Postprint: Bioinformatics Analysis of CBL-CIPK Signaling System Involvement in Cold Resistance Formation in Jatropha curcas

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Abstract

Calcineurin B-like calcium sensor (CBL) proteins are Ca2+-binding proteins that mediate Ca2+ signal transduction by interacting with calcineurin B-like calcium sensor interacting protein kinases (CIPKs). The CBL-CIPK signaling system participates in plant responses to various abiotic stresses. To deeply explore the mechanisms of cold resistance in Jatropha curcas, this study identified the CBL and CIPK gene families in Jatropha curcas at the whole-genome level based on sequence alignment methods, and analyzed their phylogenetic evolution, gene structure, expression characteristics, and functional interactions. The results showed: (1) A total of 8 CBL genes and 18 CIPK genes were identified in the Jatropha curcas genome, with CBL and CIPK protein lengths ranging from 211~257 aa and 422~484 aa, respectively, and isoelectric points ranging from 4.65~5.08 and 6.20~9.26, respectively. (2) Additionally, the CBL gene family all contained 8~10 exons, whereas the CIPK gene family was divided into two distinct groups: 1~2 exons (11 genes) and 12~15 exons (7 genes). (3) Multiple sequence alignment revealed that all Jatropha curcas CBL proteins contained one atypical EF-hand motif composed of 14 amino acid residues and three typical EF-hand motifs with varying degrees of substitution, while CIPK proteins all contained an N-terminal kinase domain and a C-terminal autoinhibitory FISL/NAF domain. (4) Chromosomal localization showed that the 26 Jatropha curcas CBL and CIPK genes were unevenly distributed across 9 chromosomes. (5) Transcriptome data analysis indicated that most CBL and CIPK genes were highly expressed in Jatropha curcas leaves, roots, and seeds, among which JcCIPK14 and JcCIPK18 showed extremely significant upregulation ($P<0.01$) under low-temperature treatment, participating in the cold resistance process of Jatropha curcas. These results provide a foundation for functional identification of Jatropha curcas CBL and CIPK genes and research on low-temperature signal transduction mechanisms.

Full Text

Bioinformatics Analysis of CBL-CIPK Signaling System Involved in Cold Resistance Formation in Jatropha curcas

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Abstract: Calcineurin B-like proteins (CBLs) are Ca^{2+}-binding proteins that mediate Ca^{2+} signal transduction by interacting with CBL-interacting protein kinases (CIPKs). The CBL-CIPK signaling system participates in plant responses to various environmental stresses. To elucidate the mechanisms underlying cold resistance in Jatropha curcas, this study identified CBL and CIPK gene families at the whole-genome level through sequence alignment and analyzed their phylogenetic relationships, gene structures, expression profiles, and functional interactions. The results revealed: (1) A total of 8 CBL genes and 18 CIPK genes were identified in the J. curcas genome, with CBL and CIPK protein lengths ranging from 211–257 aa and 422–484 aa, respectively, and isoelectric points ranging from 4.65–5.08 and 6.20–9.26, respectively. (2) The CBL gene family contained 8–10 exons, whereas the CIPK gene family was divided into two distinct groups: 11 genes with 1–2 exons and 7 genes with 12–15 exons. (3) Multiple sequence alignment revealed that all J. curcas CBL proteins possessed one atypical 14-amino-acid EF-hand motif and three typical EF-hand motifs with varying degrees of substitution, while CIPK proteins contained an N-terminal kinase domain and a C-terminal autoinhibitory FISL/NAF domain. (4) Chromosomal mapping showed that the 26 CBL and CIPK genes were unevenly distributed across 9 chromosomes. (5) Transcriptome data analysis demonstrated that most CBL and CIPK genes were highly expressed in J. curcas leaves, roots, and seeds, with JcCIPK14 and JcCIPK18 showing extremely significant upregulation ($P<0.01$) under cold treatment, suggesting their involvement in cold resistance. These findings provide a foundation for functional characterization of J. curcas CBL and CIPK genes and investigation of cold signaling transduction mechanisms.

