Phyton-
International Journal of
Experimental Botany

Tech Science Press

DOI: 10.32604/phyton.2022.025467

ARTICLE

Genome Wide Characterization of *CBL-CIPK* Family Genes and Their Responsive Expression in *Rosa chinensis*

Lunzeng Huang[1,2,#], Hongsheng Gao[1,#], Ning Jiang[1,2], Yunhong Xu[1,2], Zijian Gong[1,2], Lele Chen[1,2], Shijie Xue[1,2], Xiaoyan Li[1], Ruichao Liu[1,2], Bei Li[1], Hongxia Zhang[1], Chunyan Yu[1,*] and Xiaotong Guo[1,2,*]

[1]The Engineering Research Institute of Agriculture and Forestry, Ludong University, Yantai, 264025, China

[2]College of Agriculture, Ludong University, Yantai, 264025, China

[*]Corresponding Authors: Chunyan Yu. Email: abcdef20061548@126.com; Xiaotong Guo. Email: guoxtina@126.com

[#]These authors contribute equally to this work

Received: 14 July 2022 Accepted: 29 August 2022

ABSTRACT

Calcium (Ca^{2+}) plays a pivotal role in various signal transduction pathways. Calcineurin B-like proteins (CBLs) are a unique group of Ca^{2+} sensors that decode Ca^{2+} signals by activating the plant specific protein kinase known as the CBL-interacting protein kinase (CIPK). In plants, the CBL-CIPK signaling network regulates multiple signals in response to different extracellular cues including abiotic stress. However, the genome wide annotation and expression patterns of CBLs and CIPKs in woody cutting flower plants are still unclear. In this study, a total number of 7 *CBL*s (*RcCBLs*) and 17 *CIPKs* (*RcCIPKs*) genes, divided into four and five subfamilies, respectively, were identified from the rose genome. All RcCBLs possess a classic elongation factor-hand (EF-hand) domain, while all RcCIPKs possess both the classic kinase and NAF domains. Most RcCBLs were predicted to be plasma membrane localized, whereas most RcCIPKs were predicted to be cytoplasmic localized. Synteny analysis showed that one *RcCBL* gene pair and five *RcCIPK* gene pairs have gone through whole genome duplication events. Promoter *cis*-element prediction assays indicated that *RcCBLs* and *RcCIPKs* could function in different abiotic stress responses in rose plants. Further quantitative real-time PCR analysis demonstrated that *RcCBLs* and *RcCIPKs* were expressed in different organs with overlapped but distinct patterns in response to various abiotic stresses. The findings in this work will provide fundamental information and gene resources for further functional research on *RcCBLs* and *RcCIPKs*.

KEYWORDS

Calcineurin B-like proteins; CBL-interacting protein kinase; abiotic stress; rose

1 Introduction

Calcium (Ca^{2+}) functions as a second messenger to regulate physiological and developmental processes, and its level in plants is affected by biotic and abiotic stresses [1]. A diverse of environmental factors can evoke Ca^{2+} responses with specific temporal and spatial characteristics. These Ca^{2+} signals encoded as spikes, waves and oscillations, were interpreted by Ca^{2+} sensors and effectors, leading to specific responses [2]. Ca^{2+} signals are first perceived by Ca^{2+} sensors, such as calmodulin-like proteins (CMLs), calmodulins (CaMs), calcium-

dependent protein kinases (CDPKs) and calcineurin B-like proteins (CBLs), and then relayed into the downstream responses by interacting with the downstream proteins and phosphorylation cascades [3,4].

CBLs are a class of proteins that sense and transmit Ca^{2+} signals. Typically, a CBL protein contains three or four EF-hands (elongation factor-hands) to capture Ca^{2+} signals and interact with other proteins [1,4–6]. Usually, CBL does not act alone but forms a complex with a protein called CIPK (CBL-interacting serine-threonine protein kinase), which possesses a serine/threonine protein kinase structural domain, a 24-amino acid NAF structural domain, and a PPI structural domain. The NAF domain mediates the interaction with CBL, while the kinase structural domain performs other functions [5,7]. In the CBL-CIPK pathway, CBL protein binds with Ca^{2+}, thereby activates CIPK and forms a CBL-CIPK complex to phosphorylate and modify the downstream acting proteins, thus achieves the transduction of various signals [8–10]. CBLs contain N-myristoylation and palmitoylation sites and are generally localized on cell membranes, whereas CIPKs are found free in cytoplasm [6,7,11,12]. Both CBLs and CIPKs were first identified in Arabidopsis and subsequently studied in different plant species such as rice, honeysuckle, wheat, poplar, pepper, walnut, rapeseed and grape [6,13–16]. To date, a total number of 10 CBLs and 26 CIPKs in Arabidopsis, 10 CBLs and 33 CIPKs in rice, 10 CBLs and 27 CIPKs in poplar, 9 CBLs and 30 CIPKs in pecan, 6 CBLs and 17 CIPKs in honeysuckle, and 8 CBLs and 20 CIPKs in grape, have identified [6,13–18]. Previous studies have reported that CBL-CIPK complex plays a vital role in plant growth and response to numerous stresses. The response of CBLs and CIPKs in plants have been found to be different under different stress conditions such as salinity, osmotic, drought, cold, oxidative, pathogenic, and other abiotic and biotic stresses [11,15,19,20]. In Arabidopsis, expressions of both *AtCBL4* and *AtCBL10* were sensitive to salt stress. AtCIPK24 phosphorylated AtCBL10 at the Ser237 site, and the interaction of AtCBL4 and AtCIPK24 activated the activity of Na^+/H^+ antiporter located on cell membrane to alleviate salt stress [21–23]. Meanwhile, AtCIPK8, a homolog of AtCIPK24, bound to AtCBL10 and activated AtSOS1 to alleviate salt stress [24–26]. *AtCBL1* was also induced by various stresses, and overexpression of *AtCBL1* increased salt and drought tolerance but decreased cold resistance in transgenic plants [27–31]. In rice, silencing of *OsCIPK23* improved the salt and drought resistance of transgenic plants [32]. In apple, MdCIPK13 and MdCIPK22 acted on the Ser254 and Ser381 phosphorylation sites of MdSUT2.2, and enhanced drought and salt resistance [33,34]. In *Hordeum brevisubulatum*, *HbCIPK2* was induced by salt, drought and ABA treatments, and ectopic expression of *HbCIPK2* in wild type Arabidopsis and *sos2-1* mutant increased the salt tolerance of transgenic plants [35]. CIPK can bind with CBL even in the absence of Ca^{2+} ions. In Arabidopsis, Ca^{2+} bound with AtCBL1 and AtCBL9 to activate AtCIPK3 under low iron condition [36]. Overexpression of *AtCBL10* influenced potassium (K^+) the transport, seed germination, stomatal opening and closing, and lateral root development [37–44]. In cotton, GhTST2 interacted with GhCIPK6 to regulate plasma membrane sugar homeostasis [45].

Due to its commercial, economic and application values, Rose (*Rosa chinensis*) have been grown worldwide as one of the most popular ornamental flower crops. However, its shoot growth and flower production are severely affected by adverse environmental changes. Although the functions of CBLs and CIPKs in several plant species have been studied, the genome wide characterization and the regulatory roles of CBLs and CIPKs in rose still remain unknown. We identified 7 CBL and 17 CIPK proteins in the rose genome, and analyzed their gene structure, chromosome position, evolutionary relationships, promoter *cis*-acting elements and gene expression pattern in response to different abiotic stresses. The information presented here will provide a theoretical basis for the future researches on the accurate functions of CBL-CIPK proteins in response to adverse abiotic stress in woody cutting flower plants.

2 Materials and Methods

2.1 Identification and Classification of CBL-CIPK Genes in Rose

The information for genomic analysis and gene annotation of rose CBL and CIPK genes were downloaded from NCBI (https://www.ncbi.nlm.nih.gov/data-hub/taxonomy/74649/) [46]. The gene

sequences of CBLs and CIPKs were obtained from the Arabidopsis Information Resource (TAIR) (https://www.arabidopsis.org/) and the Rice Genome Annotation Project (RGAP) (http://rice.uga.edu/annotation_community_families.shtml), respectively [6,5,17]. The calcium binding EF-hand motif (PF00036) of CBL proteins was obtained based on the hidden Markov models (HMMs) in the Pfam database (http://pfam.xfam.org/) [47–49], and the kinase domain (PF00069) and NAF domain (PF03822) of CIPK proteins were determined based on the HMMs [50]. The candidate *CBL-CIPK* genes were identified using the HMM parameters of *E*-value < 0.05 and identity > 50% for the non-redundant sequences [51]. All the candidate genes were further validated using SMART (http://smart.embl-heidelberg.de) and NCBI protein BLAST tool in SWISS-PORT database (*E*-value < 0.05, Identity > 50%) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) [16,52,53]. The molecular weight (MW) and isoelectric point (pI) were calculated using the online tool ExPASy (https://web.expasy.org/compute_pi/) [54], and subcellular localization of proteins was predicted using the online tool Plant-PLoc server (http://www.csbio.sjtu.edu.cn/bioinf/plant-multi/) [55].

2.2 Phylogenetic Analysis and Chromosomal Mapping of CBL-CIPK Members

The full length amino acid sequences of all the identified CBLs and CIPKs from *Rosa chinensis* (Rc), *Arabidopsis thaliana* (At), and *Oryza sativa* (Os) were analyzed with Clustal X [56–58]. The phylogenetic trees were constructed using the MEGA7.0 software following the neighbor-joining (NJ) statistical method, with 1000 bootstrap replicates and the Poisson model [56]. The online tool iTOL was used to display the phylogenetic trees [59]. The chromosomal localization of *RcCBLs* and *RcCIPKs* were obtained using the TBtools software, based on the rose genome annotation data [60].

2.3 Gene Structure and Conserved Motif Analysis

The online tool SMART (http://smart.embl-heidelberg.de) was used to analyze the gene structure based on the untranslated regions (UTR), full length coding sequences (CDS), and genomic sequences [52]. The online program MEME (Multiple Em for Motif Elicitation) (https://meme-suite.org/) was used to assess the conserved motifs with the number of repetition set to zero or one, the maximum number of motif set at 15 (RcCIPKs) or 7 (RcCBLs), and all other parameter set as default [61].

2.4 Cis-Element Analysis

To explore the potential interaction of *RcCBLs* and *RcCIPKs*, the *cis*-acting elements in the 2.0 kb upstream sequences of their CDS were predicted using the PlantCARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) [46,62].

2.5 Synteny Analysis of RcCBLs and RcCIPKs

The syntenic relationship between pairs of chromosomes from rose, rice and Arabidopsis were analyzed and visualized using the MCScanX program [63]. The tandem repeat events within species were displayed using the Advanced Circos, and the non-synonymous substitution rate (*Ka*), synonymous substitution rate (*Ks*) and *Ka/Ks* values were calculated using the TBtools software [60,64]. Whole genome duplication (WGD) events depending on the *Ka*/*Ks* values were judged with KaKs Calculator software [65–67].

2.6 Protein Interaction Prediction

Using Arabidopsis as the background, the STRING database (http://stringdb.org/) was used to predict the interaction between RcCBLs and RcCIPKs (minimum required interaction score: highest confidence 0.900). Cytoscape was used to visualize the interaction networks [68].

