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RESEARCH NOTE

Arabis paniculata ApHIPP3 increases Cd tolerance by interacting with ApCHC1

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Abstract. Cadmium (Cd) is a common hazardous element that shows potential chronic toxicity in plants and animals. *Arabis paniculata* functioning as a hyperaccumulator has been found in the Yunnan–Guizhou plateau in China. This study characterizes the function of ApHIPP3 in *A. paniculata* to Cd stress, showing that the expression of ApHIPP3 was highly induced by Cd stress. *Arabidopsis thaliana* overexpressing ApHIPP3 showed stronger growth potential than wild type (WT). Cd accumulation capacity was significantly higher in transgenic *A. thaliana* than in WT. In addition, transgenic *A. thaliana* showed the ability to inhibit electrolyte leakage and eliminate reactive oxygen species (ROS). A strong interaction of ApHIPP3 with the clathrin heavy chain ApCHC1, which is known to play an important role in biotic and abiotic stresses, could be detected by a yeast two-hybrid assay. This was further confirmed by pull-down and co-immunoprecipitation (Co-IP) assays. Overall, these results demonstrate the function of ApHIPP3 to Cd stress and suggest a regulatory mechanism in response to Cd stress.

Keywords. ApHIPP3; Cd stress; transgenic *Arabidopsis thaliana*; *Arabis paniculata*.

Introduction

Cadmium (Cd) in soil is absorbed by root tissues and is transported to shoot tissues. Previous studies have shown that Cd is transferred into cells through transporters specific to nutrient elements (Zhang *et al*. 2018). In eukaryotic cells, Cd is chelated by proteins and small-molecule ligands, and the complex is transported by metallochaperones (Zhang *et al*. 2015; Khan *et al*. 2019). To avoid heavy metal accumulation in plant cells, metallochaperones, which are generally soluble proteins, can tightly bind metal ions and safely transport them through the cell (Zhang *et al*. 2015).

Heavy metal-associated isoprenylated plant protein (HIPP) has been identified as a large metallochaperone-like protein family in plants (Barth *et al*. 2009). HIPP is characterized by the presence of one or two heavy metal-associated (HMA) domains and an isoprenylation motif (Rubino and Franz 2012). Studies have shown that the HMA domain is involved in heavy metal binding (Hung *et al*. 1998; Dykema *et al*. 1999). In addition, the HMA domain participates in the transport of heavy metals to maintain heavy metal homeostasis. CdI19, which was identified as a Cd-binding protein, showed a high response to Cd stress (Suzuki *et al*. 2002). Similarly, HvFP1 in barley contains an HMA domain that was highly induced by multiple abiotic stresses (Barth *et al*. 2004).

Arabis paniculata is widely distributed in southwest China. It is regarded as a hyperaccumulator with the ability to accumulate Cd, Pb, Zn and Mn (Tang *et al*. 2009, 2016; 2020). Information on *HIPP* in *A. paniculata* is still limited. In this study, *ApHIPP3* was identified in *A. paniculata*, and the function of *ApHIPP3* to Cd stress was analysed in transgenic *Arabidopsis thaliana*. The protein that interacted with ApHIPP3 was further investigated and verified. This study determines a novel metallochaperone gene responding Cd stress and provides an ideal candidate with potential application in phytoremediation programmes.

Jie Liu and Sisi Liao contributed equally to this work.

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Materials and methods

Plant materials and growth conditions

A. paniculata was collected from Guiyang, Guizhou Province of China. *A. thaliana* of Columbia (Col) was used as the transgenic target. The seeds of *A. paniculate* were germinated in sterile double distilled H_2O and then grown in ½ Murashige & Skoog (MS) solution until the six-leaf stage. The plants were then transferred to a plastic pot with pearlite. The plants were divided into control and treatment groups. The control group was treated with ½ MS solution and the treatment group was treated with ½ MS solution containing 20 μM Cd in the form of $CdCl_2$. The seeds of *A. thaliana* were germinated and grown in ½ MS solid medium containing 10 or 30 μM Cd. All of the plants were grown in an incubator at 24°C under 16/8 h light/dark conditions. After a three-day treatment, *A. paniculate* plants were harvested and washed using double distilled H_2O and then frozen in liquid nitrogen. *A. thaliana* plants were treated for two weeks and then harvested for further analysis.