Keywords: Jatropha curcas, protein kinase, CBL-CIPK, gene family, expression analysis, cold resistance

Introduction

Sucrose non-fermenting-1-related protein kinases (SnRKs) are a family of serine/threonine protein kinases ubiquitously present in plants. Based on amino acid sequence clustering, the SnRK family is divided into three subfamilies: SnRK1, SnRK2, and SnRK3. SnRK3 is also known as CBL-interacting pro-

tein kinase (CIPK) (Shi et al., 1999; Kim et al., 2000) or salt overly sensitive (SOS) protein (Ji et al., 2013). Calcineurin B-like proteins (CBLs) serve as direct upstream interacting partners of CIPKs and can also perceive intracellular Ca^{2+} signals, together forming the Ca^{2+}-CBL-CIPK cascade signaling system that participates in plant responses to various abiotic stresses such as osmotic stress, high salinity, low temperature, and high temperature (Li et al., 2009; Sanyal et al., 2016). As Ca^{2+} receptor proteins, CBLs contain four EF-hand motifs with varying conservation, arranged in a helix-loop-helix configuration that is essential for Ca^{2+} binding (Weinl & Kudla, 2009). Additionally, some CBL proteins possess N-terminal lipid modification sites for N-myristoylation or N-palmitoylation, which facilitate their transmembrane transport (Batistic et al., 2008). The N-terminal kinase domain of CIPK proteins contains an activation-loop motif located between the -DFG- and -APE- sequences, with three highly conserved Ser, Thr, and Tyr residues being essential for kinase activity (Guo et al., 2001). The C-terminal region regulates the catalytic activity of the kinase domain, with the FISL (Phe-Ile-Ser-Leu)/NAF (Asn-Ala-Phe) domain—comprising 21 or 24 amino acid residues—serving as the core sequence for CIPK-CBL interaction (Du et al., 2011). Under normal conditions, the FISL/NAF domain interacts with the N-terminal kinase domain, conferring autoinhibition. When Ca^{2+}-bound CBL proteins bind to the FISL/NAF domain, this autoinhibition is relieved, thereby activating kinase activity (Akaboshi et al., 2008). Furthermore, the C-terminus contains a 37-amino-acid PPI (protein phosphatase interaction) domain that determines the specificity of interaction with protein phosphatase 2C (PP2C) and competitively displaces CBL proteins from the FISL/NAF domain, returning CIPK to its autoinhibited state (Ohta et al., 2003).

Genome-wide identification of CBL and CIPK genes has been conducted in numerous plant species. The CBL gene family comprises 10 members in Arabidopsis thaliana (Kolukisaoglu et al., 2004), 10 in rice (Oryza sativa) (Kolukisaoglu et al., 2004), 10 in maize (Zea mays) (Li et al., 2010), 10 in poplar (Populus trichocarpa) (Zhang et al., 2008), 7 in wheat (Triticum aestivum) (Sun et al., 2015), 7 in rapeseed (Brassica napus) (Zhang et al., 2014), and 5 in eggplant (Solanum melongena) (Li et al., 2016). The CIPK gene family includes 25 members in Arabidopsis (Kolukisaoglu et al., 2004), 33 in rice (Kolukisaoglu et al., 2004; Kanwar et al., 2014), 43 in maize (Chen et al., 2011), 27 in poplar (Yu et al., 2007), 20 in wheat (Sun et al., 2015), 52 in soybean (Glycine max) (Zhu et al., 2016), 23 in rapeseed (Zhang et al., 2014), 15 in eggplant (Li et al., 2016), 34 in apple (Malus domestica) (Niu et al., 2018), 16 in grape (Vitis vinifera) (Lu et al., 2017), and 22 in tomato (Lycopersicon esculentum) (Wang & Liu, 2018). Jatropha curcas, a perennial deciduous small tree belonging to the family Euphorbiaceae and genus Jatropha, is native to Central and South America (Lin et al., 2004). As an important woody oil plant, J. curcas seeds contain 35%–60% oil that is suitable for various diesel engines and meets Euro IV emission standards, offering broad prospects for development and utilization (Makkar & Becker, 2009). However, genome-wide identification and interaction analysis of

