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Overexpression of CBL interacting protein kinase 2 improves plant tolerance to salinity and mercury

W.H. PAN¹, Z.Z. ZHENG², X. YAN^{2,3}, J.Q. SHEN², J.X. SHOU¹, L.X. JIANG⁴, and J.W. PAN^{3*}

College of Life Sciences, Shaoxing University, Shaoxing, Zhejiang 312000, P.R. China¹

College of Chemistry and Life Sciences, Zhejiang Normal University, Jinhua, Zhejiang 321004, P.R. China²

School of Life Sciences, Lanzhou University, Lanzhou 730000, P.R. China³

College of Agriculture and Biotechnology, Zhejiang University, Hangzhou 310058, P.R. China⁴

Abstract

In plants, calcineurin B-like proteins (CBLs) and CBL-interacting protein kinases (CIPKs) regulate Ca²⁺ signalling and so responses to biotic and abiotic stresses. However, the details of specific CIPKs functions in various stress responses are poorly understood. Here, we report roles of dicot and monocot *CIPK2* genes in response to salinity and heavy metals. *Arabidopsis thaliana AtCIPK2* was found to be universally expressed in different tissues and organs and furthermore induced by salinity. Overexpression of *AtCIPK2* or Tibetan Plateau wild barley (*Hordeum spontaneum*) *HsCIPK2* in *Arabidopsis* alleviated toxic effects of NaCl and mercury on seed germination and root growth. Similarly, reduced toxic effects of copper and cadmium on seed germination, but not on root growth, were observed in these transgenic lines. Live-cell fluorescence imaging analysis revealed that HsCIPK2 was predominantly distributed in the cytoplasm and nucleus and weakly localized at the plasma membrane (PM), but its PM association was rapidly enhanced upon exposure to high salinity and mercury. These results suggest an involvement of CIPK2 in plant tolerance to salinity and mercury and provide a new insight into physiological functions of CIPKs in plant response to heavy metals.

Additional key words: cadmium, copper, gene expression, NaCl, root growth, seed germination, subcellular localization.

Introduction

To adapt to a changing environment, plants established a series of regulatory mechanisms, such as sensing, decoding, and transduction of various stress signals, stress-related gene expression, and final physiological responses. Calcium is a second messenger controlling a variety of actions including gene expression (Dodd *et al.* 2010). The change of calcium signals is initially sensed and decoded by calcium sensors and, in turn, calcium sensor interacting proteins transmit resulting signals to the downstream effectors to activate the expression of early response genes, finally leading to a wide range of physiological processes (Sanders *et al.* 2002, Batistič and

Kudla 2012, Zhu *et al.* 2013). In the past decade, calcineurin B-like proteins (CBLs), calcium sensor proteins, CBL-interacting protein kinases (CIPKs), and Ca²⁺-dependent serine/threonine kinases have been identified as a plant-specific signalling system that functions in biotic and abiotic stress responses (Kolukisaoglu *et al.* 2004, Xu *et al.* 2006, Luan 2009, Weinl and Kudla 2009, Hashimoto and Kudla 2011, De la Torre *et al.* 2013).

In *Arabidopsis*, overexpression of *AtCIPK6* enhances plant tolerance to salt stress (Chen *et al.* 2013) while its down-regulation in mutants display a salt-hypersensitive

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Abbreviations: CaMV - cauliflower mosaic virus; CBLs - calcineurin B-like proteins; CDS - coding sequence; CIPKs - CBL-interacting protein kinases; DAPI - 4',6-diamidino-2-phenylindole; FM4-64 - N-(3-triethylammoniumpropyl)-4[6-(4-(diethylamino)phenyl)hexatrienyl]pyridinium dibromide; GFP - green fluorescent protein; GUS - β -glucuronidase; MS - Murashige and Skoog; PM - plasma membrane; qPCR - quantitative polymerase chain reaction; RT-PCR - reverse transcription-PCR.

