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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 *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-CsCIPK3*, *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 (NAF) 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 chitooligosaccharides 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 (Rodríguez-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 cv. 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 (pI) 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 *TBtools* 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 *TBtools* (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 *TBtools*

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 17 (SPSS 17)* (*SPSS Statistics, IBM, USA*) 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, *M_r*, and *pI* of both *CBLs* and *CIPKs*. The *CBL* protein length ranged from 199 aa (*CsCBL6*) to 319 aa (*CsCBL7*), while *pI* ranged from 4.59 (*CsCBL1*) to 6.18 (*CsCBL7*), and *M_r* ranged from 22.70 kDa (*CsCBL6*) to 36.48 kDa (*CsCBL7*). The *CIPK* protein length and *M_r* were around 450 aa and 50 kDa, respectively, while *pI* 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_r - molecular mass, aa - amino acids.

Gene Name	Original ID	Length [aa]	pI	M _r [kDa]
<i>CsCBL1</i>	Cs1g18400.1	255	4.58	29.00
<i>CsCBL2</i>	Cs2g01320.1	226	4.70	26.06
<i>CsCBL3</i>	Cs4g08230.1	223	4.81	25.67
<i>CsCBL4</i>	Cs4g18590.3	257	4.59	29.41
<i>CsCBL5</i>	Cs5g02080.1	214	5.07	24.50
<i>CsCBL6</i>	Cs5g02090.1	199	5.14	22.70
<i>CsCBL7</i>	Cs5g07690.1	319	6.18	36.48
<i>CsCBL8</i>	orange1.1t02723.1	213	4.77	24.47
<i>CsCIPK1</i>	Cs2g04710.1	482	6.66	53.91
<i>CsCIPK2</i>	Cs2g04720.1	452	9.23	50.80
<i>CsCIPK3</i>	Cs2g08190.5	465	9.10	52.34
<i>CsCIPK4</i>	Cs2g09340.1	480	9.05	54.57
<i>CsCIPK5</i>	Cs2g09400.1	437	8.79	48.93
<i>CsCIPK6</i>	Cs2g28990.1	440	6.85	50.32
<i>CsCIPK7</i>	Cs3g16840.1	448	6.38	50.51
<i>CsCIPK8</i>	Cs4g01450.1	442	9.04	49.58
<i>CsCIPK9</i>	Cs4g17990.1	444	8.91	50.28
<i>CsCIPK10</i>	Cs6g09730.1	473	8.94	53.23
<i>CsCIPK11</i>	Cs6g09770.1	433	8.46	49.00
<i>CsCIPK12</i>	Cs7g06310.1	460	8.07	52.54
<i>CsCIPK13</i>	Cs7g30070.1	451	9.36	50.88
<i>CsCIPK14</i>	Cs9g01260.2	477	6.81	53.70
<i>CsCIPK15</i>	Cs9g08000.1	480	9.58	53.55
<i>CsCIPK16</i>	orange1.1t00555.2	469	9.01	53.51
<i>CsCIPK17</i>	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*. *CsCBL5* and 6 exhibited tandem duplication, while some *CsCIPKs* (*CsCIPK4* and *CsCIPK13*, and *CsCIPK10* and *CsCIPK13*) showed segmental duplication (Fig. 1 Suppl.). To further investigate the genome synteny of *CBLs* and *CIPKs*, a comparative syntenic map of citrus was constructed in association with *Arabidopsis*. Three *CsCBLs* localized on chromosomes 4 and 5 showed syntenic relationships with *AtCBLs*, where *CsCBL7* 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. *CsCBL2* and 3 contained all seven motifs, while *CsCBL1*, 4, 5, 7, and 8 did not have motif 7 and *CsCBL6* exhibited five motifs (motifs 1, 2, 3, 5, and 6) (Fig. 2A). *CsCIPK2*, 4, 8, 10, 12, 13, and 17 contained all 10 motifs, while *CsCIPK1*, 3, 5, 6, 7, 9, 11, 14, and 16 possessed nine motifs, except for motif 9 and *CsCIPK15* 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 *CsCBL4*, 5, 6, and 7 was induced by AMF colonization under both well-watered and drought

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.

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.

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 had a synergistic effect, as seen in the drought-induced *CsCBL7* expression under non-AMF condition, but the inducing effect was weakened under AMF colonization (Fig. 3A, Table 3 Suppl.). The expression of *CsCIPK1*, 3, 6, 8, 9, and 12 was repressed, while *CsCIPK2*, 4, 10, 11, 13, 14, and 16 were induced by AMF. Drought treatment significantly promoted the expression of *CsCIPK4* and 7. Furthermore, AMF colonization and drought stress exerted a synergistic effect on *CsCIPKs*, in which the expressions of *CsCIPK2* and 11 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. 3. Expression profiles of *Citrus sinensis* calcineurin B-like proteins (*CsCBLs*) and calcineurin B-like protein interacting protein kinases (*CsCIPKs*) responding to arbuscular mycorrhizal fungi (AMF) colonization and/or drought stress. The color scale represents $\log_2$ expression values, where red and blue indicate higher or lower transcript abundances, respectively, compared to the relevant control (non-AMF plants under well-watered conditions). W_Non - well-watered with non-AMF, W_AMF - well-watered with AMF, D_Non - drought stress with non-AMF, D_AMF - drought stress with AMF.

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* subsp. *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 Ca^{2+} 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 $2^{-\Delta\Delta C_t}$ method, where β -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.

Fig. 6. Co-expression networks of *Citrus sinensis* calcineurin B-like proteins (*CsCBLs*) and calcineurin B-like protein interacting protein kinases (*CsCIPKs*) in response to arbuscular mycorrhizal fungi colonization and/or drought stress. The co-expression networks were established based on the Pearson correlation coefficients (PCC) of the *CsCBL* and *CsCIPK* pairs. All the PCC of co-expression gene pairs were significant at $P < 0.05$, whereas the different line sizes indicate different relevance levels of co-expression gene pairs. Positive correlations are indicated with red lines ($PCC \geq 0.6$), whereas blue lines indicate a negative correlations ($PCC \leq -0.6$).

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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