RNA preparation and ApHIPP3 cloning

Total RNA and cDNA were prepared from root and shoot tissues of *A. paniculate* according to Wang *et al*. (2021). The sequence of *ApHIPP3* was screened from the transcriptome data of *A. paniculate* produced by our group (https://bigd.big.ac.cn/gsub/; accession no. CRA002602). The open reading frame (ORF) of *ApHIPP3* was amplified using the primer F1+R1 (table 1 in electronic supplementary material at http://www.ias.ac.in/jgenet/). Vector construction and sequencing were performed according to Wang *et al*. (2021).

Sequence analyses of ApHIPP3 with published HIPPs

Multiple alignments of deduced amino acid sequences of *HIPP* were performed using DNAMAN software (v. 6.0.2). *HIPP* genes from *A. thaliana* (NM120417), *Raphanus sativus* (XP018472178), and *Brassica rapa* (XP009125502) were used in the multiple alignment assay.

Quantitative real-time fluorescence PCR (qRT-PCR)

The specific primer sequences for qRT-PCR of *ApHIPP3* were designed using the Primer 5.0 program (table 1 in electronic supplementary material). *β-actin* was used as the reference gene. The CFX96 fluorescence quantitative PCR detection system (Bio-Rad, Hercules, USA) was used for qRT-PCR experiments with the UltraSYBR One Step RT-qPCR kit (CWBio, Beijing, China). All data were analysed using the $2^{-\Delta\Delta}$CT method (Livak and Schmittgen 2001).

Generation of transgenic A. thaliana overexpressing ApHIPP3

The ORF of *ApHIPP3* was inserted into a plasmid (pCAMBIA3201), and transgenic *A. thaliana* lines were generated according to Peng *et al*. (2018). Transgenic lines were detected using PCR and western blot (Hou *et al*. 2017). The antibody against GUS (Invitrogen, Shanghai, China) was involved in the western blot assay. GUS histochemical assays and GUS activity detection were carried out according to Zhao *et al*. (2019).

Measurement of electrolytic leakage and antioxidant enzyme activity

Electrolyte leakage was analysed as described by Lutts *et al*. (1996). H_2O_2 and malondialdehyde (MDA) content were measured according to Zhang *et al*. (2020). Superoxide dismutase (SOD) activity, catalase (CAT) activity, and peroxidase (POD) activity were evaluated using the testing kit (Solarbio, Beijing, China).

Detection of Cd distribution and concentration in A. thaliana

Fluorescence detection of Cd in *A. thaliana* was performed using Leadmium Green AM Dye (Invitrogen, Shanghai, China) according to the operating instructions of the kit. The images were captured by a fluorescence microscope (TE2000, Nikon, Japan). Cd concentrations were measured using inductively coupled plasma mass spectroscopy (ICP-MS) according to Zhang *et al*. (2019).

Y2H, pull-down and Co-IP assays

Root tissues of the plants under 20 μM Cd stress was used for cDNA library construction. Then the cDNA library was constructed by a commercial corporation (Invitrogen, Shanghai, China). Y2H and pull-down assays were performed according to Wang *et al*. (2021). The Co-IP assay was carried out as described by Zhang *et al*. (2019).

Statistical analysis

Data were statistically analysed by *t*-test on the basis of three biological replicates.

