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Characterization of the calcineurin B-Like (CBL) gene family in maize and functional analysis of *ZmCBL9* under abscisic acid and abiotic stress treatments

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ABSTRACT

In plants, calcineurin B-like proteins (CBLs) play crucial roles in regulating calcium-signaling in response to various abiotic stresses by interacting with specific CBL-interacting protein kinases (CIPKs). However, the identities and functions of *CBL* gene family members in maize are largely unknown. Here, we identified from the maize genome 12 *CBL* genes. All 12 CBLs have conserved EF-hand domains, and half harbor myristoylation motifs. We further characterized the function of one *CBL* gene, *ZmCBL9*, which can be induced by salt, dehydration, glucose and abscisic acid (ABA) treatments. Overexpression of *ZmCBL9* enhanced resistance or tolerance to ABA, glucose, salt and osmotic stress in *Arabidopsis* and complemented the hypersensitive phenotype of the *Arabidopsis cbl9* mutant in response to ABA and abiotic stress. The *ZmCBL9* gene negatively regulates the expression of genes in the ABA signaling, biosynthesis and catabolism pathways. Moreover, the ZmCBL9 protein is found to interact with eight maize CIPKs and these *ZmCIPK* genes were up-regulated by different stress treatments, including salt, dehydration, glucose, low potassium and ABA. These results suggest that ZmCBL9 may interact with various ZmCIPKs to regulate the abiotic stress and ABA response signaling in plants.

1. Introduction

Calcium ions (Ca^{2+}) are crucial second messengers that are involved in signal transduction in response to numerous environmental and developmental stimuli. In response to these stimuli, spatial and temporal changes in cellular Ca^{2+} levels are generated, which are referred to as "Ca^{2+} signatures" [1]. These "Ca^{2+} signatures" are sensed and decoded by Ca^{2+} sensors including Ca^{2+}-dependent protein kinases (CDPKs), calmodulins (CaMs) and calcineurin B-like proteins (CBLs) [2].

CBLs are plant-specific Ca^{2+} sensor relay proteins that interact with CBL-interacting protein kinases (CIPKs); the CBL-CIPK system is widely considered to be an important Ca^{2+} signal transmission pathway [2,3]. A total of 10 CBLs have been each identified in the *Arabidopsis* and rice genomes [3,4]. In *Arabidopsis*, two closely related CBLs, AtCBL1 and AtCBL9, that share approximately 90% sequence identity are well studied for their roles in stress responses. AtCBL1 functions as a positive regulator of salt and drought responses and as a negative regulator of cold responses in plants [5] while AtCBL9 acts as a negative regulator of abscisic acid (ABA) signaling and is involved in ABA biosynthesis under stress [6]. AtCBL1 and AtCBL9 likely function through various CIPKs. AtCBL9 is found to interact with AtCIPK3, with which it plays a role in ABA responses during seed germination [7]. AtCBL1/9 and AtCIPK23 are shown to form protein complexes to regulate potassium (K^+) uptake by activating AKT1 under low-K^+ conditions [8,9]. AtCBL1/9 also form complexes with AtCIPK26 to regulate *respiratory burst oxidase homolog F* (*AtRbohF*) in the ROS signaling pathway [10]. In addition, *CBL1* and *CBL9* function in the regulation of pollen germination and pollen tube growth through regulating K^+ homeostasis [11]. The functions of *CBLs* have also been studied in other species. For example, overexpression of *Thellungiella halophila ThCBL9* increases salt and osmotic stress tol-

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erance in transgenic *Arabidopsis* [12]. Constitutive expression of *Populus euphratica PeCBL1* in the *Arabidopsis cbl1*/*cbl9* mutant complements the low K^+ sensitivity phenotype of the mutant [13]. The BnCBL1-BnCIPK6 complex from Brassica napus is involved in plant responses to high-salinity and phosphorous deficiency as well as ABA signaling [14].

The phytohormone ABA is involved in regulating various plant developmental processes such as seed dormancy, germination and adaptive responses to environmental stresses [15]. In recent decades, numerous genes involved in ABA signal transduction, biosynthesis and catabolism have been identified using genetic, molecular, biochemical and pharmacological approaches [16]. *ABI1* and *ABI2* encode PP2C-type protein phosphatases, which are negative regulators in the ABA receptor complex [17,18]. *ABI3*, *ABI4* and *ABI5* encode transcription factors containing the B3 domain, APETALA2 (AP2) domain and bZIP factor classes, respectively, and they are involved in regulating ABA-mediated gene expression and controlling seed germination and development [19–21]. Endogenous ABA concentrations in plants are controlled by the balance between ABA biosynthesis and catabolism [22]. Almost all of the genes encoding enzymes involved in the ABA biosynthesis pathway in *Arabidopsis* have been identified. The 9-*cis*-epoxycarotenoid dioxygenase (NCED) catalyzes oxidative cleavage of 9-*cis*-violaxanthin and/or 9-*cis*-neoxanthin to produce xanthoxin which is a rate-limiting step in ABA biosynthesis [16]. Nine NCED-related genes have been identified, five of which (*NCED2, 3, 5, 6* and *9*) are thought to play a role in ABA biosynthesis [23]. The hydroxylation pathway is the main ABA catabolic pathway, and 8′-hydroxylation, which is catalyzed by ABA 8′-hydroxylase, is considered to be the first committed step in the major ABA catabolism pathway [16]. In *Arabidopsis*, 8′-hydroxylation is performed by four members of the cytochrome P450 family, CYP707A1, 2, 3 and 4 [24,25].

