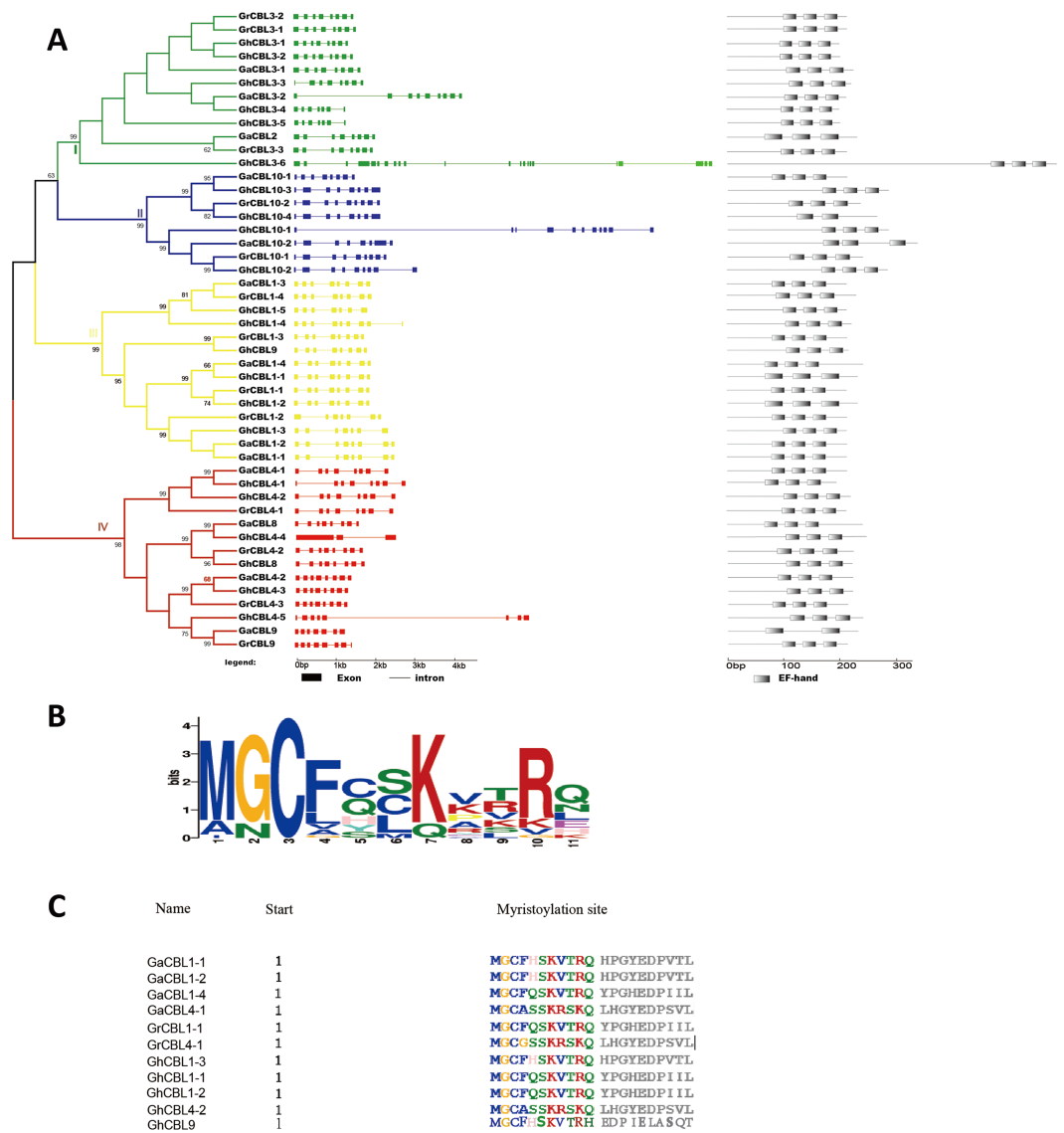
Figure 2 **Analysis of phylogenetic relationship, gene architecture and conserved domains of CBLs in**
Gossypium. (A) The phylogenetic tree, exon-intron architecture and EF-hand domains of CBLs in *G. arboreum*, *G. raimondii* and *G. hirsutum*. The four major subfamilies are numbered I–IV. The color boxes indicate exons, and the color lines indicate introns; (B) The logo of the myristoylation motif. The capital letters stand for the amino acids, the higher the letter, the higher the conservation; (C) Multiple sequences containing the myristoylation motif in *Gossypium* CBLs.