2.7 Plant Materials, Growth Conditions and Stress Treatments

In this study, rose (*Rosa chinensis*) cv. Red Leonardo da Vinci was used. One-year-old cutting plants at the same size and growth state were selected and cultured in greenhouse set at 28°C/26°C day/night

temperature with a 16 h/8 h light/dark photoperiod cycle and 65% relative humidity. For gene expression analysis, root, stem, leaf, flower, petiole and calyx samples were collected. To analyze the gene expression in response to different abiotic stresses, plantlets were respectively subjected to 4°C, 37°C, 300 mM NaCl, 15% (w:v) PEG 6000 and 5 μM ABA treatments for 0, 3, 6, 9, 12, 24 and 48 h. Subsequently, leaf samples were collected at relative time points, frozen in liquid nitrogen, and stored at −80°C for further RNA extraction and quantitative real-time PCR (qRT-PCR) analysis.

2.8 RNA Extraction and qRT-PCR Assays

Total RNA was extracted using the Plant RNA Kit (OMEGA, USA) following the manufacturer's instructions. First-strand cDNA was synthesized using HiScript® III RT SuperMix for qPCR (+gDNA wiper) (Vazyme, China). qRT-PCR was performed in 96-well plates on a BIO-RAD CFX Connect Real-Time System (BIO-RAD, USA) using the SYBR qPCR Master Mix (Vazyme, China) according to the manufacturer's instructions. Primers used for qRT-PCR were designed using the Primer3plus online tool (http://www.primer3plus.com/cgi-bin /dev/primer3plus.cgi) and validated by NCBI-primer blast (https://www.ncbi.nlm.nih.gov/tools /primer-blast/index.cgi). The rose *RcActin* gene (LOC112192124) was used as the reference gene to calculate the relative expression levels of *RcCBLs* and *RcCIPKs* [69]. Specific primers used for qRT-PCR are listed in Table S1.

2.9 Statistics Analysis

All data were analyzed for variance using the IBM SPSS Statistics 21. Differences between mean values were analyzed using the Student's *t*-test and considered statistically significant at a probability level of 5% (*$P < 0.05$) or 1% (**$P < 0.01$) [70].

3 Results

3.1 Seven RcCBLs and Seventeen RcCIPKs Are Identified in the Rose Genome

To identify the *CBL* and *CIPK* genes in rose genome, we blast searched the NCBI database (https://www.ncbi.nlm.nih.gov/data-hub/taxonomy/74649/), using the amino acid sequences of 10 Arabidopsis CBLs and 23 CIPKs as references. A total number of 7 sequences containing the calcium-binding and EF-hand domain, and 17 sequences containing the kinase and NAF domains, were confirmed as rose *CBL* and *CIPK* genes, respectively. They were named as *RcCBL1* to *RcCBL7* and *RcCIPK1 to RcCIPK17* (Table 1; Table S2). We investigated the chromosome distribution of these *RcCBLs* and *RcCIPKs*, and found that they were mapped to four and seven chromosomes, respectively. Among the seventeen *RcCIPKs*, six of them, *RcCIPK10* to *RcCIPK15*, were located on chromosome 5. Physiological and biochemical property analyses indicated that the seven identified RcCBL proteins were comprised of 211 to 231 amino acids, with a predicted isoelectric points (pI) from 4.55 to 4.9 and molecular weight (MW) from 24.45 to 28.54 kDa, whereas the seventeen identified RcCIPK proteins were comprised of 336 to 494 amino acids, with a predicted pI from 5.67 to 9.15 and MW from 37.78 to 53.47 kDa. The pI points of all RcCBL proteins were less than 7, while the pI points of most RcCIPK proteins (88%) were greater than 7 (Table 1). Subcellular localization prediction with the online tool Plant-PLoc server revealed that six out of the seven RcCBLs (RcCBL1 to RcCBL6) were plasma membrane localized, except RcCBL7, which was localized to both plasma membrane and cytoplasm. Whereas most RcCIPKs (RcCIPK1, RcCIPK3 to RcCIPK8, RcCIPK10 and RcCIPK14 to RcCIPK17) were predicted to be cytoplasmic localized, except RcCIPK2, RcCIPK9, RcCIPK11 to RcCIPK13, which were predicted to be both cytoplasmic and nucleic localized (Table 1). In addition, alternative splicing was predicted in *RcCBL1*, *RcCBL3*, *RcCBL5*, *RcCIPK1*, *RcCIPK4*, *RcCIPK8*, *RcCIPK9* and *RcCIPK13* (Table 1).

Table 1: *RcCBL-RcCIPK* genes and their encoded proteins identified in the rose genome

Proposed name	Gene ID	Chr	Localization (bp)		Molecular weight	Isoelectric point	Size (aa)	Spliced variant	Subcellular localization
			Start	End					
RcCBL1	LOC112181992	1	51477474	51480302	24852.39	4.9	218	2	Cell membrane
RcCBL2	LOC112195565	1	66255704	66259524	24448.91	4.77	213	1	Cell membrane
RcCBL3	LOC112201641	5	81335633	81339013	26542.28	4.88	230	3	Cell membrane
RcCBL4	LOC112169286	6	68037678	68039923	28537.71	4.88	246	1	Cell membrane
RcCBL5	LOC112175788	7	12406341	12409611	24786.37	4.9	217	4	Cell membrane
RcCBL6	LOC112175789	7	12427450	12429297	24482.82	4.55	211	1	Cell membrane
RcCBL7	LOC112179801	7	23608507	23612852	24526.96	4.68	213	1	Cell membrane, Cytoplasm
RcCIPK1	LOC112182121	1	52747417	52751609	50834.26	8.82	444	2	Cytoplasm
RcCIPK2	LOC112183275	1	60801440	60803647	37777.73	8.43	336	1	Cytoplasm, Nucleus
RcCIPK3	LOC112186650	2	1111137	1112857	48126.7	9.2	428	1	Cytoplasm
RcCIPK4	LOC112189249	2	2877661	2882529	50707.61	9.1	448	2	Cytoplasm
RcCIPK5	LOC112187954	2	49678318	49680402	52379.81	6.91	463	1	Cytoplasm
RcCIPK6	LOC112192642	3	8967587	8969396	49290.82	8.51	435	1	Cytoplasm
RcCIPK7	LOC112191999	3	9007454	9010327	53466.71	9	474	1	Cytoplasm
RcCIPK8	LOC112199630	4	22862150	22865645	52732.94	6.15	469	2	Cytoplasm
RcCIPK9	LOC112199860	4	52782529	52791425	52368.07	7.19	467	2	Cytoplasm, Nucleus
RcCIPK10	LOC112164476	5	7325368	7327340	52551.26	9.15	467	1	Cytoplasm
RcCIPK11	LOC112164475	5	7330470	7332350	55096.17	6.93	494	1	Cytoplasm, Nucleus
RcCIPK12	LOC112203098	5	10181894	10197026	158854.9	5.67	1472	1	Cytoplasm, Nucleus
RcCIPK13	LOC112164111	5	48471187	48478918	50167.74	6.41	439	3	Cytoplasm, Nucleus
RcCIPK14	LOC112165010	5	78412682	78415103	51872.55	8.34	452	1	Cytoplasm
RcCIPK15	LOC112164722	5	78498010	78499887	50271.57	8.32	449	1	Cytoplasm
RcCIPK16	LOC112174730	6	60564407	60566496	49261.85	8.94	439	1	Cytoplasm
RcCIPK17	LOC112176986	7	57769412	57776174	50717.33	6.9	446	1	Cytoplasm

3.2 CIPK and CBL Proteins Are Respectively Classified into Four and Five Subgroups

To understand the possible functions and evolutionary relationships of the CBL and CIPK proteins in rose, we constructed a rootless phylogenetic tree consisting of 27 CBLs and 75 CIPKs from *Arabidopsis thaliana*, *Oryza sativa* and *Rosa chinensis* (Fig. 1). Based on their sequence similarity, the seven RcCBLs were classified into four subgroups, including 2 RcCBLs (RcCBL2, RcCBL6) in group I, 1 RcCBL (RcCBL3) in group II, 2 RcCBLs (RcCBL4, RcCBL5) in group III and 2 RcCBLs (RcCBL1, RcCBL7) in group IV (Fig. 1A). The seventeen RcCIPKs were divided into five subgroups, including 2 RcCIPKs (RcCIPK6, RcCIPK15) in group I, 3 RcCIPKs (RcCIPK5, RcCIPK11, RcCIPK16) in group II, 1 RcCIPK (RcCIPK3) in group III, 4 RcCIPKs (RcCIPK2, RcCIPK7, RcCIPK10, RcCIPK14) in group IV and 7 RcCIPKs (RcCIPK1, RcCIPK4, RcCIPK8, RcCIPK9, RcCIPK12, RcCIPK13, RcCIPK17) in group V (Fig. 1B).

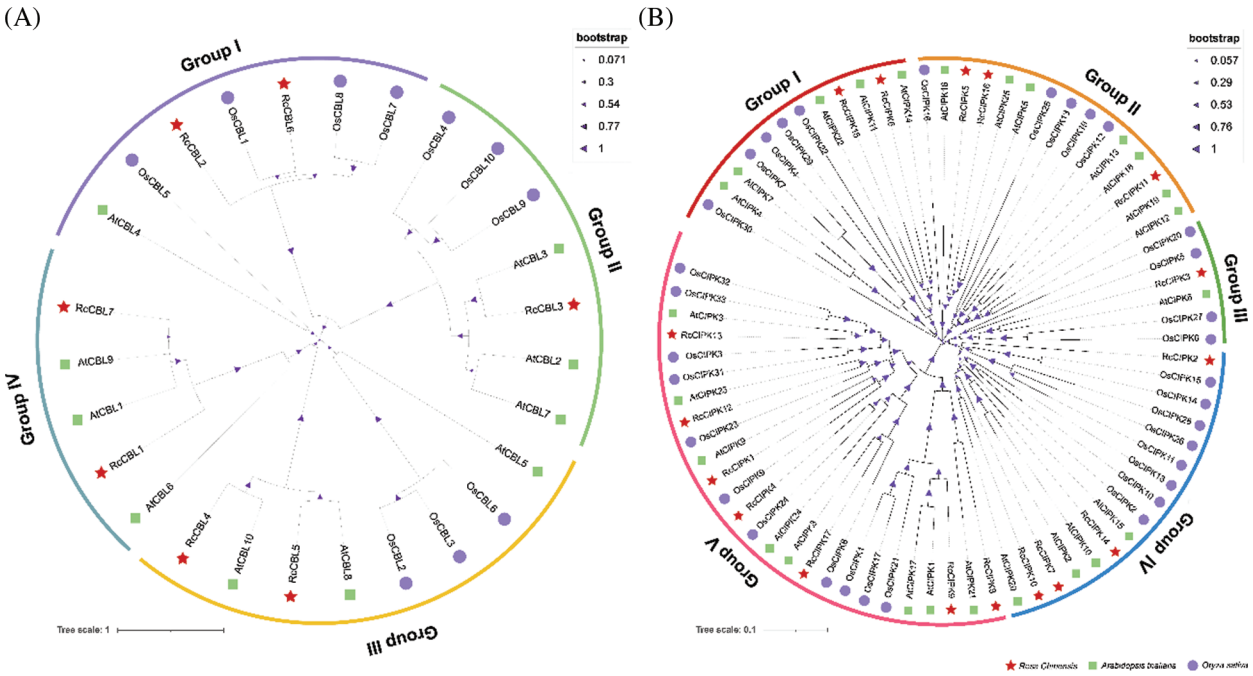

Figure 1: Phylogenetic relationship analysis of CBL and CIPK genes from Arabidopsis, rice and rose. Full length amino acid sequences of CBLs and CIPKs were used to construct the neighbor-joining (NJ) tree using MEGA7.0 with 1000 bootstrap replicates. Subfamilies are highlighted in different colors. (A) Phylogenetic tree of CBL proteins. (B) Phylogenetic tree of CIPK proteins. At, *Arabidopsis thaliana*; Os, *Oryza sativa*; Rc, *Rosa chinensis*

