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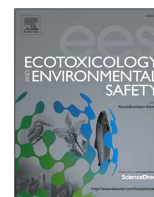

Effects of Ca addition on the uptake, translocation, and distribution of Cd in Arabidopsis thaliana

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A R T I C L E I N F O

Keywords:
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Cd uptake and translocation
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Exogenous Ca

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

polymerase chain reaction analysis was performed in optical 96-well plates using the ABI 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA), SYBR® Premix Ex Taq™ (Takara Bio), and the following steps: pre-incubation at 95 °C for 10 min, 45 cycles at 95 °C for 30 s, 95 °C for 3 s, and 60 °C for 34 s, according to the manufacturer's recommendations.

The primers for NCBI Primer-Blast were listed in Sup–Table 1. The expression of target genes was normalized relative to that of the APT1 housekeeping gene (Gutierrez et al., 2008), and the results were calculated using the $2^{-\Delta\Delta CT}$ method, in which data are presented as the fold change in gene expression normalized to an endogenous reference gene and relative to the untreated control (Livak and Schmittgen, 2001).

2.7. Statistical analysis

Statistical analyses were carried out using SPSS statistical software (SPSS Inc., Chicago, IL, USA). There were five replicates in most tests, except for the gene expression (n = 3). One-way analysis of variance followed by Tukey's multiple comparison tests was performed to determine the differences between means. A $P < 0.05$ was considered significant.

3. Results

3.1. Effect of Ca on the physiology of *A. thaliana* under Cd stress

With Ca addition (5 mM), Cd-induced toxic symptoms, such as smaller leaves, early bolting and root browning were ameliorated to some extent (Fig. 1) and the biomass of these plants was also increased compared to plants with Cd but no Ca addition (Table 1). In addition, Cd + Ca-treated plants showed longer primary roots, an increase of 23% compared to 8.6 cm length in the Cd-treated group (Table 1 and Sup–Fig. 1). Lignin in cell wall was stained red to show its content., After applied various treatments, we mostly focus on this newly-developed part. Root tips of Cd-treated plants were stained partially red compared to those in the control group while no lignin deposits were detected in the root tips of Cd + Ca-treated plants (Fig. 2). Additionally, Cd damaged the root cell membrane according to the results of the uptake of Evans blue. Absorbance (OD 600) for Cd-treated plants was 0.91 ± 0.040; while for Cd + Ca treated plants, it was 0.53 ± 0.047, quite close to the control group (0.54 ± 0.088) and the Ca-treated plants (0.62 ± 0.063).

3.2. Uptake of Cd and Ca

In Cd + Ca treatment, Cd contents in shoot and root were decreased by 55.6% and 66.4%, respectively, compared to that in Cd-treated plants (Fig. 3A). Further analysis of the subcellular fraction contents (cell wall, soluble fraction, and cell organelles) in Cd + Ca-treated plants showed that the quantity of Cd decreased in each of these three fractions (Fig. 3C), while Cd content in the cell wall fraction of roots was lowered by 86.5% with Ca addition. Additionally, in Cd-treated plants, Cd was retained mainly in the root, especially in the rhizodermis and root hair, but in the Cd + Ca-treated roots we could seldom find such a large amount of Cd (Sup–Fig. 2). Root Ca content was unaffected by the Cd treatment, regardless of whether Ca was added (Fig. 3B), indicating that the affinity or adhesion of Ca to roots was not high, though there was an increase in the root cell organelles of Cd + Ca-treated plants (Fig. 3D).

We also detected Ca and Cd in the apoplastic and symplastic saps of roots (Sup–Fig. 3). The symplastic pathway was less involved in the transport of Cd in Cd + Ca-treated plants, whereas it was the dominant pathway to transport Ca. Additionally, we found that Cd was actively absorbed through the transporter *ZIP2* and *ZIP4* under Cd stress for their expression were increased by 10-fold and 193-fold respectively, but in Cd + Ca-treated plants *ZIP2* and *ZIP4* expression decreased significantly whereas *IRT1* expression dropped slightly (Fig. 4A).

3.3. Cd translocation

The Cd TF was 0.43 ± 0.041 in Cd-treated plants, and higher (0.57 ± 0.083) in Cd + Ca-treated plants, indicating that Ca improved transport of Cd from roots to shoots, although the amount taken up decreased. The heavy-metal-associated domain (HMA) transporter family, particularly HMA2 and HMA4, are involved in xylem loading of Cd in plants. *HMA2* and *HMA4* expression increased significantly in Cd-treated plants but increased less in Cd + Ca-treated plants (Fig. 4B). The reduction was significant and the amount of Cd in Cd + Ca-treated plants was also significantly lower. It can be inferred that Ca increased the accumulation of Cd in the above-ground parts. Additionally, shoot Ca content in Cd + Ca-treated plants was increased (Fig. 3B), with a significantly higher TF (7.14 ± 0.11). PCR1 is involved in radial transport of Ca in roots and its further upward translocation. Expression of *PCR1* in the leaves of Cd + Ca-treated plants was also increased significantly (Fig. 4B). These results suggest an important role for Ca in above-ground plant parts.

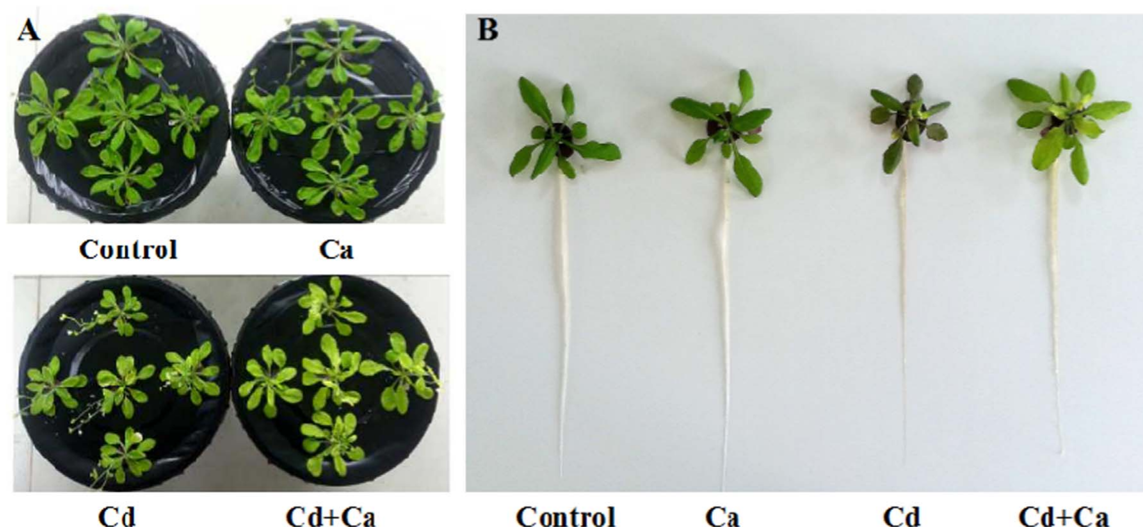

Fig. 1. A. Four-week-old *Arabidopsis* were exposed to $CdCl_2$ (50 μM) for 7 days with or without supplemental $CaCl_2$ (5 mM). B. Morphological changes of *Arabidopsis* plants induced by Cd exposure indicating root growth inhibition.

Table 1
Weight of the shoot and root, respectively, and primary root length of *Arabidopsis* under different treatments.

