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Rapeseed calcineurin B-like protein CBL4, interacting with CBL-interacting protein kinase CIPK24, modulates salt tolerance in plants

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ABSTRACT

Calcium is a ubiquitous intracellular secondary messenger in eukaryotes. Upon stress challenge, cytosolic Ca^{2+} fluctuation could be sensed and bound by calcineurin B-like proteins (CBLs), which further regulate a group of Ser/Thr protein kinases called CBL-interacting protein kinases (CIPKs) to relay the signal and induce cellular responses. Although the CBL-CIPK network has been demonstrated to play crucial roles in plant development and responses to various environmental stresses in Arabidopsis, little is known about their function in rapeseed. In the present study, we characterized *CBL4* gene from rapeseed. We found that CBL4 is localized at the plasma membrane and it interacted with CIPK24 in both yeast two-hybrid and bimolecular fluorescence complementation (BiFC) assays. Unlike the orthologs in Arabidopsis, rapeseed CIPK24 did not interact with CBL10. Furthermore, expression of rapeseed *CBL4* rescued the salt-sensitive phenotype of *sos3-1* mutant and overexpression of rapeseed *CBL4* in Arabidopsis showed enhanced tolerance of salt stress than wild-type. Overall, the results clarified the function of CBL4 in rapeseed.

1. Introduction

Salt stress in one of the major environmental factors limiting growth and productivity of plants. For glycophytes, high salt concentrations impose both ionic and osmotic stresses [1]. Ca^{2+} is a ubiquitous second messenger that is involved in the signaling of a variety of environmental and developmental stimuli. In response to these stimuli, there is fast and significant changes in the intracellular Ca^{2+} concentration and, these changes are sensed and decoded by Ca^{2+} sensors including calmodulins (CaMs), calmodulin-like proteins (CMLs), calcineurin B-like proteins (CBLs) and calcium-dependent protein kinases (CPKs) [2].

CBL genes were originally identified in the model plant Arabidopsis [3]. As a structural basis for Ca^{2+} binding, CBLs contain four EF-hand domains that can bind at most four Ca^{2+} ions [4]. To relay the signals, CBLs specifically interact with a group of SNF1 (Sucrose non-fermenting 1)-related serine/threonine kinases, group 3 (SnRK3s), namely CBL-interacting protein kinases (CIPKs) [5,6]. It is known that CIPK proteins consist of a conserved N-terminal kinase domain, a short variable junction domain and a C-terminal regulatory domain. Once binding Ca^{2+}, CBLs could interact with and activate the catalytic activity of targeting CIPKs through a conserved NAF or FISL motif within the rather divergent C-terminal regulatory domain [7,8]. Over the past more than one decade, the CBL-CIPK networks in Arabidopsis and a few other plants have been demonstrated to play important roles in regulating sodium (Na^+), potassium (K^+) and nitrate (NO^{3-}) transport across the plasma membrane (PM) and/or tonoplast [3,9–11], auxin and abscisic acid (ABA) signaling, as well as many other developmental processes [12]. The first genetically established CBL-CIPK network was identified in a screening for salt overly sensitive (SOS) phenotype, and molecular and biochemical characterization of the coding SOS proteins discovers that SOS3 (CBL4) interacts with SOS2 (CIPK24), and the SOS2–SOS3 complex phosphorylates and activates the plasma membrane-localized Na^+/H^+ antiporter (SOS1) to promote salt tolerance [13]. Later on, it was reported that Arabidopsis CBL10 (SCaBP8) also interacts with SOS2 and CBL10-SOS2 complex functions both in salt storage in vacuoles and in protecting shoots from salt stress [14,15].

So far, bioinformatic analyses have identified a total of 10 *CBLs* and 26 *CIPKs* in Arabidopsis, and 10 *CBLs* and 30 *CIPKs* in rice (*Oryza sativa*) [12]. The selective interactions between CBLs and CIPKs are proposed to allow for a complex interplay of different CBL-CIPK

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combinations that, in turn, could decode the Ca^{2+} signals from different stimuli through spatiotemporal regulation of downstream signaling cascades [16]. Furthermore, recent evidence demonstrates that phosphorylation of CBL proteins by their interacting CIPKs is required for full activity of CBL-CIPK complexes toward their target proteins [17,18].

Despite extensive studies of the CBL-CIPK network in *Arabidopsis* and a few other plants, little is known with CBL-CIPK network in the important oilcrop, rapeseed until very recently [19,20]. Rapeseed oil has the virtue of low erucic acid and low glucosinolates. Understanding role of calcium signal and function of Ca^{2+}-decoding proteins as well as the underlining molecular mechanisms of rapeseed responses to salt stress is a prerequisite for improving salt tolerance to meet the increasing demanding for edible oil.

2. Materials and methods

2.1. Plant materials and growth condition

Rapeseed, *Arabidopsis thaliana* and *N. benthamiana* plants were grown in a soil mix in the greenhouse with a photoperiod of 14 h light (light intensity of ~120 μE m^{-2} s^{-1})/10 h dark, and a temperature of 22 °C day/20 °C night.