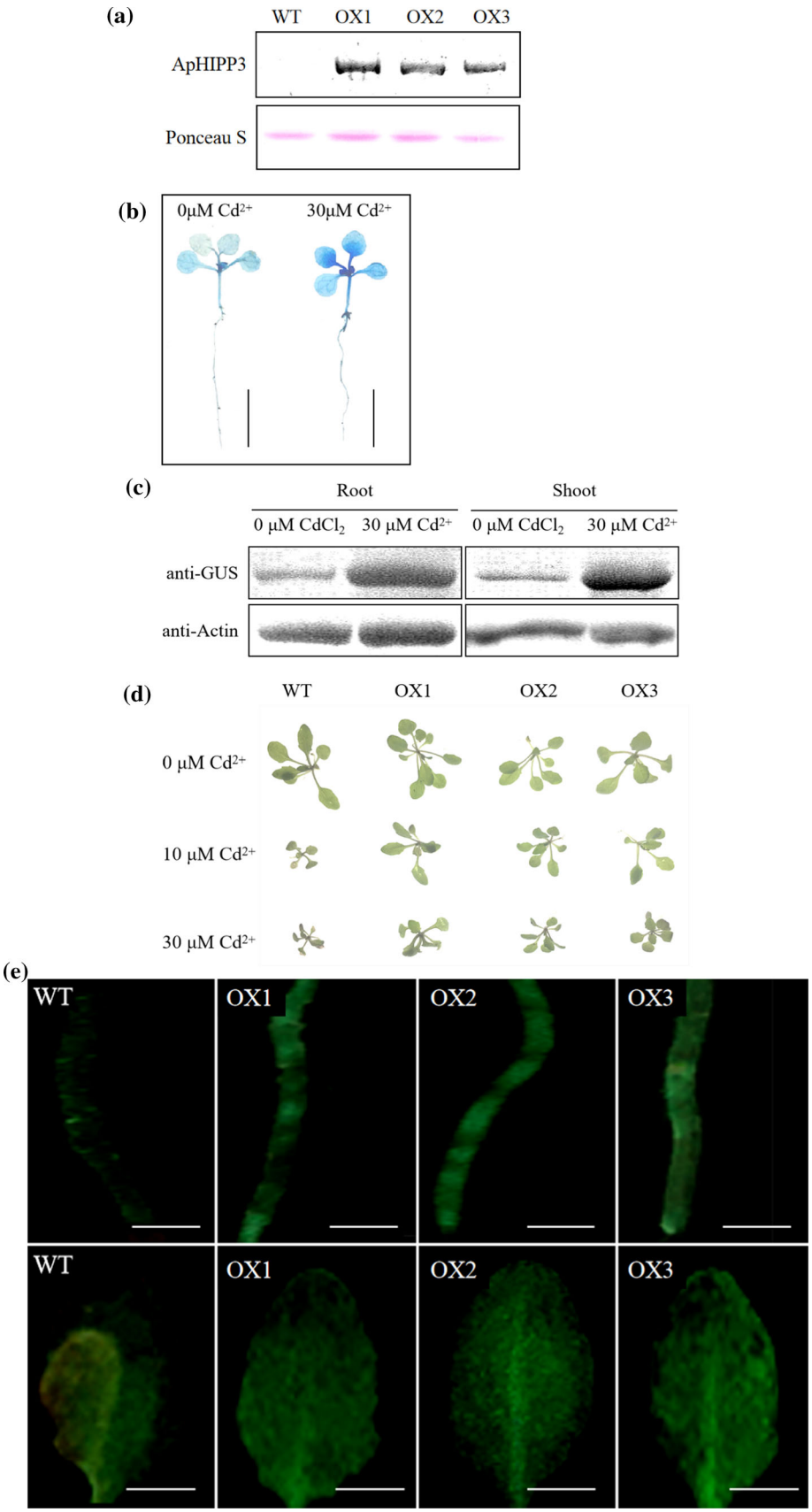
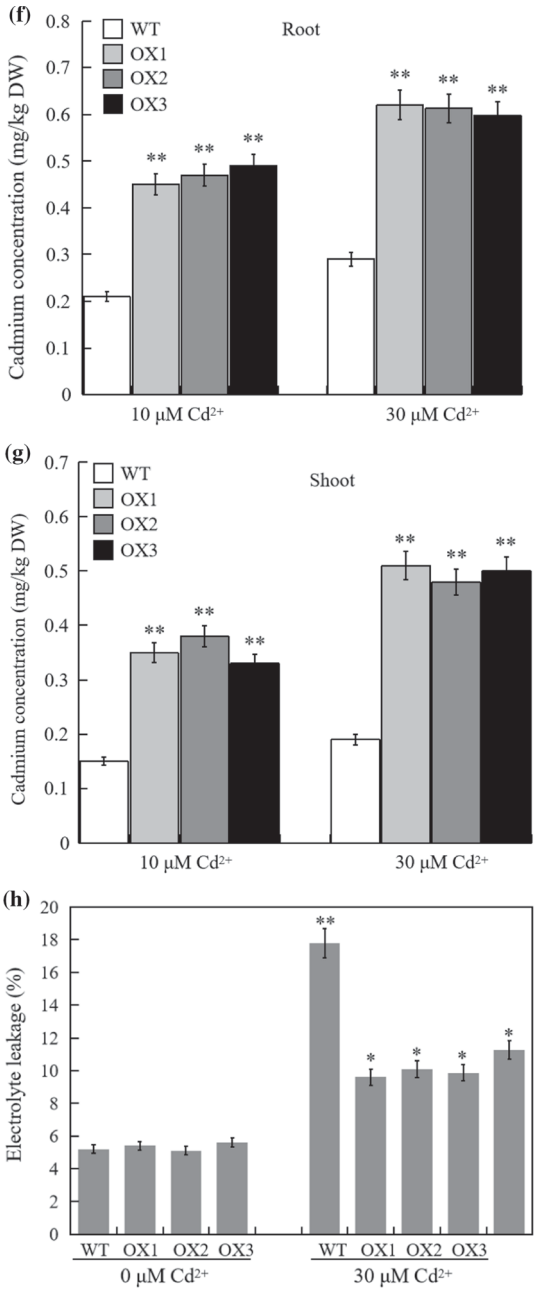