3.3 Gene Structures of RcCBLs and RcCIPKs Are Highly Conserved

We further analyzed the structures of *RcCBL and RcCIPK* genes and found that members in the same subfamily demonstrated similar motif distribution and gene structures (Fig. 2). A total number of seven and fifteen conserved motifs in RcCBL and RcCIPK proteins were respectively identified (Fig. 2A). All RcCBL proteins, except RcCBL4, which lacked motif 7, possessed all the seven motifs (motifs 1 to 7). In RcCBL proteins, motif 1, motif 3 and motif 4 contained the essential phosphorylation site for the calcium ion EF-hand structure, and motif 7 was identified as the myristoylation site (Fig. 2C). Almost all RcCIPK proteins, except RcCIPK2, which lacked motifs 5 and 6, possessed motifs 1 to 7. Six of the seven RcCIPKs (RcCIPK1, RcCIPK4, RcCIPK8, RcCIPK12, RcCIPK13, and RcCIPK17) in group V contained motif 11 (Fig. 1C). Motif 12 was not only observed in the C-terminus of RcCIPK7, RcCIPK10 and

RcCIPK14, but also in the N-terminal of RcCIPK5 and RcCIPK15. In addition, motifs 8, 9 and 13 were detected in most RcCIPK proteins. However, RcCIPK2, RcCIPK5 and RcCIPK15 lacked motif 8, RcCIPK2, RcCIPK8, RcCIPK9 and RcCIPK15 lacked motif 9, and RcCIPK6, RcCIPK7, RcCIPK14 and RcCIPK15 lacked motif 13 (Fig. 2A). All the motifs in RcCIPK proteins were listed in Table S3, and the detailed sequences of the six representative motifs were exhibited in Fig. 2D. Motifs 1, 2 and 5 possessed the kinase catalytic activation loop, while motifs 7, 8 and 14 contained the basic NAF motif regulatory domain.

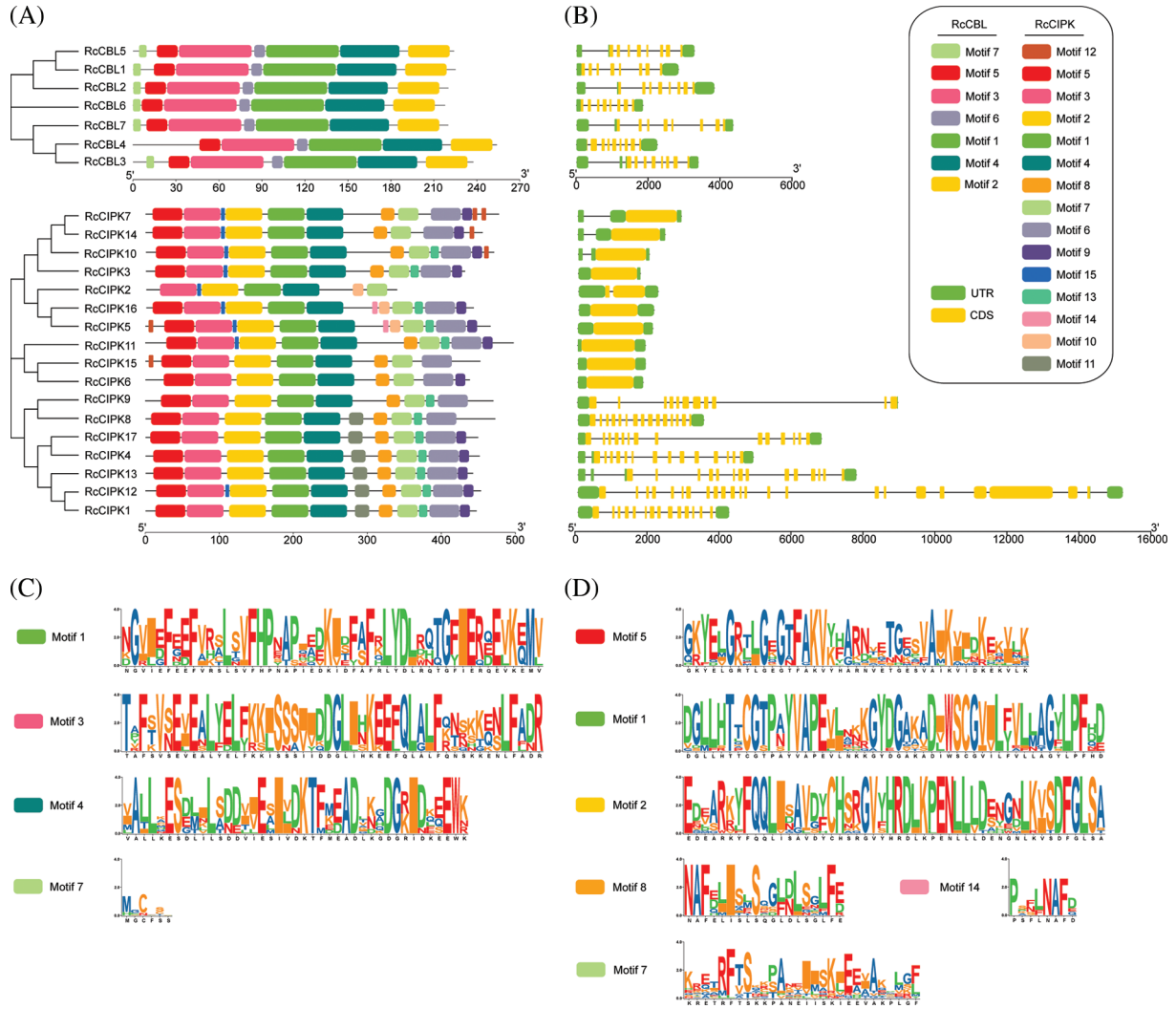

Figure 2: Phylogenetic relationship, conserved motifs and gene structure of RcCBLs and RcCIPKs. A. Phylogenetic trees of RcCBL and RcCIPK proteins generated with the MEGA 7.0. The conserved motifs of RcCBLs and RcCIPKs were analyzed with the MEME program. B. Exon-intron structures of *RcCBLs* and *RcCIPKs*. Untranslated regions (UTR), exons, and introns are represented with green boxes, yellow boxes and gray lines, respectively. C. Sequence logos of the four core motifs of *RcCBLs*. Motif 1, motif 3 and motif 4, the EF-hand motifs; motif 7, the transmembrane domain. D. Sequence logos of the six core motifs of the *RcCIPKs*. Motifs 1, 2 and 5, the serine/threonine protein kinases; motifs 8 and 14, the NAF motifs; motif 7, the PPI motif

Furthermore, the CDS and UTR of the *RcCBL* and *RcCIPK* genes were analyzed to determine the exon and intron distribution. We observed that all *RcCBLs* contained 8–9 introns, whereas most *RcCIPKs* contained 0–1 intron, except those in group V, which contained 10–22 introns (Fig. 2B). Consistent with the results of phylogenetic analysis, *RcCBLs* and *RcCIPKs* on the same developmental branch showed similar exon-intron organization, despite the differences of intron numbers and distances in each gene (Fig. 2B). In particular, *RcCIPK3, RcCIPK5, RcCIPK6, RcCIPK7, RcCIPK10, RcCIPK11, RcCIPK14, RcCIPK15* and *RcCIPK16* contained contiguous CDS sequences that converged on two evolutionary branches at the same time. The continuity or discontinuity of the CDS region of *RcCIPKs* might be correlated with the evolutionary changes.

3.4 Promoters of RcCBLs and RcCIPKs Contain the Key Cis-Elements for Plant Hormone and Stress Response

We further analyzed the promoter regions of *RcCBL* and *RcCIPK* genes. We found that they all contained the basic *cis*-acting elements related to stress response, light response, anaerobic regulation, plant hormone (ABA, SA, MeJA, GA), mechanical injury defense and developmental regulation (Fig. 3, Table S4). All *cis*-acting elements detected in the promoter regions of *RcCIPKs*, except HD-Zip 1, were also found in the promoter regions of *RcCBLs*. In addition, a number of light responsive elements, ABA responsive and MYB binding sites were found in the promoter region of *RcCBLs* and *RcCIPKs*, implying their multiple roles in flowering, morphogenesis and stress response in rose.

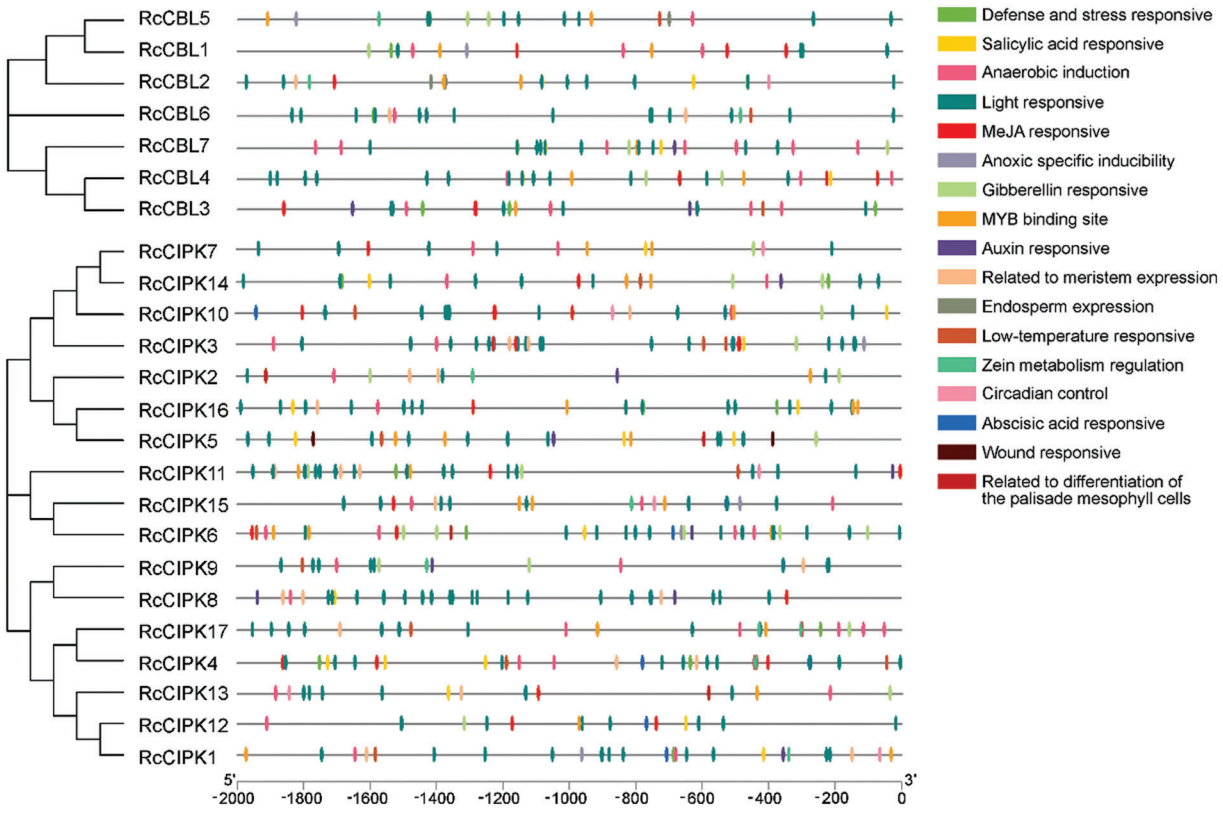

Figure 3: Phylogenic relationship and *cis*-acting elements in the promoter regions of *RcCBLs* and *RcCIPKs*. Phylogenetic tree of RcCBL and RcCIPK proteins was constructed with the MEGA 7.0 software. The *cis*-acting elements were predicted with PlantCARE and displayed with boxes in different colors