gene pairs were found between *G. raimondii* and *G. Hirsutum* (Fig. 3A). Using the same method, seven homologous gene pairs were observed between *G. arboreum* and *G. raimondii*. They were distributed on five chromosomes in *G. arboreum* and five chromosomes in *G. raimondii*, respectively (Fig. 3B). Moreover, 212 homologous gene pairs (both based on orthology and paralogy) were found among the CBLs from the three *Gossypium* species (Table S1). These results imply that many cotton CBL genes may have evolved through segmental duplication.
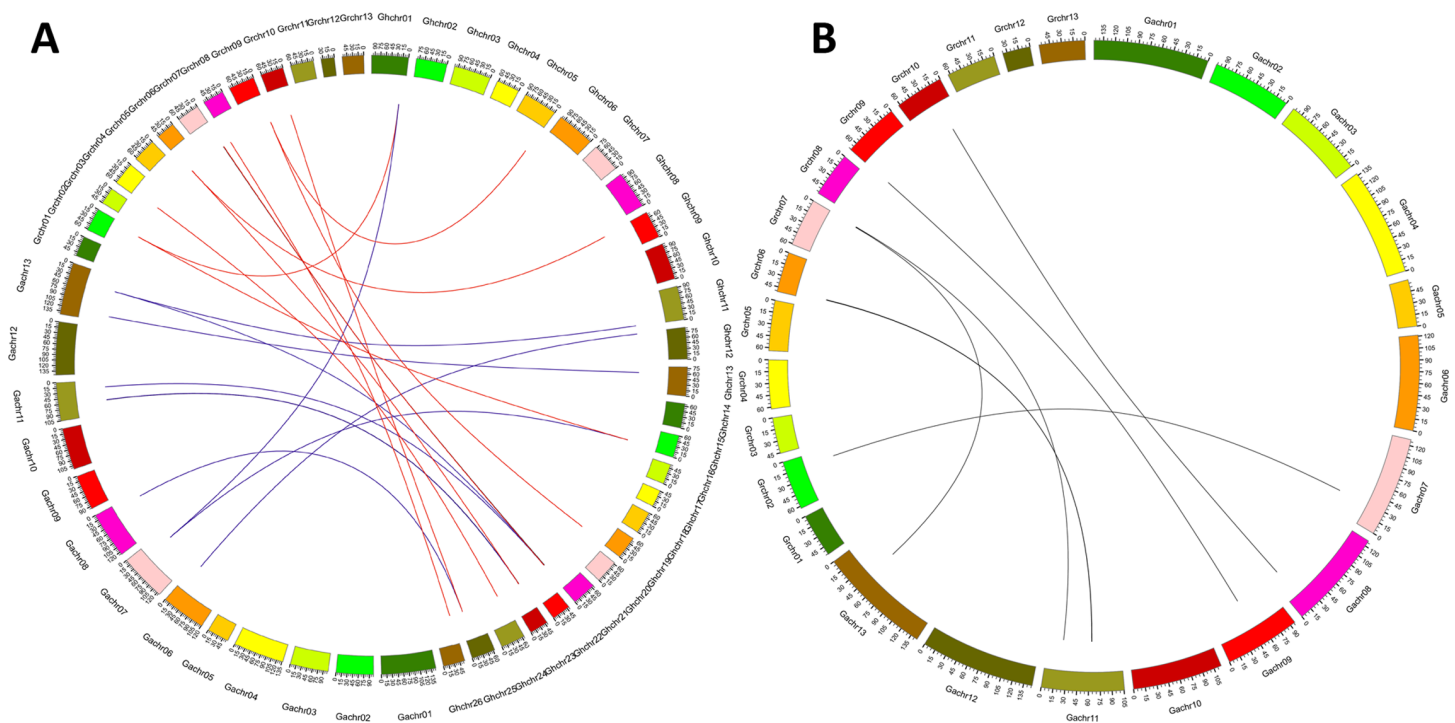
Figure 3 **Genome-wide synteny analysis of *Gossypium* CBL genes.** (A) Synteny analysis between *G. hirsutum* and two diploid species *G. arboreum* and *G. raimondii*. Blue lines link gene pairs between *G. arboreum* and *G. hirsutum*, and red lines link gene pairs between *G. raimondii* and *G. hirsutum*; (B) Synteny analysis between *G. arboreum* and *G. raimondi*.

Analysis of Ka/Ks values of the CBLs

To better understand the divergence of the *Gossypium 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, Table S2). The results showed that the Ka/Ks values among most of the homologous genes were less than 1, indicating they evolved under the purifying selection effect. Only GhCBL10-2/GrCBL10-1 has a Ka/Ks ratio more than 1, hinting that the gene pair may have been generated via the directional selection.

Phylogenetic relationship of CBLs in *Gossypium* and other plant species

To gain insight into the evolutionary relationships among GaCBLs, GrCBLs, GhCBLs and CBLs of other plant species, we constructed a phylogenetic tree. Full-length amino acid sequences of 126 predicted CBL proteins were obtained from *G. arboretum*, *G. raimondii*, *G. hirsutum*, *A. thaliana*, *C. papaya*, *G. max*, *V. vinifera*, *T. cacao*, *P. trichocarpa*, *R. communis* and *O. sativa*. Phylogenetic trees were generated using the neighbor-joining method and MEGA 5.0 software. The CBLs family was divided into thirteen subfamilies according to the topology of the phylogenetic tree (Fig. 5). As expected, the three *Gossypium* CBLs commonly clustered closely in a subfamily. Most of them belonged to subfamily two, eight and thirteen. We found that the CBL members from different dicotyledon species
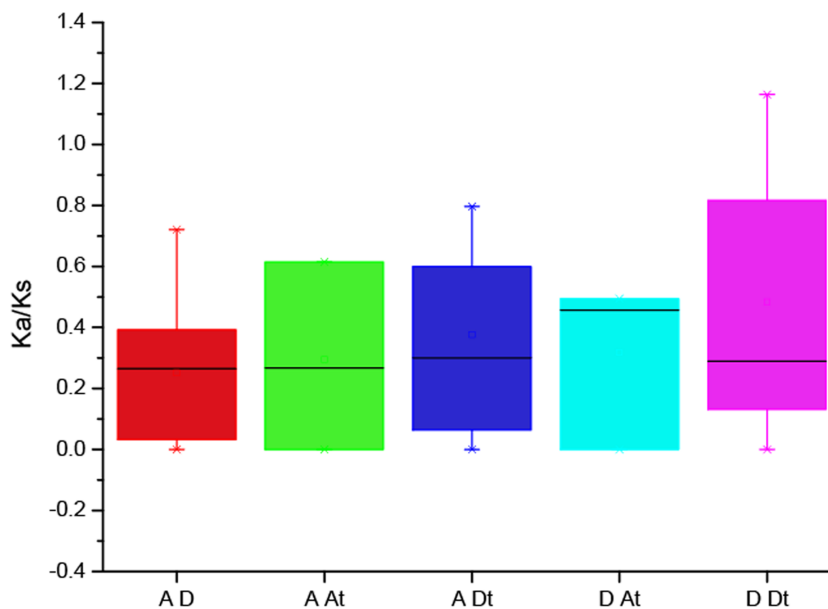
Figure 4 The Ka/Ks values of the CBL homologous genes between the A genome, D genome and subgenomes of *G. hirsutum* (A_tD_t).

and rice always clustered in a subfamily, suggesting that the CBLs shared an ancestral sequence before the divergence of eudicots and monocots or convergent evolution events for these CBLs might have occurred in adaptations to drastic changes in the environment. Moreover, the CBLs from *Gossypium* plants often clustered together with those from *T. cacao* (Fig. 5). These results are expected because both *Gossypium* and *T. cacao* are in the *Malvaceae* family.

