Identifying citrus CBL and CIPK gene families and their expressions in response to drought and arbuscular mycorrhizal fungi colonization

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Abstract

The calcineurin B-like protein (CBL)-interacting protein kinase (CIPK) complex is an essential calcium sensor and contributes to biotic and abiotic stress responses. However, citrus CBL and CIPK gene family members and their underlying roles during drought and arbuscular mycorrhizal fungi (AMF) colonization remain relatively unknown. In the present study, CBLs and CIPKs were characterized in Citrus sinensis by analyzing the presence of specific domains such as the elongation factor (EF)-hand motif in CBLs, and a protein kinase and an Asn-Ala-Phe domain in CIPKs. After mining the C. sinensis genome, we identified 8 CsCBLs and 17 CsCIPKs. Among these genes, three CsCBLs and nine CsCIPKs showed syntenic relationships with the Arabidopsis thaliana homologs AtCBLs and AtCIPKs, respectively. According to gene expression and cis-acting element analysis, all 8 CsCBLs and 16 CsCIPKs were expressed in the roots, where the regulation of expression was not consistent with their promoter cis-elements. Drought treatment remarkably downregulated the expression of CsCBL8 and upregulated CsCBL7, CsCIPK4, and CsCIPK7 expressions. The AMF colonization induced CsCBL4, 5, 6, and 7 as well as CsCIPK2, 4, 10, 11, 13, 14, and 16 expressions and repressed CsCBL1, 2, 3, and 8 and CsCIPK1, 3, 6, 8, 9, and 12 expressions. Based on the expression data and co-expression analysis, CsCBL1-CsCIPK1, CsCBL1-CSIPK3, CsCBL1-CsCIPK6, and CsCBL1-CsCIPK9 showed the significant positive correlations to drought and AMF responses.

Additional key words: calcineurin B-like protein, CBL-interacting protein kinase, gene structure, phylogenetic analysis, transcriptional regulation.

Introduction

Calcium is a common plant cell signaling second messenger that regulates various signal transduction pathways (Kolukisaoglu et al. 2004), where the calcineurin B-like protein (CBL)-interacting protein kinase (CIPK) complex is a primary component of calcium sensors in perceiving various stress signals (Liu et al. 2013). All CBL proteins contain four elongation factor (EF)-hand motifs that can bind four Ca²⁺ ions (Sanchez-Barrena et al. 2005). In addition, this requires an interaction with a separate kinase, a CIPK, to transduce the perceived calcium signal into a cellular response. CIPK possesses a conserved N-terminal kinase domain and a less conserved C-terminal regulatory domain that are separated by a junction domain (Akaboshi et al. 2008). The regulatory domain of CIPK contains a conserved 24 amino acid Asn-Ala-Phe (NAP) domain, which is self-inhibiting and mediates the interaction with CBL (Guo et al. 2001). The calcium signature can bind to the EF-hand domains of the CBL proteins, where they bind to the NAF domain of the C-terminus of CIPKs (Hashimoto et al. 2012). Afterwards, the CIPK can phosphorylate its targets and transduce the Ca²⁺ signal downstream (Luan 2009).

In plants, the CBL-CIPK signaling pathways play key roles in the response to stresses, such as drought and microbial interactions. Most CBL and CIPK show positive effects with respect to drought tolerance. As Zea mays ZmCIPK8 interacts with ZmCBL1, 4, and 9, overexpression of ZmCIPK8 was shown to improve drought tolerance (Fuju et al. 2016). Vitis vinifera VaCIPK2 strongly interacted with VaCBL1, 4, 5, and 8, where constitutive expression of VaCIPK2 in Arabidopsis thaliana displayed increased drought tolerance accompanied by abscisic acid (ABA)-hypersensitive stomatal closure (Xu et al. 2020). In addition, Malus domestica MdCIPK22 in transgenic

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Abbreviations: ABA - abscisic acid; AMF - arbuscular mycorrhizal fungi; CBL - calcineurin B-like protein; CIPK - CBL-interacting protein kinase; EF - elongation factor; FPKM - fragments per kilobase per million mapped reads; NAF - Asn-Ala-Phe; ROS - reactive oxygen species.