2.2. Subcellular localization and confocal microscopy

The coding regions of genes were amplified through PCR using *Pfu* DNA polymerase with primers listed in Table S1. After restriction, they were cloned before the *GFP* gene in the pYJGFP binary vector with a hydrophobic linker between CDSs and GFP. After confirmed by sequencing, these constructs and silencing suppressor p19 of tomato bushy stunt virus were transferred into *Agrobacterium tumefaciens* GV3101 before infiltrated into the leaves of *N. benthamiana* [20]. The leaf discs near the injection site were cut 2 d after infiltration and, the signal of GFP was examined under an LSM510meta confocal microscope (Carl Zeiss, Germany).

2.3. Yeast two-hybrid (Y2H) analysis

The coding regions of *CBL* and *CIPK* genes were cloned into pGBKT7 and pGADT7 vectors, respectively, using primers listed in Table S1. Then, the plasmids were sequentially transformed into yeast strain AH109 through the lithium acetate method following the protocol described in Yeast Protocols Handbook (Clontech). Cotransformants were plated on non-selective SD-LW(synthetic dropout medium lacking Leu and Trp) and selective SD-LWHA (lacking Leu, Trp, His and Adenine). For the deletion assay, different fragments of *BnaCIPK24* gene were cloned into pGADT7 vector through restriction-ligation method using the primers listed in Table S1. Serial 1:10 dilutions were prepared in water and 2 μl of each dilution was used to yield one spot. Plates were incubated at 30 °C for 3–7 d before scored and photographed. X-Gal staining assay was conducted following the instruction in the Yeast Protocols Handbook.

2.4. Bimolecular fluorescence complementation (BiFC) assay

The coding region of *BnaCIPK24* was fused with the N-terminal half of yellow fluorescence protein (YFP) at the N-terminus of the coded protein in the binary 35S-SPYNE(R)173 vector, and the coding regions of *BnaCBLs* were fused with the C-terminal half of YFP at the C-terminus of the coded proteins in the binary 35S-SPYCE(M) vector [21]. Binary plasmids were transformed into *Agrobacterium tumefaciens* GV3101 and coinfiltrated together with the p19 strain at OD_{600} of 0.26:0.26:0.26 into leaves of 5-week-old *N. benthamiana*. YFP signals of the lower epidermal cells of leaves cut 4 d after infiltration were examined on a Nikon A1 confocal

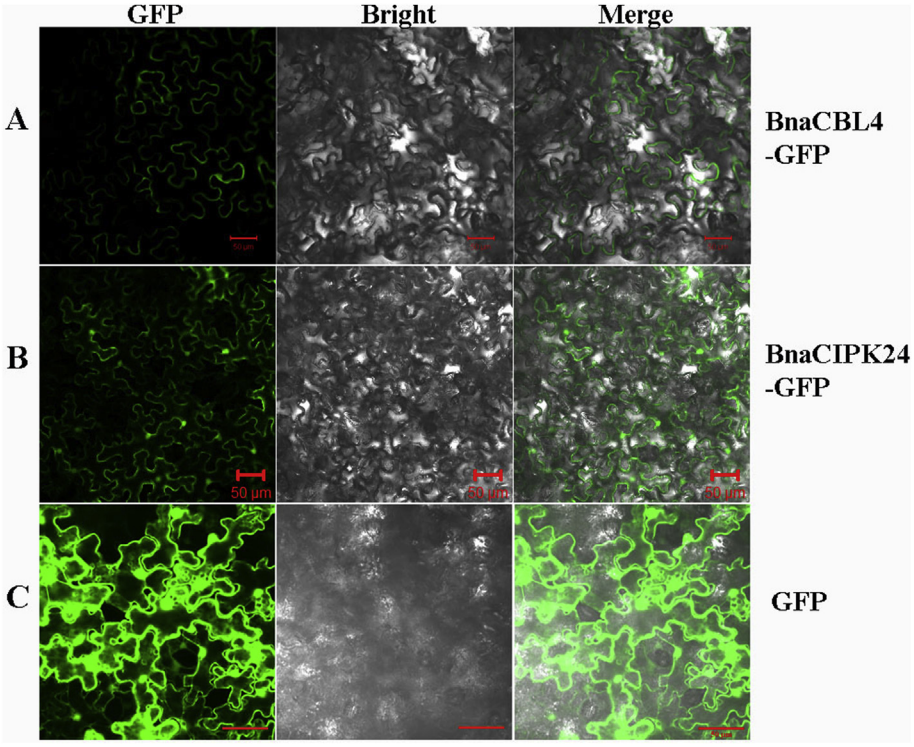

Fig. 1. Subcellular localization of fusion proteins in *N. benthamiana* leaf cells. (A) Subcellular localization of BnaCBL4-GFP protein. (B) Subcellular localization of BnaCIPK24-GFP fusion protein. (C) Subcellular localization of GFP protein. GFP signals were examined 2 days after agroinfiltration. The extreme left panel is GFP fluorescence, the middle bright field and the right represents an overlay of the two images. Bar = 50 μm.