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

Tingting Lu ¹, ², Gaofeng Zhang ¹, Lirong Sun ¹, Ji Wang ¹, Fushun Hao Corresp. ¹

¹ College of Life Sciences, Henan University, State Key Laboratory of Cotton Biology, Henan Key Laboratory of Plant Stress Biology, Kaifeng, Henan, China
² Henan University of Animal Husbandry and Economy, College of Pharmaceutical Engineering, Zhengzhou, Henan, China

Corresponding Author: Fushun Hao
Email address: haofsh@henu.edu.cn

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 3 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.
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Authors:
Tingting Lu\textsuperscript{1,2*}, Gaofeng Zhang\textsuperscript{1*}, Lirong Sun\textsuperscript{1}, Ji Wang\textsuperscript{1} and Fu-Shun Hao\textsuperscript{1}

\textsuperscript{1}State Key Laboratory of Cotton Biology, Henan Key Laboratory of Plant Stress Biology, College of Life Sciences, Henan University, Kaifeng 475004, China

\textsuperscript{2}Henan University of Animal Husbandry and Economy, Zhengzhou 450011, China

* These authors contributed equally to this work

Corresponding author
Fu-Shun Hao, haofsh@henu.edu.cn
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¹ State Key Laboratory of Cotton Biology, Henan Key Laboratory of Plant Stress Biology, College of Life Sciences, Henan University, Kaifeng 475004, China
² Henan University of Animal Husbandry and Economy, Zhengzhou 450011, China

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 3 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.
Keywords Gossypium; calcineurin B-like proteins (CBLs); gene family; phylogeny; gene expression

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 et al., 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, et al., 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 et al., 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 et al., 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 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 et al., 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 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 *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 et al., 2010).

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). 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 *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 were 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://asgpb.mhpcc.hawaii.edu) 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 and Skoog, 1962) in the growth chamber described above for 3 weeks. Then, the plants were watered with K⁺-lacking liquid 1/2 MS medium (KNO₃ was replaced by NH₄NO₃ and KH₂PO₄ was replaced by NH₄H₂PO₄) 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 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) following the instructions in the Promega kit.