Annotation analysis of GhCBLs

Putative functions of GhCBLs were analyzed using KOG (EuKaryotic orthologous groups (KOG) database (ftp://ftp.ncbi.nih.gov/pub/COG/KOG). Only the information on GhCBL3-6 was obtained. 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 the 22 GhCBLs was also assessed. The result showed that these GhCBL members were capable of binding calcium ion, like those of other plant species. These analyses 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 and *GhCBL3-6* strongly expressed in leaves. Moreover, the transcripts of *GhCBL1-1*, *GhCBL1-4*, *GhCBL1-5*, *GhCBL3-4*, *GhCBL3-5*, *GhCBL3-6* 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, *GhCBL3-6* mainly
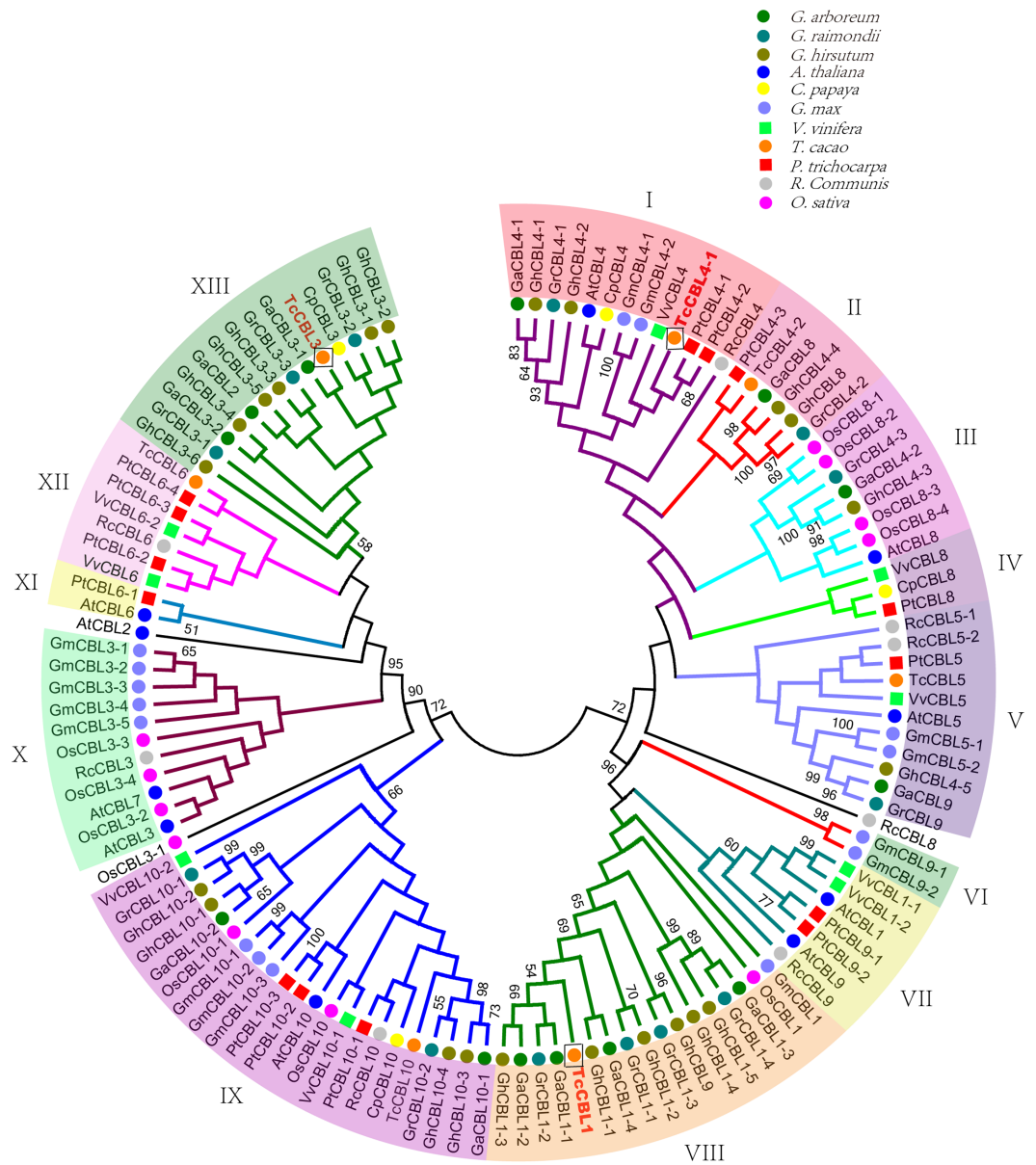
Figure 5 **Phylogenetic tree of CBLs in *Gossypium* and other plant species.** The plants in the square frame indicate that the CBL genes outside of *Gossypium* have the closest evolutionary relationship with *Gossypium* CBLs.

functions in leaves and other genes may chiefly act in flowers. *GhCBL1-1*, *GhCBL1-4*, *GhCBL1-5*, *GhCBL3-4*, *GhCBL3-5*, *GhCBL3-6* and *GhCBL9* also probably play a part in fiber development in cotton.