3.5 Gene Duplication Events in RcCBLs and RcCIPKs Are Observed

We investigated the chromosome distribution of these *RcCBLs and RcCIPKs*, and found that *RcCBLs* were mapped to chromosome 1, chromosome 5, chromosome 6 and chromosome 7, whereas *RcCIPKs* were distributed unevenly on all the seven chromosomes (Fig. 4). We also examined gene duplication events in *RcCBLs* and *RcCIPK*s using MCScanX. Collinear analyses based on the annotation information revealed that 2 gene pairs *RcCBL5* and *RcCBL6*, and *RcCIPK10* and *RcCIPK11*, have undergone tandem duplication events, and 6 gene pairs *RcCBL2* and *RcCBL5*, *RcCIPK2* and *RcCIPK6*, *RcCIPK2* and *RcCIPK14*, *RcCIPK3* and *RcCIPK16*, *RcCIPK7* and *RcCIPK13*, and *RcCIPK6* and *RcCIPK14*, have undergone whole genome duplication events (Fig. 4).

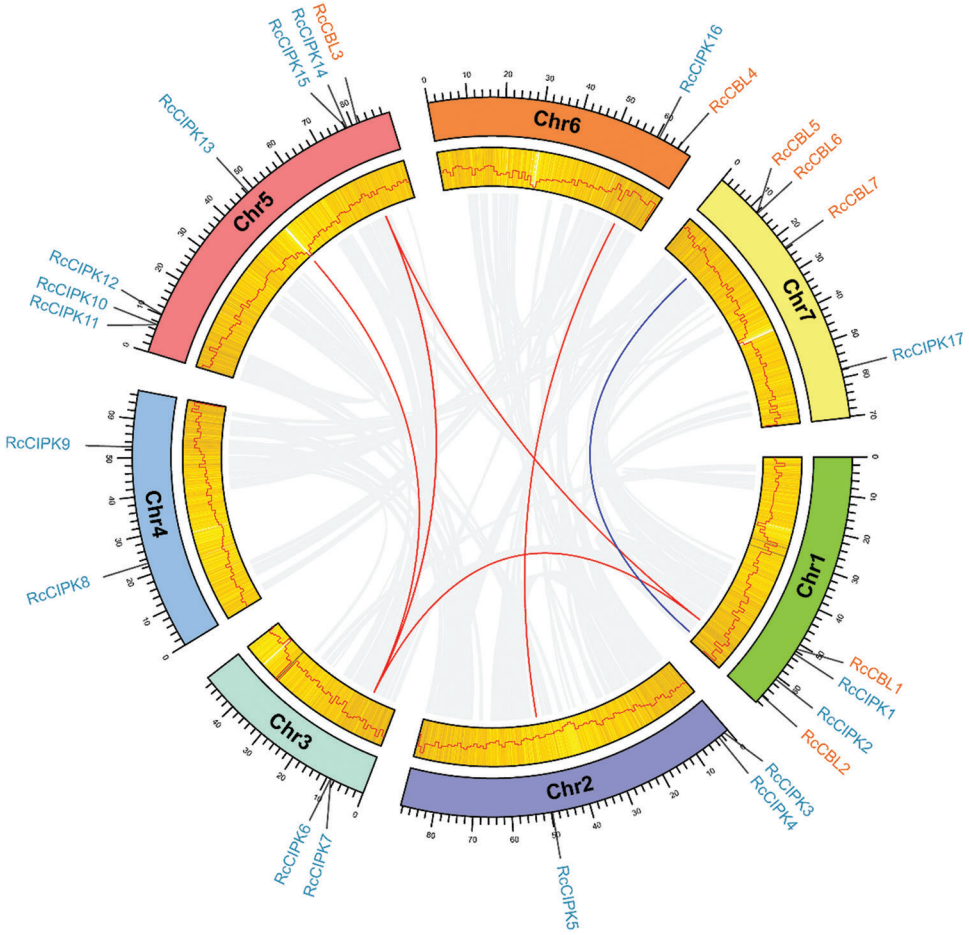

Figure 4: Chromosome distribution and syntenic analysis of *CBLs* and *CIPKs*. Syntenic relationships between *RcCBL*s and *RcCIPK*s were visualized using Circos. The rose chromosomes are represented with colored bars. The length of chromosomes is marked in scale, and the density of genes is demonstrated in a heatmap and a line chart. Gray lines indicate synteny blocks in the rose genome. Duplicated *RcCBL* and *RcCIPK* gene pairs were indicated with blue and orange lines, respectively

To further understand the evolution of *RcCBL* and *RcCIPK* genes in details, *Ka*/*Ks* ratios were calculated. The *Ka*/*Ks* values of all the seven gene pairs were less than 1, indicating the influence of purifying selection on the evolution of these genes with limited functional divergence after the duplication events (Table 2). Therefore, the replication events in these seven gene pairs have increased

the number and functional diversity of *RcCBL* and *RcCIPK* family members. Then, we constructed a comparative syntenic map using the *CBL* and *CIPK* genes of Arabidopsis, rice and rose. We found that 19 *RcCBL* and *RcCIPK* genes had homologs in rice, while 24 *RcCBL* and *RcCIPK* genes had homologs in Arabidopsis, implying that these genes might have played significant roles in the evolution of *CBL* and *CIPK* gene families (Fig. 5).

Table 2: Selection type analysis of the duplicated *RcCBL* and *RcCIPK* genes

Duplicated gene 1	Duplicated gene 2	*Ka*	*Ks*	*Ka/Ks*	D-time (MYA)	Duplication type
CBL2	CBL5	0.182845	1.017222	0.179749	3.39	WGD
CIPK2	CIPK6	0.302303	2.199711	0.137428	7.33	WGD
CIPK2	CIPK14	0.324891	1.440210	0.225586	4.8	WGD
CIPK3	CIPK16	0.329178	1.974645	0.166702	6.58	WGD
CIPK7	CIPK13	0.266252	1.047401	0.254203	3.49	WGD
CIPK6	CIPK14	0.191320	2.482406	0.077071	8.27	WGD

Note: MYA, Million years ago; WGD, whole genome duplication.

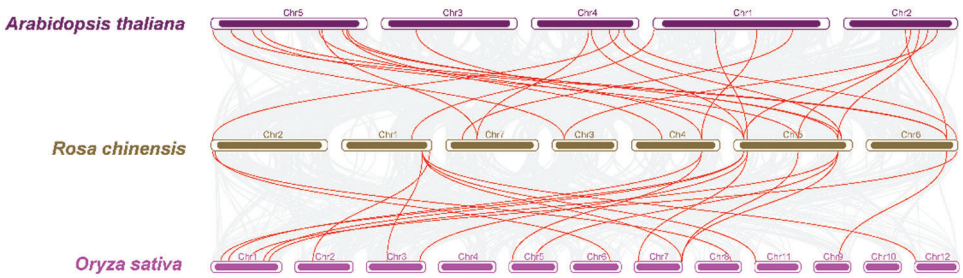

Figure 5: Collinear correlations of Arabidopsis, rice, and rose. The five chromosomes of Arabidopsis, seven chromosomes of rose and 12 chromosomes of rice were presented in different colors. The collinear blocks and syntenic *RcCBLs* and *RcCIPKs* gene pairs among Arabidopsis, rice, and rose are respectively shown in gray and red lines

3.6 Protein Interaction Are Predicted in RcCBLs and RcCIPKs

In the protein interaction network, RcCBL and RcCIPK proteins showed 34 nodes and 44 edges among themselves (Fig. 6). In particular, RcCBL3, RcCBL6, RcCIPK12 and RcCIPK15 demonstrated potential interactions with most other members. In addition, ten proteins were predicted to potentially interact with RcCBLs and RcCIPKs (Table S5).

3.7 RcCBL and RcCIPK Genes Show Overlapped but Distinct Expression Patterns in Rose

To understand the possible functions of *RcCBLs* and *RcCIPKs* in the growth and development of rose, we examined their expression levels in various organs including roots, stems, leaves, petioles, flowers and calyxes. They showed overlapped but distinct expression patterns (Fig. 7, Table S6). *RcCIPK3* and *RcCIPK7* were highly expressed in all tested organs, while expressions of *RcCIPK2*, *RcCIPK4* and *RcCIPK10* were hardly detected. All *RcCBLs* displayed a higher expression in the vegetative organs such as roots, stems, leaves and petioles, and a lower expression in flowers. A higher expression of *RcCBL2*, *RcCIPK4*, *RcCIPK5*, *RcCIPK12*, *RcCIPK13* and *RcCIPK14* in leaves, *RcCBL5* in roots, *RcCBL4* and *RcCIPK3* in stems, and *RcCBL6*, *RcCIPK1*, *RcCIPK2, RcCIPK7*, *RcCIPK9* and *RcCIPK10* in petioles,

was detected than in the other organs. Meanwhile, *RcCIPK15* and *RcCIPK16* were predominantly expressed in flowers, indicating their possible role in flowering regulation.

