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Arabidopsis CIPK14 positively regulates glucose response

Jingli Yan [1], Fangfang Niu [1], Wu-Zhen Liu, Hanfeng Zhang, Boya Wang, Bo Yang, Yuan-Qing Jiang *

State Key Laboratory of Soil Erosion and Dryland Farming on the Loess Plateau and, College of Life Sciences, Northwest A & F University, Yangling, Shaanxi 712100, China

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ABSTRACT

Calcium is a ubiquitous intracellular secondary messenger in plants. Calcineurin B-like proteins (CBLs), which contain four Ca^{2+}-binding EF hand motifs, are Ca^{2+} sensors and regulate a group of Ser/Thr protein kinases called CBL-interacting protein kinases (CIPKs). 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 glucose signaling.

In the present study, we identified CIPK14 gene from Arabidopsis that play a role in glucose signaling. The subcellular localization of CIPK14 was determined using green fluorescence protein (GFP) as the reporter. Furthermore, the expression levels of *CIPK14* in response to salt, drought, cold, heat, ABA, methyl viologen (MV) and glucose treatments were examined by quantitative RT-PCR and it was found to respond to multiple stimuli, suggesting that CIPK14 may be a point of convergence for several different signaling pathways. Moreover, knock-out mutation of *CIPK14* rendered it more sensitive to glucose treatment. Yeast two-hybrid assay demonstrated that CIPK14 interacted with three CBLs and also with two key kinases, sucrose non-fermenting 1-related kinase (SnRK) 1.1 and SnRK1.2 implicated in glucose signaling. This is the first report to demonstrate that CIPK also plays a role in glucose signaling.

1. Introduction

Plants are sessile and frequently challenged with environmental stresses. Therefore plants have developed complex signal transduction pathways to cope with a fluctuating environment throughout their life cycle. Ca^{2+} is a ubiquitous second messenger involved in the signaling of a variety of environmental and developmental stimuli. In response to these stimuli, cells generate transient 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) [1].

CBL and CBL-interacting protein kinase (CIPK) proteins are plant specific, which were firstly identified in the model plant Arabidopsis [2,3]. As a structural basis for Ca^{2+} binding, CBLs contain four EF-hand domains that can bind at most four Ca^{2+} ions [4,5]. CBLs specifically target a group of SNF1 (Sucrose non-fermenting 1)-related serine/threonine kinases, group 3 (SnRK3), namely CIPKs, to transduce the sensed calcium signal [2,3]. Commonly, CIPK proteins consist of a conserved N-terminal kinase domain, and a C-terminal regulatory domain, which is separated from the kinase domain by a variable junction domain. Ca^{2+}-bound CBLs 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 [6,7]. So far, bioinformatic analyses of both CBL and CIPK families have identified a total of 10 CBLs and 26 CIPKs in Arabidopsis, and 10 CBLs and 30 CIPKs in rice (*Oryza sativa*), respectively, many of which have been reported to show distinct and selective interactions among these complementary partners [8]. 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 [9,10].

Over the past decade, the CBL–CIPK network in Arabidopsis has been demonstrated to play an important role in regulating sodium (Na^+), potassium (K^+) and nitrate (NO_3^-) transport across the plasma membrane (PM) and/or tonoplast [11–14]. In Arabidopsis, a few members of CBL and CIPK family genes have also been identified to participate in auxin and abscisic acid (ABA) signaling, as well as many other developmental processes in Arabidopsis [8,15].

As plants are sessile, they need to integrate an array of stimuli with their metabolic activity, growth and development. Sugars, generated by photosynthetic carbon fixation, are central in

* Corresponding author. Fax: +86 29 87092262.
E-mail addresses: yanjingli512@163.com (J. Yan), fangfangyouran@163.com (F. Niu), 1264353722@qq.com (W.-Z. Liu), 330545594@qq.com (H. Zhang), miao_kk@126.com (B. Wang), yangwl@nwafu.edu.cn (B. Yang), jiangyq@nwafu.edu.cn (Y.-Q. Jiang).
[1] Equal contribution.