We previously identified a function of the maize *CBL* gene *ZmCBL4* in the regulation of the salt stress response [26]. However, functions of most maize *CBLs* remain unclear. In the present study, we analyzed maize *CBL* genes at the genome-wide scale and identified 12 *CBL* genes based on the maize B73 genome. Subsequent expression analysis showed that five genes, *ZmCBL2-1, 4, 5, 6-2* and *9*, were up-regulated by both ABA and NaCl treatments. Further study showed that *ZmCBL9* could improve the tolerance to ABA, glucose, salt and osmotic stress in *Arabidopsis*, and it negatively regulated the expression of genes in ABA signaling, biosynthesis and catabolism in the *Arabidopsis.* Moreover, we found that eight maize CIPKs interacted with ZmCBL9 in yeast two-hybrid and bimolecular fluorescence complementation (BiFC) system. These findings shed light on the functions of the CBL-CIPK complex signaling network in maize in response to abiotic stress.

2. Materials and methods

2.1. Identification and phylogenetic analysis of the CBL genes in maize

To identify maize *CBL* gene family members, the protein sequences of 10 CBLs from *Arabidopsis* and rice were used to search the maize sequence database (http://blast.maizegdb.org/) with the BLAST program using default parameters (E-value < e^{-10}). The full-length amino acid sequences of CBL proteins were aligned with Clustal X2.1 software via pairwise and multiple alignment using default parameters. The phylogenetic tree was constructed based on the alignment results using the neighbor-joining method and 1000 bootstrap trials with the Clustal X tool in conjunction with MEGA software (http://www.megasoftware.net/).

2.2. Motif display and gene structure analyses

MEME was used to identify the motifs of CBL proteins from maize (http://meme.sdsc.edu/meme/intro.html). Information about the maize *CBL* genes (including the genomic sequences, full coding sequence and protein sequences) was downloaded from the maizeGDB website (http://www.maizegdb.org/), and theoretical pI (isoelectric point) and MW (molecular weight) values were calculated using the ExPASy Compute pI/Mw tool. The structures of the maize CBL proteins were visualized using GSDStool (http://gsds.cbi.pku.edu.cn/).

2.3. Reverse-transcription quantitative PCR analysis of ZmCBL gene expression

Seeds of maize inbred line B73 were germinated and grown in greenhouse under controlled conditions (27 °C day/23 °C night, 16 h photoperiod, 300 μmol m^{-2} s^{-1} photons, 30–50% relative humidity). After the three-leaf stage, the seedlings were exposed to abiotic stress treatments. For NaCl, glucose and ABA treatments, the seedlings were transferred to Hoagland solution containing 250 mM NaCl, 8% glucose and 100 μM ABA respectively. For dehydration treatment, the seedlings were transferred to filter paper [27]. Whole seedlings were harvested at the indicated time points, immediately frozen in liquid nitrogen and stored at −80 °C until analysis.

For tissue-specific expression analysis, leaves and roots of seedlings at the three-leaf stage, as well as root (aerial root), stalk (below the highest node), leaf (tip of the ninth leaf), tassel, immature ear, bract and immature cob tissue at the pre-flowering stage were subjected to total RNA extraction.

Quantitative reverse-transcription PCR (qRT-PCR) was conducted with SYBR Premix ExTaq^{TM} (Takara) on a Bio-Rad system. *Tubulin* (accession number AY103544) was used as the internal control. The reaction procedure was 95 °C for 2 min followed by 40 cycles of 95 °C for 10 s, 60 °C for 15 s and 72 °C for 30 s.

2.4. Subcellular localization

N-terminal myristoylation of ZmCBL9 was predicted using the PlantP program [28]. The open reading frame (ORF) of *ZmCBL9* was cloned into the pEarleyGate-101 vector [29], which contains the yellow fluorescent protein (YFP) reporter gene, to generate the ZmCBL9-YFP fusion construct under the control of the CaMV 35S promoter. The construct was used for transient transformation of onion (*Allium cepa*) epidermis via microprojectile bombardment with the gene gun system (Bio-Rad). After incubation on MS medium for 16 h at 28 °C, YFP fluorescence was observed under a confocal laser scanning system (Nikon) and examined at 514 nm (excitation) using an argon laser with an emission band of 515–530 nm. Plasmolysis was induced in the onion epidermis by incubating the samples in 20% sucrose solution for 15 min.

2.5. Cloning and sequence analysis of ZmCBL9

To obtain the full-length cDNA of *ZmCBL9* (GenBank accession number EU676042.1), primers *ZmCBL9-F* and *ZmCBL9-R* were used to amplify full-length *ZmCBL9* cDNA from *Zea mays* (Table S1). Total RNA was extracted from three-leaf-stage seedlings using the TRIzol reagent (Invitrogen), and first-strand cDNA was synthesized with a SuperScript Kit (Invitrogen). The thermocycling conditions for amplification were as follows: an initial denaturation at 94 °C for 5 min, followed by 30 cycles of 94 °C for 35 s, 58 °C for 35 s, 72 °C extension for 90 s and a final extension at 72 °C for 7 min. The PCR