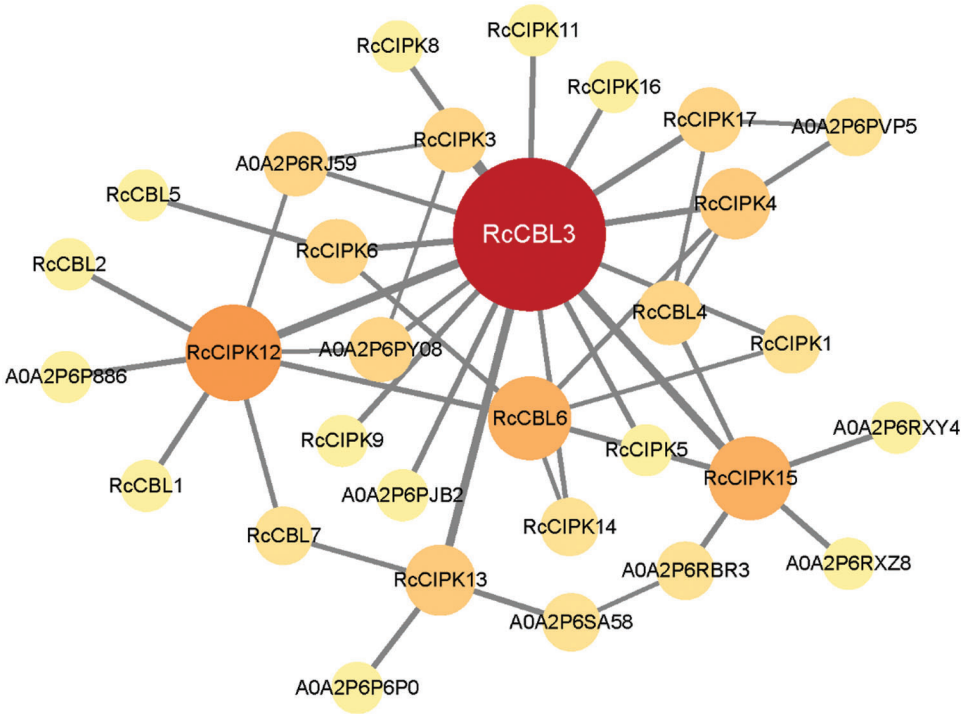

Figure 6: A predicted protein interaction network of RcCBLs and RcCIPKs. Knot sizes in different colors indicate the frequency of interacting proteins. The higher the protein-protein interaction frequency is, the deeper the color and the larger the size are. The line thickness shows the intension of data support

3.8 Expressions of RcCBLs and RcCIPKs Are Regulated by Different Abiotic Stresses

To further understand the possible functions of *RcCBLs* and *RcCIPKs* in the response to environment stresses, we examined their expression levels in the leaves of rose plants in response to 4°C, 37°C, NaCl, PEG and ABA treatments (Fig. 8, Table S7). One-year-old rose plants grown in green house were subjected to 4°C, 37°C, 300 mM NaCl, 15% PEG and 5 μM ABA treatments for 0, 3, 6, 12, 24 and 48 h, respectively. All *RcCBLs* and *RcCIPKs* showed responsive expression to at least one treatment. They all showed up- or down-regulated expression in response to low temperature (4°C), high temperature (37°C), salt stress, PEG and ABA treatments. However, expression of *RcCBL4* was not affected by high temperature, and *RcCBL2* not by ABA and heat stress. Compared with that at 0 h, low temperature induced *RcCBL5* expression at 6, 9, 12 h, *RcCBL3* expression at 6 h, and *RcCBL2* expression at 6 and 9 h, whereas suppressed *RcCBL1* and *RcCBL4* expression at 48 h, *RcCBL2* and *RcCBL7* expression at 3 h, and *RcCBL6* expression at 9 h. Meanwhile, heat stress induced the expression of *RcCBL1*, *RcCBL3*, *RcCBL5*, *RcCBL6* and *RcCBL7* at 6, 3, 3, 3 and 9 h, and suppressed the expression of *RcCBL6* at 12 h and 24 h, and *RcCBL3* at 12 h, respectively. ABA treatment increased the expression levels of *RcCBL1*, *RcCBL3*, *RcCBL6* and *RcCBL7* at 3 h, and *RcCBL1*, *RcCBL5 and RcCBL7* at 6 h, but suppressed the expression of *RcCBL5* at 48 h. Salt stress increased the expression of *RcCBL2*, *RcCBL3, RcCBL4* and *RcCBL7* at 3 h, and *RcCBL4, RcCBL5* and *RcCBL6* at 9 h, but suppressed the expression of *RcCBL4* at 24 and 48 h. PEG treatment increased the expression of all seven *RcCBLs* at one or more time points. Most *RcCIPKs* showed different expressions upon different stress treatments, and at least six of the seventeen *RcCIPK*s showed fluctuated

expression levels under each stress treatment. Only *RcCIPK9* and *RcCIPK15* showed responsive expression to all the five treatments, while fifteen *RcCIPKs* responded to at least one stress treatment. *RcCIPK11* responded to low temperature. *RcCIPK7* was up-regulated by PEG and salt stress. Expression of *RcCIPK2* was induced by high temperature, salt stress and PEG treatment. Expression of *RcCIPK5* was up- or down-regulated by high temperature, salt stress, PEG and ABA treatments (Fig. 8, Table S7).

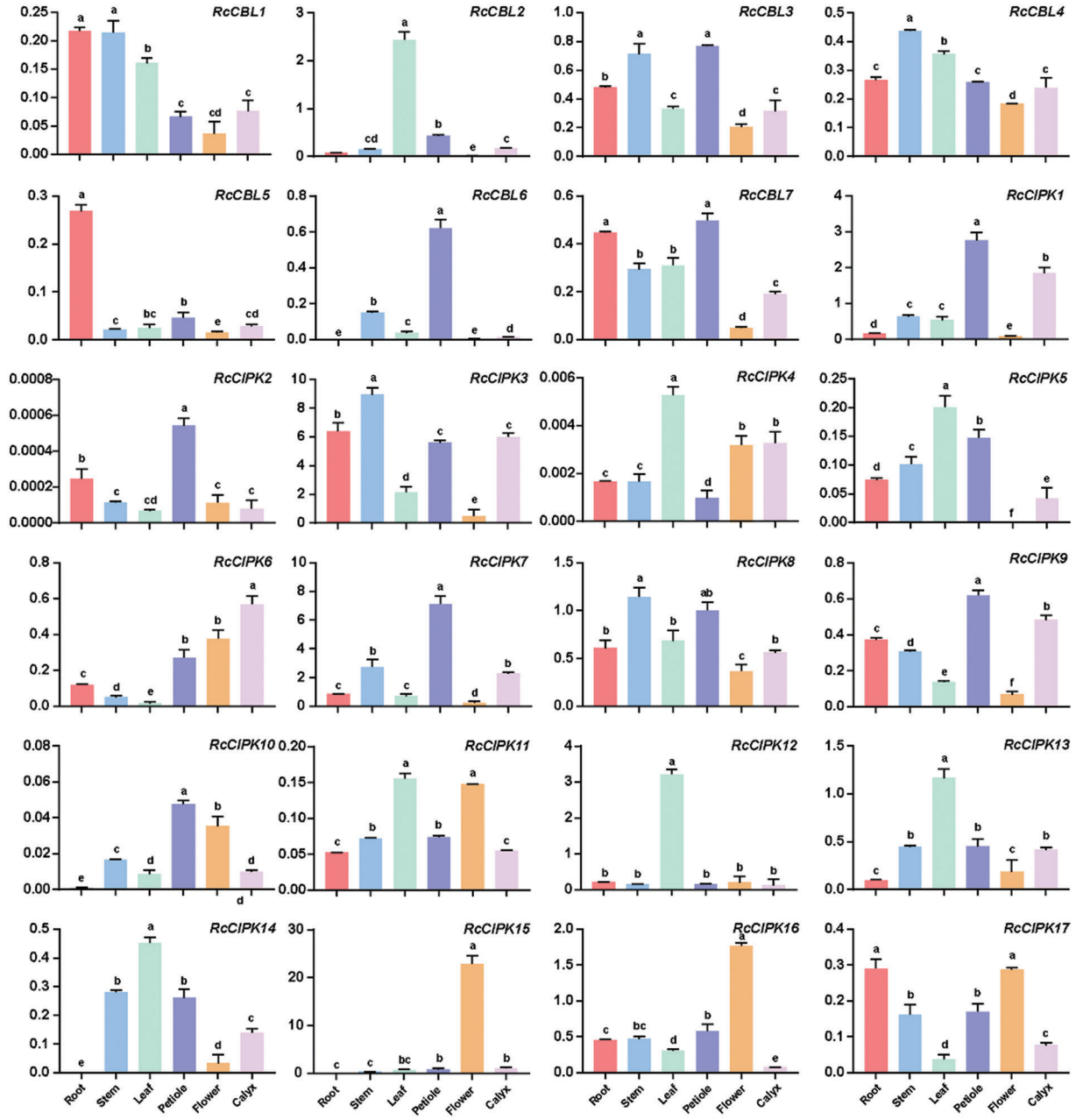

Figure 7: Expression patterns of *RcCBLs* and *RcCIPKs* in rose plants grown under normal condition. The relative expression levels of *RcCBL* and *RcCIPK* genes in roots, stems, leaves, flowers, petioles and calyxes were normalized with the reference gene *RcActin*. Data are represented as the mean ± SD of three biological replicates. Different letters (a, b, c, d, and e) indicate significant differences in the gene expression levels among roots, stems, leaves, flowers, petioles and calyxes (n = 9; $P < 0.05$, Student's *t*-test)

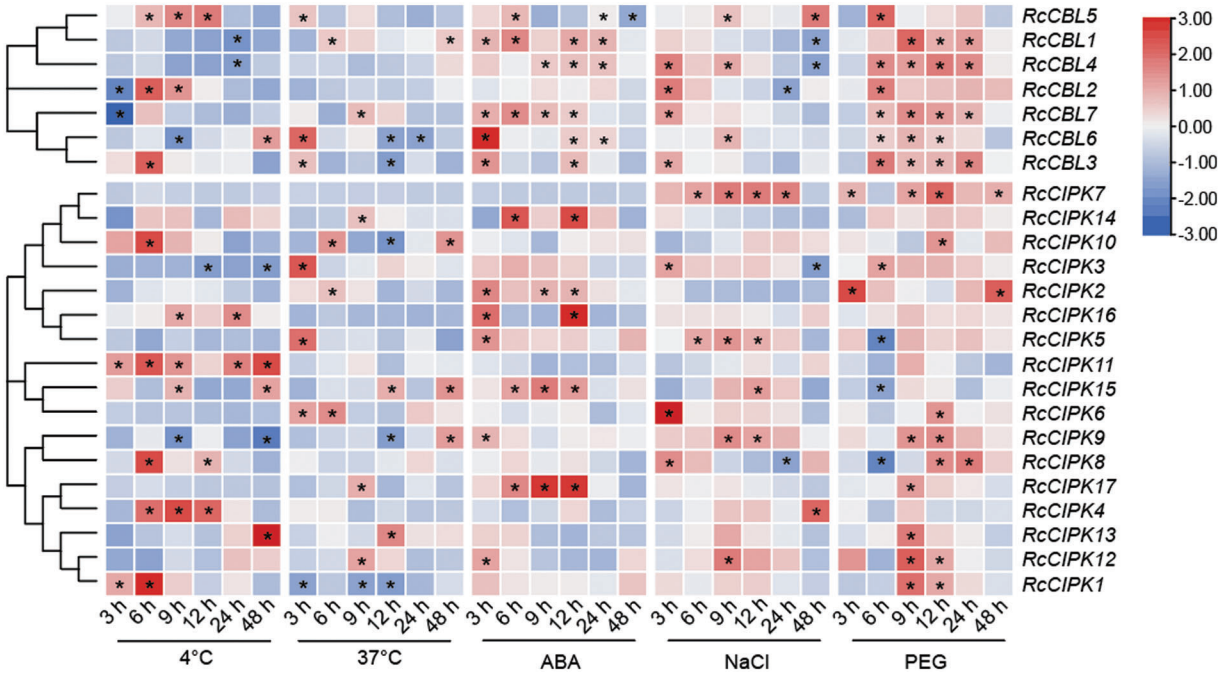

Figure 8: Hierarchical clustering of the relative expressions of *RcCBL*s and *RcCIPK*s at six time points under different stress (4°C, 37°C, ABA, NaCl and PEG) conditions. The transcript levels at 0 h were used as the internal control. The expression of each gene relative to *RcActin* was used as the internal standard. The average of three independent experiments was used to analyze the SD (n = 9; $P < 0.05$, Student's *t*-test)