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plants promoted sugar accumulation and improved drought tolerance (Ma et al. 2017). Furthermore, *A. thaliana* AtCBL5, *Triticum aestivum* TaCIPK23, *Oryza sativa* OsCIPK12 and 23, and *Brachypodium distachyon* BdCIPK31, all resulted in improved drought tolerance in transgenic plants (Xiang et al. 2007, Yang et al. 2008, Cheong et al. 2010, Luo et al. 2017, Cui et al. 2018). In contrast, AtCIPK11 seems to act as a negative regulator of drought tolerance (Ma et al. 2019), where over-expression of AtCIPK11 in *A. thaliana* resulted in decreased drought tolerance, by regulating Di19-3, a Cys2/His2-type zinc-finger transcription factor. Moreover, AtCIPK11 over-expression in plants causes higher leaf water loss and higher accumulation of reactive oxygen species (ROS) after drought relative to control (Ma et al. 2019). Aside from abiotic stresses, the CBL-CIPK signaling pathway is also involved in plant-microbe interactions, where these signaling pathways were identified in rice, wheat, sugarcane, and cassava (Kurusu et al. 2010, Liu et al. 2018, Yan et al. 2018, Su et al. 2020). The OsCIPK14 and 15 were shown to interact with several OsCBLs in yeast cells with the strongest interaction being of OsCBL4 in rice. OsCIPK14 and 15 were rapidly induced by microbe-associated molecular patterns, including chitoosigosaccharides and xylanase (*Trichoderma viride*/ethylene-inducing xylanase (TvX/EIX)). Furthermore, OsCIPK14/15 RNAi transgenic cell lines exhibited reduced sensitivity to TvX/EIX with the induction of a wide range of defense responses, including hypersensitive cell death, mitochondrial dysfunction, phytoalexin biosynthesis, and pathogenesis-related gene expression. In addition, TvX/EIX-induced cell death was enhanced in OsCIPK15 over-expression lines (Kurusu et al. 2010). TaCBL4-TaCIPK5 positively contributed to wheat resistance in *Puccinia striiformis* f. sp. *tritici* in a ROS-dependent manner (Liu et al. 2018). Sugarcane (*Saccharum spp.*) transient over-expression of ScCBL genes in *Nicotiana benthamiana* leaves resulted in a different expression of immunity-associated marker genes in tobacco as well as increased resistance to infection with *Ralstonia solanacearum* (Su et al. 2020). *Manihot esculenta* MeCIPK23 interacted with MeCBL1 and 9, where over-expression of these genes conferred an improved defense response to *Xanthomonas axonopodis pv. manihotis* infection (Yan et al. 2018).

Drought stress is known to restrict vegetative growth and yield of citrus (Rodriguez-Gamir et al. 2010). Owing to their fewer root hairs and high arbuscular mycorrhizal dependency, citrus plants are strongly associated with mycorrhizal fungi, where arbuscular mycorrhizal fungi (AMF) can enhance drought tolerance in citrus plants (Wu et al. 2013). How CBL-CIPK genes respond to both drought and AMF in citrus remains unclear. Thus, we attempted to identify members of the CBL and CIPK family genes in citrus. The exon-intron organization, motif compositions, gene duplications, chromosome distribution, phylogeny, synteny, and cis-elements of citrus CBLs and CIPKs were further investigated. Global expression and co-expression analyses were performed to identify the involvement of CBLs and CIPKs in drought stress and AMF interactions. Hence, this study aimed to provide valuable insights into the functional characterization of CBL and CIPK members in citrus.

**Materials and methods**

**Investigation of CBL and CIPK sequences:** The complete genome assembly of *Citrus sinensis* (L.) Osbeck Valencia was downloaded from the *Citrus Genome Database* (https://www.citrusgenomedb.org/organism/Citrus/sinensis; *C. sinensis* genome v. 2.0). A total of 10 CBL and 26 CIPK protein sequences were obtained from the *Arabidopsis Information Resource (TAIR)* (https://www.arabidopsis.org/). citrus CBL and CIPK were searched using *Arabidopsis* query sequences via the *Protein Basic Local Alignment Search Tool (BLASTP)*. Repeated sequences were manually deleted based on their Eigen values. The relative molecular mass (Mr) and isoelectric point (pl) of the candidate protein sequences were determined using ExPaSy (https://web.expasy.org/compute_pi/) (Gasteiger et al. 2003). The presence of conserved CBL and CIPK domains was verified for all potential proteins using the *National Center for Biotechnology Information (NCBI)* Batch CD-Search (https://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi) program.