Figure 1. (*continued*)



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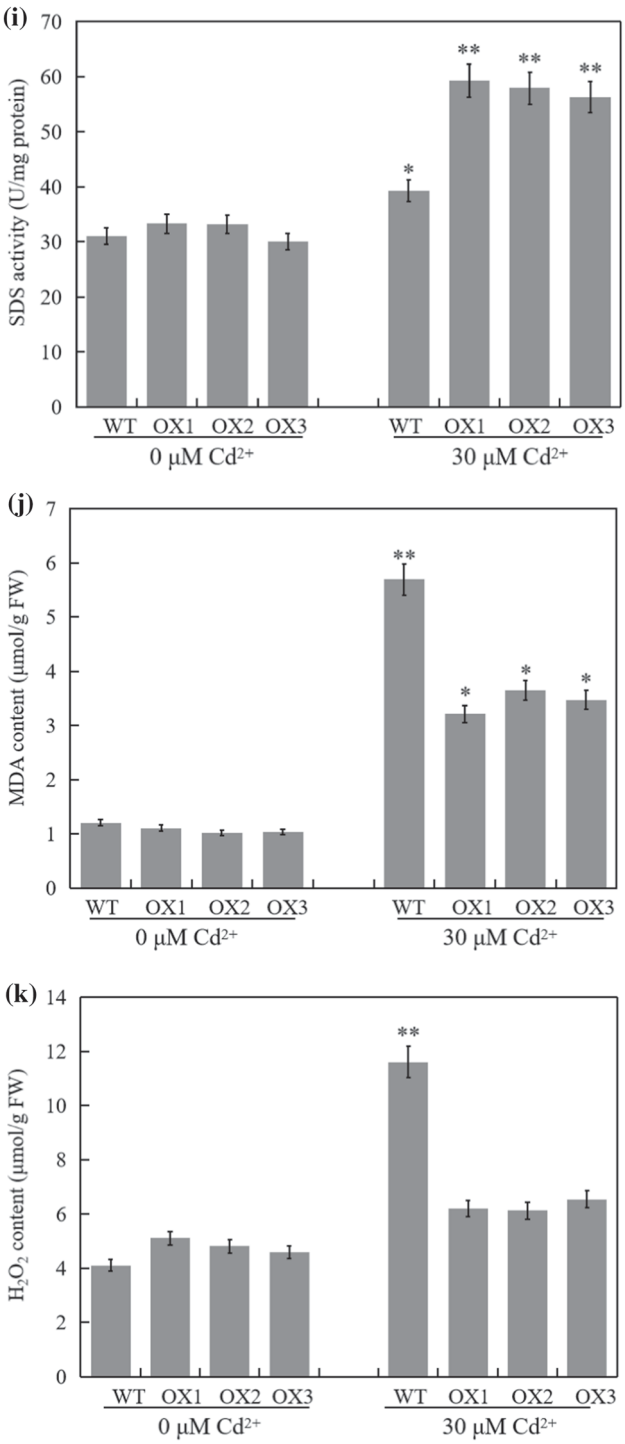

Figure 1. (*continued*)

