



Article

Genome-Wide Analysis and Expression Tendency of Banana (*Musa acuminata* L.) Calcineurin B-Like (MaCBL) Genes under Potassium Stress

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Abstract: Calcineurin B-like (CBL) proteins are reported to play significant roles in plant development and ion-transport regulation. Potassium shortages are a serious problem in banana cultivation. However, to date, the members of the banana CBL gene family, and their function in regulating potassium stress, remain unclear. In this study, 11 CBL genes were identified from the banana genome and grouped into four groups (Group I–IV) based on their phylogenetic relationships. The genomic features of these MaCBL genes were analyzed, focusing on their gene structures, standpat motifs, chromosomal distributions, and evolutionary history. Expression pattern analysis revealed that the MaCBLs were function-specific. Further qRT-PCR analysis indicated that the presence of MaCBL2 was indeed a response to potassium deficiency stress. The MaCBL2 gene was cloned, and sequence alignment indicated that it contained four elongation factor hand (EF-hand) domains, the conserved N-terminal myristoylation domain “MGCXXS/K(T)” and the “FPSE” motif. Subcellular location analysis showed that MaCBL2 was located in the plasma membrane, nucleus and cytoplasm. The overexpression of MaCBL2 could restore the growth of the yeast mutant R5421 on a K⁺-deficient medium. The overexpression of MaCBL2 could promote the root length of transgenic seedlings on K⁺-deficient medium. These findings indicate that MaCBL2 was, in our study, the key gene of the CBL family in responding to potassium deficiency in bananas. Our discoveries have established a considerable basis for the further study and application of MaCBL genes.

Keywords: banana; CBL; potassium stress; gene expression; functional complementation; yeast mutant; subcellular location

1. Introduction

Calcineurin B-like (CBL) proteins, a family of Ca²⁺ transducers, are famous for their interactions with the CBL-interacting protein kinases (CIPK), enacting numerous regulatory roles in plant development and physiological activity according to CBL–CIPK models [1]. CBLs are found in both monocots and dicots, and also belong to a complex, polygenic family. According to published data, the numbers of CBL genes vary between different species. For instance, only 5 CBL members are found in eggplants [2], while there are 8 in pineapples [3], 9 in peppers [4], 10 in both *Arabidopsis* and rice [5], and 22 in *Gossypium hirsutum* [6]. Evolutionary analysis has suggested that the number of CBL members in the genome of each species is independent of its genome size [7]. For example, among monocotyledonous

plants, the genome size of maize and rice is 2500 and 372 Mbs, respectively, while the number of *CBL* genes in maize is less than that in rice, which is 9 and 11, respectively [7]. *CBL*s feature four elongation factor hand (EF-hand) motifs and bear Ca^{2+} -binding sites in their protein sequences [7]. However, the EF-hand structure varies across different *CBL*s. For example, there is only one unmutated classical EF-hand structure in *AtCBL6*, -7, -8 and -10, and there two unmutated EF-hand structures in *AtCBL1* and *AtCBL9*, while *AtCBL2–5* have no classical EF-hand structure [5]. Although the EF-hand structures of different *CBL* proteins vary to different degrees, the quantities of EF-hand motifs have remained stable in all the previously studied *CBL*s [5]. In addition, some *CBL* proteins contain domains associated with protein interaction and cell anchoring, such as the myristoylation domain [8] and tonoplast-targeting sequences [9].

CBL family genes can be involved in signal transduction either by interacting with *CIPK*s or in a manner independent of them [10]. The *CBL–CIPK* model is reported to characterize many forms of abiotic stress response. The *CBL4–CIPK24–SOS1* and *CBL10–CIPK24–NHX* pathways are the two salt-stress signal-transduction pathways in *Arabidopsis* [11,12], and the *CBL1/9–CIPK23–AKT1*, *CBL3–CIPK9*, and *CBL4–CIPK6–AKT2* pathways are involved in signal transduction for potassium absorption [13–16]. Alternatively, *CBL1/9–CIPK1* is involved in responding to drought stress, *CBL1/9–CIPK26* is involved in the regulation of ROS signals, and *CBL2–CIPK7* is involved in the light signaling pathway [17–20]. Several studies have indicated that some *CBL* genes can directly activate downstream genes without any association with *CIPK* genes. For example, *AtCBL3* can directly act on its downstream target gene *AtMTAN* and inhibit its enzymatic activity [21], and *AtCBL10* can directly act on *AtAKT1* to balance the K^+ levels in plant cells [10]. However, previous studies have mainly focused on the model plant *Arabidopsis thaliana*, and the issue of whether the homologous genes in other plant species have similar functions remains to be investigated.

The banana (*Musa acuminata* L.) is a traditional fruit tree that grows in tropical or subtropical areas around the world. It typically suffers from potassium shortages during its cultivation. It is well known that potassium availability and supply are vital for the banana and usually the most crucial factors aiding or restricting the physiology and development of the fruit. Decades of research has provided deep insight into the molecular components and mechanisms of potassium-ion absorption, distribution and storage in the plants. Some *CBL* family members have been reported to control the potassium absorption process [13–16]; however, whether the *CBL* family members in bananas have the same function and which one is the key factor remain to be determined. In this study, publicly accessible banana genomic data [22] and our previous transcriptomic data [23] were used to perform genome-wide mining, as well as evolutionary and gene expression analysis, of the *CBL* family genes. The selected *MaCBL2* gene was further cloned from the banana plant and studied for its function in the response to potassium via functional complementarity experiments with yeast mutation and the growth analysis in tobacco transgenic seedlings under potassium shortage treatments. The results provide novel insights into the potassium-deficient stress responses of *MaCBL* gene members and may be valuable for further understanding the utilization of *CBL* genes in bananas or other plants.