CBL and CIPK families in J. curcas have not been reported. Based on the J. curcas genome sequence (Sato et al., 2011), this study employed bioinformatics approaches to identify CBL and CIPK genes and analyze their physicochemical properties, gene structures, protein motifs, phylogenetic relationships, cold-responsive expression, and functional interactions, aiming to establish a foundation for investigating the stress signaling transduction mechanisms of the CBL-CIPK gene families in J. curcas.

Materials and Methods

1.1 Identification of J. curcas CBL and CIPK Gene Families

Based on the CBL and CIPK gene families identified in the model plants Arabidopsis thaliana, rice, and the closely related species poplar (Kolukisaoglu et al., 2004; Zhang et al., 2008; Yu et al., 2007), protein sequences of 10 Arabidopsis CBL and 25 CIPK genes were downloaded from the TAIR database (https://www.arabidopsis.org/), 10 rice CBL and 33 CIPK genes from the PlantBiology database (http://rice.plantbiology.msu.edu/), and 10 poplar CBL and 27 CIPK genes from the Phytozome database (https://phytozome.jgi.doe.gov/pz/portal.html). Multiple sequence alignment was performed using ClustalX, and the Hmmbuild program in HMMER3.0 was used to generate hidden Markov models (HMMs) for the CBL and CIPK domains. Meanwhile, the latest annotated protein databases of J. curcas were downloaded from GenBank (http://www.ncbi.nlm.nih.gov/genome/915/) and Kazusa (http://www.kazusa.or.jp/jatropha/) (Sato et al., 2011). The Makeblastdb program from NCBI was used to create a local database. Local BlastP searches against the J. curcas protein database were conducted using NCBI Blast (E-value threshold <1e-10, sequence similarity >50%) to obtain preliminary CBL and CIPK protein sequences. Duplicate sequences were removed through self-BLAST, and non-redundant candidate sequences were further screened using the Pfam (http://pfam.sanger.ac.uk/) and CDD (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) online tools to analyze the EF-hand motifs in CBLs and the protein kinase domains in CIPKs, yielding the final J. curcas CBL and CIPK family protein sequences. Corresponding gene and mRNA sequences were also downloaded for subsequent gene structure analysis.

1.2 Sequence Analysis of J. curcas CBL and CIPK Gene Families

The ExPASy online tool ProtParam (http://web.expasy.org/protparam/) was used to analyze basic parameters of J. curcas CBL and CIPK proteins, including amino acid number, theoretical molecular weight (Mw), and isoelectric point (pI). The identified J. curcas CBL and CIPK protein sequences were aligned with those from Arabidopsis, rice, and poplar using ClustalX. Phylogenetic trees were constructed using the neighbor-joining (NJ) method in MEGA6.0 software with bootstrap validation. Conserved domains in CBL and CIPK proteins were analyzed from the ClustalX alignment results using GenDOC soft-

ware. Gene structures, including intron-exon organization, were determined by aligning coding sequences (CDS) with genomic sequences, and gene structure diagrams were generated using the Gene Structure Display Server (GSDS, http://gsds.cbi.pku.edu.cn/). Chromosomal localization was anchored to the J. curcas genetic linkage map constructed by Wu et al. (2015), and gene mapping diagrams were drawn using MapChart (version 2.1). Protein-protein interaction networks for CBL and CIPK were analyzed using STRING (http://string-db.org) with a confidence threshold >0.7.