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| GAAAGGCATTCCACCTGAGCAAC  | CTTGACCCTTCTTCTTTTG                    |
|        |            | GAGCGTAACGGAGGTCAAGCAAA  | TGCTTG                                  |
|        |            | TTTTGTTGAGCAGCTCAAATGTTT | CTCC                                    |
|        |            | GACATTCTGGGAAGGCGATA     | TTGCCCTCAATCGTTTTCATAG                |
|        |            | AGAGATAGACCCTTACCATACTAA | ATCAGGTATGGGAGGGTCACT                    |
|        |            | GGATGCCGACACTAACCAGCGG   | CGAGCGAGATATTCTCCGA                    |
|        |            | AGTTTGCTCGTGCTCTCTCTG    | TCCAACAACGTTAAGCGGCC                    |
|        |            | ATGTTGCTCGTGCTCTCTCTG    | ATCATCTGAAAAGGTTTCAT                    |
|        |            | GCAAGAGAGAGCCCTT          | GCCA                                    |
|        |            | AGAAGGCTTCTTCTTTCTTG     | AATCTTATCGTCAATGGG                     |
| GhCBL1-1 | Gh_A11G0257| GAAAGGCATTCCACCTGAGCAAC  | CTTGACCCTTCTTCTTTTG                    |
| GhCBL1-2 | Gh_D11G0276| GAGCGTAACGGAGGTCAAGCAAA  | TGCTTG                                  |
| GhCBL1-3 | Gh_A03G0043| TTTTGTTGAGCAGCTCAAATGTTT | CTCC                                    |
| GhCBL1-4 | Gh_D09G1875| GACATTCTGGGAAGGCGATA     | TTGCCCTCAATCGTTTTCATAG                |
| GhCBL1-5 | Gh_A09G1766| AGAGATAGACCCTTACCATACTAA | ATCAGGTATGGGAGGGTCACT                    |
| GhCBL3-1 | Gh_A01G0740| GGATGCCGACACTAACCAGCGG   | CGAGCGAGATATTCTCCGA                    |
| GhCBL3-2 | Gh_D01G0760| AGTTTGCTCGTGCTCTCTCTG    | TCCAACAACGTTAAGCGGCC                    |
|        |            | ATGTTGCTCGTGCTCTCTG    | ATCATCTGAAAAGGTTTCAT                    |
|        |            | GCAAGAGAGAGCCCTT          | GCCA                                    |
|        |            | AGAAGGCTTCTTCTTTCTTG     | AATCTTATCGTCAATGGG                     |
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-
dioxyl-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                 | CGCGGATCTTGGAACACCTCTCA                        |
| GhCBL1-3(BD)| Gh_A03G0043| CCGGAATTCATGGGCTGCTTTCAATCT                 | CGCGGATCCTGGAACACCTCTCA                       |
| GhCBL1-4(BD)| Gh_D09G1875| CCGGAATTCATGGGCTGCTTTCAATCT                 | CGCGGATCCTGGAACACCTCTCA                       |
| GhCBL1-5(BD)| Gh_A09G1766| CCGGAATTCATGGGCTGCTTTCAATCT                 | CGCGGATCCTGGAACACCTCTCA                       |
| GhCBL3-2(BD)| Gh_D01G0760| CCGGAATTCATGGGCTGCTTTCAATCT                 | CGCGGATCCTGGAACACCTCTCA                       |
| GhCBL3-4(BD)| Gh_D13G1364| CCGGAATTCATGGGCTGCTTTCAATCT                 | CGCGGATCCTGGAACACCTCTCA                       |
| GhCBL4-1(BD)| Gh_A11G0126| CCGGAATTCATGGGCTGCTTTCAATCT                 | CGCGGATCCTGGAACACCTCTCA                       |
| GhCBL4-3(BD)| Gh_A12G2144| CCGGAATTCATGGGCTGCTTTCAATCT                 | CGCGGATCCTGGAACACCTCTCA                       |
| GhCBL4-4(BD)| Gh_A09G1696| CCGGAATTCATGGGCTGCTTTCAATCT                 | CGCGGATCCTGGAACACCTCTCA                       |
| GhCBL8(BD)  | Gh_D09G1801| CCGGAATTCATGGGCTGCTTTCAATCT                 | CGCGGATCCTGGAACACCTCTCA                       |
| GhCBL9(BD)  | Gh_D08G1764| CCGGAATTCATGGGCTGCTTTCAATCT                 | CGCGGATCCTGGAACACCTCTCA                       |
| GhCBL10-3(BD)| Gh_A05G0335| CCGGAATTCATGGGCTGCTTTCAATCT                 | CGCGGATCCTGGAACACCTCTCA                       |
| GhCIPK23(AD)| Gh_A06G1219| CCGGAATTCATGGGCTGCTTTCAATCT                 | CGCGGATCCTGGAACACCTCTCA                       |

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<sub>t</sub>D<sub>t</sub> 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 play a special role in cotton.

**Table 3** The CBL family genes in *Gossypium*

| Gene name | Gene ID | pI  | MW (kDa) | Hydrophilicity | Predicted subcellular localization | amino acid residues | coding sequence |
|-----------|---------|-----|--------|----------------|-----------------------------------|--------------------|-----------------|
| Protein  | Species       | Accession     | M.Wt | PI   | Cell Location | M.Wt | PI   |
|----------|---------------|---------------|------|------|---------------|------|------|
| 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       | 1326 | 3981 |
| Gene Name | Accession | p-value | FDR | Localisation | Chromosome | Start | End |
|-----------|-----------|---------|-----|--------------|------------|-------|-----|
| GhCBL4-1  | Gh_A11G0126 | 4.77    | -0.059 | Cell membrane | 201        | 606   |
| GhCBL4-2  | Gh_D11G0140 | 4.82    | -0.185 | Cell membrane | 220        | 663   |
| GhCBL4-3  | Gh_A12G2144 | 4.97    | -0.175 | Cell membrane | 215        | 648   |
| GhCBL4-4  | Gh_A09G1696 | 5.27    | -0.184 | Cell membrane | 248        | 747   |
| GhCBL4-5  | Gh_D12G2320 | 5.64    | 0.023  | Cell membrane | 218        | 657   |
| GhCBL8    | Gh_D09G1801 | 4.85    | -0.177 | Cell membrane | 217        | 654   |
| GhCBL9    | Gh_D08G1764 | 4.74    | -0.032 | Cell membrane | 209        | 630   |
| GhCBL10-1 | Gh_A06G0800 | 5.18    | -0.143 | Cell membrane | 293        | 882   |
| GhCBL10-2 | Gh_D06G0922 | 4.95    | -0.159 | Cell membrane | 265        | 798   |
| GhCBL10-3 | Gh_A05G0335 | 5.16    | -0.114 | Cell membrane | 262        | 789   |
| GhCBL10-4 | Gh_D05G0440 | 5.01    | -0.08  | Cell membrane | 262        | 789   |