2. Materials and Methods

2.1. Mining of *CBL* Genes in Banana Genome

Phytozome13 [24] was employed to search for genes encoding *CBL* proteins in the banana (*Musa acuminata*) genome [22]. First, the well-studied *CBL* protein sequences for *Arabidopsis* [5] were employed in searching for the homologous proteins in the banana genome through BLASTP. The *CBL* genes obtained were named *MaCBL*s and numbered based on their chromosomal locations in ascending order. The gene sequences, annotations and information on chromosome location were obtained from the genomic data. The physicochemical parameters of the *MaCBL*s were calculated with the ProtParam tool in ExPasy (<http://web.expasy.org/protparam> accessed on 18 January 2021) [25].

2.2. Phylogenetic Analysis and Multiple Sequence Alignment

The sequences of the CBL proteins from banana (*Musa acuminata*), *Arabidopsis thaliana* [5], rice (*Oryza sativa*) [5], pineapple (*Ananas comosus*) [3], wheat (*Triticum aestivum*) [26], pepper (*Capsicum annuum*) [4], cassava (*Manihot esculenta*) [27], *Brassica napus* [28], and *Populus trichocarpa* were collected to perform multiple alignment via ClustalW within the MEGA7 software. The phylogenetic tree was constructed in MEGA7, and the evolutionary history was extrapolated with the neighbor-joining method. Multiple alignment and visualization were performed via the JGI-CIG Jalview procedure in Phytozome [24].

2.3. Gene Structure, Conserved Motif, Chromosome Localization, and Syntenic Relationship Analysis

Conserved motifs in the MaCBL protein sequences were analyzed with the MEME platform (accessed date on 18 January 2021, <https://meme-suite.org/meme/tools/meme>), and the gene structure map was obtained with TBtools [29]. The combined visualization of the phylogenetic tree, the motifs, and the gene structures of the MaCBLs were also achieved with TBtools, as were the visualizations of the chromosome localization and syntenic relationships [29]. The Ka and Ks were calculated with the DnaSP6 software [30].

2.4. Plant Treatments and RNA Extraction

The cultivation and treatment of the banana plants was undertaken in a manner similar to that in our previous report, with some variation [23]. In this study, the banana plants were treated with four potassium concentrations: 0, 0.03, 0.3, and 3 mM K₂SO₄. After treatment for 30 days, the roots were collected and used for RNA extraction. The RNAprep Pure Plant Kit (TIANGEN BIOTECH (BEIJING) CO. LTD) was used to extract the total RNA. The PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time) (Takara) was used to reverse transcription of mRNA into cDNA. The obtained cDNA was stored at −20 °C for later use.

2.5. Expression Analysis of MaCBLs

For the integral analysis of the *MaCBL* genes in terms of their responses to potassium stress, the FPKM (kilobase/million mapped reads) data of the *MaCBLs* were extracted from our previously derived RNA-seq data (PRJNA589855) [23]. For assessing the expression of the selected *MaCBL2* gene in response to potassium-shortage stress, qRT-PCR analysis was performed. The reagents and method were similar to those in a previous report [23]. The *MaActin* gene was used as the internal reference gene. The specific primer pairs of *MaCBL2* and *MaActin* were QMaCBL2-F: GGCAAACAGGCTACATAGAGCG, QMaCBL2-R: AAGCTCGGAAAGGCCATGCTTA, QMaActin-F: CGAGGCTCAATCAAAGA, QMaActin-R: ACCAGCAAGGTCCAAAC. The Real Time PCR Easy™-SYBR Green I Kit (FORE GENE) was used to perform the qRT-PCR. The relative gene expression levels were calculated by the 2^{−ΔΔCt} method. In this experiment three biological replicates were performed. Values were presented as mean ± SD. The significance of the data was evaluated using one-way ANOVA (analysis of variance) with Duncan's multiple range test (DMRT). The significance level was set at $p \leq 0.05$ indicated by one asterisk and $p \leq 0.01$ indicated by two asterisks.

2.6. Cloning and Function Analysis of MaCBL2

To clone the *MaCBL2* gene from banana, the following primer pairs were used for PCR: MaCBL2-F1 (CGGAGACCGTCTTCTCACAG) and MaCBL2-R (ATGCAGTGGTCTG-GATTGGA). The Ex Taq (TaKaRa, Dalian, China) was used to perform the RT-PCR reactions. The obtained PCR product was detected by 1% agarose gel electrophoresis and then sequenced by Shenggong company.

For performing the subcellular localization experiment for *MaCBL2*, the following primer pairs were used to clone the *MaCBL2* CDS without the stop codon: MaCBL2-F2 (AGAACACGGGGGACTCTAGAATGGGTTGTGTGTCTTCTAAG) and MaCBL2-R2

(GCCCTTGCTCACCATGGATCCAGTCGTGTCTGGATCATCAC). The obtained sequence was subcloned into the pCambia1300–35S–GFP vector to construct the pCambia1300–35S–*MaCBL2*–GFP vector, which was subsequently used for the transient transfection of tobacco leaves with *Agrobacterium tumefaciens*. Green fluorescence detection was performed with a Laser Scanning Confocal Microscope FV1000 (OLYMPUS) at 488 nm. The pCambia1300–35S–GFP vector was used as the control.