1.3 Expression Analysis of J. curcas CBL and CIPK Gene Families

Illumina high-throughput sequencing data for different J. curcas organs were downloaded from the NCBI SRA database (leaf: SRR1639660, root: SRR1639659, seed: SRR1639661). The identified J. curcas CBL and CIPK gene families were aligned to the sequencing data using Bowtie2 and Samtools to obtain read counts for each gene. Expression levels were quantified as fragments per kilobase per million (FPKM) values using Cufflinks, $\log_2$-transformed, and subjected to hierarchical clustering based on both genes and organs. Additionally, using our previously generated J. curcas transcriptome (Wang et al., 2014) and digital gene expression (DGE) data (Wang et al., 2013), raw clean tag data for CBL and CIPK genes were extracted from control and 12°C cold-treated samples (12, 24, and 48 h). Standardized expression levels were obtained as transcripts per million clean tags (TPM) (Thoen et al., 2008; Morrissy et al., 2009) to analyze differential expression under cold stress. Heatmaps for clustering analysis were generated using the gplots and pheatmap functions in R software (version 3.4.1).

Results

2.1 Identification and Sequence Characteristics of J. curcas CBL and CIPK Genes

Through homology-based sequence searching, 8 CBL genes (JcCBL1–JcCBL8) and 18 CIPK genes (JcCIPK1–JcCIPK18) were identified in the J. curcas genome (Table 1). Physicochemical parameter analysis using ExPASy tools revealed that CBL family genes ranged from 2,172 bp (JcCBL7) to 9,344 bp (JcCBL2) in length, with protein sequences of 211 aa (JcCBL8) to 257 aa (JcCBL7) and strongly acidic isoelectric points ranging from 4.59 (JcCBL5) to 5.08 (JcCBL4). In contrast, CIPK family genes ranged from 1,522 bp (JcCIPK16) to 9,348 bp (JcCIPK9) in length, with protein sequences of 422 aa (JcCIPK16) to 484 aa (JcCIPK2). Except for JcCIPK9, JcCIPK14, JcCIPK15, and JcCIPK17, all CIPKs had strongly basic isoelectric points ranging from 8.03 (JcCIPK2) to 9.26 (JcCIPK4).

2.2 Phylogenetic Relationships and Gene Structures of J. curcas CBL and CIPK Genes

Phylogenetic trees for CBL and CIPK gene families were constructed using MEGA, incorporating J. curcas, Arabidopsis, rice, and poplar sequences (Figure 1 [Figure 1: see original paper]). The CBL family clustered into four subgroups (I–IV), with J. curcas genes distributed as follows: 3 genes in subgroup I (JcCBL4, JcCBL6, JcCBL8), 1 in subgroup II (JcCBL3), 2 in subgroup III (JcCBL5, JcCBL7), and 2 in subgroup IV (JcCBL1, JcCBL2) (Figure 1A). This classification was consistent with the phylogenetic tree constructed using only J. curcas CBL genes (Figure 2A). Notably, JcCBL3 clustered closely with JcCBL1 and JcCBL2 in the single-species tree but formed a separate subgroup II in the multi-species analysis, corresponding to its distinct gene structure (9 exons) compared to JcCBL1 and JcCBL2 (10 exons). The CIPK family clustered into six subgroups, with J. curcas genes distributed as 3, 1, 5, 2, 2, and 5 members respectively (Figure 1B), consistent with the J. curcas-only CIPK phylogenetic tree (Figure 2B).

Based on the phylogenetic clustering, gene structures were analyzed using the GSDS tool. The subgroup classification correlated well with gene architecture. All eight J. curcas CBL genes contained 8–10 exons and both 5 -UTR and 3 -UTR regions (Table 1). Subgroup III genes JcCBL5 (2,501 bp) and JcCBL7 (2,172 bp) contained 9 exons and were relatively short, whereas subgroup IV genes JcCBL1 (7,811 bp) and JcCBL2 (9,344 bp) contained 10 exons and were considerably longer (Figure 2A). Consistent with reported patterns in other species, the J. curcas CIPK gene family also contained 5 -UTR and 3 -UTR regions. The 18 members were divided into two major groups based on gene structure: 11 JcCIPK genes contained 1–2 exons (JcCIPK4, JcCIPK5, JcCIPK8, JcCIPK10, JcCIPK13, JcCIPK16, and JcCIPK18 had a single exon; JcCIPK1, JcCIPK2, JcCIPK3, and JcCIPK6 had two exons), while the remaining 7 genes contained 12–15 exons (JcCIPK7, JcCIPK9, JcCIPK11, JcCIPK12, and JcCIPK17 had 15 exons; JcCIPK14 had 14 exons; JcCIPK15 had 12 exons) (Figure 2B).