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
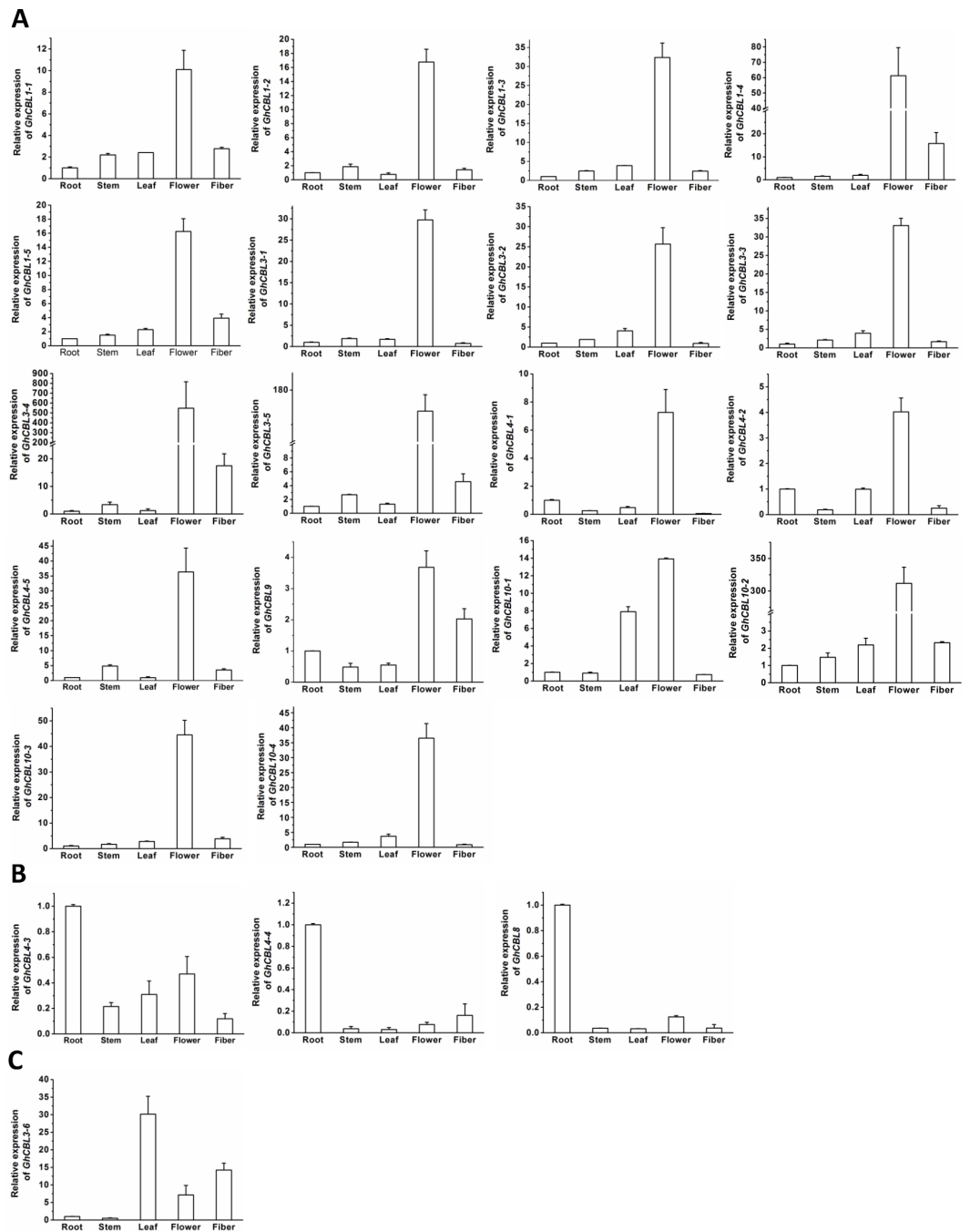
Figure 6 **Expression of 22 *GhCBL* genes in tissues of cotton.** The genes preferentially expressed in flowers (A), roots (B) and leaves (C) are shown. The relative expression of genes was calculated from 3 independent replicates. The expression value of the gene in roots was set as 1. The vertical bars represent the standard error.

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, the transcripts of many genes were reduced at 6 h, but increased at 2 d and/or 5 d. These gene included *GhCBL3-1*, *GhCBL3-2*, *GhCBL3-3*, *GhCBL3-4*, *GhCBL4-4*, and *GhCBL10-3*. The expression levels of *GhCBL3-5*, *GhCBL3-6*, *GhCBL4-3*, *GhCBL4-5*, *GhCBL8* and *GhCBL9* were decreased while those of other genes were unchanged after shortage of potassium (Fig. 7). The effects of K^+ resupply on the abundances of *GhCBL* transcripts were also investigated. Compared with 5 d of low-K^+ treatments, 3 h of K^+ refeeding clearly resulted in decreases in the expression of many genes such as *GhCBL1-3*, *GhCBL1-5*, *GhCBL3-2*, *GhCBL3-3*, *GhCBL3-4*, *GhCBL10-1* and *GhCBL10-3*. However, K^+ resupply increased the expression of *GhCBL4-1*. The transcriptional levels of other genes did not significantly alter upon K^+ resupply (Fig. 7). These results suggest that a number of GhCBLs may play roles in response to potassium starvation in cotton.

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 directly regulate GhCIPK23 in cotton.