**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 7 chromosomes. Among them, 3 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 9 chromosomes. Each of the 4 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. Each of the 5 chromosomes Ghchr09, Ghchr11, Ghchr19, Ghchr21 and Ghchr23 had 2 CBL members, and other chromosomes individually carried 1 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 play a role in generating these genes during evolution.
Fig.1 Distributions of the CBL family genes on chromosomes in *Gossypium*

The GaCBLs, GrCBLs and GhCBLs are from *G. arboreum*, *G. raimondii* and *G. hirsutum*, respectively.

**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 (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 the 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 9 and GaCBL9, GrCBL1-2 had 7. 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 *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 3 EF-hand motifs except for GaCBL9, which contained 2 such motifs (Fig. 2A). Furthermore, a conserved myristoylation motif (MGXXS/T) was detected in the N-terminal regions of 11 CBL proteins. These proteins included 4 GaCBLs, 2 GrCBLs and 5 GhCBLs (Fig. 2B, C). 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).
Fig. 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 to 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.

### Syntenic 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 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 were distributed on 5 chromosomes in *G. arboreum* and 5 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 3 *Gossypium* species (Table S1). These results imply that many cotton CBL genes may have evolved through segmental duplication.

![Fig. 3 Genome-wide synteny analysis of *Gossypium* CBL genes](image)

(A) Syntenic 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) Syntenic analysis between *G. arboreum* and *G. raimondii*. 
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.

![Fig 4. The Ka/Ks values of the CBL homologous genes between the A genome, D genome and subgenomes of *G. hirsutum* (A,D)](image)

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. arboreum*, *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 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.

Fig. 5 Phylogenetic tree of CBLs in Gossypium and other plant species
The plants in the square frame indicated that the CBL genes outside of Gossypium have the closest evolutionary relationship with Gossypium CBLs.
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 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.
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.

**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, 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.
The relative expression of GhCBLs was examined under potassium deficiency or resupply 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. 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.
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.

![Yeast two-hybrid analysis of interactions between GhCBLs and GhCIPK23](image)

**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 when proceeding from left to right. The first row represents a positive control, the 2nd and 3rd 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\textsubscript{t} and D\textsubscript{t} subgenome, respectively. They were similar to the number of CBLs 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 the 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* diverged from their orthologs in *G. hirsutum* during evolution. Moreover, we observed 5 *GhCBLs* (*GhCBL1-3, GhCBL3-5, GhCBL4-1, GhCBL4-4, GhCBL10-1*) in A\textsubscript{t} subgenome and 2 *GhCBLs* (*GhCBL3-4, GhCBL3-6*) in D\textsubscript{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 et al., 2010). They are distributed in quite different places during evolution. Moreover, *G. arboreum* and *G. hirsutum* are two domasticated species (Wendel et al., 2010). Hence, geographic separation of the three species, and human selection may be essential for the diversity of the CBLs in *Gossypium*.

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 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\textsuperscript{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, and D, 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, or D, 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 (Fig. 1; Fig. 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., 2016; 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 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., 2016). 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 \textit{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 interact with and modulate \(GhCIPK23\) in various growth and/or stress responses in cotton. The cotton homologues of \textit{Arabidopsis} \(CBL1\) and \(CBL9\) suggest that \(GhCBL1\) and \(GhCBL9\) probably play similar roles to \(CBL1\) and \(CBL9\) in cotton.

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