2.3 Amino Acid Sequence and Domain Analysis of J. curcas CBL and CIPK Proteins

Previous studies have reported that CBL proteins contain four EF-hand motifs with varying conservation. The helix-loop-helix structure represents the typical EF-hand configuration, with the central loop (12 amino acid residues, consensus sequence -DKDGDGKIDFEE-) having conserved residues at positions 1 (X), 3 (Y), 5 (Z), 7 (-Y), 9 (-X), and 12 (-Z) that are essential for Ca^{2+} binding (Figure 3A). Analysis of the eight J. curcas CBL protein sequences revealed that the first EF-hand (EF1) comprised 14 amino acid residues and was atypical, whereas EF2–EF4 contained the canonical 12 residues. Except for absolutely conserved Asp at position 1 and Glu at position 12, other residues (positions 3, 5, 7, and 9) exhibited partial substitutions, with substitution rates increasing progressively

from EF4 to EF2. Specifically, Asp (D) at position 3 was substituted by Lys (K) in EF2, Lys/Arg (K/R) in EF3, and Lys/Asn (K/N) in EF4. Asp (D) at position 5 was replaced by Asn/Lys (N/K) in EF2 and Gln/Asn (Q/N) in EF3. Lys (K) at position 7 was substituted by Val/Ile (V/I) in EF2 and Phe/Tyr (F/Y) in EF3. Asp (D) at position 9 was replaced by Glu (E) in both EF2 and EF3 (Figure 3B). These results indicate that similar types of amino acid substitutions occurred across EF-hands 2–4, preserving both the Ca^{2+}-binding capacity and functional diversity. Based on N-terminal length, JcCBL1, JcCBL2, JcCBL3, JcCBL4, JcCBL6, and JcCBL8 contained short N-terminal sequences of 16–34 aa, with JcCBL3 and JcCBL8 harboring N-myristoylation motifs (-MGCXXSK/T-) that enhance membrane association, whereas JcCBL5 and JcCBL7 possessed longer N-terminal regions (Figure 3A).

Plant-specific CIPK proteins, also known as SnRK3, are homologous to yeast SNF1 and mammalian AMPK and contain an N-terminal kinase domain with a central activation-loop motif. All 18 J. curcas CIPK proteins contained activation-loop motifs flanked by -DFG- and -APE- sequences (Figure 3C) and conserved Ser, Thr, and Tyr residues (Figure 3C, arrows). Additionally, the C-terminal 21-aa autoinhibitory FISL/NAF motif with the conserved -NAF- sequence was identified in all CIPKs (Figure 3D), ensuring their autoinhibited state under normal conditions.

2.4 Chromosomal Localization of J. curcas CBL and CIPK Gene Families

Based on the high-density genetic linkage map of J. curcas constructed by Wu et al. (2015), CBL and CIPK genes were mapped at the chromosomal level. The results showed that 26 J. curcas CBL and CIPK genes were unevenly distributed across 9 chromosomes, with no genes located on chromosomes 5 and 7. Chromosomes 3 and 11 contained the highest number of genes (5 each), while chromosome 10 had the fewest with only JcCIPK6. Tandemly duplicated gene pairs were identified, including JcCBL6/JcCBL8 on chromosome 9 in the CBL family and JcCIPK2/JcCIPK4, JcCIPK3/JcCIPK13, and JcCIPK1/JcCIPK16 on chromosomes 3, 4, and 6, respectively, in the CIPK family (Figure 4 [Figure 4: see original paper]), suggesting gene duplication events.