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coordinating metabolic fluxes under changing environment and in providing cells and tissues with the necessary energy for continued growth and survival [16]. Glucose is a universal carbon and energy source, which is preferred by organisms ranging from unicellular microbes to plants and animals [16]. While sucrose is the most important transport sugar in plants, most regulatory effects can be ascribed to glucose. Previous studies have uncovered nuclear hexokinase 1 (HXK1) as a pivotal and conserved glucose sensor, which directly mediates transcription regulation, while the Arabidopsis SnRK1.1 (AKIN10) and SnRK1.2 (AKIN11) energy sensor protein kinases function as master regulators of transcription networks under sugar and energy deprivation conditions [17,18]. However, whether and how calcium-associated protein kinases are involved in glucose response and signaling is yet unknown.

In this study, we identified an Arabidopsis CIPK gene, *CIPK14*, which plays an important role in glucose response. We found it may fulfill its role through interacting with SnRK1.1 and SnRK1.2. To our knowledge, this is the first report that the CIPK also participates in glucose response and signaling.

2. Materials and methods

2.1. Plant materials and growth condition

Arabidopsis, wild type (Col-0) or mutant plants were grown in Pindstrup soil mix (Denmark) in the greenhouse with a photoperiod of 16 h light (T8 fluorescent tubes with a light intensity of approximately 100 μE m−2 s−1)/8 h dark, and a temperature of 22 °C day/20 °C night with a relative humidity of 60–70%. For hydroponics, 7-d-old seedlings were transferred from ½ × MS medium plates onto a foam rack floating in ¼ × MS solution, which was changed every 5 d, until tissues and organs were harvested separately.

2.2. Gene cloning

7-d-old Arabidopsis seedlings were harvested for RNA isolation using the Plant RNA kit (Omega bio-tek, USA). RNA integrity was checked by electrophoresis on an agarose gel and quantified using the NanoDrop 1000 (Thermo Sci, USA). 2.5 μg of total RNA were used to synthesize cDNAs by using RNase H minus MMLV (Fermentas, USA) and oligo(dT)$_{18}$ (Fermentas). PCR was conducted in a 50 μL final volume including 0.5 μL of cDNA template, 1 × *Pfu* buffer, 200 μM deoxynucleotide triphosphates (dNTPs), 400 nM of each primer, and 1.25 units of *Pfu* DNA polymerase. The PCR conditions included an initial denaturation at 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 50 °C for 30 s, 72 °C for 1 min per kb, with a final extension at 72 °C for 8 min. Clones were confirmed by sequencing. The primers used are listed in the Table S1.

2.3. Subcellular localization and confocal microscopy

The coding region of *CIPK14* was subcloned and fused before the GFP gene in the pCsGFPBT binary vector (GenBank: DQ370426) with a Gly-Ala rich peptide linker in between. *Agrobacterium tumefaciens* GV3101 cells harboring the recombinant plasmid were infiltrated into leaves of *Nicotiana benthamiana* with the signal of GFP examined 2 d later under an A1 confocal microscope (Nikon, Japan) [19]. Three independent squashes were examined and representative images were presented.

2.4. Yeast two-hybrid (Y2H) analysis

Y2H analysis was performed using the MatchMaker yeast two-hybrid system (Clontech, USA). In brief, the coding regions of CIPK, CBL or SnRK genes were fuse with DNA-binding domain (BD) and activation domain (AD) of transcription factor GAL4, respectively. Then, the plasmids were sequentially transformed into yeast strain AH109 according to Yeast Protocols Handbook (Clontech). After plated on three sets of media, SD–Leucine–Tryptophan (SD–LW), SD–Leucine–Tryptophan–Histidine supplemented with 5 mM 3-amino-1,2,4-triazole(3-AT, Sigma)(SD–LWH+3-AT), and SD–Adenine–Histidine–Leucine–Tryptophan (SD–LWHA), the yeast colonies were grown at 30 °C for 2–7 d before photographed. The titration and X-gal staining assays were performed as previously described [19].