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* Corresponding author; e-mail: jwpan@lzu.edu.cn

phenotype (Tripathi *et al.* 2009). In *Oryza sativa*, overexpression of *OsCIPK3*, *OsCIPK12*, and *OsCIPK15* improves the resistance to cold, drought, and salt stresses (Xiang *et al.* 2007), while loss-of-function *OsCIPK31* mutants exhibit hypersensitive responses to multiple abiotic stresses including abscisic acid (ABA), salt, mannitol, and glucose during seed germination and early seedling stages (Piao *et al.* 2010). Overexpression of *Zea mays* *ZmCIPK16* or *Hordeum brevisubulatum* *HbCIPK2* in *Arabidopsis* *sos2* mutants improves their tolerance to salt and/or osmotic stresses (Zhao *et al.* 2009, Li *et al.* 2012). Furthermore, expression of halophytic shrub (*Nitraria tangutorum*) *NtCIPK2* in *Escherichia coli* improves cell tolerance to high salinity, alkalinity, osmotic stress, dehydration, heat, and cold stresses (Zheng *et al.* 2014).

In addition to the roles of CIPKs in above-mentioned

various stresses, however, it is no evidence showing that CIPKs function in plant response to heavy metal toxicity.

As a result of industrial activity, heavy metal contamination in soil and water has become a major environmental concern due to its impacts on plant growth and yield. Heavy metals are commonly categorized as essential and non-essential ones on the basis of their roles in plant growth (DalCorso *et al.* 2013, Sharma *et al.* 2016). Essential heavy metals (e.g. Cu^{2+} , Zn^{2+} , Mn^{2+} , Fe^{2+} , Ni^{2+} , and Co^{2+}) at low concentrations are necessary for plant growth, but their excessive amounts are toxic to plant cells, whereas non-essential heavy metals (e.g. Hg^{2+} , Cd^{2+} , and Pb^{2+}) are more hazardous to plant growth even at low concentrations. The aim of this work was to characterize the physiological role of *Arabidopsis* *AtCIPK2* and wild barley *HsCIPK2* in response to heavy metals and salinity.

Materials and methods

Plants and growth conditions: *Arabidopsis thaliana* L. (ecotype Columbia-0) seeds were surface sterilized and imbibed in the dark at 4 °C for 3 d and then sown onto 0.5× Murashige and Skoog (MS) agar plates [1.5 % (m/v) agar and 1 % (m/v) sucrose, pH 5.6]. Seedlings, unless specified, were vertically grown on agar plates in a growth room (22/20 °C day/night temperature, 16/8-h photoperiod, 80 $\mu\text{mol m}^{-2} \text{s}^{-1}$ irradiance, and 70 % air humidity; for details see Wang *et al.* 2013). Grains of Tibetan Plateau annual wild barley (*Hordeum spontaneum* C. Koch) cv. X74, were surface sterilized and subsequently germinated between layers of wet filter papers with 0.1 mM CaCl_2 (pH 5.8) in the dark and 25 °C for 1 d and then germinated grains were incubated in 0.1 mM CaCl_2 solution for 4 d (Pan *et al.* 2004).

Bioinformatic analysis, cloning, and constructs of *HsCIPK2* and *AtCIPK2*: Full-length cDNAs of *AtCIPK2* (AAF86506; At5G07070) and *OsCIPK2* (ACD76974) as probes were used to search their homologous gene in full-length cDNA library of *Hordeum vulgare* (http://earth.lab.nig.ac.jp/~dclust/cgi-bin/barley_pub/). Based on a cDNA sequence with the highest identity with *AtCIPK2* and *OsCIPK2*, reverse transcription (RT)-PCR was used to amplify full-length coding sequence (CDS) of *HsCIPK2*. The total RNA of wild barley was isolated by RNeasy plant mini kit (Qiagen, Valencia, CA, USA) and subsequently, the first-strand of cDNA was synthesized with SuperScript III first-strand synthesis kit (Invitrogen, Carlsbad, CA, USA), as previously described (Wang *et al.* 2016). Bioinformatic analyses of CIPK2 including amino acid alignment and transmembrane domain prediction were performed using Lasergene software (MegAlign) and TMHMM2.0 server (<http://www.cbs.dtu.dk/services/TMHMM-2.0/>), respectively.

