Cadmium and zinc uptake and translocation in dwarf Polish wheat seedlings as affected by calcium and potassium combination

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Abstract

Combination of calcium and potassium (Ca-K) influences cadmium and zinc uptake and translocation in dwarf Polish wheat (*Triticum polonicum*), but its effects remain unclear. In the present study, a high concentration of Ca-K reduced uptake of Cd and Zn by roots and promoted their translocations to shoots under Cd and Zn excess. Whatever under a low or high concentration of Ca-K, Zn inhibited Cd uptake and translocation under Cd+Zn stress when compared with Cd stress alone. However, the reduced Cd content caused by Zn under the high concentration of Ca-K was significantly lower than under the low concentration of Ca-K. Under both Ca-K treatments, Cd promoted Zn uptake and inhibited Zn translocation under Cd+Zn stress when compared with those under Zn stress. The high concentration of Ca-K reinforced the promotion of Zn uptake and the inhibition of Zn translocation caused by Cd. The Ca-K or Zn affected the expression of several metal transporters and influenced cell wall metabolism, the subcellular distribution of Cd, and the Cd chemical forms. Meanwhile, Ca-K or Cd also affected the expressions of several metal transporters and changed the subcellular distribution of Zn. The differentially expressed metal transporters and changes in subcellular distributions and Cd chemical forms were associated with Cd and Zn uptake and translocation. In summary, the application of Ca-K caused changes in gene expression, Cd and Zn uptake, translocation, and subcellular distribution.

Additional key words: Cd chemical forms, Cd/Zn interaction, gene expression, metal transporters, subcellular distribution, *Triticum polonicum*.

Introduction

Cadmium is one of the most toxic heavy metals for all living organisms, even at low concentrations. An increasing proportion of arable soils worldwide have been contaminated with Cd released as a result of human activities or environmental causes (Perrier *et al.* 2016, Li *et al.* 2017). Given that Cd is a non-essential element, no specific transporter is responsible for Cd uptake and translocation by plants (Sasaki *et al.* 2012). Plants absorb and distribute Cd *via* the pathway(s) responsible for the uptake and transport of essential ions, such as calcium, iron, and zinc (Clemens 2006, Sasaki *et al.* 2012, Sharma *et al.* 2016, Wang *et al.* 2017a). Thus, Zn and Fe may affect Cd uptake or accumulation (Hart *et al.* 2002, 2005, Sun *et al.* 2005, Balen *et al.* 2011, Cheng *et al.* 2018), although

the effects depend on the exogenous bioavailable Cd/Zn concentrations, species, tissues, developmental stages, and nutritional conditions.

In wheat, application of Zn fertilizer has been used as an agronomic practice to reduce Cd uptake and accumulation. Hart *et al.* (2002, 2005) and *Sun et al.* (2005) reported that Cd and Zn mutually inhibited their uptake and translocation in roots and shoots in some genotypes of durum and bread wheat. However, mutual promotion of Cd and Zn uptake has been observed in bread wheat (Nan *et al.* 2002). Thus, Zn inhibition or promotion of Cd uptake differs among wheat species and genotypes. In dwarf Polish wheat (DPW; *Triticum polonicum* L., AABB, $2n = 4x = 28$), we previously observed mutual inhibition of Cd and Zn uptake by each other and that Cd translocation was influenced by Zn. In addition, a number of genes involved

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in Ca and K ion channels were differentially regulated by Cd, Zn, and Cd+Zn stresses (Wang *et al.* 2016, 2017b). Thus, Ca and K may participate in the Cd/Zn interactions.

As essential elements, Ca and K are required for nitrogen (N) metabolism, play diverse roles in cell wall and membrane structure, and cellular signal transduction (Siddiqui *et al.* 2012, Ahmad *et al.* 2016). Thus, the two elements play important roles in plant growth and development. In addition, Ca and K may reduce Cd and/ or Zn uptake in plants (such as tobacco, soybean, wheat, chickpea, and faba bean), thereby alleviating Cd toxicity and enhancing tolerance of Cd and Zn (Siddiqui *et al.* 2012, Song *et al.* 2015, Yang and Juang 2015, Ahmad *et al.* 2016, Wang *et al.* 2017a). Interestingly, combined application of Ca and K (hereafter Ca-K) is more effective in reducing Cd uptake and alleviating Cd toxicity compared with the individual effects of Ca and K applications (Siddiqui *et al.* 2012, Ahmad *et al.* 2016). Thus, Ca-K is a desirable strategy to inhibit Cd entry into the food chain. These studies have mainly focused on Ca-K alleviation of Cd toxicity through investigating several physiological and biochemical indexes (Siddiqui *et al.* 2012, Ahmad *et al.* 2016).