2.7. Functional Complementation of *MaCBL2* in Yeast

The following primer pairs were used to clone the *MaCBL2* gene for constructing the yeast expression vector: *MaCBL2*-F3 (CGCGGATCCATGGGTTGTGTCTTCTAAG-CATG) and *MaCBL2*-R3 (ATAGTTTAGCGGCCCGCCTAAGTCGTGTCTGGATCATC). The obtained PCR product was subcloned into the pYES2 vector to construct the pYES2–*MaCBL2* vector. Then, the pYES2–*MaCBL2* vector and pYES2 vector (control) were transfected into the yeast mutant strain R5421 (CY162), which is a chemically competent cell line (Shanghai Weidi Biotechnology Co., Ltd.) [31].

To perform the yeast complementation assays, the yeast liquid contained pYES2–*MaCBL2*, and the pYES2 empty vectors were first cultured in SD-Ura liquid medium at 30 °C to an OD 600 value of about 1.0. The yeasts were collected, washed, and diluted with sterile water by factors of 1, 10^{-1} , 10^{-2} , and 10^{-3} and then cultured on the AP solid medium with different KCl levels: 50, 10, and 1 mM. Five days later, the growth of the yeast was observed, and photos were taken.

2.8. Functional Identification of *MaCBL2* in Tobacco

To further test the function of *MaCBL2* in response to potassium deficiency stress, the pCambia1300–35S–*MaCBL2*–GFP vector was permanently expressed in tobacco. For the tobacco transformation experiment, the agrobacterium contained the vector pCambia1300–35S–*MaCBL2*–GFP was co-cultivated with the tobacco leaf disks on 1/2 MS medium containing 1 mg/L 6-BA for 3 days, and then transferred to the 1/2 MS medium containing 1 mg/L 6-BA, 50 mg/L hygromycin (hyg), and 500 mg/L carbenicillin (car) for screening for 1 month. After subcultured the callus on the same medium for 1~2 times to get the regeneration plants, the regenerated plants were transferred onto the 1/2 MS medium with 50 mg/L hyg, 500 mg/L car, and 0.1 mg/L NAA for rooting and planting in soil to obtain the T1 seeds. The seeds were grown on the hygromycin containing 1/2 MS medium to screen the homozygous strain.

Wild type and three overexpression *MaCBL2* transgenic homozygous lines (OE1~3) were cultured for 5 days and then treated with potassium deficiency stress on 1/2 MS medium without K⁺ and 1/2 MS medium add 0.05 mM KCl. The seedlings growing on 1/2 MS medium were used as control. The phenotype was observed and photographed after the 15th day of treatment, and the root length was measured. The values were presented as mean ± SD. The significance of the data was evaluated using one-way ANOVA similar to the method in Section 2.5 part.

3. Results

3.1. Identification of *MaCBL* Gene Members from Banana Genomic Data

In total, 11 *CBL*-related gene members were initially recognized in the banana genome, all containing the conserved EF-hand motif. Based on their genomic serial numbers, in ascending order, these *CBLs* were named *MaCBL1* to *MaCBL11* (Table S1). Their genomic DNA lengths ranged from 1853 bp (*MaCBL5*) to 10,640 bp (*MaCBL11*), the coding regions ranged from 588 bp (*MaCBL1*) to 795 bp (*MaCBL4*) in length, and the amino acid lengths ranged from 195 aa (*MaCBL1*) to 264 aa (*MaCBL4*). Their theoretical molecular weights ranged from 22,475.82 Da (*MaCBL1*) to 30,476.21 Da (*MaCBL4*), while their isoelectric points varied from 4.56 (*MaCBL3*) to 5.19 (*MaCBL1*)—all below 7, indicating that these banana *CBL* proteins are rich in strong amino acids. Instability index analysis indicated that three *CBLs* (*MaCBL4*, *MaCBL5*, and *MaCBL7*) are unstable proteins, as their instability

indices were greater than 40, while the others are stable proteins. Hydrophobicity analysis showed that the GRAVY value of MaCBL4 was 0.033, indicating that it is a hydrophobic protein, while the GRAVY values of all the other MaCBLs were negative, indicating they are hydrophilicity (Table S1).

3.2. Phylogeny and Motif Examination of MaCBL Members in Bananas

To assess the evolutionary relationships among the CBLs in banana and other plant species, a phylogenetic tree was constructed (Figure 1). Based on previous studies of CBL proteins in *Arabidopsis* models, the MaCBL proteins were classified into four groups (I–IV). Four MaCBLs (MaCBL1, 2, 3, and 5) were placed in Group I; MaCBL4 was placed in Group II; MaCBL8, 7 and 10 were placed in Group III; and MaCBL6, 9 and 11 were placed in Group IV (Figure 1). From the tree, we can also infer that the MaCBLs in the same group have a close relationship with the CBLs from monocotyledonous species, such as *Oryza sativa*, *Ananas Comosus*, and *Triticum aestivum*, while being slightly further away from the CBLs of dicotyledonous plants, such as *Arabidopsis thaliana*, *Manihot esculenta*, *Capsicum annuum*, *Brassica napus*, and *Populus trichocarpa*.