2.5 Differential Expression Analysis of J. curcas CBL and CIPK Genes

Based on J. curcas transcriptome data from GenBank, organ-specific expression profiles for all 26 CBL and CIPK genes were obtained using Cufflinks (Figure 5 [Figure 5: see original paper]). Except for JcCBL6 and JcCBL8, which were not expressed in seeds, the remaining 24 genes were expressed in leaves, roots, and seeds. JcCBL2, JcCIPK1, JcCIPK3, JcCIPK5, JcCIPK7, JcCIPK8, JcCIPK13, and JcCIPK18 showed high expression levels ($\log_2$FPKM > 3.5) across all three organs, with JcCIPK5 exhibiting the highest expression, suggesting a central role in the J. curcas Ca^{2+}-CBL-CIPK signaling system. Other genes displayed

organ-specific expression patterns: JcCIPK4 was highly expressed in leaves but low in seeds; JcCBL7 was expressed exclusively in roots with minimal expression in leaves and seeds; and JcCBL4 showed high expression in leaves and roots but was nearly absent in seeds (Figure 5).

DGE data analysis revealed expression patterns for nine J. curcas CBL and CIPK genes under cold stress (Figure 6 [Figure 6: see original paper]). Compared to the control, JcCIPK14 and JcCIPK18 were extremely significantly upregulated ($P<0.01$) at 12, 24, and 48 h of 12°C treatment, indicating their direct involvement in cold resistance. Additionally, JcCIPK4 and JcCIPK16 showed gradually increasing expression with prolonged cold treatment, reaching 9.92-fold ($P<0.01$) and 2.10-fold upregulation at 48 h, respectively. JcCIPK1 and JcCIPK2 were early cold-responsive genes, peaking at 12 h with 5.21-fold and 2.87-fold upregulation, respectively, followed by gradual downregulation (Figure 6).

2.6 Protein Interaction Network Analysis of J. curcas CBL and CIPK Proteins

Based on homologous CBL and CIPK proteins from J. curcas and Arabidopsis, protein-protein interaction networks were analyzed using STRING 10.5 to elucidate their involvement in signaling pathways and potential functions. At a confidence threshold of 0.7, 8 JcCBLs and 14 JcCIPKs (excluding JcCIPK1, JcCIPK3, JcCIPK4, and JcCIPK6) participated in canonical CBL-CIPK signaling pathways, exhibiting one-to-many and many-to-one interaction patterns. Notably, JcCBL1/2 and JcCBL3 could interact with 12 and 10 JcCIPKs, respectively, suggesting key roles in the Ca^{2+}-CBL-CIPK signaling network, whereas JcCBL6 interacted only with JcCIPK7 and JcCIPK16. JcCIPK7 could bind to all eight JcCBLs, while JcCIPK17 and JcCIPK18 interacted exclusively with JcCBL1/2 (Figure 7 [Figure 7: see original paper]).

Discussion

CBL proteins decode and perceive changes in Ca^{2+} concentration and distribution (Scrase-Field & Knight, 2003; Batistic & Kudla, 2012) and specifically interact with downstream CIPKs to form the Ca^{2+}-CBL-CIPK signaling system that participates in cold resistance in J. curcas (Sanders et al., 2002). This study identified 8 CBL and 18 CIPK genes in the J. curcas genome, with conserved protein lengths and gene structures. Notably, the isoelectric points of CBL and CIPK proteins showed distinct family-specific patterns: CBL proteins were acidic, whereas most CIPK proteins were basic (Table 1). Under physiological pH conditions, these interacting proteins carry opposite charges, suggesting that electrostatic Coulomb forces may play an important role in CBL binding to the FISL/NAF domain of CIPKs. Yeast two-hybrid experiments have demonstrated that CBL-CIPK interactions in both J. curcas and Arabidopsis exhibit cross-reactivity and binding preferences (Kim et al., 2000; Guo et al., 2001). For instance, JcCBL1/2 could interact with 12 JcCIPKs, JcCIPK7