	Shoot (mg/plant)		Root (mg/plant)		Root length (cm)
	Fresh weight	Dry weight	Fresh weight	Dry weight	
Control	28.54 ± 1.06 a	1.93 ± 0.05 ab	3.47 ± 0.26 a	0.22 ± 0.01 a	15.62 ± 1.29 a
Ca	26.88 ± 1.73 ab	2.03 ± 0.17 a	3.63 ± 0.30 a	0.21 ± 0.02 a	15.80 ± 1.21 a
Cd	17.56 ± 0.89c	1.27 ± 0.11 b	2.55 ± 0.32 a	0.13 ± 0.09 b	8.61 ± 1.43c
Cd + Ca	22.06 ± 1.73 BCE	1.67 ± 0.19 ab	2.87 ± 0.32 a	0.19 ± 0.03 ab	10.63 ± 1.35 b

Different letters indicate significant difference between different treatments ($P < 0.05$). Data are means ± SE (n = 5).

3.4. Cd distribution

Dithizone staining clearly revealed the occurrence of Cd in plants. Cd was mainly deposited in guard cells of stomata after plants exposed to Cd for 3 days (see Fig. 5 Cd (a)), whereas Cd was distributed randomly in the inner tissues of Cd + Ca-treated plants except for accumulation in the intercellular space around the trichomes (Fig. 5 Cd + Ca (a)). These results are consistent with the differences in stomatal conductance (Gs) and transpiration rate (Tr). Gs and Tr decreased dramatically by 88% and 83%, respectively, in Cd-treated plants compared with those in the control group, but they were increased significantly in Cd + Ca-treated plants (see Sup–Fig. 4), indicating the alleviating effect of Ca addition.

Minimal precipitation was observed in young leaves of Cd-treated plants on day 6 but was clearly presented in its mature leaves. In contrast, in Cd + Ca-treated plants, Cd precipitation was observed in most leaves, but more in mature leaves and always accumulated around the trichome, later formed a ring in the base of it. Subsequently, Cd was eliminated as treatment was extended (Fig. 6).

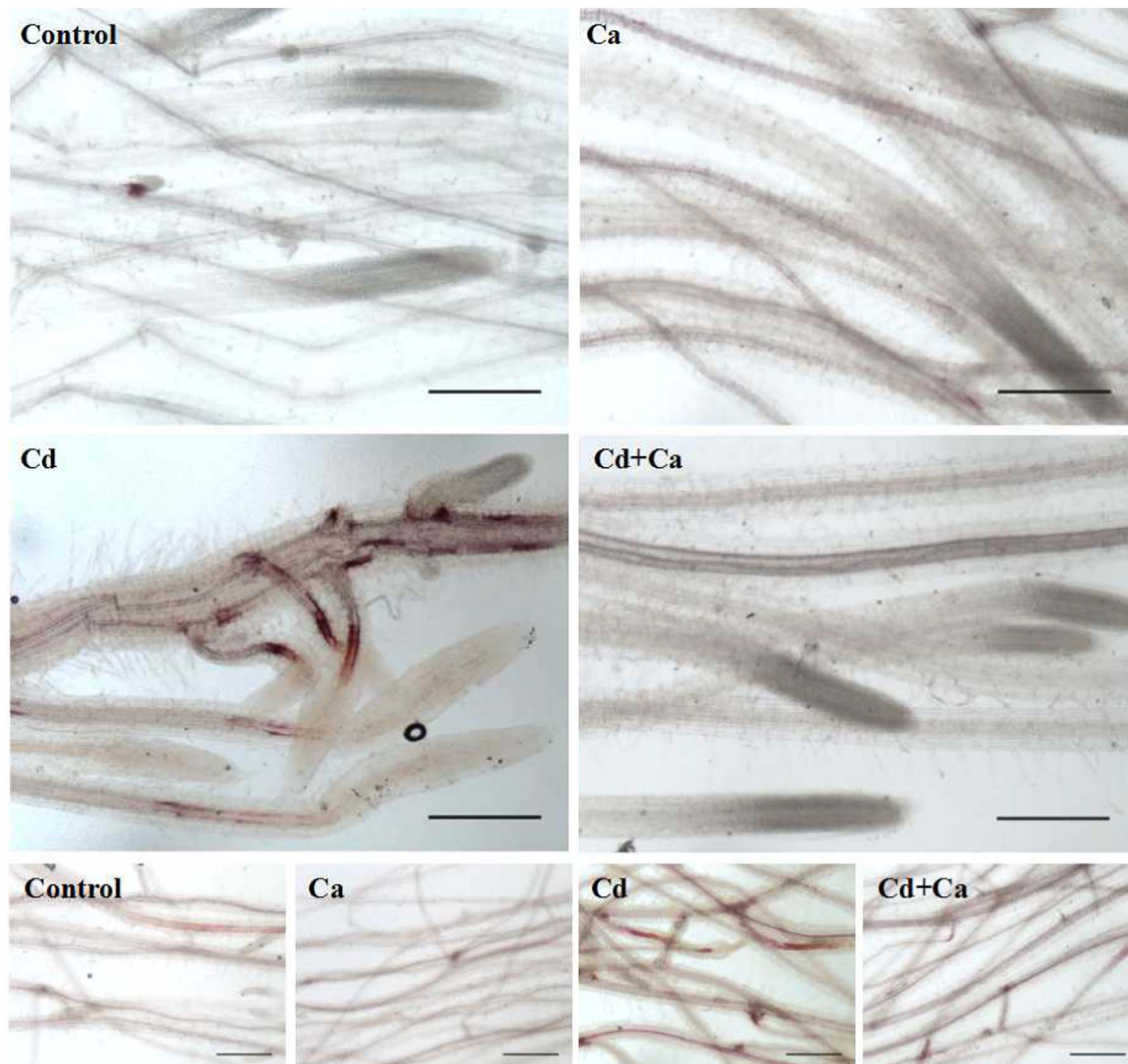

Fig. 2. Effects of Cd and Ca on the lignin deposition in *Arabidopsis* root. Red-stained zone showed the lignin deposition. The lower row displayed the root system above the newly-born root tip of different treatments. Scale bar = 50 µm.