Constructs of *AtCIPK2* and *HsCIPK2* under the control of the constitutive cauliflower mosaic virus (CaMV) 35S promoter were generated using PCR

amplification with gene-specific primers (Table 1 Suppl.), restriction digestion, and ligation reaction with transformation vector (pCambia2300) containing 2× 35S promoter. Vectors pBI121 with *GUS* (encoding β -glucuronidase) and pCambia1300 with 35S promoter and *GFP* (encoding green fluorescent protein; Wang *et al.* 2013) were used to generate the *ProAtCIPK2::GUS* construct (1381 bp; Table 1 Suppl.) and the constructs of *Pro35S::HsCIPK2-GFP* and *Pro35S::GFP* (as a negative control; Table 1 Suppl.), respectively. All resulting constructs were confirmed by sequencing and consequently transformed into wild-type *Arabidopsis* ecotype (Col-0) via *Agrobacterium*-mediated floral dipping.

PCR-based gene expression assay: Reverse transcription (RT) PCR was used to examine whether exogenous genes are expressed in *Arabidopsis* transgenic lines. The total RNA isolation from T2 kanamycin-resistant transgenic lines and the first-strand cDNA synthesis were carried out as described above. The cDNA templates were used in PCR amplification for *AtCIPK2* (34 cycles), *HsCIPK2* (34 cycles), and *AtActin11* (AT3G12110; 28 cycles; as an internal control) transcripts with the gene-specific primers (Table 1 Suppl.). PCR products were analyzed by 1 % (m/v) agarose gel electrophoresis.

Real-time quantitative (q) PCR was performed with Thunderbird SYBR qPCR mix (Toyobo, Japan) and StepOnePlus real-time PCR system (Applied Biosystems, Foster City, CA, USA). Reactions were performed in a 20- μm^3 volume contained 10 μm^3 of 2× SYBR qPCR mix (Toyobo), 40 ng cDNA, and 1 μM of gene-specific primer (Table 1 Suppl.). PCR cycles were performed as: 95 °C for 5 min; 40 cycles of 95 °C for 5 s and 60 °C for 45 s. The resulting data were collected and analyzed using the StepOne software v2.1 and consequently normalized with the housekeeping gene *AtActin2* (At3G18780) (Table 1 Suppl.). The transcriptions in all treatments with NaCl or

HgCl₂ were standardized to the corresponding mock control or 0 min time point.

Determination of seed germination and seedling growth: Seeds of Col-0 and two independent homozygous transgenic lines overexpressing *HsCIPK2* and *AtCIPK2* (T3 generation) after 3-d cold treatment were sown on 0.5× MS agar plates (0.7 % agar and 1 % sucrose, m/v, pH 5.6) supplemented with different concentrations of NaCl, anhydrous HgCl₂, CuSO₄·5 H₂O, and anhydrous CdCl₂. Agar plates were horizontally incubated for 7 d in the growth room. Seed germination rates [%] were calculated by a formula: the number of germinated seeds/the total number of seeds × 100. All germination rates were standardized to the corresponding mock control (100 %) and thereby presented as relative germination rates.

To assay the effects of NaCl on root growth, two methods were used: 5-d-old vertically grown seedlings were transferred to fresh 0.5× MS agar plates supplemented with 125 mM NaCl, and subsequently rotated for 180° (roots vertically upward) and vertically incubated for additional two weeks before imaging; alternatively, 5-d-old vertically grown seedlings were transferred to the same agar plates supplemented with 125 mM NaCl, and subsequently vertically incubated for another 3 d without 180° rotation before imaging, the latter of which was also similarly used to examine the effects of heavy metals on root growth. For quantification of root growth, root elongation of the primary roots was measured during 2-week or 3-d incubation using *ImageJ* software (<http://rsb.info.nih.gov/ij>). Average values of root elongation were standardized to the corresponding mock control and presented as relative root elongation (%). In addition, a total fresh mass of seedlings in each treatment and an average fresh mass per seedling were presented.