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 eight *GaCBLs* and nine *GrCBLs* were homologous genes of *GhCBLs*. However, homologues of five GaCBLs and four GrCBLs were not discovered in the genome of *G. hirsutum*. These findings indicate that the eight *GaCBLs* and nine *GrCBLs* have been maintained in *G. hirsutum* after polyploidization event, while the five *GaCBLs* and four *GrCBLs* diverged from their orthologs in *G. hirsutum* during evolution. Moreover, we observed five *GhCBLs* (*GhCBL1-3*, *GhCBL3-5*, *GhCBL4-1*, *GhCBL4-4*, *GhCBL10-1*) in A_t subgenome and two *GhCBLs* (*GhCBL3-4*, *GhCBL3-6*) in D_t had no homologues in A genome of *G. arboreum* and D genome of *G. raimondii*, respectively. It is conceivable because selection pressures in diploids per loci are different than in the allotetraploid. Relaxed selection allows for development of novel and new functional alleles, but may also accumulate non functional, both at a higher rate possible that within the diploids. *G. arboreum* originates in the Africa/Arabia while *G. raimondii* and *G. hirsutum* originate in the Americas (*Wendel, Brubaker & Seelanan, 2010*). They are distributed in quite different places during evolution. Moreover, *G. arboreum* and *G. hirsutum* are two domasticated species (*Wendel, Brubaker & Seelanan, 2010*). Hence, geographic separation of the three species, and human selection may be essential for the diversity of the CBLs in *Gossypium*.
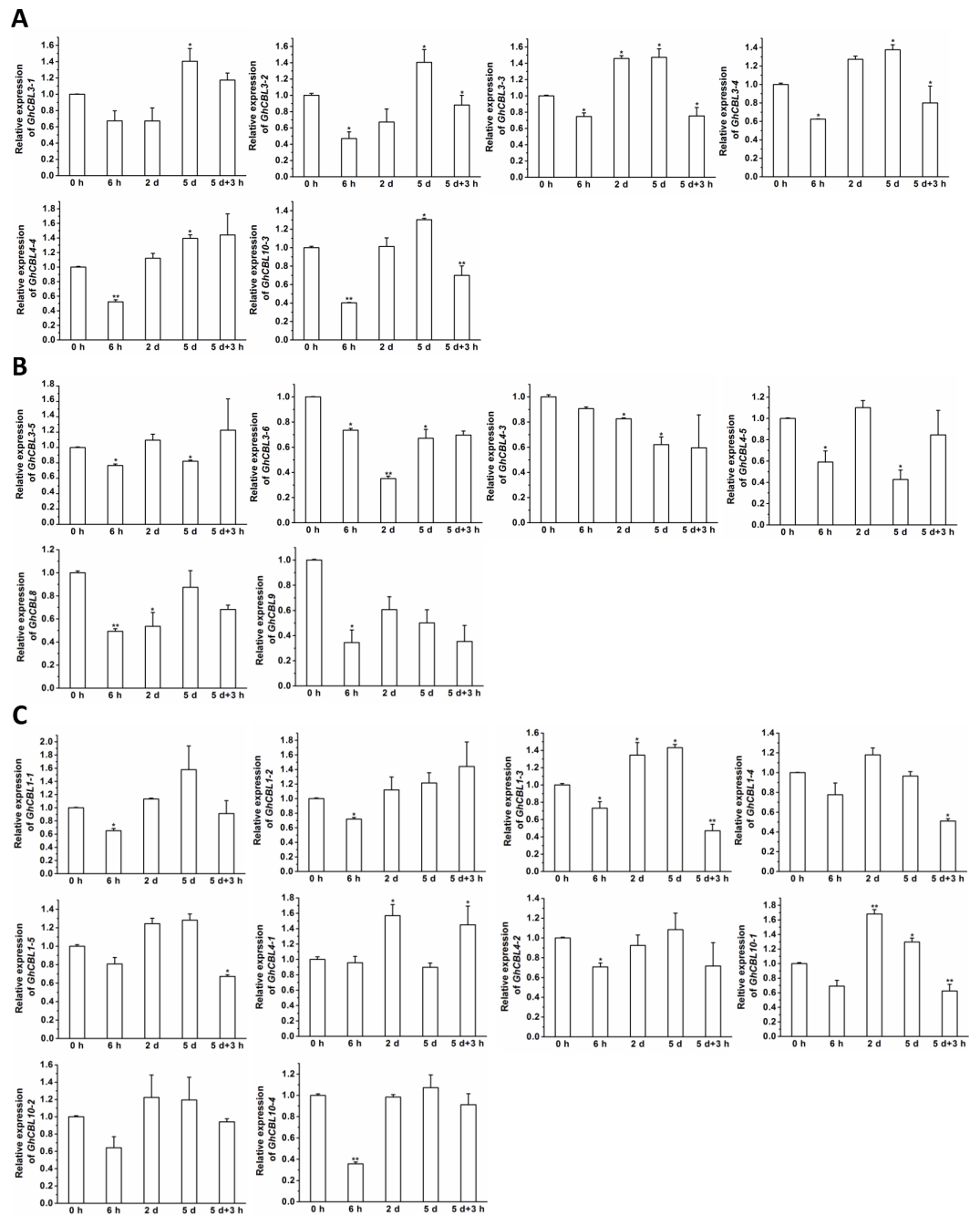
Figure 7 Expression of 22 *GhCBL* genes under potassium deprivation. The relative expression of *GhCBLs* was examined under potassium deficiency or resupply for indicated period of time. Under K deficiency, the expression levels of the genes were decreased at 6 h but increased at 2 d and/or 5 d (A), were reduced (B), and were not altered (C) significantly. The expression value of the gene at 0 h was set as 1. The vertical bars mean the standard error. Statistical analyses were conducted by student's *t* test to assess the differences between the samples at 0 h and those at 6 h, 2 d, or 5 d as well as between the samples at 5 d and those upon resupplying potassium for 3 h (5 d + 3 h). The single and double asterisks means that the differences are significant ($P \leq 0.05$) and extremely significant ($P \leq 0.01$), respectively.

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 remain highly conserved during evolution. The majority of *Gossypium* 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 *Gossypium*. 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. GhCBL3-6 also gives obvious proof of the evolutionary advantage of being tetraploid. It may be a product of significant human intervention because nothing like it was seen in either diploid.

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 appear on their corresponding A_t or D_t chromosomes, suggesting that complex exchange events of chromosome segments occurred in *G. hirsutum* during evolution. Additionally, separated (e.g., *GaCBL4-1* and *GaCBL4-2*; *GrCBL1-1* and *GrCBL1-2*) and jointed (*GaCBL4-2* and *GaCBL9*) distributions of the *Gossypium* CBL homologous genes in chromosomes in combination with the colinearity results of these genes (Figs 1; 3) 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 CBL genes in *Gossypium* was 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., 2016a*; *Li et al., 2016b*; *Zhang et al., 2016*), reflecting the rather conserved structure of CBL genes in different species. Moreover, nearly all of the *Gossypium* CBLs shared three conserved EF hand domains with other higher plants (Fig. 2). In addition, many *CBLs* from *Gossypium* 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*).

Measurement of the ratio of Ka to Ks indicated that majority of *Gossypium* CBL homologous genes have undergone purifying selection whereas *GhCBL10-2/GrCBL10-1* has experienced directional selection after polyploidization (Fig. 4). These results suggest that most *GhCBLs* have very high similarity in gene sequences and highly conserved functions to their orthologs from *G. arboretum* and *G. raimondii* during evolution. By contrast, a large divergence between *GhCBL10-2* and *GrCBL10-1G* has happened. *GhCBL10-2* may have evolved some novel functions through natural selection and human selection.