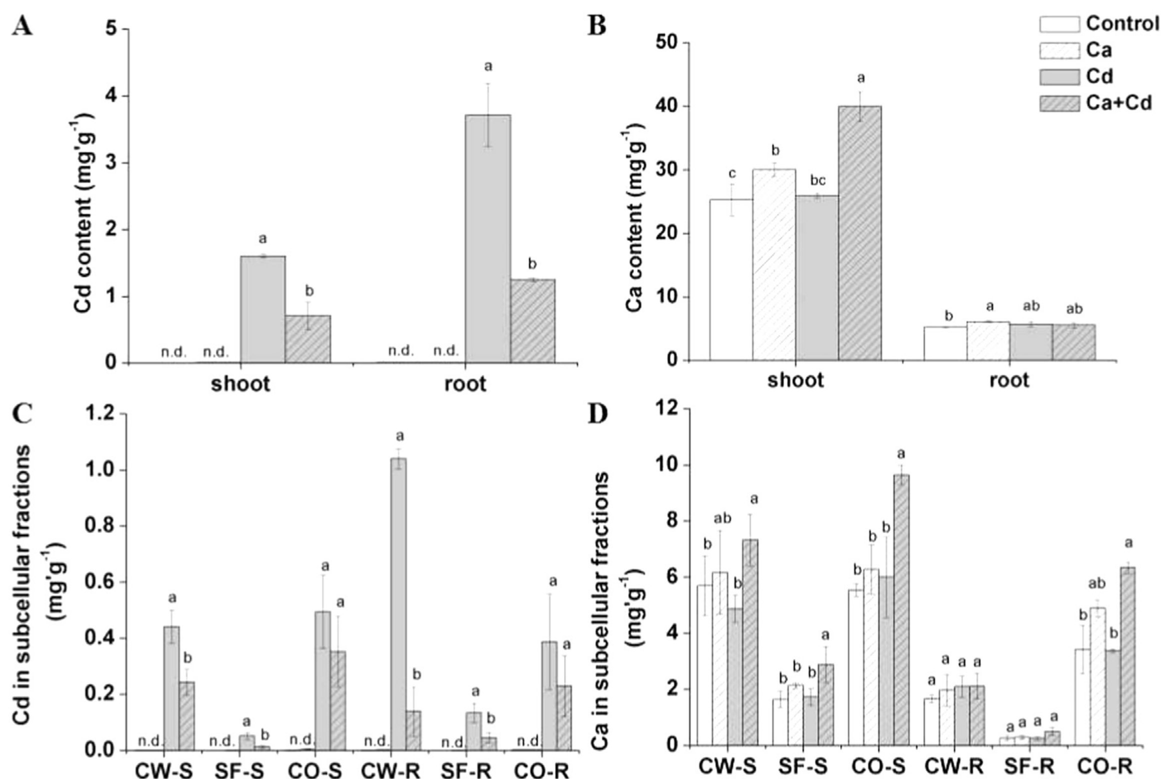

Fig. 3. Contents of Cd and Ca in *Arabidopsis* after 7 days treatment. A and B, show Cd and Ca contents in the shoots and roots, respectively. C and D show Cd and Ca contents in subcellular fractions, CW (cell wall), SF (soluble fraction), CO (cell organelle), S (Shoot), R (Root). Data are means ± SE (n = 5). Means labeled with different letters are significantly different according to Tukey's multiple comparison tests ($P < 0.05$). n.d. stands for not detected.

4. Discussion

4.1. Effects of Ca addition on Cd uptake and accumulation

It is believed that Ca has an inhibitory effect on Cd absorption because of competition for binding sites (Andersson and Nilsson, 1974), or well appretiated that Cd^{2+} participates in a number of Ca^{2+}-dependent pathways (Choong et al., 2014).

In Cd-treated plants, Ca content was not reduced neither in the shoot nor in the root when camparing to those in the non-Cd treatment (control and Ca-treated plants). It could be concluded that Cd would not interfere the absorption of this macro-element Ca. But there was a subtle change in other divalent cations, even a slight increase in the shoot Mg content and root Fe content in the Cd-treated plants(Sup–Fig. 5). We could not exclude the possible competition between Cd and Ca, since the variation could not be detected due to the difference in their organ content amounting to 1 order of magnitude. However, it seemed that the competition between different ions were not strong during the absorption.

It is certain that Cd content of Cd + Ca-treated plants was much lower than that of Cd-treated plants but the role of the Ca addition played in the uptake of Cd remains unclear. The cell wall is the first barrier to protect protoplasts from heavy metal toxicity. Plants exposed to heavy metals tend to increase suberin and lignin contents (Schreiber et al., 1999). Lignin is mainly found in the cell wall of the inner and outer cortex of the roots, and also plays an important role to regulate the absorption of water and mineral elements (Baxter et al., 2009). Under Cd stress, the expression of genes involved in lignin synthesis and cell-elongation were up-regulated in Arabidopsis thaliana (Cd-sensitive plant) compared to Thlaspi caerulescens (Cd-tolerant plant) (Van De Mortel et al., 2008). Accumulation of lignin in the root tips and loss of root cell membrane integrity in Cd-treated plants indicated the stronger

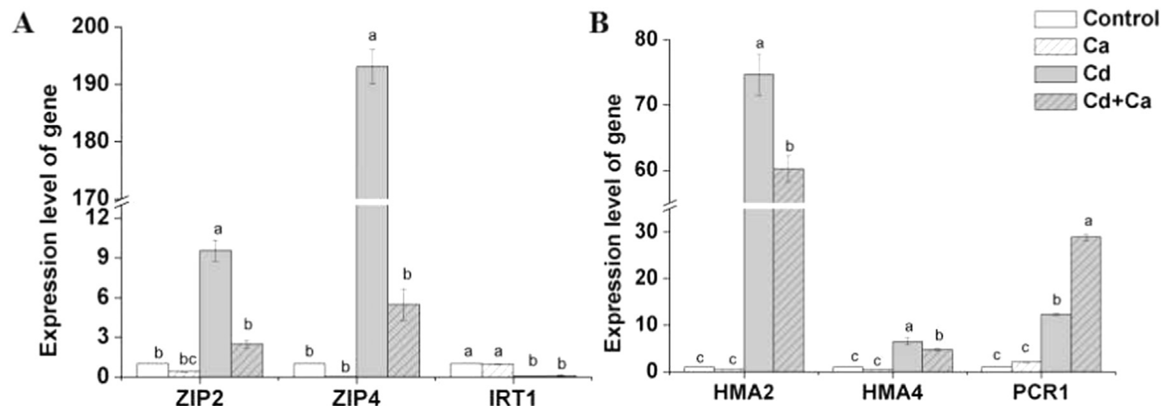

Fig. 4. RT-PCR analysis of expression of the genes involved in the root uptake (A), like *AtZIP2*, *AtZIP4* and *AtIRT1*, xylem loading (B), like *AtHMA2* and *AtHMA4* of Cd and also the gene involved in Ca transport, *AtPCR1*. Data are means ± SE (n = 3).