2.5. Stress treatments and quantitative RT-PCR (qRT-PCR)

Wild type (Col-0) seeds were surface sterilized and sown in medium (1/2 × MS plus 1% sucrose) plates, cold treated at 4°C for 2 d and then placed in a greenhouse with a photoperiod of 16 h light/8 h dark. 7-d-old seedlings were subjected to various treatments on stress medium beginning at 9:45 in the morning (2.75 h after light on). Salinity was increased by adding NaCl to a final concentration of 200 mM. Cold and heat treatments were applied by putting the plants in 4 °C and 38 °C chambers, respectively, with light. ABA and oxidative stress were applied through providing 50 μM (±)-ABA (Invitrogen, USA) and 10 μM Paraquat (methyl viologen, Sigma), respectively. Water deficit was subjected by transferring seedlings to plates soaked with 15% PEG8000. Glucose treatment was applied through substituting 1% sucrose in the medium with 4% glucose (MP Biomedicals). The control plants were allowed to continue to grow in fresh-made 1/2 × MS medium. Whole tissues were harvested at 6 and 24 h time points after treatments and flash-frozen in liquid nitrogen and stored at −80 °C. The planting, treatments and harvesting were repeated three times independently.

Total RNA samples were isolated from treated and non-treated control tissues using the Plant RNA kit (Omega, USA). RNA was transcribed into cDNA by using RevertAid H minus reverse transcriptase (Fermentas) and Oligo(dT)18 primer (Fermentas). Primers used for qRT-PCR were designed using PrimerSelect program (DNASTAR Inc.) targeting 3'UTR of each genes with an amplicon size between 80 and 250 bp (Table S1). The reference genes used were *UBC* and *UBQ10*. The specificity of each pair of primers was checked through regular PCR followed by 1.5% agarose gel electrophoresis, and also by primer test in CFX96 qPCR machine (Bio-Rad, USA) followed by melting curve examination. The amplification efficiency (E) of each primer pair was calculated following that described previously [19]. Three independent biological replicates were run.

2.6. Phenotypic assay

Seeds of the wild type (Col-0) and mutants were sterilized in 2.65% bleach containing 0.03% Tween-20, then planted in triplicate on ½ × MS medium with 1% sucrose or ½ × MS medium supplemented with 4% glucose (without sucrose) solidified with 0.8% Phytoblend. After stratified in 4 °C for 2–3 d, seed plates were transferred to a growth chamber with a photoperiod of 16 h light/8 h dark at the temperature 22–23 °C. Emergency of green cotyledon was scored 2 d after and continued until 7 d, and plates were photographed.

3. Results and discussion

3.1. Expression pattern and stress response of AtCIPK14

We first examined the tissue specificity of *AtCIPK14* in various Arabidopsis tissues and organs through quantitative RT-PCR. We

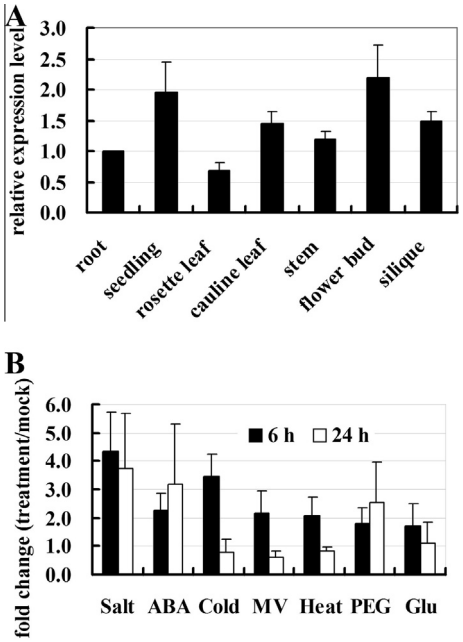

Fig. 1. Expression analyses of *AtCIPK14* in various tissues and in response to abiotic stress treatments through qRT-PCR. (A) *AtCIPK14* expression in various tissues. The relative expression of *AtCIPK14* was normalized to the expression of *UBC* and *UBQ10* and expressed relative to the level in roots. (B) Analysis of the expression pattern of *AtCIPK14* under various abiotic stress conditions. The relative expression of *AtCIPK14* was normalized to the expression of *UBC* and *UBQ10* and expressed relative to the level in mock-treated seedlings. Data is the mean (treatment/mock, linear scale) of three biological replicates ± S.E.