Results

Cd-responsive expression of ApHIPP3 in A. paniculata

Multiple alignments were performed using the deduced amino acid sequence of *ApHIPP3* with *HIPPs* from three crucifer species: *A. thaliana*, *R. sativus* and *B. rapa* (figure 1a in electronic supplementary material). High homology and similarity of sequences suggest that *HIPP* genes were quite conservative in these species of the cruciferous family. Considering that *ApHIPP3* might show a different responsive expression to Cd stress in root and shoot tissues, we monitored the expression of *ApHIPP3* in both root and shoot tissues under 20 μM Cd stress. *ApHIPP3* was strongly upregulated upon Cd stress in these tissues

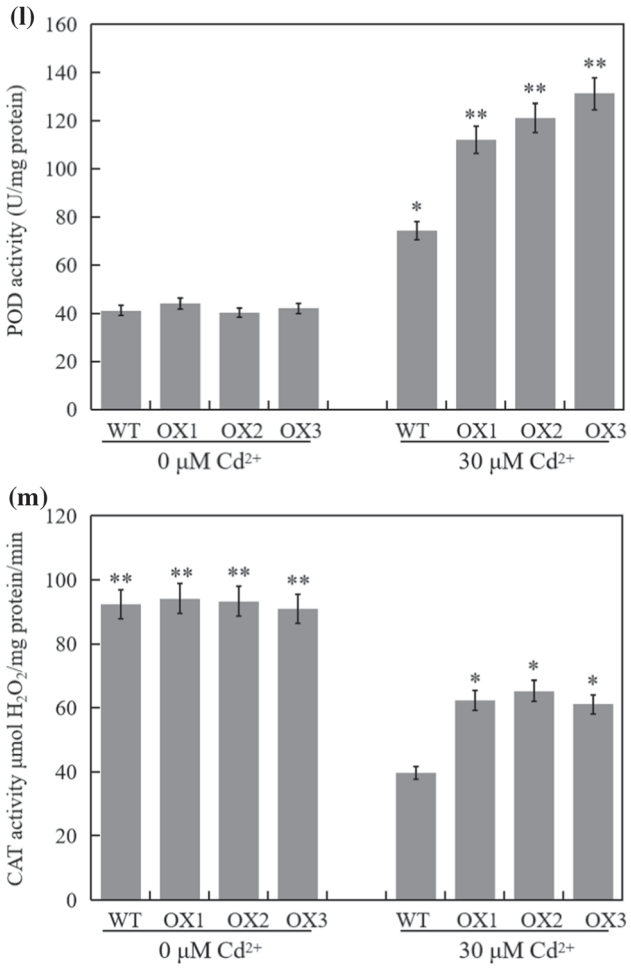

Figure 1. (a) Verified transgenic *A. thaliana* using western blot. The western blot assay was performed using the antibody against GUS. Rubisco was stained using Ponceau S. WT, wild type; OX, transgenic line. (b) GUS staining of transgenic *A. thaliana* under 0 μM or 30 μM Cd^{2+} stress. (c) Identification of GUS abundance in *A. thaliana* under 0 μM or 30 μM Cd stress by western blot. (d) Morphology of WT and transgenic lines under Cd^{2+} stress. (e) Fluorescence of Cd in root and leaf of WT and transgenic lines under 30 μM Cd^{2+} stress. (f) Cd concentration in root tissues of WT and transgenic *A. thaliana* under 10 μM or 30 μM Cd^{2+} stress. (g) Cd concentration in shoot tissues of WT and transgenic *A. thaliana* under 10 μM or 30 μM Cd^{2+} stress. (h) Electrolyte leakage of WT and transgenic *A. thaliana* under 0μM or 30 μM Cd^{2+} stress. (i) SDS activity of WT and transgenic *A. thaliana* under 0 μM or 30 μM Cd^{2+} stress. (j) MDA content of WT and transgenic *A. thaliana* under 0 μM or 30 μM Cd^{2+} stress. (k) H_2O_2 content of WT and transgenic *A. thaliana* under 0 μM or 30 μM Cd^{2+} stress. (l) POD activity of WT and transgenic *A. thaliana* under 0 μM or 30 μM Cd^{2+} stress. (m) CAT activity of WT and transgenic *A. thaliana* under 0 μM or 30 μM Cd^{2+} stress. The data are the means ± SD of three independent measurements from three individual plants. * And ** indicate significantly different at P=0.05 and 0.01, respectively.