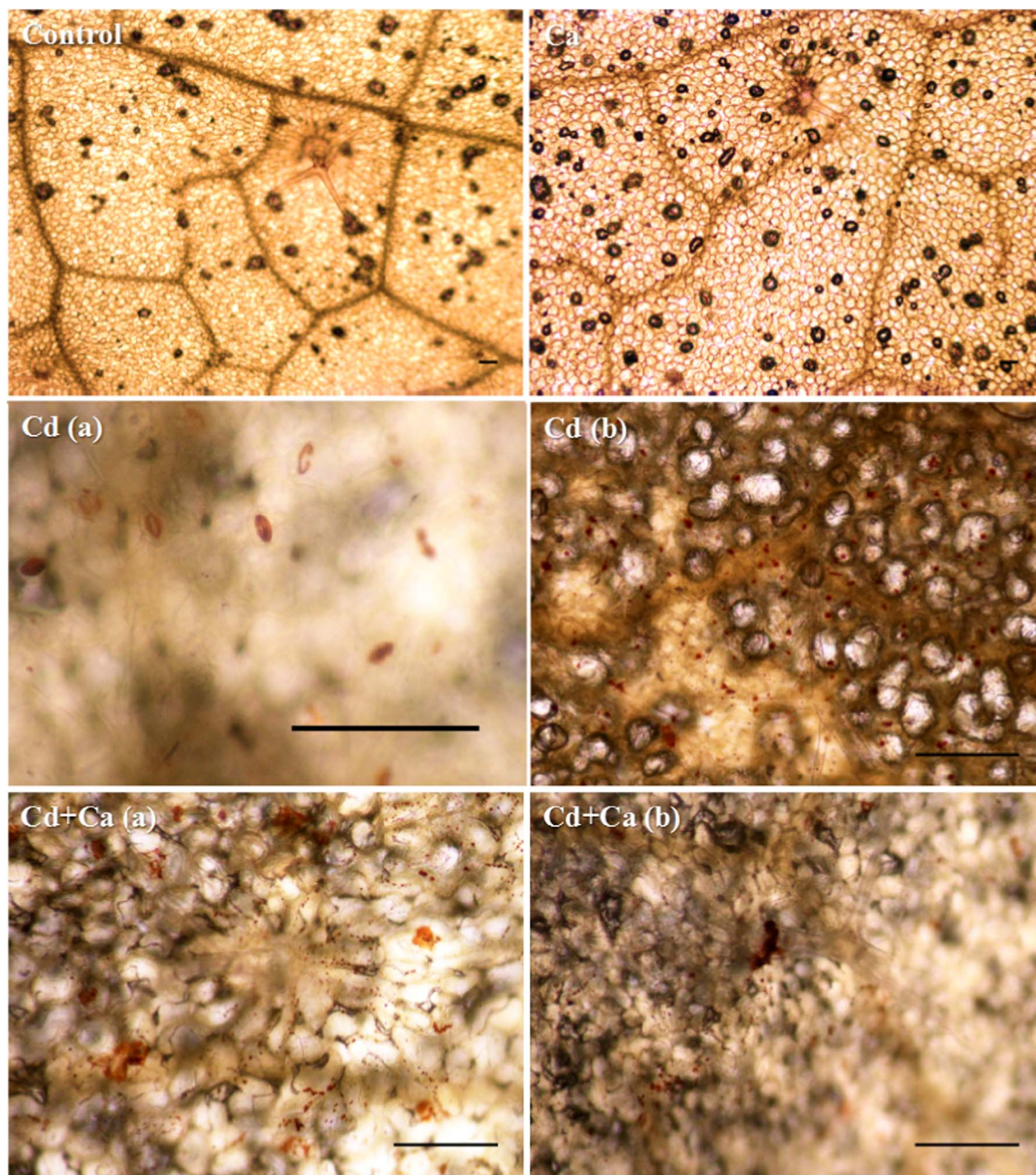

Fig. 5. Distribution of Cd in leaves of *Arabidopsis.* Reddish spots indicate the precipitates of Cd and dithizone. After 3 days of treatment, spots accumulated in the guard cell in Cd treatment (Cd(a)) some in the inner tissue (Cd(b)), while in the Cd + Ca treatment, they largely accumulatedin the intercellular space around the trichome (Cd + Ca(a-b)). Scale bar = 50 µm.

adherence of Cd to its root surface than that to Cd + Ca-treated plants, which would inhibit the growth of primary root, while in roots of Cd + Ca-treated plants less Cd was retained.

We assumed that Ca addition maintained the root cell membrane and reduced lignin deposition to reduce the physical adsorption of Cd.

In Cd + Ca-treated plants, the symplastic pathway of Cd uptake was weakened, while for Ca it was enhanced. No specific Cd transporter has been identified in plants. Therefore, Cd may be absorbed primarily by the existing ZIP transporters or through a similar mechanism (Pence et al., 2000). Milner (2013) reported that *AtZIP2* is involved in Mn uptake. *ZNT1* (*AtZIP4* orthologue) in *Noccaea caerulescens* is highly expressed under treatment with various Zn concentrations, which is consistent with high Zn accumulation (Van De Mortel et al., 2006; Assunção et al., 2001). *AtZIP2* and *AtZIP4* expression increased significantly in Cd-treated plants, indicating the strong uptake by these two transporters, whereas their expression decreased significantly in Cd + Ca-treated plants. The subtle change in *IRT1* might be explained by the finding of Nakanishi et al. (2006), which reported that Fe transporter would be involved in Cd uptake only when the plant was in the condition of lacking Fe and suffering Cd. But in our study Cd-treated plants were not Fe-deficient. Hence, we inferred that Ca decreased uptake of Cd mainly by reducing physical adsorption of Cd and changing its transport mode, as opposed to mutual competition for absorption sites. Besides, it is worth noting that the gene expression results from this study revealed one important mechanism that may cause the difference in Cd absorption under various treatment, though it was only analyzed in one particular time point, and the differential expression in mRNA level do not necessarily lead to the difference in protein expression (Herbette et al., 2006; Weber et al., 2006).

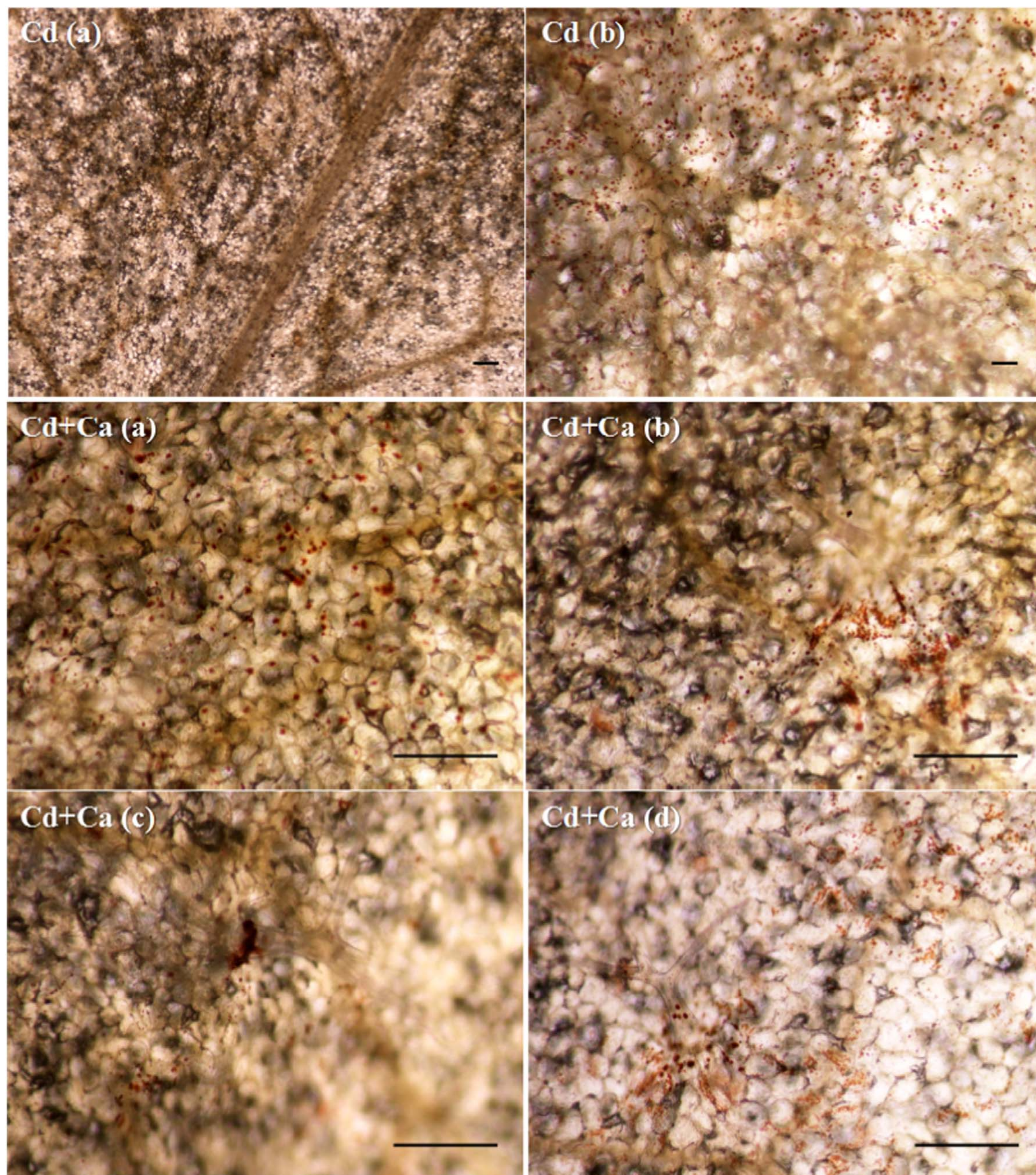

Fig. 6. Distribution of Cd in leaves of *Arabidopsis.* Reddish spots indicate the precipitates of Cd and dithizone. After 6 days treatment, spots could seldom be observed in the newly-developed leaves (a), but in the mature leaves (b) of Cd-treated plants while in Cd + Ca treatment, spots could be found in both kinds of leaves (a, young leaves. b–d, mature leaves) and always accumulated around the trichome (b), later formed a ring in the base of it (c) and was eventually eliminated. Scale bar = 50 μm.