found *AtCIPK14* mRNA was more abundant in 7-d-old seedlings and flower buds, followed by that in cauline leaves and young siliques (Fig. 1A). To investigate whether *CIPK14* is involved in responses to abiotic stresses and stress hormone-ABA, qRT-PCR was used to analyze expression patterns of it in seedlings under salt, cold, heat, oxidative (MV), drought, glucose treatments as well exogenous ABA application. We found that *AtCIPK14* transcripts increased in abundance following treatments with NaCl, ABA and PEG8000 for 6 or 24 h. Cold, heat, glucose and ROS accumulation triggered by MV also up-regulated *CIPK14* expression at the 6 h time point; however, these four treatments showed no much influence on *CIPK14* expression at the 24 h time point (Fig. 1B).

3.2. Subcellular localization of CIPK14 protein

The subcellular localization of a protein may provide evidence of its function. So far, the subcellular localization of several Arabidopsis CIPK proteins has been determined [20–23], however the subcellular localization of other CIPK proteins from Arabidopsis has not been reported. We therefore fused the coding region of *CIPK14* to the *GFP* reporter gene in a binary vector and expressed in leaves of *N. benthamiana* with the GFP signals examined two days later. We observed that CIPK14-GFP was localized in both cytoplasm and nuclei (Fig. 2A). As a control, the GFP protein alone was observed to localize in both the cytoplasm and nucleus (Fig. 2B).

3.3. CIPK14 interacts with three CBL proteins

It has been reported that different CBL proteins interact with different CIPK proteins and the specificity of this interaction determines the network outcome [8,15]. To investigate the interaction preferences of CBL proteins with CIPK14, we used a yeast two-hybrid system. *CIPK14* and ten *CBLs* were cloned in-frame with GAL4-BD and -AD domains, respectively. We found that CIPK14 interacted significantly with three CBLs, CBL2, -3 and -9, however, it did not show any interaction with any other CBLs, indicating interaction specificity. The authenticity of interactions was further confirmed by the X-gal staining (Fig. 3A).

3.4. cipk14 mutant is sensitive to glucose treatment

To dissect the biological function of CIPK14, we obtained two T-DNA insertion lines (SALK_147899 and SALK_149471, Fig. 4A) and confirmed that both are knock-out mutants as transcript level was not detectable (Fig. 4B). On normal ½ × MS medium plates, wild type (WT) and mutant seeds germinated very well and displayed similar growth phenotype (Fig. 4C). However, when the medium was supplemented with 4% glucose, germination of mutant seeds were significantly inhibited compared with WT seeds (Fig. 4C). For instance, at 4 DPS (days post-stratification), 61.3% WT seeds germinated on medium containing glucose, however, only 2.8% cipk14 mutant seeds could germinate. At 7 DPS, 93.3% WT seeds germinated, while only 26% mutant seeds germinated on medium containing glucose (Fig. 4D). These data suggest that knock-out mutation of CIPK14 increased sensitivity to glucose.

To understand the role of CIPK14 in glucose response, we further aimed to identify its interacting partners through Y2H. In Arabidopsis and other plants, SnRK1s have been demonstrated to play important roles in controlling metabolic homeostasis, stress signaling, ABA signaling as well as plant development [24,25]. There are three SnRK genes, SnRK1.1 (KIN10), -1.2 (KIN11) and 1.3 (KIN12) in Arabidopsis genome, among which SnRK1.3 expression was not detected and may be a pseudogene [26]. Yeast two-hybrid test indicated that there are indeed interactions between CIPK14 and SnRK1.1, -1.2 (Fig. 3B), suggesting that CIPK14 may form a complex with both SnRK1.1 and -1.2, and/or modulates SnRK1.1 and -1.2 activities.