Phylogenetic analysis results revealed that the CBLs in *Gossypium* have closer relationship with those in cocoa than in other plants tested (Fig. 5). These findings strongly suggest that
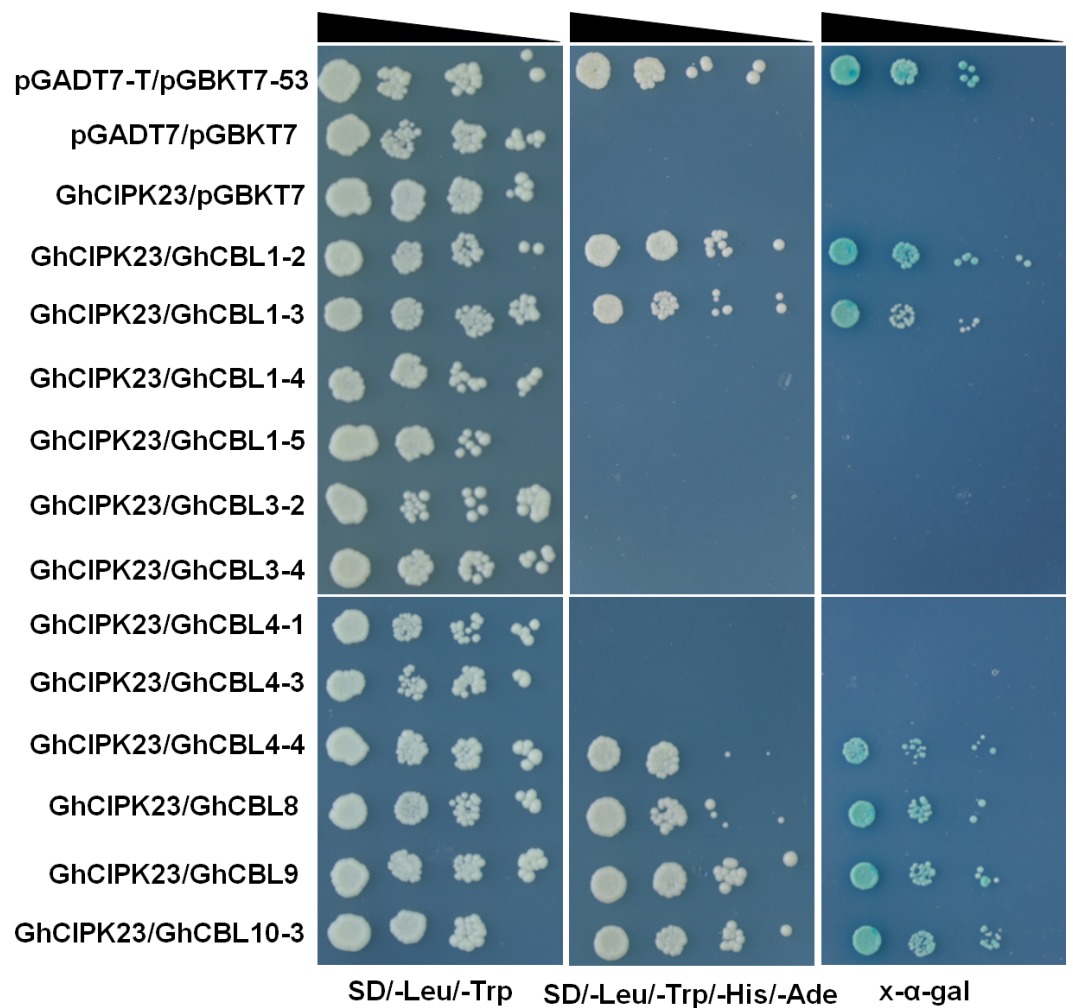
Figure 8 Yeast two-hybrid analysis of interactions between GhCBLs and GhCIPK23. The yeast cells containing the indicated plasmids were grown on the non-selective SD/-Leu/-Trp solid medium and selective SD/-Leu/-Trp/-His/-Ade solid medium, followed by X- α-Gal staining. The reduced cell densities in the dilution series are shown by narrowing triangles when proceeding from left to right. The first row represents a positive control, the 2th and 3th rows represent two negative controls.

the cotton species may have a more recent common ancestor with cacao relative to other plant species, in line with the results of other gene families in *Gossypium* (*Li et al., 2014b*; *Li et al., 2016a*; *Li et al., 2016b*). It may justify using CBL as another evolutionary model in plants because it showed highest similarity with another taxon from the same family and may help to narrow down the most vital or evolutionarily conserved or ancient sequences in *Gossypium*.

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 and fiber (Fig. 6), hinting that these genes may play important roles in the reproductive development in cotton. *G. hirsutum* is a highly domesticated plant for its seed fiber, which is developed from the flower. Preferential

expression of many *GhCBLs* in flowers and fibers suggests that human selection markedly affects the genetic variation and expression profiles of *GhCBLs*. Besides, the expression levels of *GhCBL4-3 GhCBL4-4* and *GhCBL8* in roots were clearly higher than those of other genes. These data imply that the three genes may function in modulation of ion transport or acclimation to diverse abiotic stresses in roots. Their detailed actions and mechanisms will be examined in the future.

The expression of 22 *GhCBLs* in responding to potassium starvation was determined. The transcription of most genes was moderately promoted 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 induced by low-potassium stress also likely play a part in adaptations to potassium deprivation in cotton. However, which sequences and how GhCBLs regulate potassium starved responses remains to be investigated in the future.

CIPK23 has been observed to function in diverse cellular processes in *Arabidopsis* (Mao et al., 2016). In this study, six out of 12 GhCBLs could interact with GhCIPK23 in yeast (Fig. 8), indicating that different GhCBL members may interact 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.

ADDITIONAL INFORMATION AND DECLARATIONS

Competing Interests

The authors declare there are no competing interests.

Author Contributions

- Tingting Lu conceived and designed the experiments, analyzed the data, wrote the paper, prepared figures and/or tables, reviewed drafts of the paper.

- Gaofeng Zhang performed the experiments, wrote the paper, prepared figures and/or tables, reviewed drafts of the paper.
- Lirong Sun analyzed the data, contributed reagents/materials/analysis tools, technical support.
- Ji Wang performed the experiments.
- Fushun Hao conceived and designed the experiments, analyzed the data, wrote the paper, reviewed drafts of the paper.

Data Availability

The following information was supplied regarding data availability:

The raw data has been uploaded as a Supplemental File.

Supplemental Information

Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.3653#supplemental-information.

REFERENCES

Allen RD. 2010. Opportunities for engineering abiotic stress tolerance in cotton plants. In: *Cotton.* Berlin: Springer Verlag, 127–160.

Cheong YH, Pandey GK, Grant JJ, Batistic O, Li L, Kim BG, Lee SC, Kudla J, Luan S. 2007. Two calcineurin B-like calcium sensors, interacting with protein kinase CIPK23, regulate leaf transpiration and root potassium uptake in *Arabidopsis. The Plant Journal* **52**:223–239.