4.2. Effects of Ca addition on Cd upward-translocation

It seemed that for most species Ca addition would reduce the amount of Cd that uptaken. As for *A. thaliana* (Suzuki, 2005), *Sedum alfredii* (Tian et al., 2011), citrus plant (López-Climent et al., 2014), it was reported that Ca addition decreases the amount of Cd in the whole plants. Some researchers found that Cd contents in both the above-ground part and root were decreased by Ca, as reported for *Zea mays* (Kurtyka et al., 2008), *Trifolium repens* (Wang and Song, 2009), *Brassica napus* (Wang et al., 2011), *Lens culinaris* (Talukdar, 2012), *Matricaria chamomilla* (Farzadfar et al., 2013), *Boehmeria nivea* (Gong et al., 2016) though exception occurred for *Spartina alterniflora* (He et al., 2013). However, it is worth noting that most of these studies did not proceed with a calculation of the translocation factor. We calculated back to their original data and discovered that the translocation factor of Cd in the Cd + Ca treated plants was always higher compared to those treated with Cd alone, irrespective of dose, time course or species. Transport of Zn as well as Cd is mediated by *AtHMA2* and *AtHMA4* (Hussain et al., 2004; Verret et al., 2004; Wong and Cobbett, 2009; Mills et al., 2010; Wu et al., 2015). Over-expression of *HMA4* in yeast and *E. coli* leads to the efflux of Zn and Cd, which demonstrates the important role of HMA4 in xylem loading (Mills et al., 2005, 2012). These results were confirmed in our study. It was further proved that Ca facilitated the upward-translocation of Cd from the aspect of gene expression.

In Cd + Ca-treated plants, Ca content in shoots was significantly higher, and *AtPCR1* expression in leaves also supported this results. *Brassica juncea* Plant Cadmium Resistance 1 (BjPCR1) protein facilitates radial transport of Ca in roots and its translocation to shoots, and its knock-out would disturb the transport of Ca to shoot and inhibit plant growth (Song, 2004; Song et al., 2011). The amino acids sequence

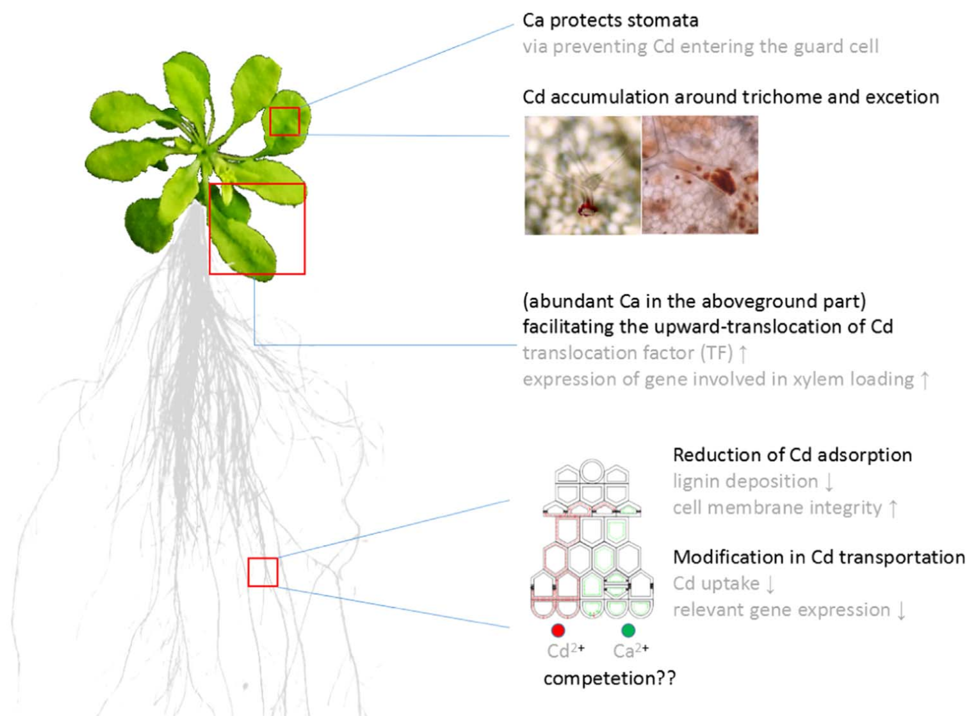

Fig. 7. Schematic diagram of alleviating effects of Ca on Cd-induced toxicity in Arabidopsis.

comparison showed that AtPCR1, AtPCR2 and BjPCRs had a high similarity and the GUS staining indicated that PCR2 is strongly expressed in roots, whereas PCR1 is expressed only in the aboveground parts of *A. thaliana*, particularly in trichomes (Song and Martinoia, 2010). The high expression of *PCR1* and high Ca content in leaves suggests its important role. There must be some reasons for transporting such a large amount of Ca upwards. Previous studies revealed that Ca always accumulates in either the mesophyll cells or trichomes, depending on the plant species (Karley et al., 2000), and plays an important role in some hyperaccumulator species. Investigations into why some plants were resistant to heavy metal toxicity revealed their use of organic acids, such as calcium oxalate (Mazen and Maghraby, 1997; Yang et al., 2000). Trichome density was increased and Ca/Zn mineral grains were produced on the head cells of trichomes in the Zn + Ca-treated tobacco plants, which was proposed as a novel Zn detoxification mechanism (Sarret et al., 2006).

4.3. Effects of Ca addition on Cd distribution

We observed the distribution of Cd in leaves using the dithizone-staining to determine if Ca was involved in its modification in *A. thaliana.* Considering that much lower Gs and Tr and Cd accumulation in the guard cell of Cd-treated plants, it was inferred that Ca addition changed the distribution of Cd by prohibiting it from entering guard cell, thus protecting the stomata and restoring the photosynthesis. The number of defective and undeveloped stomata was increased in plants that treated with a lower dose of Cd (Greger and Johansson, 1992). A Cd-dependent decrease in leaf conductance was observed at a higher dose, despite that the photosynthetic apparatus appeared not to be affected (Perfus-Barbeoch et al., 2002), consistent with our findings (Data not shown).

In this study, accumulated Cd in the base of trichome was obviously decreased following extended time of Ca treatment. Trichome has been thought to be a sink that store metals during detoxification in *Arabidopsis thaliana* (Domínguez-Solís et al., 2004). In tobacco, trichome is a pathway that excrete the metal compounds, as revealed by scanning electron microscopy coupled with the energy-dispersive X- ray (Choi et al., 2004; Choi and Harada, 2005; Sarret et al., 2006; Isasure et al., 2010); while in some members of the Brassicaceae, trichome-associated cells are known to accumulate high levels of metals under heavy metal contamination conditions, like *Arabidopsis halleri* (Küpper et al., 2000), Alyssum (Broadhurst et al., 2004; McNear et al., 2005). The results from this study suggested the trichome as a detoxification mechanism in *Arabidopsis thaliana*, and implied that Ca addition may help the excretion of Cd in it.