**Sequence alignment and phylogenetic analysis:** Full-length CBL and CIPK protein sequences of *C. sinensis, A. thaliana,* and *Oryza sativa* were aligned using Muscle (Edgar 2004) in MEGA version 7.0, with default parameters (Kumar et al. 2016), after which a neighbor-joining (NJ) tree was generated with the bootstrap test replicated 1 000 times. The phylogenetic relationships among the gene family members of citrus CBL and CIPK were estimated from another generated phylogenetic tree according to the alignment of citrus proteins separately.

**Conserved motif and gene structure analysis:** Motif analysis was carried out using the *Multiple EM for Motif Elicitation (MEME)* website (http://meme-suite.org/tools/meme) to identify conserved motifs with a limit of seven and ten motifs for CsCBLs and CsCIPKs, respectively. Sequences that had zero or one occurrence per sequence were deemed as motif sites. The gene structures of citrus CBL and CIPK protein sequences were determined using TTools by comparing the coding sequences and their corresponding genomic sequences (Chen et al. 2018). The citrus generic feature format (GFF) file was downloaded from the *Citrus Genome Database* and used to elucidate the structure information of CBL and CIPK genes. In addition, an illustration depicting the citrus CBL and CIPK protein motifs, conserved domain, gene structures, and phylogenetic tree was constructed using TTools (Chen et al. 2018).

**Chromosomal distribution, gene duplication, and collinearity relationships:** Chromosome locations of the citrus candidate CBL and CIPK genes were analyzed based on the GFF information and visualized using the TTools.
software (Chen et al. 2018). Gene duplication events of the citrus CBL and CIPK proteins with the collinearity relationships between CBL and CIPK sequences from Arabidopsis and citrus were investigated using MCScanX (Wang et al. 2012). The results were then visualized using TBtools (Chen et al. 2018).

Expression analysis of CsCBL and CsCIPK genes during AMF and drought stress responses: A completely randomized two-factor block experiment was performed using different treatments causing drought stress and AMF colonization. In drought assay, the samples were either well-watered or experienced drought, while samples were grown with or without Funneliformis mosseae in the AMF treatment. Four treatments (well-watered with non-AMF (W_Non), well-watered with AMF (W_AMF), drought stress with non-AMF (D_Non), drought stress with AMF (D_AMF)) were therefore designed. Treatments were performed using three biological replicates, where each treatment consisted of nine pots with three seedlings per pot, and three pots per biological replicate. The experimental protocol was adapted from Zhang et al. (2020a,b). The seeds of citrus were germinated in autoclaved sand for 4 weeks. Subsequently, three seedlings having 4 leaves were transplanted into 2.3-dm³ pots containing 2.7 kg of an autoclaved (0.11 MPa for 2 h) mixture of soil and sand with the same volume ratio. For the AMF treatment, approximately 2 000 spores of Funneliformis mosseae (Nicol. & Gerd.) were added into each pot at transplantation of seedlings. For non_AMF treatment, an equal amount of autoclaved inoculums with an addition of 2 cm³ of inoculum filtrate (filtered using 25 μM filter) was added. The treated seedlings were exposed to a 12-h photoperiod, a photon flux density of 900 μmol m⁻² s⁻¹, 28/20 °C day/night temperatures, and a 68 % relative humidity in a greenhouse. After transplantation of the seedlings, the inoculated and un-inoculated seedlings were subjected to 75 % of maximum water holding capacity of pot substrates for ten weeks. After that, the water status in half of the plants were changed to 50 % of maximum water holding capacity of substrates (drought stress) for seven weeks, while the other plants were kept in well-watered condition (75 % of maximum water holding capacity of pot substrates) for seven weeks and then harvested. The substrate water content was daily monitored through weighing, and water lost from pots was supplied.