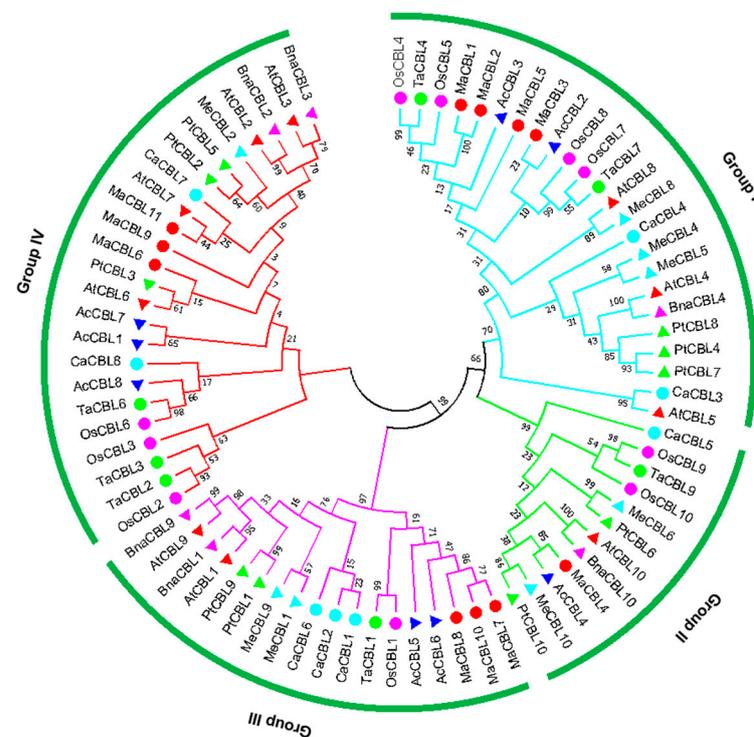


Figure 1. Phylogenetic tree of banana calcineurin B-like proteins (CBLs), with specific CBLs that characterize different species. Ma, At, Os, Ac, Ta, Ca, Me, Bna, and Pt denote *Musa acuminata*, *Arabidopsis thaliana*, *Oryza sativa*, *Ananas comosus*, *Triticum aestivum*, *Capsicum annuum*, *Manihot esculenta*, *Brassica napus*, and *Populus trichocarpa*, respectively.

To more effectively assess the conservation and diversification of the MaCBLs, 10 putative motifs for each of the MaCBL proteins were predicted. All of the MaCBLs contained motifs 1, 2, and 4, while motifs 3, 5, 6, 7, 8, 9, and 10 were present in 10, 9, 7, 2, 6, 2, and 2 MaCBLs, respectively, suggesting that these motifs play specific roles in matching proteins. It was also evident that the motif compositions of MaCBLs in the same group were highly similar (Figure 2A,B).

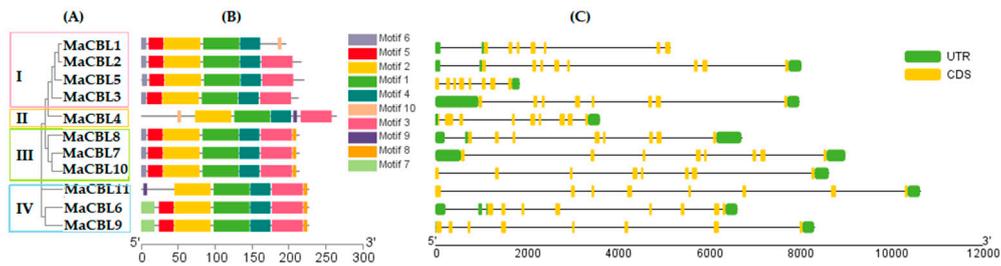


Figure 2. Phylogenetic clustering, conserved motifs and exon/intron organization of MaCBL genes. (A) The phylogenetic tree was constructed based on MaCBL proteins. (B) The motifs of each of the MaCBLs, and 10 different motifs, are each denoted by different colored boxes. (C) Exon/intron organization of MaCBLs. Untranslated (UTR) 5'- and 3'-regions are indicated by green boxes, exon regions are indicated by yellow frames, and intron regions are indicated by black lines.

3.3. Exon/Intron Organization within MaCBL Genes

Gene structure is one of the factors that reflects the evolution of a multigene family. Thus, the exon/intron organization of each *MaCBL* gene was analyzed, revealing that most of the *MaCBLs* contained eight exons, while *MaCBL1* had seven exons, and *MaCBL4* contained nine exons (Figure 2C).

3.4. Chromosomal Assignment and Evolutionary Analysis of MaCBL Members

The 11 *MaCBL* members were disproportionately located across five chromosomes of the banana genome (Figure 3A). Chr1 and chr3 each presented two *MaCBLs*, chr4 presented four, and chr9 had one, while two *MaCBLs* were located at unknown chromosomal positions (chrUn). To further investigate the syntenic relationships between the banana CBL genes, a synteny analysis was performed. Two pairs of syntenic genes, *MaCBL4–MaCBL6* and *MaCBL7–MaCBL8*, were found in the banana *MaCBLs* (Figure 3B). The nonsynonymous (Ka) and synonymous (Ks) substitution rates were calculated to reveal the selective pressure and duplication types of the *MaCBL* gene pairs. The Ka/Ks ratios of 18 gene pairs were below 1.00, indicating that they exhibited purifying selection, while the Ka/Ks ratios of 25 pairs were greater than 1.00, reflecting that they showed positive selection (Table S2).