4.4. Interactions between Cd and Ca

Opinions differ regarding the interactions between Cd and Ca. In our study, we propose that the action of Cd was coordinated with Ca which facilitated its movement, in consideration of the higher translocation factor of both Cd and Ca and modifications in Cd distribution in leaves. Other researchers found that metal-hyperaccumulating species tend to take up specific metals and Ca simultaneously (Broadhurst et al., 2004; Nayidu et al., 2014) or have a high demand for Ca (Wang et al., 2011). Domínguez-Solís et al. (2004) reported that Cd preferentially accumulated in the base of trichome underneath the branching point, whereas Ca was highly abundant in the middle and upper parts of trichome cells after long-term (14 days) treatment of *A. thaliana* with a high Cd concentration (200 μM) without any addition of Ca. Küpper et al. (2000) reported a similar finding in the hyperaccumulator *A. halleri*. Another evidence was given by our discovery that more Cd precipitation was observed in the newly developed leaves of Cd + Ca-treated plants than that of Cd-treated ones, which seemed to be contradictory with the statement that smaller amount of Cd was detected in Cd + Ca-treated plants. It is likely that higher Ca contents in young leaves which resulted from the abundant Ca in the shoot of Cd + Ca-treated plant also led to a higher content of Cd.

But why is such a large amount of Ca being transported to the shoot only when the plants are exposed to Cd and Ca simultaneously? It seems that in this condition Cd has initiated the mechanism of Ca regulation, which is an economic way for plants to detoxify. Previous studies indicated that calcium oxalate formation is a mechanism for regulating free calcium levels in tissues and organs. Excessive calcium must be efficiently compartmentalised or eliminated to prevent stomatal closure by extracellular Ca^{2+} and interference with cell signaling pathways (Wu and Kuo-Huang, 1997; Franceschi and Nakata, 2005).

5. Conclusion

In summary, Ca addition reduced Cd uptake mainly by reducing its root adsorption and by changing its mode of transport. Ca supplementation also facilitated Cd accumulation in shoots, and further modified Cd distribution in leaves to alleviate the toxicity caused by Cd exposure (as summarized in Fig. 7). This study found that Ca addition could alleviate the relevant toxicity and reduce Cd absorption, suggesting the important prospects of Ca addition to improve the reduction in food supply caused by Cd contamination. However, Ca addition increased the upward-translocation of Cd. Given that the edible portion of crops is usually the aboveground part, the safety of Ca addition to crops grown in Cd contaminated soil requires further investigations. Considering that grain crops, particularly rice, are the primary source of Cd to some Asian populations, it is necessary to explore the effects of Ca addition on Cd uptake, transportation and related mechanisms for grain crops (such as rice) in the follow-up research. Given that the concentration of Cd supply employed in the present study was relatively higher than that reported in global farmlands, further studies are also needed to investigate the alleviating effects of Ca addition to chronic and/or lower dose of Cd exposure.

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Appendix A. Supporting material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2017.01.023.