Transcriptomic data of seedling roots from the four treatments were analyzed as described by Shu et al. (2016). Twelve libraries of seedling roots were sequenced using the Illumina HiSeq 2000 system. Reads that contained adapters, more than 10 % unknown nucleotides, and more than 50 % bases with a quality value ≤ 5 were removed to obtain uncontaminated sequences based on the raw data. Uncontaminated sequences were mapped to the genome of C. sinensis (status: draft, version2draft, version2) for annotation. The transcriptomic data were uploaded to the NCBI Sequence Read Archive using SRR10413223, SRR10413224, SRR10413225, and SRR10413226. Gene expression was analyzed based on the transcriptomic data, where the transcriptional abundance of CsCBLs and CsCIPKs was calculated as fragments per kilobase of exon model per million mapped reads (FPKM) using the Cufflinks package cuffdiff v. 2.2.1. The FPKM value of W_Non treatment was considered the relevant control. Heat maps were created using TBtools software based on the transformed data of log2(FPKM+1) values (Chen et al. 2018).

Real time quantitative PCR was performed following the method of Shu et al. (2012) using three independent biological samples with three technical replications each. Twelve genes were selected for RNA-seq verification, where the primers used for real time quantitative PCR are summarized in Table 1 Suppl. Relative gene expression was calculated using the 2^(-ΔΔCt) method as described by Livak and Schmittgen (2001), where β-actin was used as the reference gene. The measured transcripts were normalized to the relative expression value in the W_Non treatment. Significant differences between treatments were determined by Duncan’s multiple range tests at P = 0.05 with Statistical Analysis Software (SAS) (SAS Institute, Inc., Cary, NC, USA). Different letters indicate significant differences.

Cis-acting element analysis of CsCBLs and CsCIPKs: The 2 000-bp sequences upstream of the transcription start site of candidate genes were extracted from the citrus genome sequence. The PlantCare software (http://sphinx.rug.ac.be:8080/PlantCARE/) was used for investigating the cis-acting elements (Rombauts et al. 1999). Results were then visualized using TBtools (Chen et al. 2018).

Co-expression network analysis: A co-expression network analysis of CsCBLs and CsCIPKs was performed and displayed based on the Pearson correlation coefficient (PCC) of gene expression. The PCC of CsCBL and CsCIPK gene pairs was calculated using Statistical Package for the Social Sciences (SPSS) 17 following Tang et al. (2016). All CsCBLs and CsCIPKs gene pairs with PCC of P < 0.05 were collected and used to create the co-expression networks using Cytoscape 3.6.0 (Shannon et al. 2003).

Results

A total of 8 CBLs and 17 CIPKs were identified from the citrus genome after probing for CBL and CIPK domain sequences, respectively. The CBLs and CIPKs were named according to their positions on each chromosome. Considerable variation was observed in protein length, Mᵣ, and pl of both CBLs and CIPKs. The CBL protein length ranged from 199 aa (CsCBL6) to 319 aa (CsCBL7), while pl ranged from 4.59 (CsCBL1) to 6.18 (CsCBL7), and Mᵣ ranged from 22.70 kDa (CsCBL6) to 36.48 kDa (CsCBL7). The CIPK protein length and Mᵣ were around 450 aa and 50 kDa, respectively, while pl ranged from 6.38 (CsCIPK7) to 9.58 (CsCIPK15) (Table 1). Eight CsCBLs were distributed across four chromosomes, where one CsCBL is on an undetermined chromosome. Chromosome 5 contained the largest number of
Table 1. Basic information on calcineurin B-like proteins (CsCBLs) and calcineurin B-like protein interacting protein kinases (CsCIPKs) identified in the Citrus sinensis. M, - molecular mass, aa - amino acids.