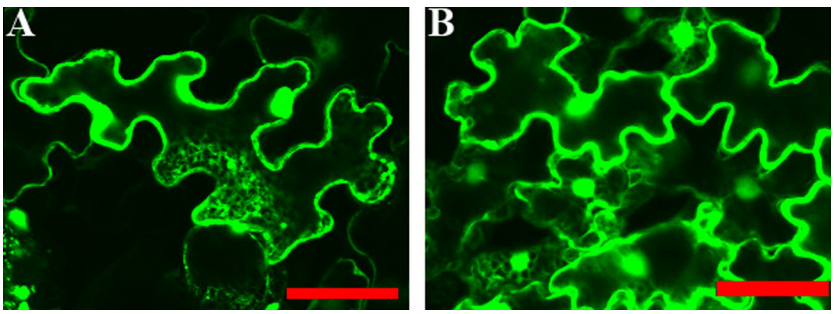

Fig. 2. Subcellular localization of CIPK14-GFP fusion protein in *N. benthamiana* leaf cells. (A) Subcellular localization of CIPK14-GFP fusion protein. (B) Subcellular localization of GFP protein alone. Bar = 50 μm.

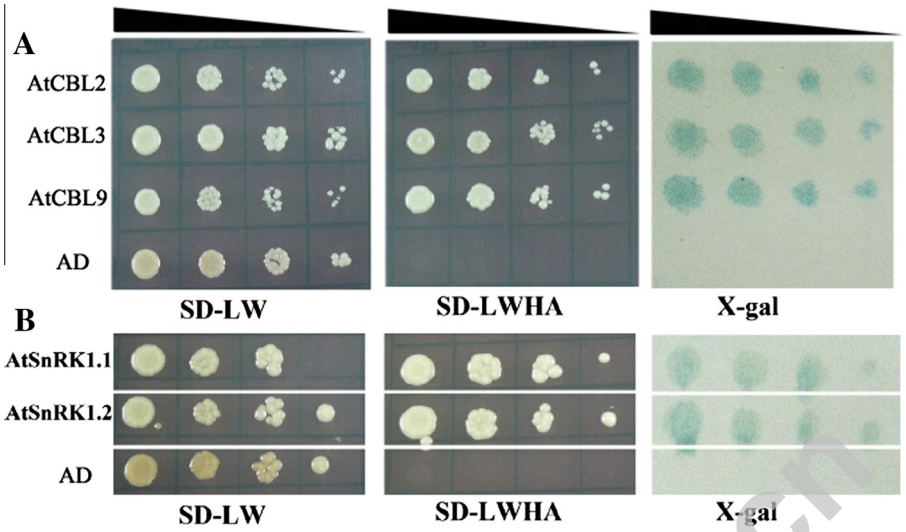

Fig. 3. Yeast two-hybrid screening of interacting partners of AtCIPK14 protein. (A) The titration assay of interactions between CIPK14 and CBLs. (B) The titration assay of interactions between CIPK14 and SnRK1s. In each panel, the yeast cells of strain AH109 harboring the indicated plasmid combinations were grown on either the nonselective (SD–LW) or selective (SD–LWHA) media, followed by β-galactosidase assay (X-gal staining). Decreasing cell densities in the dilution series are illustrated by narrowing triangles. The last row in each panel is the control test using pGADT7 empty vector (AD).