References

Andersson, A., Nilsson, K.O., 1974. Influence of lime and soil pH on Cd availability to plant. Ambio 3, 198–200.
Andosch, A., Affenzeller, M.J., Lütz, C., Lütz-Meindl, U., 2012. A freshwater green alga under cadmium stress: ameliorating calcium effects on ultrastructure and photosynthesis in the unicellular model *Micrasterias*. J. Plant Physiol. 169, 1489–1500.
Assunção, A.G.L., Martins, P.D.C., Folter, S.D., Vooijs, R., Schat, H., Aarts, M.G.M., 2001. Elevated expression of metal transporter genes in three accessions of the metal hyperaccumulator *Thlaspi caerulescens*. Plant Cell Environ. 24, 217–226.
Baker, C.J., Mock, N.M., 1994. An improved method for monitoring cell death in cell suspension and leaf disc assays using evans blue. Plant Cell Tissue Organ Culture 39, 7–12.
Baxter, I., Hosmani, P.S., Rus, A., Lahner, B., Borevitz, J.O., Muthukumar, B., Mickelbart, M.V., Schreiber, L., Franke, R.B., Salt, D.E., 2009. Root suberin forms an extracellular barrier that affects water relations and mineral nutrition in *Arabidopsis*. Plos Genet. 5, 188–192.
Broadhurst, C.L., Chaney, R.L., Angle, J.S., Maugel, T.K., Erbe, E.F., Murphy, C.A., 2004. Simultaneous hyperaccumulation of nickel, manganese, and calcium in *Alyssum* leaf trichomes. Environ. Sci. Technol. 38, 5797–5802.
Carro, L., Barriada, J.L., Herrero, R., Vicente, M.E.S.D., 2015. Interaction of heavy metals with Ca-pretreated Sargassum muticum algal biomass: characterization as a cation exchange process. Chem. Eng. J. 264, 181–187.
Chen, J., Xue, B., Xia, X., Yin, W., 2013. A novel calcium-dependent protein kinase gene from *Populus euphratica*, confers both drought and cold Stress tolerance. Biochem. Biophys. Res. Commun. 441, 630–636.
Choi, Y.E., Harada, E., 2005. Roles of calcium and cadmium on Cd-containing intra-and extracellular formation of Ca crystals in tobacco. J. Plant Biol. 48, 113–119.
Choi, Y.E., Harada, E., Wada, M., Tsuboi, H., Morita, Y., Kusano, T., Sano, H., 2001. Detoxification of cadmium in tobacco plants: formation and active excretion of crystals containing cadmium and calcium through trichomes. Planta 213, 45–50.
Choi, Y.E., Harada, E., Kim, G.H., et al., 2004. Distribution of elements on tobacco trichomes and leaves under cadmium and sodium stresses. J. Plant Biol. 47, 75–82.
Choong, G., Liu, Y., Templeton, D.M., 2014. Interplay of calcium and cadmium in mediating cadmium toxicity. Chem.-Biol. Interact. 211, 54–65.
Clemens, S., Aarts, M.G.M., Thomin, S., Verbruggen, N., 2013. Plant science: the key to preventing slow cadmium poisoning. Trends Plant Sci. 18, 92–99.
Conn, S.J., Hocking, B., Dayod, M., Xu, B., Athman, A., Henderson, 2013. Protocol: optimising hydroponic growth systems for nutritional and physiological analysis of *Arabidopsis thaliana* and other plants. Plant Methods 9, 120–125.
Domínguez-Solís, J.R., López-Martín, M.C., Ager, F.J., Ynsa, M.D., Romero, L.C., Gotor, C., 2004. Increased cysteine availability is essential for cadmium tolerance and accumulation in *Arabidopsis thaliana*. Plant Biotechnol. J. 2, 469–476.
Farzadfar, S., Zarinkamar, F., Modarres-Sanavy, S., Hojati, M., 2013. Exogenously applied calcium alleviates cadmium toxicity in *Matricaria chamomilla* L. plants. Environ. Sci. Pollut. Res. Int. 20, 1413–1422.
Franceschi, V.R., Nakata, P.A., 2005. Calcium oxalate in plants: formation and function. Annu. Rev. Plant Biol. 56, 41–71.
Gallego, S.M., Pena, L.B., Barcia, R.A., Azpilicueta, C.E., Iannone, M.F., Rosales, E.P., Zawoznika, M.S., Groppaa, M.D., Benavidesa, M.P., 2012. Unravelling cadmium toxicity and tolerance in plants: insight into regulatory mechanisms. Environ. Exp. Bot. 83, 33–46.
Gong, X., Liu, Y., Huang, D., Zeng, G., Liu, S., Tang, H., Zhou, L., Hu, X., Zhou, Y., Tan, X., 2016. Effects of exogenous calcium and spermidine on cadmium stress moderation and metal accumulation in *Boehmeria nivea* (L.) Gaudich. Environ. Sci. Pollut. Res. Int. 23, 8699–8708.
Greger, M., Johansson, M., 1992. Cadmium effects on leaf transpiration of sugar beet (*Beta vulgaris*). Physiol. Plant. 86, 465–473.
Guerinot, M.L., 2000. The ZIP family of metal transporters. Biochim. Et. Biophys. Acta 1465, 190–198.
Gutierrez, L., Mauriat, M., Guénin, S., Pelloux, J., Lefebvre, J.F., Louvet, R., Rusterucci, C., Moritz, T., Guerineau, F., Bellini, C., Van Wuytswinkel, O., Laurent, G., Mélanie, M., Stéphanie, G., Jérôme, P., Jean-François, L., Romain, L., 2008. The lack of a systematic validation of reference genes: a serious pitfall undervalued in reverse transcription-polymerase chain reaction (RT-PCR) analysis in plants. Plant Biotechnol. J. 6, 609–618.
Hart, J.J., Welch, R.M., Norvell, W.A., Sullivan, L.A., Kochian, L.V., 1998. Characterization of cadmium binding, uptake, and translocation in intact seedlings of bread and durum wheat cultivars. Plant Physiol. 116, 1413–1420.
He, Z.Z., Cai, M.W., Wei, Y., Liu, Y., Shi, F., 2013. Effects of $CaCl_2$ on the Cd accumulation and stress of *Spartina alterniflora*. Chin. J. Ecol. 32, 1571–1577.
Herbette, S., Taconnat, L., Hugouvieux, V., Piette, L., Magniette, M.L., Cuine, S., Auroy, P., Richaud, P., Forestier, C., Bourguignon, J., Renou, J., P., Vavasseur, A., Leonhardt, N., 2006. Genome-wide transcriptome profiling of the early cadmium response of Arabidopsis roots and shoots. Biochimie 88, 1751–1765.
Hussain, D., Haydon, M.J., Wang, Y., Wong, E., Sherson, S.M., Young, J., Camakaris, J., Harper, J.F., Cobbett, C.S., 2004. P-type ATPase heavy metal transporters with roles in essential zinc homeostasis in *Arabidopsis*. Plant Cell 16, 1327–1339.
Ikegawa, H., Yamomoto, Y., Matsumoto, H., 2002. Cell death caused by a combination of aluminum and iron in cultured tobacco cells. Physiol. Plant. 104, 474–478.
Isasure, M.P., Sarret, G., Harada, E., Choic, Y.E., Marcusb, M.A., Fakra, S.C., Geoffroy, N., Pairis, S., Susini, J., Clemens, S., Manceau, A., 2010. Calcium promotes cadmium elimination as vaterite grains by tobacco trichomes. Geochim. Et. Cosmochim. Acta 74, 5817–5834.
Karley, A.J., Leigh, R.A., Sanders, D., 2000. Where do all the ions go? The cellular basis of differential ion accumulation in leaf cells. Trends Plant Sci. 5, 465–470.
Kim, Y.Y., Yang, Y.Y., Lee, Y., 2002. Pb and Cd uptake in rice roots. Physiol. Plant. 116, 368–372.
Küpper, H., Andresen, E., 2016. Mechanisms of metal toxicity in plants. Metallomics 8, 269–285.
Küpper, H., Lombi, E., Zhao, F.J., McGrath, S.P., 2000. Cellular compartmentalization of cadmium and zinc in relation to other elements in the hyperaccumulator *Arabidopsis halleri*. Planta 212, 75–84.
Kurtyka, R., Małkowski, E., Kita, A., Karcz, W., 2008. Effect of calcium and cadmium on growth and accumulation of cadmium, calcium, potassium and sodium in maize seedlings. Pol. J. Environ. Stud. 17, 51–56.
Li, P., Zhao, C., Zhang, Y., Wang, X.M., Wang, X.Y., Wang, J., Wang, F., Bi, Y., 2016. Calcium alleviates cadmium-induced inhibition on root growth by maintaining auxin homeostasis in *Arabidopsis* seedlings. Protoplasma 253, 185–200.
Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-delta delta c(t)) method. Methods 25, 402–408.
López-Climent, M.F., Arbon, V., Pérez-Clemente, R.M., Zalinas, S.I., Gómez-Cadenas, A., 2014. Effect of cadmium and calcium treatments on phytochelatin and glutathione levels in citrus plants. Plant Biol. 16, 79–87.
Lozano-Rodríguez, E., Hernández, L.E., Bonay, P., Carpena-Ruiz, R.O., 1997. Distribution of cadmium in shoot and root tissues of maize and pea plants: physiological disturbances. J. Exp. Bot. 48, 123–128.
Mazen, A.M.A., Maghraby, O.M.O.E., 1997. Accumulation of cadmium, lead and strontium, and a role of calcium oxalate in water hyacinth tolerance. Biol. Plant. 40, 411–417.
McNear Jr., D.H., Peltier, E., Everhart, J., Chaney, R.L., Sutton, S., Newville, M., et al., 2005. Application of quantitative fluorescence and absorption-edge computed microtomography to image metal compartmentalization in *Alyssum murale*. Environ. Sci. Technol. 39, 2210–2218.
Mills, R.F., Francini, A., Ferreira da Rocha, P.S., Baccarini, P.J., Aylett, M., Krijger, G.C., Williams, L.E., 2005. The plant P_{1B}-type ATPase AtHMA4 transports Zn and Cd and plays a role in detoxification of transition metals supplied at elevated levels. FEBS Lett. 579, 783–791.
Mills, R.F., Peaston, K.A., Runions, J., Williams, L.E., 2012. HvHMA2, a P_{1B}-ATPase from barley, is highly conserved among cereals and functions in Zn and Cd transport. Plos One 7, e42640.
Mills, R.F., Valdes, B., Duke, M., Peaston, K.A., Lahner, B., Salt, D.E., Williams, L.E., 2010. Functional significance of AtHMA4C-terminal domain in planta. Plos One 5, e13388.
Milner, M.J., 2013. Transport properties of members of the ZIP family in plants and their