| Gene Name | Original ID | Length [aa] | pI | M. [kDa] |
|-----------|-------------|-------------|----|---------|
| CsCBL1    | Cs1g18400.1 | 255         | 4.58 | 29.00   |
| CsCBL2    | Cs2g01320.1 | 226         | 4.70 | 26.06   |
| CsCBL3    | Cs4g08230.1 | 223         | 4.81 | 25.67   |
| CsCBL4    | Cs4g18590.3 | 257         | 4.59 | 29.41   |
| CsCBL5    | Cs5g02080.1 | 214         | 5.07 | 24.50   |
| CsCBL6    | Cs5g02090.1 | 199         | 5.14 | 22.70   |
| CsCBL7    | Cs5g07690.1 | 319         | 6.18 | 36.48   |
| CsCBL8    | orange1.1t02723.1 | 213 | 4.77 | 24.47 |
| CsCIPK1   | Cs2g04710.1 | 482         | 6.66 | 53.91   |
| CsCIPK2   | Cs2g04720.1 | 452         | 9.23 | 50.80   |
| CsCIPK3   | Cs2g08190.5 | 465         | 9.10 | 52.34   |
| CsCIPK4   | Cs2g09340.1 | 480         | 9.05 | 54.57   |
| CsCIPK5   | Cs2g09400.1 | 437         | 8.79 | 48.93   |
| CsCIPK6   | Cs2g28990.1 | 440         | 6.85 | 50.32   |
| CsCIPK7   | Cs3g16840.1 | 448         | 6.38 | 50.51   |
| CsCIPK8   | Cs4g01450.1 | 442         | 9.04 | 49.58   |
| CsCIPK9   | Cs4g17990.1 | 444         | 8.91 | 50.28   |
| CsCIPK10  | Cs6g09730.1 | 473         | 8.94 | 53.23   |
| CsCIPK11  | Cs6g09770.1 | 433         | 8.46 | 49.00   |
| CsCIPK12  | Cs7g06310.1 | 460         | 8.07 | 52.54   |
| CsCIPK13  | Cs7g30070.1 | 451         | 9.36 | 50.88   |
| CsCIPK14  | Cs9g10260.2 | 477         | 6.81 | 53.70   |
| CsCIPK15  | Cs9g08000.1 | 480         | 9.58 | 53.55   |
| CsCIPK16  | orange1.1t00555.2 | 469 | 9.01 | 53.51 |
| CsCIPK17  | orange1.1t02758.1 | 433 | 8.75 | 48.98 |

CsCBLs (three genes), while no CsCBLs were found on chromosomes 3, 6, 7, and 8. A total of 17 CIPKs were distributed on six chromosomes. Chromosome 2 included six CsCIPKs, whereas chromosomes 1, 5, and 8 had no CsCIPK. CsCBL 5 and 6 exhibited tandem duplication, while some CsCIPKs (CsCIPK 4 and CsCIPK 13, and CsCIPK 10 and CsCIPK 13) showed segmental duplication (Fig. 1 Suppl.). To further investigate the genome synteny of CBLs and CIPKs, a comparative synteny map of citrus was constructed in association with Arabidopsis. Three CsCBLs localized on chromosomes 4 and 5 showed syntenic relationships with AtCBLs, where CsCBL 7 has two syntenic genes in Arabidopsis. Three CsCIPKs localized on chromosomes 4 and 5 showed syntenic relationships with AtCIPKs, where CsCIPK 7 has two syntenic genes in Arabidopsis (Fig. 2 Suppl.). Nine CsCIPKs localized on chromosomes 2, 3, 4, 6, 7, and 9 demonstrated syntenic relationships with AtCIPKs. Among these genes, five CsCIPKs exhibited more than one syntenic gene in Arabidopsis (Fig. 2 Suppl., Table 2 Suppl.). The observed synteny suggests that these three CsCBLs and nine CsCIPKs may have greatly contributed to evolution.

The phylogenetic relationships of CBLs and CIPKs were analyzed based on the phylogenetic tree constructed using citrus, Arabidopsis and rice protein sequences, respectively. As shown in Fig. 1A, CBLs were clustered into five groups, where groups I and IV had the largest number of members of CBLs. Three branches in group II, IV and V possessed one CsCBL and one AtCBL. CIPKs were clustered into five major groups, where the number of members in group I was larger than in other groups (Fig. 1B).

