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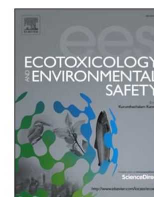

Effects of Ca addition on the uptake, translocation, and distribution of Cd in Arabidopsis thaliana

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A B S T R A C T

Cadmium (Cd) pollution poses a risk to human health for its accumulation in soil and crops, but this can be alleviated by calcium (Ca) addition. However, its mechanism remains unclear yet. In this study, *Arabidopsis thaliana* was used to explore the alleviating effects of Ca on Cd toxicity and its specific function during uptake, upward-translocation, and distribution of Cd. Supplementing plants with 5 mM $CaCl_2$ alleviated the intoxication symptoms caused by 50 μM $CdCl_2$, such as smaller leaves, early bolting and root browning. Ca addition decreased uptake of Cd, possibly by reducing the physical adsorption of Cd since the root cell membrane was well maintained and lignin deposition was decreased as well, and by decreasing symplastic Cd transport. Expression of the genes involved (*AtZIP2* and *AtZIP4*) was also decreased. In addition, Ca accumulated in the plant shoot to help facilitating the upward-translocation of Cd, with evidence of higher translocation factor and expression of genes that were involved in Ca transport (*AtPCR1*) and Cd xylem loading (*AtHMA2* and *AtHMA4*). Dithizone-staining of Cd in leaves showed that in Cd + Ca-treated plants, Ca addition initially protected the leaf stomata by preventing Cd from entering guard cells, but with prolonged Cd treatment facilitated the Cd accumulation around trichomes and maybe its excretion. We conclude that Ca promotes the upward-translocation of Cd and changes its distribution in leaves. The results may have relevance for bioremediation.

1. Introduction

Cadmium (Cd) is a non-essential element that is highly toxic and persistently poisonous to plants and animals. Accumulation of Cd often results in visible plant symptoms, such as inhibited growth, chlorosis, browning of roots, or death of the entire plant. These Cd-induced toxic injuries are largely attributed to interference of other ions by Cd, such as uptake and translocation of ions, stronger affinity to Cd, and substitution of the binding site within the protein, eventually leading to metabolic dysfunction (Clemens et al., 2013; Wang and Björn, 2014). According to the newly published 2014 Report on China's Soil Pollution Survey, Cd content in about 7% of the land in China has exceeded the criteria, which was increasing annually in the past century, and Cd has been formally identified as a primary pollutant. Yang et al. (2013) reported that Cd concentrations in over half of the soil in Guangdong Province of China exceeded 0.05 mg kg^{-1}. Therefore, abatement of Cd pollution has aroused worldwide concern.

Calcium (Ca) is an essential element involved in most physiological processes in the growth and development of plants. Numerous studies have indicated that Ca plays a vital role in plant stress resistance, such as drought (Chen et al., 2013) and low temperature (Zhang et al., 2014). Ca alleviation of Cd toxicity has been described for many plant species. For example, by addition of Ca, the inhibition of photosynthesis and respiration was reduced (Andosch et al., 2012), and antioxidant enzyme activities were well maintained to avoid generation of reactive oxygen species and malondialdehyde (Farzadfar et al., 2013). As a result of Ca addition, glutathione synthesis was increased (López-Climent et al., 2014), while auxin level was stable (Li et al., 2016) and ionic disorders resulted from Cd were alleviated (Min et al., 2012). Besides, trichome was supposed to be a pathway that plants would used to actively exclude toxic metals by forming and excreting Ca-containg crystals, like Cd in tobacco plants (Choi et al., 2001; Choi and Harada, 2005) and Zn in *Arabidopsis* (Sarret et al., 2006).