could bind to all identified JcCBLs, JcCIPK17 and JcCIPK18 preferentially bound JcCBL1/2, and JcCIPK8/10 showed preference for JcCBL4/8 (Figure 7). Similar patterns have been observed in Arabidopsis, where AtCIPK7/17 preferentially binds AtCBL9, AtCIPK24 prefers AtCBL4, and AtCIPK9 favors AtCBL2 (Kim et al., 2000). This specificity and preference are primarily determined by the Ca^{2+}-binding capacity conferred by the EF-hand motifs in CBL proteins (Nagae et al., 2003; Sanchez-Barrena et al., 2005) and structural differences in the FISL/NAF domain and its flanking sequences in CIPK proteins (Kim et al., 2000; Guo et al., 2001; Halfter et al., 2000). Unlike the typical 12-amino-acid EF-hand motif, the first EF-hand motif in all eight J. curcas CBL proteins consisted of 14 amino acid residues with greater variation, particularly Asp (D) being frequently replaced by Ser (S) (Figure 3B). This motif likely determines distinct Ca^{2+} affinities, enabling J. curcas CBL proteins to decode different Ca^{2+} signals simultaneously (Weinl & Kudla, 2009; Sanchez-Barrena et al., 2007). Binding of the CBL protein to the FISL/NAF domain of CIPK leads to phosphorylation of conserved residues in the activation loop, thereby activating the kinase (Weinl & Kudla, 2009). In this study, the activation loops of J. curcas CIPK proteins were located between the conserved -DFG- and -APE- motifs and contained three conserved phosphorylatable Ser (S), Thr (T), and Tyr (Y) residues (Figure 3C).

The evolution of the CBL-CIPK signaling system from lower to higher plants is coordinated with environmental adaptation to biotic and abiotic stresses (Weinl & Kudla, 2009). Jatropha curcas originates from tropical regions and possesses strong drought and salt tolerance but limited cold tolerance. The CBL-CIPK signaling system was first characterized in Arabidopsis through the salt overly sensitive (SOS) pathway. Under high salinity, AtCBL4 (SOS3) and AtCBL10 bind Ca^{2+} and interact with AtCIPK24 (SOS2) to form protein complexes that regulate Na^+-K^+ antiporters (SOS1) on the plasma and vacuolar membranes, pumping excess Na^+ out of the cell or sequestering it into vacuoles to mitigate salt stress (Zhu, 2002; Quan et al., 2007). Based on homology with Arabidopsis, the corresponding SOS cascade in J. curcas is JcCBL4/5/7-JcCIPK12 (Figure 7). Similarly, a drought signaling pathway JcCBL3-JcCIPK15 was identified (Figure 7), analogous to the Arabidopsis AtCBL1/9-AtCIPK1 pathway that perceives drought signals and promotes CBF family gene expression to achieve osmotic balance (Dangelo et al., 2006). As a cold-sensitive plant, J. curcas also activates the CBL-CIPK signaling system under low temperature, transmitting signals to downstream cold-responsive transcription factors or key rate-limiting proteins. In this study, transcriptome data from J. curcas treated at 12°C identified JcCIPK14 and JcCIPK18 as genes closely associated with cold resistance (Figure 6), representing important candidate genes for future cloning and functional characterization. Previous reports have shown that under cold stress, Arabidopsis perceives signals through AtCBL1-AtCIPK3 to activate downstream cold-responsive transcription factors such as RD29A (Kim et al., 2003), while rice and maize employ OsCIPK3 and ZmCIPK3 to regulate osmotic-related genes, enhancing proline and soluble sugar content to improve

cold tolerance (Xiong et al., 2007; Bian et al., 2008). These transcription factors and metabolic pathways represent primary research directions for validating downstream mechanisms of the CBL-CIPK cold signaling pathway in J. curcas.

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