Confocal microscopy and quantification: To analyze the subcellular localization of *HsCIPK2*-GFP, 5-d-old vertically grown seedlings of transgenic lines expressing *HsCIPK2*-GFP or free *GFP* were incubated in 0.5× MS liquid medium containing 5 µg cm⁻³ 4',6-diamidino-2-phenylindole (DAPI) in darkness for 10 min, washed with fresh 0.5× MS liquid medium, and then incubated for 2 min in 0.5× MS liquid medium containing 1 µM N-(3-triethylammoniumpropyl)-4[6-(4-(diethylamino)phenyl)hexatrienyl] pyridinium dibromide (FM4-64; Wang

et al. 2013) before confocal imaging.

To determine the effects of NaCl and Hg²⁺ on subcellular distribution of *HsCIPK2*-GFP, 5-d-old vertically grown seedlings expressing *HsCIPK2*-GFP or free *GFP* were incubated in 0.5× MS liquid medium supplemented with 200 mM NaCl or 1 µM HgCl₂ for different duration (as indicated in the figure legends) before confocal imaging.

Images were captured using confocal laser scanning microscope (*Leica TCS SP5 AOBS*, Bensheim, Germany). Excitation wavelengths were 488 nm (argon laser) for GFP, 543 nm (helium/neon laser) for FM4-64, and 355 nm (UV laser) for DAPI. Emission was detected at 496 - 532 nm for GFP, 651 - 761 nm for FM4-64, and 420 - 470 nm for DAPI. For quantification of GFP fluorescence intensities, laser, pinhole and gain settings of the confocal microscope were identical in the same treatment or genotype.

To measure the intensities of GFP fluorescence signals at the plasma membrane (PM), digital images were analyzed by regions of interest (ROIs) using a freehand line tool of *ImageJ* software as shown in Fig. 6 and relative fluorescence intensities were presented as percentages of the corresponding 30-min mock control (100 %) as previously described (Wang *et al.* 2016).

GUS staining assay: After 3-d cold treatment in darkness, seeds were sown on 0.5× MS 1.5 % (m/v) agar plates supplemented with different concentrations of NaCl as indicated in the text. Seven-d-old whole seedlings treated with or without NaCl or tissues and organs at different developmental stages of plants grown in the soil were vacuumed for 15 min in X-gluc staining solution [10 mM EDTA, 0.1 % (?/v) *Triton X-100*, 2 mM Fe²⁺CN, 2 mM Fe³⁺CN, 100 µg cm⁻³ chloramphenicol, 1 mg cm⁻³ X-gluc in 50 mM phosphate buffer (pH 7.0)] (Yu *et al.* 2016) and subsequently incubated at 37 °C overnight. After staining, resulting materials were stored in 70 % (v/v) ethanol solution before microscope imaging.

Statistical analyses: All quantitative data from at least three independent repeats were statistically evaluated using a Student's *t*-test (two tails; type 2). Numbers of seeds, seedlings, and cells examined are indicated in the figure legends.

Results

Although previous studies have shown that wild barley *HbCIPK2* is involved in salt and osmotic stress tolerance (Li *et al.* 2012), tissue-specific expression pattern of *CIPK2* in plants remains unknown. To this end, we generated transgenic lines expressing *GUS* under control of *AtCIPK2* native promoter (*ProAtCIPK2*:*GUS*) in *Arabidopsis*. As shown in Fig. 1, GUS signals were detectable in the root (root-shoot junction and root tip), hypocotyl, cotyledon, stem, rosette leaf, and flower organs

including sepal, stamen (filament), pistil (stigma), and petal (base), indicating that *AtCIPK2* is universally expressed in various plant tissues and organs.