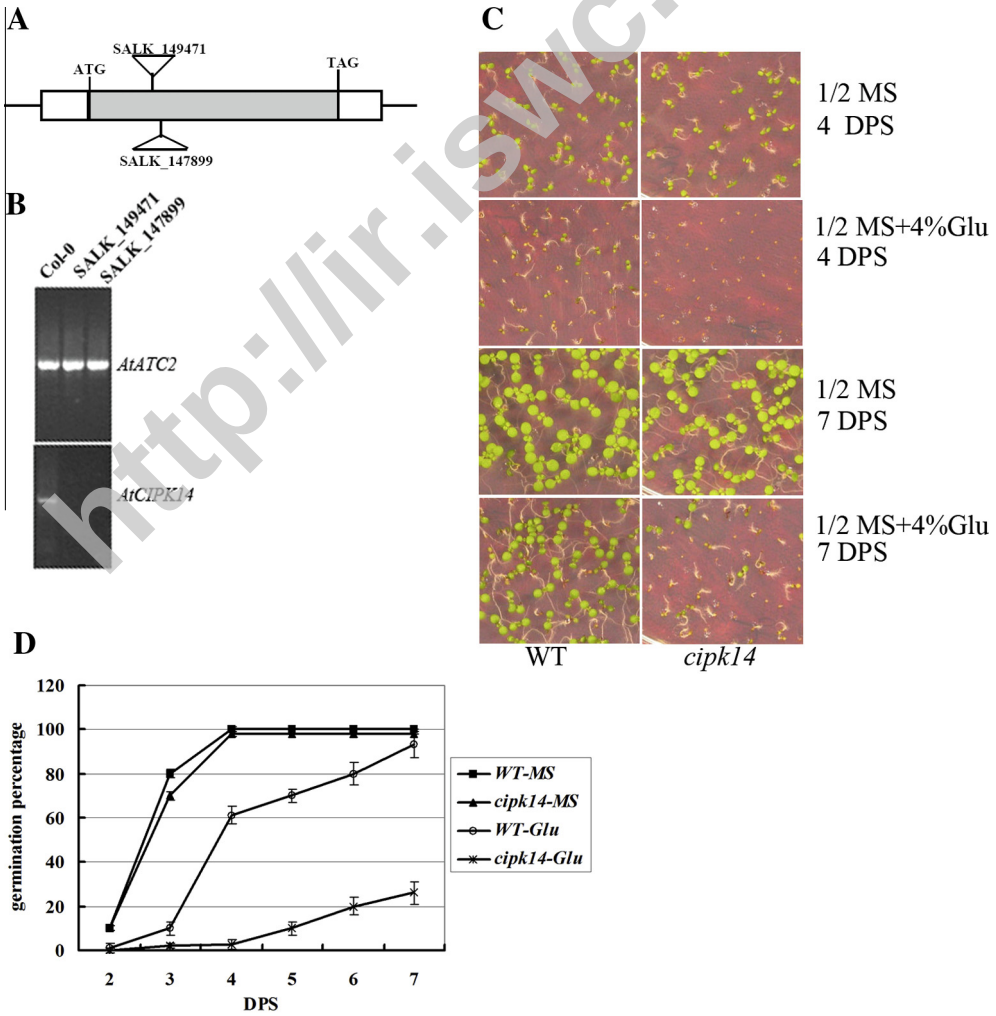

Fig. 4. Identification and phenotypic assay of *cipk14* mutant. (A) Schematic representation of CIPK14 gene structure and T-DNA insertion positions. (B) RT-PCR analysis of *CIPK14* transcript levels in WT (Col-0) and mutant plants. *ACT2* is the endogenous control. (C) Germination of WT and *cipk14* mutant seeds 4 and 7 DPS (days post-stratification) in ½ × MS medium containing 0% and 4% glucose, respectively. (D) The germination percentages of WT and *cipk14* mutant seeds plated on MS medium supplemented with or without 4% glucose. Approximately 80 seeds for each genotype were used in each of three replicates. Error bars represent S.E.

In conclusion, we have identified and functionally characterized an Arabidopsis CIPK gene, CIPK14, as a novel kinase gene positively regulating glucose response. Our data suggest that expression of CIPK14 gene responded to multiple abiotic stress treatments and CIPK14 protein interacts with CBL2, -3, and -9. AtCIPK14 also showed strong interaction with both SnRK1.1 and -1.2, adding to the complex regulation of SnRK1 network. To our knowledge, this is the first report that a CIPK regulates plant sensitivity to glucose, possibly through interacting with SnRK1.1 and SnRK1.2.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.07.064.

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