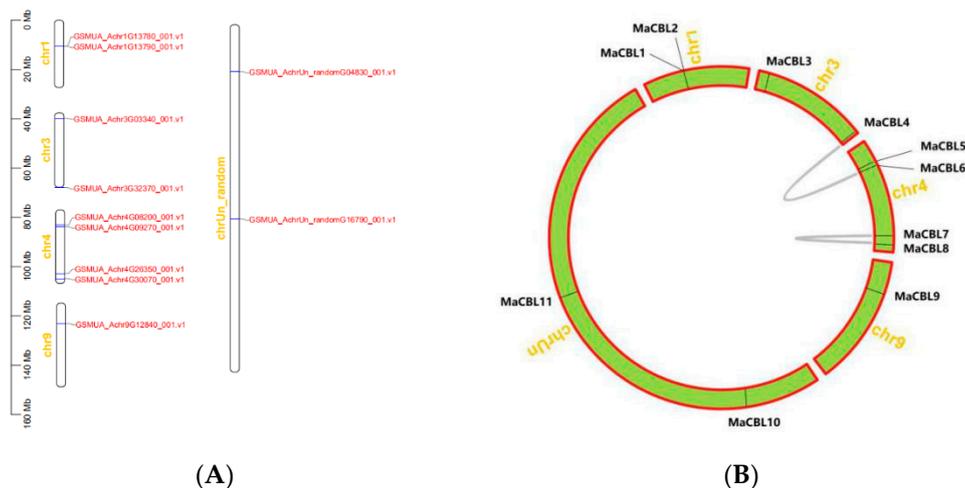


Figure 3. Distribution of CBL genes on chromosomes in bananas (A) and the syntenic relationships (B) of CBL genes.

3.5. Expression Pattern of MaCBLs under Different Potassium Stresses

The RNA-seq data we previously derived were used to reveal the expression patterns of the *MaCBLs* in bananas after 0, 0.03, 3, and 200 mM K⁺ stress (Figure 4A). Among the 11 *MaCBLs* analyzed, the expression of *MaCBL5* was very low, or undetectable, after different K⁺-stress treatments. *MaCBL10* showed the highest expression under each treatment;

however, there were no significant differences in gene expression among the different treatments. Notably, the expression of *MaCBL2* was strongly induced in roots subjected to the 0 and 0.03 mM K⁺ treatments, with expression levels of 8.23 and 13.20, respectively, while under both the 3 and 200 mM K⁺ treatments, the expression level was 3.61. The *MaCBL2* expression differences between 0 and 3, and between 0.03 and 3 mM K⁺ were 2.28- and 3.66-fold, respectively. However, the differences in the expression of the other *MaCBL* genes in the same pairs were no more than twofold.

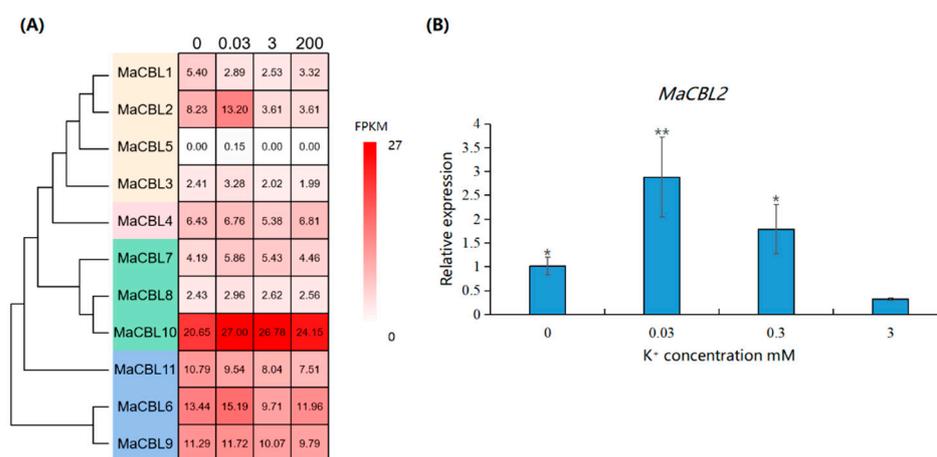


Figure 4. Expression tendency analysis of the *MaCBL* genes in bananas under potassium stress. **(A)** The expression pattern of *MaCBLs* in bananas under different potassium-stress levels, based on FPKM data. **(B)** The relative expression tendency of *MaCBL2* as assessed by qRT-PCR. The asterisks indicate statistically significant difference, (*) for $p \leq 0.05$, (**) for $p \leq 0.01$.

To confirm the responsiveness of *MaCBL2* to low-potassium stress, roots cultured in 0, 0.03, 0.3, and 3 mM K⁺ were collected for qRT-PCR. The results indicated that the *MaCBL2* expression levels under the 0, 0.03, and 0.3 mM K⁺ treatments were all higher than under the 3 mM K⁺ treatment (Figure 4B). This result was consistent with the rest of the results, and it implies that *MaCBL2* may play an important part in the response to low-potassium stress.

3.6. Cloning and Characterization of *MaCBL2* Gene

MaCBL2 was cloned for further study because it was induced by low-K⁺ stress. Its protein was found to contain 216 amino acid residues and have a calculated molecular weight of 24,703.17 Da (Table S1). The protein sequences of *MaCBL2* and the CBLs of other species in the same group (Group I), such as *AcCBL3* and -4; *OsCBL4*, -5, -7, and -8; *TaCBL4* and -7; and *AtCBL4* and -8, were used to perform a multiple alignment analysis. As can be seen from Figure 5, *MaCBL2* contained the conserved Ca²⁺-binding-related EF-hand domains, the N-terminal myristoylation domain "MGCXXS/K(T)", and the "FPSF" motif, as did most of the homologous proteins in other species.