Next, to address whether salt stress induces the expression pattern of endogenous *AtCIPK2* in *Arabidopsis*, a GUS staining assay was performed in the transgenic seedlings treated with different concentrations of NaCl (0, 40, 60, and 80 mM). Salt treatment elevated the GUS signals in the root, hypocotyl, and cotyledon,

especially in their vascular tissues, relative to the mock control (Fig. 2A-H). Consistently, RT-qPCR analysis showed that salt treatment enhanced the transcriptional expression of endogenous *AtCIPK2* in the seedlings under the same conditions (Fig. 2I). These results together demonstrate that *AtCIPK2* expression pattern is regulated by salt stress.

To characterize a role of *HsCIPK2* in response to various abiotic stresses, we used full-length cDNAs of *OsCIPK2* and *AtCIPK2* as a probe to perform a *BLAST* search for their homologue in full-length cDNA library of *Hordeum vulgare* and finally isolated *HsCIPK2* CDS from the total cDNA pool of wild barley seedlings by RT-PCR- and sequencing-based approaches. Alignment of the predicted amino acid sequence revealed that, similar to

OsCIPK2 and *AtCIPK2*, *HsCIPK2* contained two CIPK-specific highly conserved domains including a N-terminal kinase domain with an activation loop and a C-terminal regulatory domain with a NAF motif (Fig. 1 Suppl.). Furthermore, *HsCIPK2* shared ~86 and ~64 % amino acid identity with *OsCIPK2* and *AtCIPK2*, respectively, indicating that different monocot CIPK2 homologues display a higher level of evolutionary conservation, relative to dicot CIPK2. Consistent with this, a potentially conserved transmembrane helix domain in *HsCIPK2* and *OsCIPK2* but not in *AtCIPK2* was predicted (Fig. 2 Suppl.). Sequencing of PCR products amplified from wild barley, rice, and *Arabidopsis* genomic DNAs (data not shown) confirmed the previous finding that plant-specific CIPK2 does not have any introns (Li

Fig. 1. GUS staining analysis of *AtCIPK2* expression pattern. A - Seven-d-old seedling expressing *ProAtCIPK2::GUS*. Inset shows GUS signals in the root tip. B to H - Individual organs including stem (B), rosette leaf (C), flower (D), pistil and stigma (E), sepal (F), anthers and filaments (G), and petal (H) in soil-grown transgenic plants expressing *ProAtCIPK2::GUS*. Arrowheads show GUS signals in the root and flower organs. Bars = 5 mm (A), 200 µm in inset (A), 10 mm in (B), 5 mm in (C), 1 mm (D), and 200 µm in (E) to (H).

et al. 2012, Zheng *et al.* 2014). Thus, CDS and deduced amino acid sequence of *HsCIPK2*, as a novel homologue of plant-specific *CIPK2*, were deposited into the GenBank (accession No. KP638475).

To address whether *AtCIPK2* and *HsCIPK2* are functionally involved in plant salt tolerance, transgenic lines overexpressing *AtCIPK2* and *HsCIPK2* under control of the 35S promoter (*Pro35S:AtCIPK2*; *Pro35S:HsCIPK2*; Fig. 3 Suppl.) were generated. Analysis of RT-PCR in the T2 generation showed that multiple transgenic lines displayed overexpression of *AtCIPK2* or *HsCIPK2* relative to the wild-type control (Fig. 3 Suppl.). In T3 generation, two independent homozygous lines *AtCIPK2*-9, 2-18, and *HsCIPK2*-7, 2-15 were used to evaluate transgenic plant response to salt stress. In the

absence of NaCl, no significant differences were observed in seed germination and root growth between the transgenic lines and the wild type (Fig. 3A,B, Fig. 4A,C Suppl., Fig. 5A,B Suppl.). Upon seed exposure to 125 or 150 mM NaCl, seed germination percentages in two independent transgenic lines overexpressing *AtCIPK2* or *HsCIPK2* were higher than in the corresponding wild-type control (Fig. 3A, Fig. 5A Suppl.). Consistently, root growth in these two independent transgenic lines overexpressing *AtCIPK2* or *HsCIPK2* was more resistant to 125 mM NaCl relative to the corresponding wild-type grown on the vertical agar plates with 180° rotation for additional two weeks (Fig. 3B; relative root elongation of Col-0: 11.8 ± 5.5 %; *AtCIPK2*-9: 20.4 ± 8.2 %; *HsCIPK2*-15: 24.0 ± 7.3 %) or without 180° rotation for another 3 d (Fig. 5B