Seven conserved motifs were identified in CsCBLs, while ten conserved motifs were identified in CsCIPKs. The motifs of the CsCBLs exhibited considerable variation. CsCBL 2 and 3 contained all seven motifs, while CsCBL 1, 4, 5, 7, and 8 did not have motif 7 and CsCBL 6 exhibited five motifs (motifs 1, 2, 3, 5, and 6) (Fig. 2A). CsCIPK 4, 8, 10, 12, 13, and 17 contained all 10 motifs, while CsCIPK 1, 3, 5, 6, 7, 9, 11, 14, and 16 possessed nine motifs, except for motif 9 and CsCIPK 15 possessed nine motifs, except for motif 6 (Fig. 2B). CsCBLs and CsCIPKs with similar motif compositions and gene structures (EF-hand/Pkinase/NAF) were clustered in the same group in the phylogenetic tree. All motifs are summarized in Fig. 3 Suppl. The EF-hand domains of CsCBLs consisted of motifs 1, 2, 3, and 6, while the Pkinase domain of CsCIPKs was motif 7, and the NAF domain of CsCIPKs was motif 1, 2, 4, and 5 (Fig. 3 Suppl.).

Expression analysis showed that 8 CsCBLs and 16 CsCIPKs were expressed in the roots. However, these genes exhibited differential expression patterns. The expression of CsCBL 4, 5, 6, and 7 was induced by AMF colonization under both well-watered and drought conditions.
Fig. 1. A phylogenetic tree of Citrus sinensis calcineurin B-like proteins (CsCBLs) and calcineurin B-like protein interacting protein kinases (CsCIPKs) based on the alignment of citrus (Cs), Arabidopsis (At), and rice (Os) proteins. The phylogenetic tree was constructed using the neighbor-joining method implemented in MEGA 7.0. The reliability of the predicted tree was tested using bootstrapping with 1 000 replicates. Branch lines with different colors represent different groups.
conditions, but the expression of CsCBL1, 2, 3, and 8 was repressed by AMF. Drought treatment significantly downregulated the expression of CsCBL8 and promoted CsCBL7 under non-AMF condition. Furthermore, AMF colonization and drought stress exerted a synergistic effect on CsCIPKs, in which the expressions of CsCIPK4 and 7 were repressed by drought under non-AMF condition, but induced by drought under AMF condition (Fig. 3B, Table 3 Suppl.). The expression of the 10 selected CsCBLs and CsCIPKs was analyzed using real time quantitative PCR. Results showed that expression of CsCBL7 and CsCIPK2, 4, and 13 were induced by AMF or/and drought stress, while the expression of CsCBL1 and CsCIPK1, 2, 3, and 9 were repressed by AMF and/or drought stress (Fig. 4). The expression pattern identified by real time quantitative PCR was in accordance with the transcriptomic data.

Several cis-elements, e.g., ‘anaerobic induction’ and ‘hormone responsive’, were identified in the upstream regulatory regions (promoters) of CsCBLs and CsCIPKs, where cis-elements belonging to ‘defense and stress responsive’ and ‘drought inducibility’ were responsible for the responses to AMF and drought. ‘Drought inducibility’ cis-elements were noted in the promoters of six CsCBLs (CsCBL1, 2, 5, 6, 7 and 8), while ‘defense and stress responsive’ cis-elements were identified in the promoters of four CsCBLs (CsCBL2, 3, 4 and 7). ‘Drought inducibility’ cis-elements were also identified in the promoters of 11 CsCIPKs (CsCIPK1, 2, 3, 6, 7, 8, 10, 12, 14, 15 and 16), while ‘defense and stress responsive’ cis-elements were observed in the promoters of two CsCIPKs (CsHDAC6 and 16). Furthermore, two ‘drought inducibility’ cis-elements were identified in CsCBL6, 7, and 8, as well as CsCIPK1, 7 and 12, indicating that these genes might be more affected by drought stress. In addition, the promoters of CsCBL2, CsCBL7, CsCIPK6, and CsCIPK16 included both ‘drought inducibility’ and ‘defense and stress responsive’ cis-elements, suggesting that these four genes might be regulated by both drought condition and microbial interaction (Fig. 5).