4 Discussion

The *CBL-CIPK* family is a class of Ca^{2+} signaling complexes widely found in plants to perceive Ca^{2+} levels under various external environments [3,4]. Previous studies have identified the CBL-CIPK members in many species and reported their responses to low temperature, ABA treatment and salt stress [23,41,71,72]. However, research on their possible roles in cutting flower crops is still limited. In this study, 7 *RcCBLs* and 17 *RcCIPKs* in the rose genome were identified, which is consistent with the number of *CBLs* but less than the *CIPKs* in other species [3,6,14]. We also compared their protein characteristics, such molecular weight, isoelectric point, protein size, spliced variant and subcellular localization (Table 1). Consistent with the Arabidopsis CBLs, RcCBLs were also predicted to be localized on plasma membrane and could activate CIPK upon the receiving of calcium signals [9,73].

Phylogenetic analysis has been used to analyze species evolution history and kinship. We constructed the phylogenetic trees which sorted *RcCBLs* into four subgroups and *RcCIPKs* into five subgroups (Fig. 1). Consistent with the grouping in other species, RcCBL proteins were evenly distributed among the four groups, while RcCIPK were mainly distributed in the fifth subgroup (42% of the total) [15,18]. Further gene structure analysis revealed that, like the CBLs in *A. thaliana* and grape, the rose CBLs also contained four EF-hand motifs (Fig. 2A) [15]. Consistent with those in other species, all the *RcCIPKs* identified in this study have the basic kinase domain and NAF motif [1,6,17,18]. *RcCIPK2*, in which motif 6 and motif 9 in the C-terminal and motif 5 in the N-terminal were missing, was shorter than the other *RcCIPKs* (Fig. 2A, Table S3). The distribution of motifs varied among *RcCIPKs*, while the members on the same branch in the phylogenetic tree shared similar motif distribution. However, the length and distribution of the motifs in *RcCIPK12* were extremely different from the length and

distribution of the motifs in the other *RcCIPKs* due to the absence of a protein structural domain at the C-terminus (Table 1). The loss of this domain may probably be due to a stop codon error in genome sequencing [46]. Moreover, the phylogeny and gene structure of *RcCBLs* and *RcCIPKs* indicated a potential functional association among them, and the number and relative position of the introns showed a correlation with the phylogeny. For example, *RcCBL3* and *RcCBL4* were on the same branch, and they showed similar gene structures. While *RcCIPK* was intuitively divided into intron poor and intron rich groups according to the phylogenetic relationship (Fig. 2B). This property is highly conserved among various species, such as rice, Arabidopsis, poplar and soybean [18,74]. The presence of introns is often accompanied by variable splicing, especially in response to environmental changes [75]. We also observed variable splicing in *RcCBLs* and *RcCIPKs*, with a quantitative distribution similar to that detected in other species, indicating that *CBLs* and *CIPKs* are conserved across species in terms of gene structure (Table 1) [13,15].

The chromosome distribution of *CBL*s and *CIPK*s varies and the regulatory relationship of them changes depending on their biological functions [76,77]. We analyzed the phylogenetic relationships and their regulatory elements of *RcCBL*s and *RcCIPK*s (Fig. 3, Table S4). Unfortunately, we did not find a significant association between phylogeny and regulatory elements in the promoters of *RcCBLs* and *RcCIPKs*, not even for the tandem repeat gene pairs (e.g., *RcCIPK10* and *RcCIPK11*) or the gene pairs with high homology but located on different chromosomes (e.g., *RcCBL3* and *RcCBL4*). However, numerous stress responsive *cis*-acting elements were found in the promoter regions of *RcCBLs* and *RcCIPKs*, which have been also identified in the promoters of *CBL*s and *CIPK*s from other plant species, and some of them have been experimentally confirmed to respond to transcription factors [78–81].

To infer evolutionary divergences of genes, covariance analysis and *Ka*/*Ks* calculation have been used to gain detailed information on the approximate timing of the replication events [65]. *RcCBLs* were present only on chromosomes 1, 5, 6 and 7, whereas *RcCIPKs* were distributed on all chromosomes. This distribution is probably related to the evolutionary function and the complex gene exchange. Consistent with previous reports in other plant species, both tandem and genome wide duplication repeats were detected in *RcCBLs* and *RcCIPKs* (Fig. 4, Table 2) [78,79]. Based on the *Ka*/*Ks* values (≤1), we inferred that *RcCBL* and *RcCIPK* genes have gone through strong purifying selection during the evolution. Furthermore, interspecies covariance analysis also indicated that both *CBL* and *CIPK* genes were continuously and unequally distributed in Arabidopsis, rice and rose (Fig. 5) [3,6]. CBL and CIPK can form a complex to perceive Ca^{2+} signal [17]. We predicted the interaction between RcCBLs and RcCIPKs based on previous reports and found that RcCBL3 and RcCIPK12 were highly related to other members, indicating that they may function as the core proteins in the CBL-CIPK protein interaction network (Fig. 6).

During different developmental stages, gene expression patterns may vary among different organs. Some genes are expressed only at the early growth and development stages of plants, while some gene act during the formation of organs. Therefore, study on gene expression patterns can provide important information for the understanding of their biological functions [69,82,83]. We observed differential expression patterns of *RcCBLs* and *RcCIPKs* in rose plants (Fig. 7). The expressions of *RcCBL2* and *RcCIPK12* were significantly higher in leaves than in other parts, implying their possible roles in leaf development. *RcCIPK15* and *RcCIPK16* were predominantly expressed in flowers, indicating their possible role in flowering regulation. Meanwhile, some genes, such as *RcCBL4* and *RcCIPK8*, were constitutively expressed in all tested organs, which indicated that they may function in the whole developmental process of rose plants [69].

Numerous studies have demonstrated that *CBL*, *CIPK* or *CBL-CIPK* complex play important roles in plant growth and response to abiotic stress. In Arabidopsis, *AtCBL1* was induced by cold, mannitol and salt treatments, and were involved in ABA-mediated salt, cold and drought stress response [18,25,27,84–

87]. AtCBL4-AtCIPK24 activated the membrane Na^+/H^+ ion channels to alleviate the toxic damage caused by salt stress in an ABA-dependent manner [87–89]. Meanwhile, AtCBL3-AtCIPK9 was reported to target PAT10 and participate in ABA-dependent stomata divergence under stress condition [43,44]. In addition, AtCBL10 and AtCIPK8 were associated with AtSOS1 in alleviating salt stress, while AtCBL5 and AtCIPK11 targeted by SLAC1 were associated with ABA signaling [12]. Overexpression of *OsCIPK23* in rice and *ZmCIPK8* in maize respectively increased plant tolerance to salt and drought [32,90]. We observed that most *RcCBLs* and *RcCIPKs* responded to cold, heat, salt and ABA treatment, although the response patterns varied among different abiotic stresses (Fig. 8). Most *RcCBL* and *RcCIPK* genes were up-regulated under osmotic stress condition, but down-regulated by high temperature treatment. These results indicate that *RcCBL* and *RcCIPK* genes may have different functions in response to different abiotic stresses at different growth stages in rose.

5 Conclusions

Taken together, a total number of 7 *CBL* and 17 *CIPK* genes in the rose genome were identified and comprehensively analyzed. The identified *RcCBLs* and *RcCIPKs* were respectively divided into four and five subfamilies. Expression pattern analyses revealed that most *RcCBLs* and *RcCIPKs* were constitutively expressed and all members were either up- and/or down-regulated by cold, heat, salt, osmotic stress and/or ABA treatment. Our findings provide valuable information for the future study on the biological functions of *CBL* and *CIPK* genes in the growth, development and response to abiotic stress in flowering crops.

Data Availability Statement: Data are contained within the article and supplementary material.

Authorship: The authors confirm contribution to the paper as follows: study conception and design: Lunzeng Huang, Hongsheng Gao and Ning Jiang; data collection: Yunhong Xu, Zijian Gong, Lele Chen, Shijie Xue; analysis and interpretation of results: Xiaoyan Li, Ruichao Liu and Bei Li; draft manuscript preparation: Hongxia Zhang, Chunyan Yu, and Xiaotong Guo. All authors reviewed the results and approved the final version of the manuscript.

Funding Statement: This research was funded by the following grants: the Natural Science Foundation of Shandong Province of China (ZR2021MC169); the Cooperation Project of University and Local Enterprise in Yantai of Shandong Province (2021XDRHXMPT09).

Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

References

1. Ma, X., Li, Q., Yu, Y., Qiao, Y., Gong, Z. (2020). The CBL-CIPK pathway in plant response to stress signals. *International Journal of Molecular Sciences, 21(16),* 5668.
2. Tian, W., Wang, C., Gao, Q., Li, L., Luan, S. (2020). Calcium spikes, waves and oscillations in plant development and biotic interactions. *Nature Plants, 6(7),* 750–759.
3. McAinsh, M. R., Pittman, J. K. (2009). Shaping the calcium signature. *New Phytologist, 181(2),* 275–294.
4. Weinl, S., Kudla, J. (2009). The CBL-CIPK Ca^{2+}-decoding signaling network: Function and perspectives. *New Phytologist, 184(3),* 517–528.
5. Luan, S., Kudla, J., Rodríguez-Concepción, M., Yalovsky, S., Gruissem, W. (2002). Calmodulins and calcineurin B-like proteins: Calcium sensors for specific signal response coupling in plants. *The Plant Cell, 14,* S389–S400.
6. Mohanta, T. K., Mohanta, N., Mohanta, Y. K., Parida, P., Bae, H. (2015). Genome-wide identification of Calcineurin B-Like (CBL) gene family of plants reveals novel conserved motifs and evolutionary aspects in calcium signaling events. *BMC Plant Biology, 15(6),* 189–204.