(figure 1b in electronic supplementary material). Thus, *ApHIPP3* functions as a Cd-responsive gene in *A. paniculata*.

Overexpression of ApHIPP3 in A. thaliana enhances tolerance to Cd stress

To better define the function of the *ApHIPP3* gene to Cd stress, we produced *ApHIPP3* overexpressing lines in *A. thaliana.* Three transgenic lines were selected for further analysis (figure 1, a–c). To evaluate if *ApHIPP3* expression increases tolerance to Cd stress in *A. thaliana*, wild type (WT) and transgenic *A. thaliana* were grown under Cd stress for three weeks. In control conditions, WT and transgenic *A. thaliana* showed similar phenotype (figure 1d). However, under Cd stress conditions, the WT showed a decrease in biomass (figure 1d). Cd distribution and concentration were further investigated in transgenic *A. thaliana.* In the visualization analysis, Cd fluorescence was stronger in transgenic *A. thaliana* than in WT (figure 1e). The fluorescence intensity was consistent with the test results measured by ICP-MS. As shown in figure 1f, transgenic *A. thaliana* accumulated significantly more Cd than WT. These results demonstrate that *ApHIPP3* overexpression enhanced the tolerance of *A. thaliana* to Cd stress.

ApHIPP3 affects the physiological parameters of transgenic A. thaliana

Physiological parameters including electrolyte leakage, MDA content, H_2O_2 content, SOD activity, POD activity, and CAT activity were measured in WT and transgenic *A. thaliana* under Cd stress. After Cd treatment, electrolyte leakage, MDA content, and H_2O_2 content substantially decreased in transgenic *A. thaliana* than in WT (figure 1, h, j & k); however, SOD activity, POD activity, and CAT activity were significantly higher in transgenic *A. thaliana* than in WT.

ApHIPP3 interacts with ApCHC1

To investigate the regulatory mechanism of *ApHIPP3* involved in Cd stress response, a Y2H assay was performed to identify ApHIPP3-interacting proteins. A clathrin heavy chain ApCHC1 was identified. Yeast cells coexpressing ApHIPP3-BD and ApCHC1-AD grew on an SD/-Trp/-Leu/-His/-Ade screening medium (figure 2a), indicating that ApHIPP3 interacts with ApCHC1 in yeast cells. Pull-down and Co-IP assays were performed to further verify the interaction between ApHIPP3 and ApCHC1. These results show that ApHIPP3-HIS interacted with ApCHC1-GST but not with GST alone (figure 2, b&c). In sum, ApHIPP3 interacted with ApCHC1 *in vivo* and *in vitro*. Finally, we investigated the expression of *ApCHC1* under 20 μM Cd stress. As shown in figure 2d, *ApCHC1* expression was highly induced by Cd stress in root and shoot tissues. Thus,