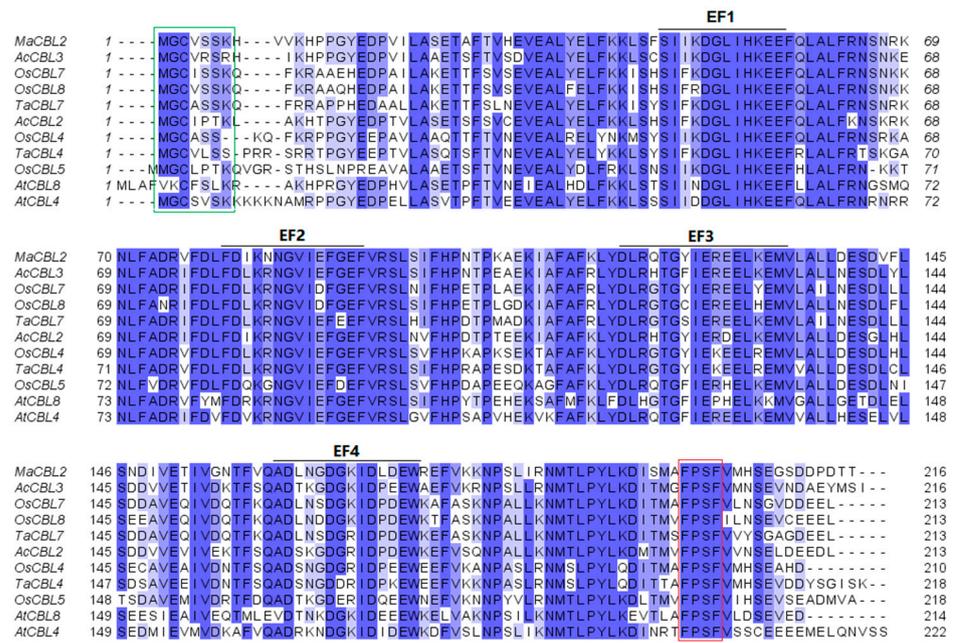


Figure 5. Multiple sequence alignment of MaCBL2 and other CBLs from other species. The blue shadow indicates similarity at $\geq 50\%$. The black lines indicate the elongation factor hand (EF-hand) domains. The green box indicates the myristoylation site, and the red box indicates the FPSF motif.

3.7. Subcellular Localization Analysis of MaCBL2

To characterize the subcellular positioning of MaCBL2, a pCambia1300–35S–MaCBL2–GFP fusion expression vector was constructed and transferred into tobacco leaf epidermal cells. Confocal fluorescence imaging was used to ascertain the fluorescence. The results indicate that MaCBL2 was located in the plasma membrane, nucleus and cytoplasm, locations similar to those for the GFP control (Figure 6).

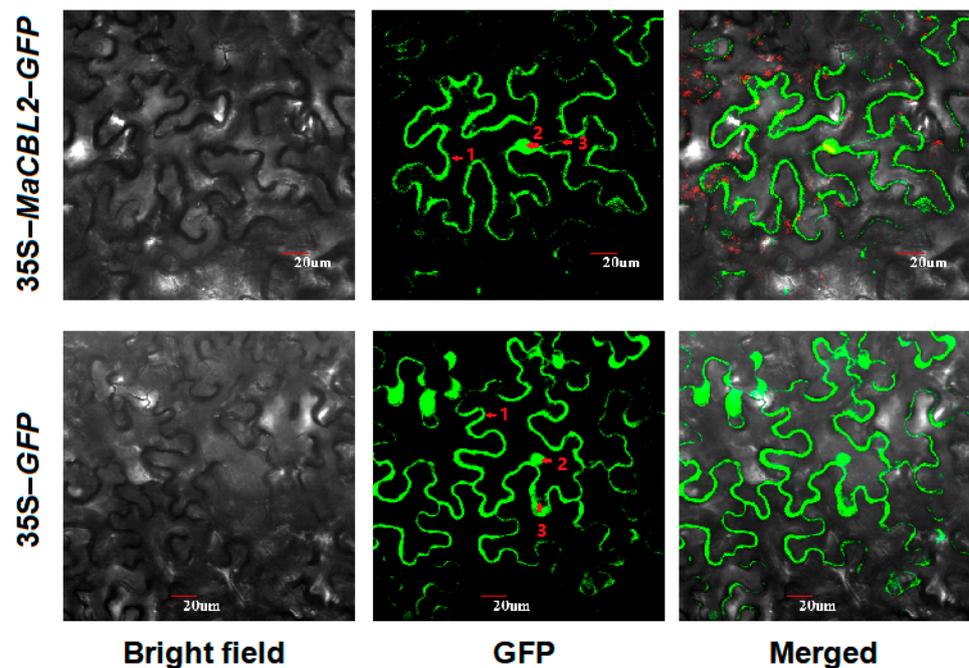


Figure 6. Subcellular localization of MaCBL2 in tobacco leaves. Red arrows and numbers indicate (1) plasma membrane, (2) nucleus, and (3) cytoplasm.

3.8. Functional Complementation Validation of *MaCBL2*

The potassium-ion-deficient yeast strain R5421 was used to study the function of *MaCBL2*. The *MaCBL2* sequence was subcloned into the pYES2 vector to obtain a pYES2–*MaCBL2* vector. The pYES2–*MaCBL2* and the pYES2 empty vector (control) were transformed into R5421 for the potassium-stress experiment. The results indicate that there was no visible growth deviation between the control pYES2 yeast and pYES2–*MaCBL2* when they were grown on AP medium containing 50 mM K⁺ (Figure 7). As the K⁺ concentration was decreased to 10 mM, the *MaCBL2*-overexpressing yeast grew more successfully than the control yeast. When the K⁺ concentration was reduced to 1 mM, the control yeast could not grow, while the yeast expressing *MaCBL2* could overcome their growth defect under the same conditions (Figure 7). This suggests that *MaCBL2* plays a certain role in potassium shortage resistance.