Co-expression analysis was employed to identify CsCBL-CsCIPK signal transduction for drought and AMF colonization, which revealed co-expression gene pairs. Within this network, 49 regulatory edges indicate 49 co-expression gene pairs of CsCBLs-CsCIPKs that were

Fig. 2. A phylogenetic tree of deduced Citrus sinensis calcineurin B-like proteins (CsCBLs) and calcineurin B-like protein interacting protein kinases (CsCIPKs) associated with motif compositions and exon-intron compositions of CsCBLs and CsCIPKs. The phylogenetic trees were constructed using the neighbor-joining method implemented in MEGA 7.0 (the left-hand side of the figure). The reliability of the predicted tree was tested using bootstrapping with 1 000 replicates. The motif composition related to each CsCBL and CsCIPK protein is displayed in the middle of the figure. The motifs are displayed in different colored boxes. The information for each motif is provided in Fig. 1 Suppl. The exon-intron structure of CsCBL and CsCIPK genes and conserved domains are displayed on the right-hand side of the figure. Green boxes indicate untranslated (UTR) 5'- and 3'-regions, yellow boxes indicate coding sequences (CDS), and black lines indicate introns. EF - elongation factor, NAF - Asn-Ala-Phe.
linked with their PCCs. In terms of relatedness, 23 out of 49 had positive and significant correlations. CsCBL1, 2, 4, and 5 showed the largest association with CsCIPKs. Among these, CsCBL1-CsCIPK1, CsCBL1-CsCIPK3, CsCBL1-CsCIPK6, and CsCBL1-CsCIPK9 showed a significant positive correlation in response to AMF and drought (Fig. 6).

**Discussion**

To date, several CBLs and CIPKs have been identified in different plant species, such as *Arabidopsis*, rice, grapevine, and pineapple (Kolukisaoglu et al. 2004, Xi et al. 2017, Aslam et al. 2019), where the numbers of CBL and CIPK proteins vary among plant species. The *Arabidopsis* genome contains 10 AtCBLs and 26 AtCIPKs, the rice genome contains 10 OsCBLs and 30 OsCIPKs (Kolukisaoglu et al. 2004), the grapevine genome contains 8 VvCBLs and 21 VvCIPKs (Xi et al. 2017), and 8 AcCBLs and 21 AcCIPKs were identified from the pineapple genome (Aslam et al. 2019). In the present study, 8 CsCBLs and 17 CsCIPKs were identified in the citrus genome. It is known that segmental (or whole-genome duplications) and tandem duplications are the two major drivers of gene family expansion in plants (Hématy and Höfte 2008). The CsCBLs has exhibited one tandem duplication, while CsCIPK has one segmental duplication (Fig. 1 Suppl.), suggesting that the expansion of CsCBL and CsCIPK families occurred a few times during evolution. In addition, three CsCBLs and nine CsCIPKs showed syntenic relationships with *AtCBLs* and *AtCIPKs*, respectively. In conjunction with gene duplication and synteny analysis, these results confirmed that CsCBLs and CsCIPKs are relatively conserved.

All 8 CsCBLs and 16 CsCIPKs were expressed in the roots, implying that they play vital roles in the development of roots or other related biological processes. However, CsCIPK15 was not expressed in the roots, suggesting that it might be involved in other biological processes, similarly to *BrrCBL10.2* and *BrrCIPK13.3* genes of turnip (*Brassica rapa* subs. *rapa*), which were expressed in the given tissue and developmental stage (Yin et al. 2017). ‘Drought inducibility’ *cis*-elements were noted in the promoters of six CsCBLs (*CsCBL1, 2, 5, 6, 7, and 8*). However, drought treatment significantly downregulated the expression of CsCBL8, while promoting CsCBL7. The defense and stress responsive *cis*-elements were identified in the promoters of four CsCBLs (*CsCBL2, 3, 4, and 7*). Furthermore, the expression of CsCBL4, 5, 6, and 7 was induced by AMF colonization, while the expression of CsCBL1, 2, 3, and 8 were repressed by AMF. These results suggest that the regulation of CsCBLs and CsCIPKs expression was not consistent with the *cis*-elements present in their promoters. Similar results were shown for CsCIPKs (Figs. 3-5). These results may be attributed to the integration of other gene regulatory elements, such as *trans*-acting factors (Chow et al. 2018, Xie et al. 2018).