role in Zn and Mn homeostasis. J. Exp. Bot. 64, 369–381.
Min, H., Cai, S., Xu, Q., 2012. Effects of exogenous calcium on resistance of *Hydrilla verticillata* (L.f.) Royle to cadmium stress. Acta Ecol. Sin. 32, 256–264.
Nakanishi, H., Ogawa, I., Ishimaru, Y., Mori, S., Nishizawa, N.K., 2006. Iron deficiency enhances cadmium uptake and translocation mediated by the Fe^{2+} transporters OsIRT1 and OsIRT2 in rice. Soil Sci. Plant Nutr. 52, 464–469.
Nayidu, N.K., Tan, Y., Taheri, A., Li, X., Bjorndahl, T.C., 2014. *Brassica villosa,* a system for studying non-glandular trichomes and genes in the *Brassicas*. Plant Mol. Biol. 85, 519–539.
Pence, N.S., Larsen, P.B., Ebbs, S.D., Ebbs, S.D., Letham, D.L., Lasat, M.M., Garvin, D.F., Eide, D., Kochian, L.V., 2000. The molecular physiology of heavy metal transport in the Zn/Cd hyperaccumulator *Thlaspi caerulescens*. Proc. Natl. Acad. Sci. USA 97, 4956–4960.
Perfus-Barbeoch, L., Leonhardt, N., Vavasseur, A., Forestier, C., 2002. Heavy metal toxicity: cadmium permeates through calcium channels and disturbs the plant water status. Plant J. 32, 539–548.
Qiu, Q., Wang, Y.T., Yang, Z.Y., Yuan, J.G., 2011. Effects of phosphorus supplied in soil on subcellular distribution and chemical forms of cadmium in two Chinese flowering cabbage (*Brassica parachinensis* L.) cultivars differing in cadmium accumulation. Food Chem. Toxicol. 49, 2260–2267.
Rodriguez-Hernandez, M.C., Bonifas, I., Alfaro-De, la., Torre, M.C., Flores-Flores, J.L., Bañuelos-Hernández, B., Patiño-Rodríguez, O., 2015. Increased accumulation of cadmium and lead under Ca and Fe deficiency in *Typha latifolia*: a study of two pore channel (TPC1) gene responses. Environ. Exp. Bot. 115, 38–48.
Rogers, L.A., Dubos, C.C., Willment, J., Cullis, I.F., Mansfield, S.D., Campbell, M.M., 2005. Comparison of lignin deposition in three ectopic lignification mutants. New Phytol. 168, 123–140.
Romè, C., Huang, X.Y., Danku, J., Salt, D.E., Sebastiani, L., 2016. Expression of specific genes involved in Cd uptake, translocation, vacuolar compartmentalisation and recycling in *Populus alba* Villafranca clone. J. Plant Physiol. 202, 83–91.
Sarret, G., Harada, E., Choi, Y.E., Isaure, M.P., Geoffroy, N., Fakra, S., Marcus, M.A., Birschwilks, M., Clemens, S., Manceau, A., 2006. Trichomes of tobacco excrete zinc as zinc-substituted calcium carbonate and other zinc-containing compounds. Plant Physiol. 141, 1021–1034.
Schreiber, L., Hartmann, K., Skrabs, M., Zeier, J., 1999. Apoplastic barriers in roots: chemical composition of endodermal and hypodermal cell walls. J. Exp. Bot. 50, 1267–1280.
Seregin, I.V., Ivanov, V.B., 1997. Histochemical investigation of cadmium and lead distribution in plants. Russ. J. Plant Physiol. 44, 791–796.
Song, W.Y., 2004. A novel family of cys-rich membrane proteins mediates cadmium resistance in *Arabidopsis*. Plant Physiol. 135, 1027–1039.
Song, W.Y., Martinoia, E., 2010. *Arabidopsis* PCR2 is a zinc exporter involved in both zinc extrusion and long-distance zinc transport. Plant Cell 22, 2237–2252.
Song, W.Y., Choi, K.S., Alexis, D.A., Martinoia, E., Lee, Y., 2011. *Brassica juncea* plant cadmium resistance 1 protein (BjPCR1) facilitates the radial transport of calcium in the root. Proc. Natl. Acad. Sci. USA 108, 19808–19813.
Suzuki, N., 2005. Alleviation by calcium of cadmium-induced root growth inhibition in Arabidopsis seedlings. Plant Tissue Cult. Lett. 22, 19–25.
Talukdar, D., 2012. Exogenous calcium alleviates the impact of cadmium-induced oxidative stress in *Lens culinaris* Medic. Seedlings through modulation of antioxidant enzyme activities. J. Crop Sci. Biotechnol. 15, 325–334.
Thomine, S., Schroeder, J.I., 2000. Cadmium and iron transport by members of a plant metal transporter family in *Arabidopsis* with homology to Nramp genes. Proc. Natl. Acad. Sci. USA 97, 4991–4996.
Tian, S., Lu, Zhang, J., Wang, K., Brown, P., 2011. Calcium protects roots of *Sedum alfredii* H. against cadmium-induced oxidative stress. Chemosphere 84, 63–69.
Van De Mortel, J.E., Villanueva, L.A., Schat, H., Kwekkeboom, J., Coughlan, S., Moerland, P.D., van Themaat, E.V.L., Koornneef, M., Aarts, M.G.M., 2006. Large expression differences in genes for iron and zinc homeostasis, stress response, and lignin biosynthesis distinguish roots of *Arabidopsis thaliana* and the related metal hyperaccumulator *Thlaspi caerulescens*. Plant Physiol. 142, 1127–1147.
Van De Mortel, J.E., Schat, H., Moerland, P.D., 2008. Expression differences for genes involved in lignin, glutathione and sulphate metabolism in response to cadmium in *Arabidopsis thaliana* and the related Zn/Cd-hyperaccumulator *Thlaspi caerulescens*. Plant Cell Environ. 31, 301–324.
Verret, F., Gravot, A., Auroy, P., 2004. Overexpression of AtHMA4 enhances root-to-shoot Cd translocation of zinc and cadmium and plant metal tolerance. FEBS Lett. 576, 306–312.
Wang, C.Q., Song, H., 2009. Calcium protects *Trifolium repens* L. seedlings against cadmium stress. Plant Cell Rep. 28, 1341–1349.
Wang, C.Y., Wang, S.J., Rong, L., Luo, X.Q., 2011. Analyzing about characteristics of calcium content and mechanisms of high calcium adaptation of common pteridophyte in Maolan karst area of China. Chin. J. Plant Ecol. 35, 1061–1069.
Wang, Y.T., Björn, L.O., 2014. Heavy metal pollution in Guangdong Province, China, and the strategies to manage the situation. Front. Environ. Sci. 2, 1–12.
Weber, M., Trampczynska, A., Clemens, S., 2006. Comparative transcriptome analysis of toxic metal responses in *Arabidopsis thaliana* and the cd(2+)-hypertolerant facultative metallophyte *Arabidopsis halleri*. Plant Cell Environ. 29, 950–963.
Weigel, H.J., Jager, H.J., 1980. Subcellular distribution and chemical form of cadmium in bean plants. Plant Physiol. 65, 480–482.
Wong, C.K.E., Cobbett, C.S., 2009. HMA P-type ATPases are the major mechanism for root-to-shoot Cd translocation in *Arabidopsis thaliana*. New Phytol. 181, 71–78.
Wu, C.C., Kuo-Huang, L.L., 1997. Calcium crystals in the leaves of some species of Moraceae. Bot. Bull. Acad. Sin. 38, 97–104.
Wu, Z., Zhao, X., Sun, X., Tan, Q., Tang, Y., Nie, Z., Hu, C., 2015. Xylem transport and gene expression play decisive roles in cadmium accumulation in shoots of two oilseed rape cultivars (*Brassica napus*). Chemosphere 119, 1217–1223.
Yang, C.L., Guo, R.P., Yue, Q.L., Zhou, K., Wu, Z.F., 2013. Environmental quality assessment and spatial pattern of potentially toxic elements in soils of Guangdong Province, China. Environ. Earth Sci. 70, 1903–1910.
Yang, Y.Y., Jung, J.Y., Song, W.Y., Suh, H.S., Lee, Y., 2000. Identification of rice varieties with high tolerance or sensitivity to lead and characterization of the mechanism tolerance. Plant Physiol. 124, 1019–1026.
Zhang, G., Liu, Y., Ni, Y., Meng, Z., Lu, T., Li, T., 2014. Exogenous calcium alleviates low night temperature stress on the photosynthetic apparatus of tomato leaves. Plos One 9, e97322.
Zhu, X.F., Lei, G.J., Wang, Z.W., Shi, Y.Z., Braam, J., Li, G.X., Zheng, S.J., 2013. Coordination between apoplastic and symplastic detoxification confers plant Al resistance. Plant Physiol. 162, 1947–1955.