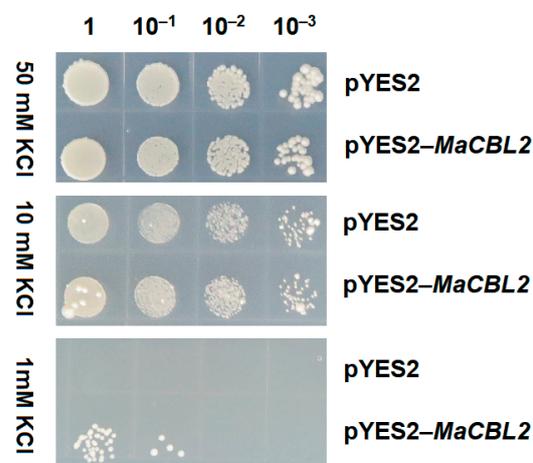


Figure 7. Growth status of the K⁺-uptake-defective yeast mutant R5421 expressing pYES2–*MaCBL2* and pYES2 (control).

3.9. Functional Identification of *MaCBL2* in Tobacco Seedling

To further test the function of *MaCBL2* in response to potassium deficiency stress, the pCambia1300–35S–*MaCBL2*–*GFP* vector was permanently expressed in tobacco. Three transgenic tobacco lines were used for comparative tests. As can be seen in Figure 8A, the root hairs of transgenic tobacco were more than that of control on 1/2 MS medium, but their root length were similar (Figure 8A,B). When the seedlings were cultivated on potassium shortage 1/2 MS (without K⁺, or plus 0.05 mM KCl), the root length of the transgenic lines were significantly longer than the wild type tobacco seedlings (Figure 8). These results indicated that overexpression of *MaCBL2* gene can improve the root growth of transgenic tobacco under potassium deficiency condition.

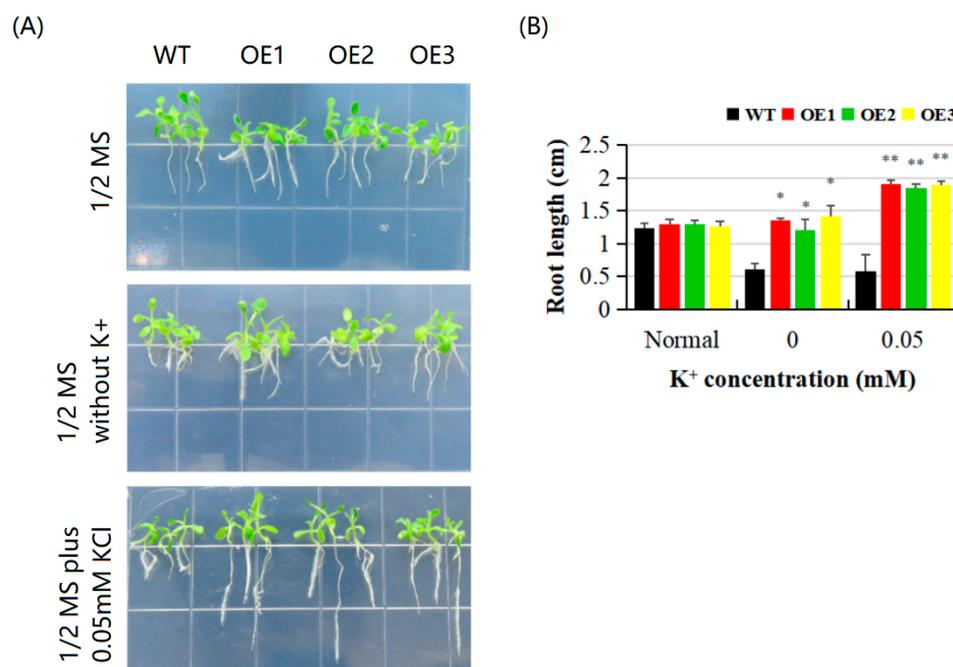


Figure 8. Seedling assay for potassium deficiency. (A): Phenotype differences in the seedling growth after 15 days treatment for potassium deficiency. WT: wild type tobacco; OE1–3: the *MaCBL2* overexpressed transgenic tobacco. (B) Differences in root length of potassium deficiency treated tobacco seedlings. The error bar indicates the mean \pm SD, (*) indicates the significant difference $p \leq 0.05$, (**) indicates the significant difference $p \leq 0.01$.

4. Discussion

Studies have suggested that *CBL* family members play vital roles in regulating signal transduction under abiotic stress, either alone [10,21] or in combination with *CIPK* [13–16], in plants. *OsCBL4* combined with *OsCIPK15* was reported to participate in low-oxygen signaling in rice [32]. *BnCBL1* interacted with *BnCIPK26* and promoted the high-salinity and low-phosphate resistance of transgenic *Arabidopsis* [33]. *CBL*–*CIPK* complexes have been regarded as the bridge between ROS signaling and Ca^{2+} signaling in plants, a hypothesis eventually proven by evidence of a *CIPK*–*RBOHF* interaction, and the *CBL*–*CIPK26*-mediated enhancement of *RBOHF* produced ROS [19]. The *CBL* family has, therefore, become a research hotspot in plants.