7. Kolukisaoglu, U., Weinl, S., Blazevic, D., Kudla, O. B. (2004). Calcium sensors and their interacting protein kinases: Genomics of the Arabidopsis and rice CBL-CIPK signaling networks. *Plant Physiology, 134(1),* 43–58.
8. Sanchez-Barrena, M. J., Fujii, H., Angulo, I., Martinez-Ripoll, M., Zhu, J. et al. (2007). The structure of the C-terminal domain of the protein kinase *At*SOS2 bound to the calcium sensor *At*SOS3. *Molecular Cell, 26(3),* 427–435.
9. Shi, J., Kim, K., Ritz, O., Albrecht, V., Gupta, R. et al. (1999). Novel protein kinases associated with calcineurin B-like calcium sensors in *Arabidopsis*. *The Plant Cell, 11(12),* 2393–2405.
10. Tang, R., Wang, C., Li, K., Luan, S. (2020). The CBL-CIPK calcium signaling network: Unified paradigm from 20 years of discoveries. *Trends in Plant Science, 25(6),* 604–617.
11. Batistic, O., Waadt, R., Steinhorst, L., Held, K., Kudla, J. (2010). CBL-mediated targeting of CIPKs facilitates the decoding of calcium signals emanating from distinct cellular stores. *The Plant Journal, 61(2),* 211–222.
12. Saito, S., Hamamoto, S., Moriya, K., Matsuura, A., Sato, Y. et al. (2018). N-myristoylation and S-acylation are common modifications of Ca^{2+}-regulated *Arabidopsis* kinases and are required for activation of the SLAC1 anion channel. *New Phytologist, 218(4),* 1504–1521.
13. Ma, X., Gai, W. X., Qiao, Y. M., Ali, M., Wei, A. M. et al. (2019). Identification of *CBL* and *CIPK* gene families and functional characterization of *CaCIPK1* under *Phytophthora capsici* in pepper (*Capsicum annuum* L.). *BMC Genomics, 20(1),* 775–793.
14. Sun, T., Wang, Y., Wang, M., Li, T., Zhou, Y. et al. (2015). Identification and comprehensive analyses of the *CBL* and *CIPK* gene families in wheat (*Triticum aestivum* L.). *BMC Plant Biology, 15(269),* 269.
15. Xi, Y., Liu, J., Dong, C., Cheng, Z. (2017). The *CBL* and *CIPK* gene family in grapevine (*Vitis vinifera*): Genome-wide analysis and expression profiles in response to various abiotic stresses. *Frontiers in Plant Science, 8,* 978.
16. Zhang, H., Yang, B., Liu, W., Li, H. W., Wang, L. et al. (2014). Identification and characterization of *CBL* and *CIPK* gene families in canola (*Brassica napus* L.). *BMC Plant Biology, 14(8),* 1769–1781.
17. Kanwar, P., Sanyal, S. K., Tokas, I., Yadav, A. K., Pandey, A. et al. (2014). Comprehensive structural, interaction and expression analysis of *CBL* and *CIPK* complement during abiotic stresses and development in rice. *Cell Calcium, 56(2),* 81–95.
18. Yu, Y., Xia, X., Yin, W., Zhang, H. (2007). Comparative genomic analysis of *CIPK* gene family in *Arabidopsis* and *Populus*. *Plant Growth Regulation, 52(2),* 101–110.
19. Hu, W., Xia, Z., Yan, Y., Ding, Z., Tie, W. et al. (2015). Genome-wide gene phylogeny of *CIPK* family in cassava and expression analysis of partial drought-induced genes. *Frontiers in Plant Science, 6,* 914.
20. Liu, H., Wang, Y., Li, H., Teng, R., Wang, Y. et al. (2019). Genome-wide identification and expression analysis of calcineurin B-like protein and calcineurin B-like protein-interacting protein kinase family genes in tea plant. *DNA and Cell Biology, 38(8),* 824–839.
21. Halfter, U., Ishitani, M., Zhu, J. (2000). The *Arabidopsis* SOS2 protein kinase physically interacts with and is activated by the calcium-binding protein SOS3. *Proceedings of the National Academy of Sciences of the United States of America, 97(7),* 3735–3740.
22. Liu, J., Zhu, J. (1998). A calcium sensor homolog required for plant salt tolerance. *Science, 280(5371),* 1943–1945.
23. Qiu, Q., Guo, Y., Dietrich, M. A., Schumaker, K. S., Zhu, J. (2002). Regulation of SOS1, a plasma membrane Na^+/H^+ exchanger in *Arabidopsis thaliana*, by SOS2 and SOS3. *Proceedings of the National Academy of Sciences of the United States of America, 99(12),* 8436–8441.
24. Kim, B., Waadt, R., Cheong, Y. H., Pandey, G. K., Dominguez-Solis, J. R. et al. (2007). The calcium sensor CBL10 mediates salt tolerance by regulating ion homeostasis in *Arabidopsis*. *The Plant Journal, 52(3),* 473–484.
25. Lin, H., Yang, Y., Quan, R., Mendoza, I., Wu, Y. et al. (2009). Phosphorylation of SOS3-LIKE CALCIUM BINDING PROTEIN8 by SOS2 protein kinase stabilizes their protein complex and regulates salt tolerance in *Arabidopsis*. *The Plant Cell, 21(5),* 1607–1619.
26. Yang, Y., Zhang, C., Tang, R., Xu, H., Lan, W. et al. (2019). Calcineurin B-like proteins CBL4 and CBL10 mediate two independent salt tolerance pathways in *Arabidopsis*. *International Journal of Molecular Sciences, 20(10),* 2421.
27. Cheong, Y. H., Kim, K., Pandey, G. K., Gupta, R., Grant, J. J. et al. (2003). CBL1, a calcium sensor that differentially regulates salt, drought, and cold responses in *Arabidopsis*. *The Plant Cell, 15(8),* 1833–1845.

28. Li, Z., Xu, Z., Chen, Y., He, G., Yang, G. et al. (2013). A novel role for *Arabidopsis* CBL1 in affecting plant responses to glucose and gibberellin during germination and seedling development. *PLoS One, 8(2),* e56412.
29. Ligaba-Osena, A., Fei, Z., Liu, J., Xu, Y., Shaff, J. et al. (2017). Loss-of-function mutation of the calcium sensor CBL1 increases aluminum sensitivity in *Arabidopsis*. *New Phytologist, 214(2),* 830–841.
30. Maehs, A., Steinhorst, L., Han, J., Shen, L., Wang, Y. et al. (2013). The calcineurin B-like Ca^{2+} sensors CBL1 and CBL9 function in pollen germination and pollen tube growth in *Arabidopsis*. *Molecular Plant, 6(4),* 1149–1162.
31. Straub, T., Ludewig, U., Neuhaeuser, B. (2017). The kinase CIPK23 inhibits ammonium transport in *Arabidopsis thaliana*. *The Plant Cell, 29(2),* 409–422.
32. Yang, W., Kong, Z., Omo-Ikerodah, E., Xu, W., Li, Q. et al. (2008). Calcineurin B-like interacting protein kinase OsCIPK23 functions in pollination and drought stress responses in rice (*Oryza sativa* L.). *Journal of Genetics and Genomics, 35(9),* 531–543.
33. Ma, Q., Sun, M., Kang, H., Lu, J., You, C. et al. (2019). A CIPK protein kinase targets sucrose transporter MdSUT2.2 at Ser254 for phosphorylation to enhance salt tolerance. *Plant, Cell & Environment, 42(3),* 918–930.
34. Ma, Q., Sun, M., Lu, J., Kang, H., You, C. et al. (2019). An apple sucrose transporter MdSUT2.2 is a phosphorylation target for protein kinase MdCIPK22 in response to drought. *Plant Biotechnology Journal, 17(3),* 625–637.
35. Ma, Q., Sun, M., Lu, J., Liu, Y., You, C. et al. (2017). An apple CIPK protein kinase targets a novel residue of AREB transcription factor for ABA-dependent phosphorylation. *Plant Cell And Environment, 40(10),* 2207–2219.
36. Tian, Q., Zhang, X., Yang, A., Wang, T., Zhang, W. (2016). CIPK23 is involved in iron acquisition of *Arabidopsis* by affecting ferric chelate reductase activity. *Plant Science, 246,* 70–79.
37. Ren, X., Qi, G., Feng, H., Zhao, S., Zhao, S. et al. (2013). Calcineurin B-like protein CBL10 directly interacts with AKT1 and modulates K^+ homeostasis in *Arabidopsis*. *The Plant Journal, 74(2),* 258–266.
38. Kim, K., Cheong, Y. H., Grant, J. J., Pandey, G. K., Luan, S. (2003). CIPK3, a calcium sensor-associated protein kinase that regulates abscisic acid and cold signal transduction in *Arabidopsis*. *The Plant Cell, 15(2),* 411–423.
39. Meena, M. K., Vishwakarma, N. K., Tripathi, V., Chattopadhyay, D. (2019). CBL-interacting protein kinase 25 contributes to root meristem development. *Journal of Experimental Botany, 70(1),* 133–147.
40. Pandey, G. K., Cheong, A. Y. H., Kim, K. N., Grant, B. J. J., Li, A. L. (2004). The calcium sensor calcineurin B-Like 9 modulates abscisic acid sensitivity and biosynthesis in *Arabidopsis*. *The Plant Cell, 16(7),* 1912–1924.
41. Pandey, G. K., Grant, J. J., Cheong, Y. H., Kim, B. G., Li, L. G. et al. (2008). Calcineurin-B-like protein CBL9 interacts with target kinase CIPK3 in the regulation of ABA response in seed germination. *Molecular Plant, 1(2),* 238–248.
42. Sanyal, S. K., Kanwar, P., Yadav, A. K., Sharma, C., Kumar, A. et al. (2017). Arabidopsis CBL interacting protein kinase 3 interacts with ABR1, an APETALA2 domain transcription factor, to regulate ABA responses. *Plant Science, 254,* 48–59.
43. Song, S., Feng, Q., Li, C., Li, E., Liu, Q. et al. (2018). A tonoplast-associated calcium-signaling module dampens ABA signaling during stomatal movement. *Plant Physiology, 177(4),* 1666–1678.
44. Tang, R., Zhao, F., Garcia, V. J., Kleist, T. J., Yang, L. et al. (2015). Tonoplast CBL-CIPK calcium signaling network regulates magnesium homeostasis in *Arabidopsis*. *Proceedings of the National Academy of Sciences, 112(10),* 3134–3139.
45. Deng, J., Yang, X., Sun, W., Miao, Y., He, L. et al. (2020). The calcium sensor CBL2 and its interacting kinase CIPK6 are involved in plant sugar homeostasis via interacting with tonoplast sugar transporter TST2. *Plant Physiology, 183(1),* 236–249.
46. Hibrand Saint-Oyant, L., Ruttink, T., Hamama, L., Kirov, I., Lakhwani, D. et al. (2018). A high-quality genome sequence of *Rosa chinensis* to elucidate ornamental traits. *Nature Plants, 4(7),* 473–484.
47. Finn, R. D., Tate, J., Mistry, J., Coggill, P. C., Sammut, S. J. et al. (2008). The Pfam protein families database. *Nucleic Acids Research, 32(1),* D138.
48. Kretsinger, R. H., Nakayama, S. (1993). Evolution of EF-hand calcium-modulated proteins. IV. Exon shuffling did not determine the domain compositions of EF-hand proteins. *Journal of Molecular Evolution, 36(5),* 477–488.