Most of these findings focused on the physiological aspects. Cadmium is a non-essential element for plant growth; thus, it has no specific transporters in plants. Cd is taken up via essential divalent

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cation transporters, such as the *AtIRT1* transporter for Fe and Cd (Thomine and Schroeder, 2000; Gallego et al., 2012; Romè et al., 2016), along with the ZIP family for metal transport (Guerinot, 2000) and can be loaded from the symplast into the xylem via heavy metal P1B-ATPases, such as *AtHMA2* and *AtHMA4* (Hussain et al., 2004; Mills et al., 2012; Wong and Cobbet, 2009).

It is generally believed that Cd and Ca compete for absorption sites on the root cell membrane, as they have a similar ionic radius and the same charge; hence, Ca has a great inhibitory effect on Cd absorption (Andersson and Nilsson, 1974). However, this notion lacks any direct support from experimental data. Whether Ca alleviates the toxic effects of lead (Pb), a non-essential element, remains controversial (Kim et al., 2002; Carro et al., 2015; Rodriguez-Hernandez et al., 2015). Hence, whether the actions of Cd are coordinated with Ca and how Ca helps to alleviate the Cd-induced toxicity requires more investigations. Model plant *A. thaliana* was used to explore the alleviating effect of Ca and its specific function during the uptake, translocation, and distribution of Cd in present study.

2. Materials and methods

2.1. Culture conditions, treatment and biomass measurement

Arabidopsis thaliana (Columbia, Col-0) plants were cultured hydroponically for 4 weeks (Conn et al., 2013) under short-day conditions (8 h light: 100 µmol photons m^{-2} s^{-1} /16 h dark). The plants were grown in a common nutrient solution (2 mM NH_4NO_3, 3 mM KNO_3, 0.1 mM $CaCl_2$, 2 mM KCl, 2 mM $Ca(NO_3)_2{\cdot}4H_2O$, 2 mM $MgSO_4{\cdot}7H_2O$, 0.6 mM KH_2PO_4, 1.5 mM NaCl, 50 µM NaFe(III)EDTA, 50 µM H_3BO_3, 5 µM $MnCl_2{\cdot}4H_2O$, 10 µM $ZnSO_4{\cdot}7H_2O$, 0.5 µM $CuSO_4{\cdot}5H_2O$, 0.1 µM Na_2MoO_4) and were divided into the control groups; 5 mM Ca-treated plants were the negative control group; plants exposed to 50 µM Cd by adding $CdCl_2$ were Cd-treated group; and plants treated with an additional 5 mM Ca by adding $CaCl_2$ were Cd + Ca-treated group. Pre-tests were conducted to confirm the appropriate Ca concentrations. The nutrient solution was adjusted to pH 5.8 with 0.1 M NaOH. There were five plants in each pot and more than three replicates in each group. Data regarding root length and fresh and dry weights of shoots and roots were recorded. Ten plants were used for one measurement to reduce the error caused by weighing in each treatment (n = 5).

2.2. Photosynthesis measurements

The behavior of photo-system II was assessed and recorded by Chlorophyll Fluorescence Imager (Technologica Ltd., Colchester, UK). Chlorophyll fluorescence was monitored on the whole plants after treatment of 7 and 12 days. Fluorescence measurements were conducted between 9 and 11 a.m. with a pre-adaptation in dark for 15 min at room temperature.

Stomatal conductance and transpiration rate were measured 4 days after application of the treatment, on the fully expanded leaves of plants by Li-6400 XT Portable Photosynthesis System (Li-Cor Biosciences, USA) in the growth chamber where the light was 100 mmol m^{-2} s^{-1}, relative humidity was 58% and atmospheric CO_2 was 400 $mmol^{-1}$.

2.3. Root lignin staining and quantification of plasma membrane integrity

Samples were rinsed in distilled water for 5 min and then incubated in a 1% (w/v) phloroglucinole-HCl solution for 5 min (Rogers et al., 2005), rinsed in distilled water for 3 times and then observed immediately using bright field microscopy (Nikon, Eclipse 50i, Japan).