Cho JH, Lee JH, Park YK, Choi MN, Kim KN. 2016. Calcineurin B-like protein CBL10 directly interacts with TOC34 (Translocon of the Outer membrane of the Chloroplasts) and decreases its GTPase activity in *Arabidopsis. Frontiers in Plant Science* **7**:Article 1911 DOI 10.3389/fpls.2016.01911.

Drerup MM, Schlücking K, Hashimoto K, Manishankar P, Steinhorst L, Kuchitsu K, Kudla J. 2013. The calcineurin B-like calcium sensors CBL1 and CBL9 together with their interacting protein kinase CIPK26 regulate the *Arabidopsis* NADPH oxidase RBOHF. *Molecular Plant* **6**:559–569 DOI 10.1093/mp/sst009.

Finn RD, Mistry J, Tate J, Coggill P, Heger A, Pollington JE, Gavin OL, Gunasekaran P, Ceric G, Forslund K, Holm L, Sonnhammer EL, Eddy SR, Bateman A. 2010. The pfam protein families database. *Nucleic Acids Research* **38**:D211–D222 DOI 10.1093/nar/gkp985.

Fuglsang AT, Guo Y, Cuin TA, Qiu Q, Song C, Kristiansen KA, Bych K, Schulz A, Shabala S, Schumaker KS, Palmgren MG, Zhu JK. 2007. Arabidopsis protein kinase PKS5 inhibits the plasma membrane H^+-ATPase by preventing interaction with 14-3-3 protein. *The Plant Cell* **19**:1617–1634 DOI 10.1105/tpc.105.035626.

Gao P, Zhao PM, Wang J, Wang HY, Du XM, Wang GL, Xia GX. 2008. Co-expression and preferential interaction between two calcineurin B-like proteins and a CBL-interacting protein kinase from cotton. *Plant Physiology and Biochemistry* **46**:935–940 DOI 10.1016/j.plaphy.2008.05.001.

Held K, Pascaud F, Eckert C, Gajdanowicz P, Hashimoto K, Corratgé-Faillie C, Offenborn JN, Lacombe B, Dreyer I, Thibaud JB, Kudla J. 2011. Calcium-dependent modulation and plasma membrane targeting of the AKT2 potassium channel by the CBL4/CIPK6 calcium sensor/protein kinase complex. *Cell Research* **21**:1116–1130 DOI 10.1038/cr.2011.50.

Ho CH, Lin SH, Hu HC, Tsay YF. 2009. CHL1 functions as a nitrate sensor in plants. *Cell* **138**:1184–1194 DOI 10.1016/j.cell.2009.07.004.

Kim BG, Waadt R, Cheong YH, Pandey GK, Dominguez-Solis JR, Schültke S, Lee SC, Kudla J, Luan S. 2007. The calcium sensor CBL10 mediates salt tolerance by regulating ion homeostasis in *Arabidopsis. Plant Journal* **52**:473–484 DOI 10.1111/j.1365-313X.2007.03249.x.

Kolukisaoglu Ü, Weinl S, Blazevic D, Batistic O, Kudla J. 2004. Calcium sensors and their interacting protein kinases: genomics of the arabidopsis and rice CBL-CIPK signaling networks. *Plant Physiology* **134**:43–58 DOI 10.1104/pp.103.033068.

Kudla J, Batistič O, Hashimoto K. 2010. Calcium signals: the lead currency of plant information processing. *The Plant Cell* **22**:541–563 DOI 10.1105/tpc.109.072686.

Kudla J, Xu Q, Harter K, Gruissem W, Luan S. 1999. Genes for calcineurin B-like proteins in *Arabidopsis* are differentially regulated by stress signals. *Proceedings of the National Acadamy of Sciences of the United States of America* **96**:4718–4723 DOI 10.1073/pnas.96.8.4718.

Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG. 2007. Clustal W and Clustal X version 2.0. *Bioinformatics* **23**:2947–2948 DOI 10.1093/bioinformatics/btm404.

Li F, Fan G, Wang K, Sun F, Yuan Y, Song G, Li Q, Ma Z, Lu C, Zou C, Chen W, Liang X, Shang H, Liu W, Shi C, Xiao G, Gou C, Ye W, Xu X, Zhang X, Wei H, Li Z, Zhang G, Wang J, Liu K, Kohel RJ, Percy RG, Yu JZ, Zhu YX, Wang J, Yu S. 2014b. Genome sequence of the cultivated cotton *Gossypium arboreum. Nature Genetics* **46**:567–572 DOI 10.1038/ng.2987.

Li J, Jiang MM, Ren L, Liu Y, Chen HY. 2016a. Identification and characterization of CBL and CIPK gene families in eggplant (*Solanum melongena* L.). *Molecular Genetics and Genomics* **291**:1769–1781 DOI 10.1007/s00438-016-1218-8.

Li L, Kim BG, Cheong YH, Pandey GK, Luan S. 2006. A Ca^{2+} signaling pathway regulates a K^+ channel for low-K response in *Arabidopsis. Proceedings of the National Acadamy of Sciences of the United States of America* **103**:12625–12630 DOI 10.1073/pnas.0605129103.

Li J, Long Y, Qi GN, Li J, Xu ZJ, Wu WH, Wang Y. 2014a. The Os-AKT1 channel is critical for K^+ uptake in rice roots and is modulated by the rice CBL1-CIPK23 complex. *The Plant Cell* **26**:3387–3402 DOI 10.1105/tpc.114.123455.

Li W, Shang H, Ge Q, Zou C, Cai J, Wang D, Fan S, Zhang Z, Deng X, Tan Y, Song W, Li P, Jamshed M, Lu Q, Gong W, Li J, Shi Y, Chen T, Gong J, Liu A, Yuan Y. 2016b. Genome-wide identification, phylogeny, and expression analysis of pectin

methylesterases reveal their major role in cotton fiber development. *BMC Genomics* **17**:1000 DOI 10.1186/s12864-016-3365-z.