Fig. 2. Salt-induced expression pattern of *AtCIPK2*. A to D - Whole seedlings. E to H - Magnified images of roots in (A to D). I - real time qPCR. Seeds of *ProAtCIPK2::GUS* transgenic lines were germinated and vertically grown for 7 d on 0.5× MS agar plates supplemented with different concentrations of NaCl as indicated at the top panel before GUS staining. Arrowheads show salt-induced GUS signals in roots. Bars = 5 mm in (A to D) and 1 mm in (E to H). Means $\pm$ SD, $n = 3$. Single and triple asterisks indicate significant differences at $P < 0.01$ and 0.0001, respectively (Student's t test).

Fig. 3. Effects of salt stress on seed germination and seedling growth. *A* - The wild-type, *AtCIPK2-9*, and *HsCIPK2-15* seed germination rate after 7-d exposure to 125 or 150 mM NaCl. *B* to *D* - Relative root elongation and fresh mass of the wild-type, *AtCIPK2-9*, and *HsCIPK2-15* seedlings after 2-week exposure to 125 mM NaCl. Means $\pm$ SDs, $n = 120 - 124$ seeds or 24 - 30 seedlings. Single, double, and triple asterisks indicate significant differences at $P < 0.01$, 0.001, and 0.0001, respectively (Student's *t* test; compared to the corresponding wild-type control).

Fig. 4. Toxic effects of heavy metals on seed germination and root growth. *A* to *C* - Seed germination assay in the wild-type, *AtCIPK2-9*, and *HsCIPK2-15* seeds after 7-d exposure to heavy metal stresses. *D* to *F* - Root growth assay in the wild-type, *AtCIPK2-9*, and *HsCIPK2-15* seedlings after 3-d exposure to heavy metal stresses. Relative germination rate (%) of seeds ($n = 100$ seeds each) and relative growth rate (%) of roots ($n = 30 - 34$ seedlings each) are presented. Means $\pm$ SDs. Single, double, and triple asterisks indicate significant differences at $P < 0.05$, 0.01, and 0.001, respectively (Student's *t*-test; compared to the corresponding wild-type control).

Suppl.; Col-0: 21.8 ± 3.7 %; *AtCIPK2-18*: 29.2 ± 4.3 %; *HsCIPK2-7*: 34.2 ± 4.0 %). Furthermore, the average fresh mass of transgenic plants was higher than that of the wild-type seedlings (Fig. 3C,D, Fig. 5C,D Suppl.). These results suggest that overexpression of *AtCIPK2* and *HsCIPK2* alleviates the inhibitory effect of NaCl on seed germination and seedling growth and thereby enhances salt tolerance.

Although plant-specific CIPKs are involved in multiple biotic and abiotic stresses (Shen *et al.* 2014), it remains unknown whether CIPKs function in plant responses to heavy metals. To test this, we examined the effects of heavy metals on seed germination and root growth in the transgenic lines overexpressing *AtCIPK2* or *HsCIPK2*. Upon seed exposure to HgCl₂ (2, 3, 4, 6, and 8 μ M), CuSO₄ (20, 40, 60, and 80 μ M), or CdCl₂ (25, 50, and 75 μ M), seed germination percentages in the wild type were significantly reduced (Fig. 4A-C, Fig. 6A-D Suppl.).