The *CBL* family have been systematically studied in several plant species, such as dicotyledonous plants (*Arabidopsis thaliana* [5], eggplants [2], peppers [4], and cotton [6]) and monocotyledonous plants (rice [5], pineapples [3], and sugarcane [34]). However, none of the studies to date had included a systematic analysis of *CBLs* in bananas. In this study, 11 *CBLs* were reported after genome-wide mining for bananas. These were distributed across four main phylogenetic groups (Figure 1), a finding in accord with previous studies on *Arabidopsis* and rice [5].

As imprints left by evolution, the exon/intron structures in genes are often analyzed in the systematic study of gene families [35–37]. In our study, the gene structures of most of the *MaCBLs* were similar: *MaCBL1* only had seven exons, while *MaCBL4* contained the maximum, nine (Figure 2). These data suggest that the gene structure of *MaCBLs* is relatively constant.

Duplication and divergence are the core mechanisms of gene family expansion and evolution [38]. The presence of genes on the same chromosome is referred to as tandem duplication, whereas the location of genes on various chromosomes is referred to as segmental duplication [36]. The K_a/K_s value is one of the measurements for determining the mechanisms of gene duplication evolution, and it has been widely used in recent years [36,39,40]. Our results show that 18 duplication events display a K_a/K_s ratio below

1, while 25 events show a K_a/K_s ratio greater than 1 (Figure 3); these results indicate that these genes went through positive selection [36,39,40].

The CBL family generally contains several functional regions, such as four EF-hand motifs [5], the N-terminal myristoylation domain “MGCXXS/K(T)” [41], and the “FPSP” motif [8,42,43]. In the “FPSP” motif, Ser has been proven to be a necessary CIPK phosphorylation target residue in CBL proteins [42,43]. Here, a *AtCBL4* homologous gene *MaCBL-2* gene was cloned from banana and the multiple sequence alignment was performed (Figure 5). It was shown that the MaCBL2 protein has four EF-hand domains, which is the same as in most of the CBLs in the same group [44]. MaCBL2 also has the same “FPSP” motif as the proteins in the same group, indicating it can interact with some CIPKs and be phosphorylated. It can also be observed that there is a myristoylation site in MaCBL2; this site has been reported to be important for the CBL’s association with the membrane [41]. Our results also confirmed this sequence analysis via subcellular localization experiments (Figure 6), showing that MaCBL2 can be located on a membrane.

There are numerous reports concerning the key functions of CBLs in regulating the absorption of plant nutrients [45]. The Ca^{2+} -dominated process makes a vital contribution to maintaining the homeostasis of nutrients such as potassium, nitrogen, magnesium, and iron [45,46]. The *CBL1/9–CIPK23–AKT1*, *CBL3–CIPK9*, and *CBL4–CIPK6–AKT2* pathways have been reported to participate in signal transduction for potassium absorption [13–16]. There are also some reports on the expression of CBLs in response to low-potassium stress [6,10,47]. In cotton, the expression of *GhCBLs* was the most affected by low-potassium stress [6]. In *Arabidopsis*, *CBL10* was reduced under low-potassium stress, and it can directly interact with *AKT2* to maintain the K^+ stability in plant cells [10,47]. In eggplants, low potassium treatment strongly up-regulated the expression level of five *SmCBLs*. In our study, we found that *MaCBL2* was significantly induced by low-potassium stress (Figure 4). Coincidentally, the expression of *MaCBL2*’s homologues *AtCBL4* and *TaCBL4* was also involved in the response to low-potassium stress [16,48]. In 2017, two successive and distinct Ca^{2+} signals were found to be triggered by K^+ -shortage stress in the roots [49]. One was the transient Ca^{2+} signature occurred within one minute in the root elongation zone. The second was the sustained Ca^{2+} signal in the root elongation zone and root hair differentiation zone. The secondary Ca^{2+} response was involved in plant adaptation to potassium shortage conditions [49]. Low potassium stimuli response signals trigger gene transcription and root growth to long-term adaptive response. In *Arabidopsis*, the *AtCBL4* was found to associate with *AtCIPK6* to activate the Ca^{2+} dependent *AtAKT2* K^+ channel. They found the association between *AtCBL4* and *AtAKT2* through the growth consistency in the *Arabidopsis* deficient mutants with the lost *AtCBL4* and *AtAKT2*, respectively. However, the function of CBLs in bananas remains uncertain. In this paper, we proved the function of *MaCBL2*, a *AtCBL4* homologous gene, in response to low-potassium stress in the yeast mutant R5421 and in transgenic tobacco. Overexpression of *MaCBL2* enhanced the growth ability of the yeast mutant on potassium deficiency medium (Figure 7). Overexpression of *MaCBL2* improved the transgenic tobacco seedling root growth under potassium shortage conditions (Figure 8). Our discoveries indicate that *MaCBL2* may play a key role in Ca^{2+} -signal-regulated K^+ -relevant processes in bananas, and the regulatory mechanisms of this gene require further study.

5. Conclusions

In summary, 11 CBL genes were mined from banana genomic data, and they were phylogenetically clustered into four groups (Groups I–IV). Their genomic features were analyzed, including their gene structures, start motifs, chromosomal distributions, and evolutionary history, to identify their genetic attributes. Through gene expression tendency analysis, gene cloning, functional complementary experiments in a potassium-deficient yeast mutant, and overexpression in tobacco, we singled out the gene *MaCBL2* and demonstrated that it is associated with the regulation of low-potassium stress.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/horticulturae7040070/s1>, Table S1: Basic information on the MaCBLs in bananas, Table S2: The Ka and Ks data for the MaCBLs.

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