Uptake of Evans blue is an indicator of cell death. Plasma membrane integrity was evaluated by a spectrophotometric assay to determine Evans blue retained by cells (Baker and Mock, 1994; Ikegawa et al., 2002). Harvested roots were rinsed in distilled water for 5 min and immersed in the 2 ml 0.25% Evans blue solution and stained for 15 min at room temperature. Roots were then washed 3 times with distilled water until the dye was no longer eluted, then dried with absorbent paper. The trapped Evans blue in roots was released by immersing the roots in 100% DMF for 1 h at room temperature. The extract was diluted to 3 ml with 100% DMF and later determined spectrophotometrically at 600 nm.

2.4. Element detection and subcellular distribution

After 7 days of treatment, the plants were harvested and washed thoroughly with tap water and dipped in distilled water for 30 s three times. The plants were separated into roots and shoots and dried at 80 °C for 48 h to constant weight and further ground into a powder. The detection of the target elements were based on the method described by Qiu et al. (2011). Briefly, the samples were digested in a mixture of 6 ml (5:1 [v/v]) HNO_3 and H_2O_2 in a microwave oven (Microwave Digestion System WX-8000; Peekem, Shanghai, China), and the concentrations of Ca and Cd, , K, Mg, Mn and Fe, were determined by flame atomic absorption spectrophotometry (FAAS; Z-2300; Hitachi, Tokyo, Japan). The translocation factor (TF) (Hart et al., 1998) was calculated using the following equation to identify the characteristics of Cd transport from roots to shoots: $TF = C_{shoot}/C_{root}$, where C_{shoot} and C_{root} are the Cd content in shoots and roots, respectively.

The subcellular distributions of Cd and Ca in shoots and roots, including the cell wall, soluble fraction, and cell organelles were separated and determined using the methods as suggested by Weigel and Jager (1980) and Lozano-Rodriguez et al. (1997) with some modifications. The main technique of this method is the differential centrifugation, using sucrose extraction buffer, several centrifugal speeds and times. But recently it was reported to have the risk for resulting in contamination, since all cell components were into close contact during the process of isolation (Küpper and Andresen, 2016).

Samples of each frozen root and shoot tissue were homogenized in a pre-cooled extraction buffer (50 mM Tris-HCl, 250 mM sucrose, and 1.0 mM $C_4H_{10}O_2S_2$, pH 7.5) with a chilled mortar and a pestle. The homogenate was centrifuged at 340g for 10 min. The residue contained mainly cell walls and cell wall debris. The resultant supernatant solution was centrifuged at 19890g for 45 min. The supernatant solution was referred to as the soluble fraction (including the cell sap) and the pellet was taken as the organelle fraction. All of the steps were performed at 4 °C and later all isolated fractions were dried at 80 °C to constant weight according to Zhu et al. (2013) and the following procedures were previously described.

2.5. In situ Cd localization

Histochemical determination of cadmium localization was performed according to Seregin and Ivanov (1997) by staining the leaves and roots with dithizone (diphenylthiocarbazone).

Plants were harvested and seperated, then stained with dithizone solution (30 mg dissolved in 60 ml acetone and 20 ml distilled water) for 1.5 h, rinsed in distilled water, and one whole leaf or intact root were picked and observed immediately under bright field microscopy (Nikon, Eclipse 50i, Japan). The staining process was started from the 1st day of treatment till 7th day.

2.6. Gene expression analysis

Total RNA was extracted from 100 mg of ground frozen root and leaf samples with TRIzol reagent (Qiagen, Hilden, Germany), according to the manufacturer's protocol. RNA concentration was determined by measuring optical density at 260 nm. First-strand cDNA was synthesized with the PrimeScript RT Reagent Kit and the gDNA eraser (Takara Bio, Shiga, Japan). A 10-fold dilution of cDNA was made in 1:10 diluted TE buffer (1 mM Tris-HCl, 0.1 mM Na_2-EDTA, pH 8.0; Sigma-Aldrich, St. Louis, MO, USA) and stored at −20 °C. The quantitative real-time

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