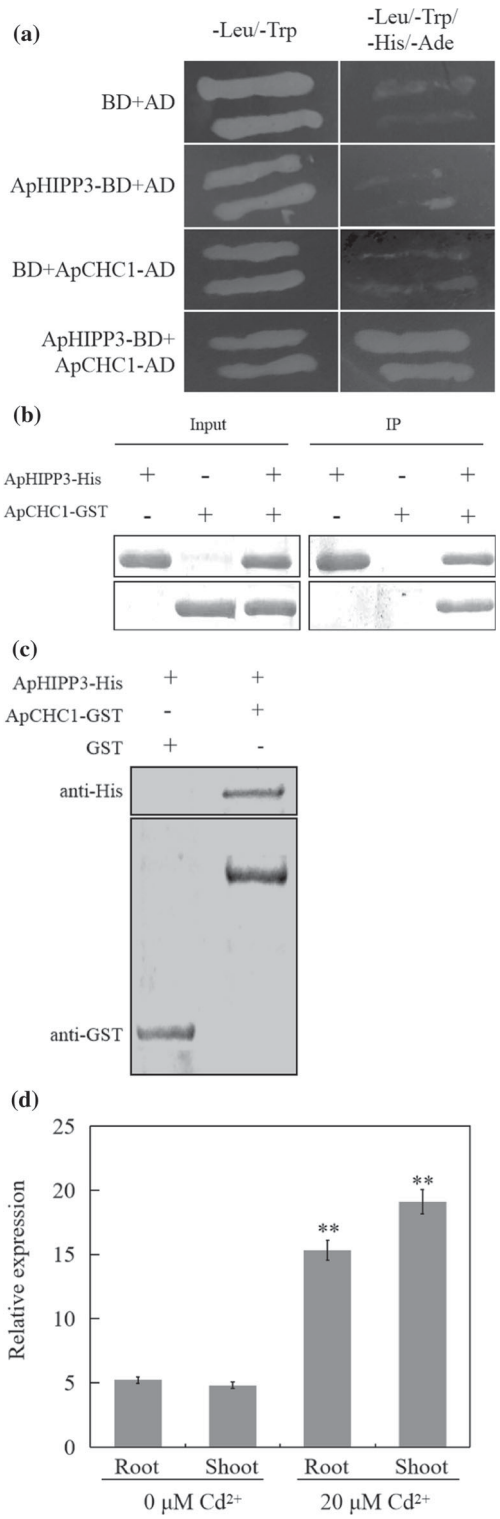

Figure 2. Identifcation of ApHIPP3 interacts with ApCHC1 by (a) yeast two-hybrid assay, (b) pull-down, and (c) co-IP. (d) Expression profiles of *ApCHC1* in response to Cd stress. The data are the means ± SD of three independent measurements from three individual plants. ** Significant differences at P=0.01.

ApCHC1 interacts with ApHIPP3 and formed a novel pathway, which is involved in the regulation of Cd responsive.

Discussion

HIPPs are special metallochaperones involved in heavy metal response in plants (DeAbreu-Neto *et al*. 2013). To date, only a few plant *HIPPs* have been functionally described. *A. paniculata*, which is widely distributed in the mining areas of the Yunnan–Guizhou plateau in China, can accumulate heavy metals (Tang *et al*. 2009, 2016, 2020). However, the function of *A. paniculata HIPP* genes in response for Cd stress is currently unclear. In this study, besides isolating *ApHIPP3* from *A. paniculata* and verifying its relation to Cd tolerance in transgenic *A. thaliana*, we demonstrated that ApHIPP3 interacts with ApCHC1.

A characteristic of HIPP is that it possesses an HMA domain and an isoprenylation motif at the C-terminal end (Khan *et al*. 2019). Both HMA domain and isoprenylation motif in one protein are only found in vascular plants (Barth *et al*. 2009). Most HIPPs studied so far have been shown to be directly involved in heavy metal homeostasis. OsHIPP34, OsHIPP60 and OsHIPP16 from rice were found to be involved in Zn, Cu and Cd tolerance (Khan *et al*. 2019). *A. thaliana AtHIPP06* expression was highly induced by Cd, Hg, Fe and Cu. In addition, *AtHIPP26*, a homologous gene of *AtHIPP06*, was promoted by Cd and Zn (Barth *et al*. 2009; DeAbreu-Neto *et al*. 2013). The present study demonstrated that overexpression of *ApHIPP3* increases Cd tolerance and accumulation in *A. thaliana* (figure 1, d–g), suggesting that *ApHIPP3* serves an important function in heavy metal stress. It is well known that in biological systems, reactive oxygen species (ROS) accumulation is related to homeostasis (Suzuki *et al*. 2012). In this study, ROS levels were analysed to elucidate the physiological mechanism underlying enhanced Cd tolerance (figure 1, h–m). The present study also indicates that overexpression of *ApHIPP3* is effective in eliminating ROS produced during Cd stress.

A previous study showed that AtHIPP26 strongly interacts with AtATHB29, a zinc-finger homeodomain transcription factor (Barth *et al*. 2009). However, in the present study, the Y2H assay indicated that ApHIPP3 interacts with the clathrin heavy chain ApCHC1 (figure 2a). This was further confirmed by pull-down and Co-IP assays (figure 2, b&c). *CHC* has been identified as an important gene involved in multiple biotic and abiotic stresses (Wu *et al*. 2015; Larson *et al*. 2017), but information on *CHC* in response to heavy metal stress is still limited. Thus, this study also revealed a novel mechanism of *A. paniculata* in response to Cd stress.

In conclusion, ApHIPP3 interacts with ApCHC1 and increases Cd tolerance and accumulation in *A. thaliana*. This study also provides a new genetic resource and promising strategy for phytoremediation.

Acknowledgements

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