By contrast, the transgenic lines overexpressing *AtCIPK2* or *HsCIPK2* displayed higher seed germination percentages than the corresponding wild-type control (Fig. 4A-C, Fig. 6A-L Suppl.). Consistently, root growth of the transgenic lines overexpressing *AtCIPK2* or *HsCIPK2* was more resistant to 2 μ M HgCl₂ (Fig. 4D; relative root elongation of Col-0: 83.1 ± 11.2 %, *AtCIPK2-9*: 104.7 ± 15.5 %; *HsCIPK2-15*: 89.1 ± 8.8 %) and 3 μ M HgCl₂ (Fig. 4D; Col-0: 8.6 ± 7.5 %, *AtCIPK2-9*: 17.9 ± 7.5 %, *HsCIPK2-15*: 14.3 ± 10.6 %) relative to the corresponding wild-type control. However, no significant differences were observed in root growth between these transgenic lines *versus* the wild-type seedlings in the presence of CuSO₄ or CdCl₂ (Fig. 4E,F). Together, these results suggest that overexpression of *AtCIPK2* and *HsCIPK2* improves seed germination tolerance to Hg²⁺, Cu²⁺, and Cd²⁺ and enhances root growth tolerance to Hg²⁺, but not to Cu²⁺ and Cd²⁺.

Fig. 5. Live-cell imaging for subcellular distribution of HsCIPK2-GFP. A to E -Subcellular localization of HsCIPK2-GFP in root epidermal cells. F to J -Subcellular localization of free GFP in root epidermal cells. Arrowheads and arrows show co-localization of HsCIPK2-GFP or free GFP with DAPI-labeled nuclei and HsCIPK2-GFP with FM4-64-labeled PM, respectively. Asterisks show cytoplasmic localization of HsCIPK2-GFP or free GFP. Bars = 10 μ m.

To analyze the subcellular distribution of CIPK2, we generated the transgenic lines expressing *HsCIPK2-GFP* under control of the 35S promoter (*Pro35S::HsCIPK2-GFP*). FM4-64, a lipophilic styryl dye and DAPI, a fluorescent dye widely used for nuclear staining, were used to label the plasma membrane (PM) and nucleus, respectively. Different from the prediction (Fig. 2 Suppl.), live-cell imaging showed HsCIPK2-GFP fluorescence signals predominantly in the cytoplasm and nucleus while only weakly at the PMs in root epidermal cells (Fig. 5A-E). Similarly, the fluorescence signals of free GFP were observed in the cytoplasm and nucleus but not at PM (Fig. 5F-J).

To address whether salt and mercury stresses influence subcellular localization of CIPK2 and/or its gene expression, we first used live-cell imaging to examine subcellular distribution of HsCIPK2-GFP upon seedling exposure to 200 mM NaCl or 1 μ M HgCl₂ for 10 - 90 min.

Both treatments caused a rapid increase in HsCIPK2-GFP fluorescence signals in the PM, cytoplasm, and nucleus of root cells (Fig. 6A-E, K-O). By contrast, the fluorescence signals of free GFP were greatly reduced after exposure to high salinity or HgCl₂ (Fig. 6F-J, P-T). Furthermore, quantitative analysis revealed that, upon 10-min exposure to 200 mM NaCl or 1 μ M HgCl₂, the PM-associated HsCIPK2-GFP fluorescence signals increased by approximately 20 and 50 % relative to the mock controls, respectively, and subsequently reached a peak (increased by ~50 and ~70 %, respectively) after 60-min exposure (Fig. 6U,V).

Next, we performed a real-time qPCR analysis to examine whether salt- and mercury-induced PM association of HsCIPK2-GFP occurs *via* a transcriptional process. Although NaCl and HgCl₂ treatments indeed enhanced the expression of endogenous *AtCIPK2* (Fig. 2I, Fig. 7A), these treatments did not alter the expression of

35S promoter-controlled *HsCIPK2-GFP* relative to the corresponding 0-min time point (Fig. 7B,C). It was consistent with previous findings that the 35S promoter does not respond to NaCl (Zhou *et al.* 2006, Sun *et al.*

2010) or HgCl₂ (Shen *et al.* 2011). Taken together, these results suggest that NaCl or HgCl₂ rapidly promote PM association of HsCIPK2.