49. Nakayama, S., Moncrief, N. D., Kretsinger, R. H. (1992). Evolution of EF-hand calcium-modulated proteins. II. Domains of several subfamilies have diverse evolutionary histories. *Journal of Molecular Evolution, 34(5),* 416–448.
50. Finn, B. E., Forsén, S. (1995). The evolving model of calmodulin structure, function and activation. *Structure, 3(1),* 7–11.
51. Kai, W., Mingyao, L., Hadley, D., Rui, L., Glessner, J. et al. (2007). PennCNV: An integrated hidden Markov model designed for high-resolution copy number variation detection in whole-genome SNP genotyping data. *Genome Research, 17(11),* 1665–1674.
52. Letunic, I. (2006). SMART 5: Domains in the context of genomes and networks. *Nucleic Acids Research, 34(90001),* D257–D260. DOI 10.1093/nar/gkj079.
53. Zhu, K., Chen, F., Liu, J., Chen, X., Hewezi, T. et al. (2016). Evolution of an intron-poor cluster of the CIPK gene family and expression in response to drought stress in soybean. *Scientific Reports, 6(1),* 28225. DOI 10.1038/srep28225.
54. Wilkins, M. R., Gasteiger, E., Bairoch., A., Sanchez, J. C., Williams., K. L. et al. (1999). Protein identification and analysis tools in the ExPASy server. *Methods in Molecular Biology, 112,* 531–552.
55. Chou, K., Shen, H. (2008). Cell-PLoc: A package of web servers for predicting subcellular localization of proteins in various organisms. *Nature Protocols, 3(2),* 153–162. DOI 10.1038/nprot.2007.494.
56. Chenna, R., Sugawara, H., Koike, T., Lopez, R., Gibson, T. J. et al. (2003). Multiple sequence alignment with the Clustal series of programs. *Nucleic Acids Research, 31(13),* 3497–3500. DOI 10.1093/nar/gkg500.
57. Kumar, S., Stecher, G., Tamura, K. (2016). MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution, 33(7),* 1870–1874. DOI 10.1093/molbev/msw054.
58. Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A. et al. (2007). Clustal W and Clustal X version 2.0. *Bioinformatics, 23(21),* 2947–2948. DOI 10.1093/bioinformatics/btm404.
59. Letunic, I., Bork, P. (2021). Interactive tree of life (iTOL) v5: An online tool for phylogenetic tree display and annotation. *Nucleic Acids Research, 49(W1),* 293–296. DOI 10.1093/nar/gkab301.
60. Chen, C., Chen, H., Zhang, Y., Thomas, H. R., Frank, M. H. et al. (2020). TBtools: An integrative toolkit developed for interactive analyses of big biological data. *Molecular Plant, 13(8),* 1194–1202. DOI 10.1016/j.molp.2020.06.009.
61. Bailey, T. L., Mikael, B., Buske, F. A., Martin, F., Grant, C. E. et al. (2009). MEME SUITE: Tools for motif discovery and searching. *Nucleic Acids Research, 37,* 202–208. DOI 10.1093/nar/gkp335.
62. Lescot, M., Déhais, P., Thijs, G., Marchal, K., Moreau, Y. et al. (2002). PlantCARE, a database of plant *cis*-acting regulatory elements and a portal to tools for *in silico* analysis of promoter sequences. *Nucleic Acids Research, 30(1),* 325–327. DOI 10.1093/nar/30.1.325.
63. Wang, Y., Tang, H., Debarry, J. D., Tan, X., Li, J. et al. (2012). *MCScanX*: A toolkit for detection and evolutionary analysis of gene synteny and collinearity. *Nucleic Acids Research, 40(7),* e49. DOI 10.1093/nar/gkr1293.
64. Krzywinski, M., Schein, J., Birol, I., Connors, J., Gascoyne, R. et al. (2009). Circos: An information aesthetic for comparative genomics. *Genome Research, 19(9),* 1639–1645. DOI 10.1101/gr.092759.109.
65. Peterson, G. I., Masel, J. (2009). Quantitative prediction of molecular clock and *Ka/Ks* at short timescales. *Molecular Biology and Evolution, 26(11),* 2595–2603. DOI 10.1093/molbev/msp175.
66. Zhang, C., Wang, J., Long, M., Fan, C. (2013). gKaKs: The pipeline for genome-level Ka/Ks calculation. *Bioinformatics, 29(5),* 645–646.
67. Zhang, Z., Li, J., Zhao, X. Q., Wang, J., Wong, G. K. et al. (2006). KaKs_Calculator: Calculating Ka and Ks through model selection and model averaging. *Genomics Proteomics Bioinformatics, 4(4),* 259–263.
68. Otasek, D., Morris, J. H., Bouças, J., Pico, A. R., Demchak, B. (2019). Cytoscape automation: Empowering workflow-based network analysis. *Genome Biology, 20(1),* 185.
69. Han, Y., Wan, H., Cheng, T., Wang, J., Yang, W. et al. (2017). Comparative RNA-seq analysis of transcriptome dynamics during petal development in *Rosa chinensis*. *Scientific Reports, 7(1),* 43382.
70. Gao, H., Huang, H., Lu, K., Wang, C., Liu, X. et al. (2021). *OsCYP714D1* improves plant growth and salt tolerance through regulating gibberellin and ion homeostasis in transgenic poplar. *Plant Physiology and Biochemistry, 168,* 447–456.

71. Hu, H., Wang, Y., Tsay, Y. (2009). AtCIPK8, a CBL-interacting protein kinase, regulates the low-affinity phase of the primary nitrate response. *The Plant Journal, 57(2),* 264–278.
72. Pandey, G. K., Kanwar, P., Singh, A., Steinhorst, L., Pandey, A. et al. (2015). Calcineurin B-like protein-interacting protein kinase CIPK21 regulates osmotic and salt stress responses in *Arabidopsis*. *Plant Physiology, 169(1),* 780–792.
73. Guo, Y., Halfter, U., Ishitani, M., Zhu, J. (2001). Molecular characterization of functional domains in the protein kinase SOS2 that is required for plant salt tolerance. *The Plant Cell, 13(6),* 1383–1399.
74. Xiang, Y., Huang, Y., Xiong, L. (2007). Characterization of stress-responsive *CIPK* genes in rice for stress tolerance improvement. *Plant Physiology, 144(3),* 1416–1428.
75. Filichkin, S. A., Hamilton, M., Dharmawardhana, P. D., Singh, S. K., Sullivan, C. et al. (2018). Abiotic stresses modulate landscape of poplar transcriptome via alternative splicing, differential intron retention, and isoform ratio switching. *Frontiers in Plant Science, 9,* 5.
76. Tran, L. P., Nakashima, K., Sakuma, Y., Simpson, S. D., Fujita, Y. et al. (2004). Isolation and functional analysis of *Arabidopsis* stress-inducible NAC transcription factors that bind to a drought-responsive cis-element in the early responsive to dehydration stress 1 promoter. *The Plant Cell, 16(9),* 2481–2498.
77. Urao, T., Yamaguchi Shinozaki, K., Urao, S., Shinozaki, K. (1993). An *Arabidopsis* myb homolog is induced by dehydration stress and its gene product binds to the conserved MYB recognition sequence. *The Plant Cell, 5(11),* 1529–1539.
78. Du, W., Yang, J., Ma, L., Su, Q., Pang, Y. (2021). Identification and characterization of abiotic stress responsive *CBL-CIPK* family genes in medicago. *International Journal of Molecular Sciences, 22(9),* 4634.
79. Wang, Q., Zhao, K., Gong, Y., Yang, Y., Yue, Y. (2022). Genome-wide identification and functional analysis of the calcineurin B-like protein and calcineurin B-like protein-interacting protein kinase gene families in Chinese cabbage (*Brassica rapa* ssp. *pekinensis*). *Genes, 13(5),* 795.
80. Cao, Y., Li, K., Li, Y., Zhao, X., Wang, L. (2020). MYB transcription factors as regulators of secondary metabolism in plants. *Biology, 9(3),* 61.
81. Lai, Y., Li, H., Yamagishi, M. (2013). A review of target gene specificity of flavonoid R_2R_3-MYB transcription factors and a discussion of factors contributing to the target gene selectivity. *Frontiers in Biology, 8(6),* 577–598.
82. Monihan, S. M., Magness, C. A., Yadegari, R., Smith, S. E., Schumaker, K. S. (2016). *Arabidopsis* CALCINEURIN B-LIKE10 functions independently of the SOS pathway during reproductive development in saline conditions. *Plant Physiology, 171(1),* 369–379.
83. Zhou, L., Lan, W., Chen, B., Fang, W., Luan, S. (2015). A calcium sensor-regulated protein kinase, CALCINEURIN B-LIKE PROTEIN-INTERACTING PROTEIN KINASE19, is required for pollen tube growth and polarity. *Plant Physiology, 167(4),* 1351–1360.
84. Huang, C., Ding, S., Zhang, H., Du, H., An, L. (2011). CIPK7 is involved in cold response by interacting with CBL1 in *Arabidopsis thaliana*. *Plant Science, 181(1),* 57–64.
85. Huang, Y., Xu, P., Hou, B., Shen, Y. (2019). Strawberry tonoplast transporter, FaVPT1, controls phosphate accumulation and fruit quality. *Plant Cell and Environment, 42(9),* 2715–2729.
86. Li, J., Long, Y., Qi, G., Li, J., Xu, Z. et al. (2014). The Os-AKT1 channel is critical for K^+ uptake in rice roots and is modulated by the rice CBL1-CIPK23 complex. *The Plant Cell, 26(8),* 3387–3402.
87. Ishitani, M., Liu, J., Halfter, U., Kim, C. S., Shi, W. et al. (2000). SOS_3 function in plant salt tolerance requires N-myristoylation and calcium binding. *The Plant Cell, 12(9),* 1667–1677.
88. Quintero, F., Ohta, M., Shi, H., Zhu, J., Pardo, J. (2002). Reconstitution in yeast of the *Arabidopsis* SOS signaling pathway for Na^+ homeostasis. *Proceedings of the National Academy of Sciences, 99(13),* 9061–9066.
89. Quintero, F. J., Martinez Atienza, J., Villalta, I., Jiang, X., Kim, W. Y. et al. (2011). Activation of the plasma membrane Na^+/H^+ antiporter Salt-Overly-Sensitive 1 (SOS1) by phosphorylation of an auto-inhibitory C-terminal domain. *Proceedings of the National Academy of Sciences, 108(6),* 2611–2616.
90. Tai, F., Yuan, Z., Li, S., Wang, Q., Liu, F. et al. (2016). ZmCIPK8, a CBL-interacting protein kinase, regulates maize response to drought stress. *Plant Cell Tissue and Organ Culture, 124(3),* 459–469.

Supporting Information:

Table S1: Primer sequences used for qRT-PCR analysis

Table S2: *CBL* and *CIPK* genes from Arabidopsis and rice

Table S3: Motifs in RcCBLs and RcCIPKs

Table S4: *Cis*-acting elements in the promoter regions of *RcCBLs* and *RcCIPKs*

Table S5: Predication of RcCBL-RcCIPK protein-protein interaction network

Table S6: Expression of *RcCBLs* and *RcCIPKs* in different organs under normal condition

Table S7: Expression of *RcCBLs* and *RcCIPKs* under various abiotic stress conditions