Ligaba-Osena A, Fei Z, Liu J, Xu Y, Shaff J, Lee SC, Luan S, Kudla J, Kochian L, Piñeros M. 2017. Loss-of-function mutation of the calcium sensor CBL1 increases aluminum sensitivity in *Arabidopsis*. *New Phytologist* **214**:830–841 DOI 10.1111/nph.14420.

Liu LL, Ren HM, Chen LQ, Wang Y, Wu WH. 2013. A protein kinase, calcineurin B-like protein-interacting protein kinase9, interacts with calcium sensor calcineurin B-like protein3 and regulates potassium homeostasis under low-potassium stress in *Arabidopsis*. *Plant Physiology* **161**:266–277 DOI 10.1104/pp.112.206896.

Luan S. 2009. The CBL-CIPK network in plant calcium signaling. *Trends in Plant Science* **14**:37–42 DOI 10.1016/j.tplants.2008.10.005.

Mao J, Manik SMN, Shi S, Chao J, Jin Y, Wang Q, Liu H. 2016. Mechanisms and physiological roles of the CBL-CIPK networking system in *Arabidopsis thaliana*. *Genes* **7**:Article 62 DOI 10.3390/genes7090062.

Mohanta TK, Mohanta N, Mohanta YK, Parida P, Bae H. 2015. Genome-wide identification of calcineurin B-Like (CBL) gene family of plants reveals novel conserved motifs and evolutionary aspects in calcium signaling events. *Plant Biology* **15**:Article 189 DOI 10.1186/s12870-015-0543-0.

Murashige T, Skoog F. 1962. A revised medium for rapid growth and bio-assays with tobacco tissue culture. *Physiologia Plantarum* **15**:473–497 DOI 10.1111/j.1399-3054.1962.tb08052.x.

Oosterhuis DM, Loka DA, Raper TB. 2013. Potassium and stress alleviation: physiological functions and management of cotton. *Journal of Plant Nutrition and Soil Science* **176**:331–343 DOI 10.1002/jpln.201200414.

Pandey GK, Cheong YH, Kim KN, Grant JJ, Li L, Hung W, D'Angelo C, Weinl S, Kudla J, Luan S. 2004. The calcium sensor calcineurin B-like 9 modulates abscisic acid sensitivity and biosynthesis in *Arabidopsis*. *The Plant Cell* **16**:1912–1924 DOI 10.1105/tpc.021311.

Ren XL, Qi GN, Feng HQ, Zhao S, Zhao SS, Wang Y, Wu WH. 2013. Calcineurin B-like protein CBL10 directly interacts with AKT1 and modulates K^+ homeostasis in *Arabidopsis*. *The Plant Journal* **74**:258–266 DOI 10.1111/tpj.12123.

Sanyal SK, Pandey A, Pandey GK. 2015. The CBL–CIPK signaling module in plants: a mechanistic perspective. *Physiologia Plantarum* **155**:89–108 DOI 10.1111/ppl.12344.

Sarwat M, Ahmad P, Nabi G, Hu XY. 2013. Ca^{2+} signals: the versatile decoders of environmental cues. *Critical Reviews in Biotechnology* **33**:97–109 DOI 10.3109/07388551.2012.672398.

Steinhorst L, Mähs A, Ischebeck T, Zhang C, Zhang X, Arendt S, Schültke S, Heilmann I, Kudla J. 2015. Vacuolar CBL-CIPK12 Ca^{2+}-sensor-kinase complexes are required for polarized pollen tube growth. *Current Biology* **25**:1475–1482 DOI 10.1016/j.cub.2015.03.053.

Straub T, Ludewig U, Neuhäuser B. 2017. The kinase CIPK23 inhibits ammonium transport in *Arabidopsis thaliana*. *The Plant Cell* **29**:409–422 DOI 10.1105/tpc.16.00806.

Sun T, Wang Y, Wang M, Li T, Zhou Y, Wang X, Wei S, He G, Yang G. 2015. Identification and comprehensive analyses of the CBL, and CIPK, gene families in wheat (*Triticum aestivum* L). *Plant Biology* **15**:Article 269 DOI 10.1186/s12870-015-0657-4.

Tang RJ, Zhao FG, Garcia VJ, Kleist TJ, Yang L, Zhang HX, Luan S. 2015. Tonoplast CBL-CIPK calcium signaling network regulates magnesium homeostasis in *Arabidopsis. Proceedings of the National Acadamy of Sciences of the United States of America* **112**:3134–3139 DOI 10.1073/pnas.142094412.

Thoday-Kennedy EL, Jacobs AK, Roy SJ. 2015. The role of the CBL-CIPK calcium signaling network in regulating ion transport in response to abiotic stress. *Plant Growth Regulation* **76**:3–12 DOI 10.1007/s10725-015-0034-1.

Tian QY, Zhang XX, Yang A, Wang TZ, Zhang WH. 2016. CIPK23 is involved in iron acquisition of *Arabidopsis* by affecting ferric chelate reductase activity. *Plant Science* **246**:70–79 DOI 10.1016/j.plantsci.2016.01.010.

Wendel JF, Brubaker CL, Seelanan T. 2010. The origin and evolution of *Gossypium.* In: Stewart JM, Oosterhuis D, Heitholt JJ, Mauney JR, eds. *Physiology of Cotton.* Berlin: Springer, 1–18.

Xu J, Li HD, Chen LQ, Wang Y, Liu LL, He L, Wu WH. 2006. A protein kinase, interacting with two calcineurin B-like proteins, regulates K^+ transporter AKT1 in *Arabidopsis. Cell* **125**:1347–1360 DOI 10.1016/j.cell.2006.06.011.

Zhang F, Li L, Jiao Z, Chen Y, Liu H, Chen X, Fu J, Wang G, Zheng J. 2016. Characterization of the calcineurin B-Like (CBL) gene family in maize and functional analysis of ZmCBL9 under abscisic acid and abiotic stress treatments. *Plant Science* **253**:118–129 DOI 10.1016/j.plantsci.2016.09.011.

Zhang H, Yang B, Liu W-Z, Li H, Wang L, Wang B, Deng M, Liang W, Deyholos MK, Jiang YQ. 2014. Identification and characterization of CBL and CIPK gene families in canola (*Brassica napus* L.). *BMC Plant Biology* **14**:1 DOI 10.1186/1471-2229-14-8.