Fig. 6. Effects of 200 mM NaCl and 1 μ M HgCl₂ on subcellular distribution of HsCIPK2-GFP or GFP at different time points (A to T). Dashed boxes in (A and K) show places for quantification of HsCIPK2-GFP signal intensities at the PM. Bars = 25 μ m. U and V - Quantification of PM-associated HsCIPK2-GFP signal intensities (U; n = 150 - 185 cells from 5 - 8 roots each; V; n = 95 - 121 cells from 5 roots each). Means $\pm$ SDs. Single and triple asterisks indicate significant differences at P < 0.05 and 0.001, respectively (Student's t -test; compared to the mock control).

Discussion

Recently, increasing evidence has shown that multiple CIPKs are involved in plant response to biotic and abiotic stresses. Previous studies have shown that overexpression of wild barley *HbCIPK2* in *Arabidopsis sos2/cipk24* mutants (Li *et al.* 2012) can rescue the salt-sensitive phenotype of *Arabidopsis* mutant *sos2-1*, indicating that CIPK2 and CIPK24 may have an overlapping function in salt tolerance. Whether CIPK2 really possesses a

significant role in the wild-type plant cell response to salt stress, however, remains unknown. Our findings showing that overexpression of *AtCIPK2* and *HsCIPK2* in *Arabidopsis* transgenic plants significantly enhances the tolerance of seed germination and plant growth to NaCl stress, together with the previous finding that expression of halophytic shrub *NtCIPK2* in *E. coli* cells enhances host cell tolerance to salt toxicity (Zheng *et al.* 2014),

Fig. 7. RT-qPCR analysis of transcriptional levels of endogenous *AtCIPK2* and exogenous *HsCIPK2-GFP*. *A* - The effect of mercury toxicity on the transcription of *AtCIPK2*. *B* and *C* - The effects of NaCl (*B*) and HgCl₂ (*C*) on the transcription of *HsCIPK2-GFP* in transgenic seedlings harboring *Pro35S:HsCIPK2-GFP*. Five-day-old vertically grown seedlings were treated with 200 mM NaCl (*B*) or 1 μ M HgCl₂ (*A* and *C*) in 0.5 $\times$ MS liquid medium for different time lengths as indicated. Shown are means $\pm$ SD. Double asterisks indicate significant difference at $P < 0.01$ (Student's *t* test; compared to the 0-min time point).

demonstrate that CIPK2 plays a significant role in plant salt tolerance. In addition, we also noticed that, based on seedling growth phenotype, the wild-type seedlings appear to be more sensitive to NaCl on 0.5 $\times$ MS agar plates (1 % sucrose) in our study than on 1 $\times$ MS agar plates (2 % sucrose) in the previous studies (Guo *et al.* 2004, Li *et al.* 2012).

In addition to the role of CIPK2 in plant tolerance to salt stress, CIPK2 functions in plant responses to heavy metals. Our findings showed that overexpression of *AtCIPK2* and *HsCIPK2* in *Arabidopsis* improved the tolerance of seed germination to Hg²⁺, Cu²⁺, and Cd²⁺. These transgenic lines, however, displayed an enhanced tolerance of plant growth only to Hg²⁺ but not to Cu²⁺ and Cd²⁺. Likely, CIPK2 played a specific role in response of seedling growth to Hg²⁺, but we cannot rule out the possibility that CIPK2 may function in responses to other heavy metals unexamined in this study.

Live-cell imaging revealed that 200 mM NaCl and 1 μ M HgCl₂ rapidly enhanced the PM association of *HsCIPK2-GFP*, indicating this as an important step for CIPK2 action mode during its mediation of stress signalling in the plant cell. Furthermore, our analyses of GUS staining and real-time qPCR showed that salt and mercury toxicities can activate the expression of *CIPK2*, but not that of 35S promoter-controlled *HsCIPK2-GFP*. These findings suggest that salt and mercury stresses may stimulate physiological functions of CIPK2 at both transcriptional and posttranscriptional levels.

Whether CIPK2 is involved in either heavy metal uptake or vacuolar detoxification or both remains to be experimentally elucidated. Further isolation and functional identification of *CIPKs* in different plant species and clarification of CIPK2 involvement in some biochemical and/or cellular processes of heavy metal toxicities will provide a better understanding of physiological roles of CIPKs during plant stress responses.

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