A co-expression network analysis revealed potential *CsCBL-CsCIPK* gene pairs for Ca2+ signal transduction during drought and AMF colonization. In this network, CsCBL1-CsCIPK1, CsCBL1-CsCIPK3, CsCBL1-CsCIPK6, and CsCBL1-CsCIPK9 showed a significant and positive correlation in response to drought and AMF (Fig. 6). A phylogenetic tree was generated based on the protein sequence alignment of CBLs and CIPKs. Members of the same group exhibited similar protein sequence length, motif composition, and gene structure, reflecting a close relationship. Thus, homologous genes in the same branch might have similar functions in plant-microbe interactions and abiotic stress. AtCBL10 (clustered with CsCBL1 in the same branch) is also related to ROS and brassinosteroids in salinity tolerance, while negatively affecting drought tolerance without ABA-induced signaling (Kang et al. 2016). The function of AtCBL10 in abiotic stresses has been well studied and demonstrated diversity, where AtCBL10-AtCIPK23 (clustered with...
Fig. 4. *Citrus sinensis* calcineurin B-like proteins (CsCBLs) and calcineurin B-like protein interacting protein kinases (CsCIPKs) expressions with arbuscular mycorrhizal fungi (AMF) colonization and/or drought stress treatment identified by real time quantitative PCR. Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method, where $\beta$-actin was used as a reference gene. Means $\pm$ SEs, $n = 3$; different letters indicate significant differences at 5% level.

Fig. 5. Predicted cis-elements in the promoters of *Citrus sinensis* calcineurin B-like proteins (CsCBLs) and calcineurin B-like protein interacting protein kinases (CsCIPKs). The “defense and stress responsive” and “drought inducibility” cis-elements are indicated by triangles with red and blue colors, respectively.
CsCIPK3 in the same branch) complex for binding to AKT1 or to directly interact with AKT1 (Fig. 1B), which negatively modulates AKT1 activity to regulate inward K⁺ currents (Ren et al. 2013). And AtCBL10-AtCIPK24 (clustered with CsCIPK9 in the same branch) complex activates downstream target proteins to respond to high salt stress (Quan et al. 2007). However, other CBL-CIPK complexes related to AtCBL10 (like AtCBL10-AtCIPK3, 12, 19) in response to stress has not been determined. Thus, whether CsCBL1 interacted with CsCIPK1, and CsCBL1 interacted with CsCIPK6, and the function of complexes in response to drought and AMF colonization requires further study.

In conclusion, 8 CsCBLs and 17 CsCIPKs distributed across over 8 chromosomes were identified in the citrus genome. A total of 3 CsCBLs and 9 CsCIPKs showed syntenic relationships with AtCBLs and AtCIPKs, respectively, while 8 CsCBLs were classified into four families, and 17 CsCIPKs were classified into two families based on their protein domains, motifs, and sequences. Gene expression analysis showed that all members of the 8 CsCBLs and 16 CsCIPKs were expressed in the roots and exhibited differential expression patterns. Drought treatment remarkably downregulated the expression of CsCBL8 and promoted CsCBL7, CsCIPK4, and 7. AMF colonization induced CsCBL4, 5, 6, 7 and CsCIPK2, 4, 10, 11, 13, 14, 16 expressions, while repressing the expressions of CsCBL1, 2, 3, and 8 and CsCIPK1, 3, 6, 8, 9, and 12. The regulation of CsCBL and CsCIPK expression was not concordant with the cis-elements found in their promoter regions. Based on the expression data and co-expression analysis, CsCBL1-CsCIPK1, CsCBL1-CsCIPK3, CsCBL1-CsCIPK6, and CsCBL1-CsCIPK9 showed a significant and positive correlation in response to both drought and AMF. This study provides valuable insights into CsCBLs and CsCIPKs, including their responses to drought and AMF.

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