Polish wheat shows low genetic similarity with *T. durum*, *T. turgidum*, and *T. aestivum* (Wiwart *et al.* 2013). A Chinese accession of DPW has been reported to show high tolerance to Cd and Zn (Wang *et al.* 2017b). In DPW seedlings, ammonium nitrogen (NH_4^+-N) reinforced the inhibition of Cd uptake and the promotion of Cd translocation caused by Zn, but alleviated the inhibition of Zn uptake and partially reduced the promotion of Zn translocation stimulated by Cd (Cheng *et al.* 2018). Additionally, previous study indicated that the Fe accumulation could alleviate Cd toxicity (Wu *et al.* 2012), because it functions in various important processes, including photosynthesis, respiration, and chlorophyll biosynthesis (Kobayashi and Nishizawa 2012). However, the effects of Ca-K on Cd and Zn uptake and translocation, and Cd/Zn interactions were not investigated. In the present study, we hypothesized that a high concentration of Ca-K, compared with a low concentration of Ca-K, would reduce Cd, Zn, and Fe uptake, promote Cd and Zn translocation, and change the Cd/Zn interaction. To test this hypothesis, we analyzed the Cd, Zn and Fe content in roots and shoots, the translocation factor and subcellular distribution for both elements, the Cd chemical forms, and gene expression profiles under Cd, Zn, and Cd+Zn stresses concomitant with a low or high concentration of Ca-K.

Materials and methods

Plants and growth conditions: Dwarf Polish wheat (*Triticum polonicum* L.) was collected from Xinjiang, China and the plants were cultivated as described by Cheng *et al.* (2018) with some modification of treatments. Briefly, after germination, uniform seedlings were grown in hydroponic culture with nutrient solution [4 mM Ca(NO$_3$)$_2$ · 4 H$_2$O, 5 mM KNO$_3$, 1 mM NH$_4$NO$_3$, 1 mM KH$_2$PO$_4$, 2 mM MgSO$_4$ · 7 H$_2$O, 10 μM H$_3$BO$_3$, 1.8 μM MnSO$_4$, 0.2 μM NaMoO$_4$, 0.31 μM CuSO$_4$, 5 μM ZnSO$_4$, and 50 μM Fe-EDDHA, pH 6.0] in a growth chamber at a temperature 25 °C, a 16-h photoperiod, an irradiance of 180 μmol m^{-2} s^{-1}, and a relative humidity of 75 %. After two weeks, all seedlings were divided into two groups (with low and high concentrations of Ca-K) each with four subgroups, which were individually treated with 0 μM CdCl$_2$ (CK), 80 μM CdCl$_2$ (Cd), 500 μM ZnSO$_4$ (Zn), or 80 μM CdCl$_2$ + 500 μM ZnSO$_4$ (Cd+Zn). For the high concentration of Ca-K group, each treatment was supplemented with 5 mM KNO$_3$ and 4 mM Ca(NO$_3$)$_2$ · 4 H$_2$O. In addition, 1 mM Ca (^{15}NO$_3$)$_2$ was incorporated in the nutrient solution to track the capacity of DPW for instantaneous absorption of NO$_3^-$. Each treatment was conducted with three independent biological replicates (60 plants per biological replicate). On the seventh day of treatment, seedlings were sampled to measure root and shoot lengths, then were dried to analyze metal (Cd, Fe, and Zn) content. The remaining plants were used to analyze the subcellular distribution of Cd and Zn, Cd chemical forms, and gene expression.

Measurement of Cd, Zn, and Fe content and translocation: As described by Cheng *et al.* (2018), dried powder (0.2 g) of the root and shoot of each sample was digested with HNO$_3$ and HClO$_4$ (4:1, v/v) at 260 °C. The Cd, Zn, and Fe content was measured using an inductively coupled plasma mass spectrometer (*7900*, *Agilent*, Santa Clara, CA, USA). The reference standard solutions of Cd, Zn, and Fe were purchased from the *Guobiao Testing and Certification Company* (Beijing, China). The translocation factor (TF) was calculated as the shoot-to-root metal content ratio.

Analysis of subcellular distribution of Cd and Zn: The subcellular distribution of Cd and Zn was measured as described by Wang *et al.* (2009) and Lai (2015). Briefly, ground powder (1 g fresh mass) was centrifuged in precooling extraction buffer [50 mM Tris-HCl (pH 7.5), 250 mM sucrose and 1.0 mM dithiothreitol (C$_4$H$_{10}$O$_2$S$_2$)]. Three fractions, namely the cell wall fraction (F$_{CW}$), the organelles fraction (F$_{CO}$), and the soluble fraction (including vacuoles; F$_{CS}$), were extracted *via* different centrifugations. The Cd and Zn content in the different fractions were measured as described in the preceding section.

Cadmium chemical forms: The Cd chemical forms were analyzed following the method described by Wu *et al.* (2005) and Cheng *et al.* (2018). Six chemical forms of Cd were differentiated: inorganic Cd (the fraction extracted by ethanol, F$_E$), water-soluble Cd (the fraction extracted by water, F$_W$), pectate- and protein-integrated Cd (the fraction extracted by NaCl, F$_{NaCl}$), undissolved Cd phosphate [the fraction extracted by acetic acid (HAc) F$_{HAc}$], Cd oxalate (the fraction extracted by HCl, F$_{HCl}$), and residual Cd (the fraction of residual Cd, F$_R$). The Cd content in each extract was measured using the afore-mentioned procedure.

Analysis of ^{15}N and total N content: The ^{15}N and total N content was determined in accordance with the method

of Preston and Owens (1983) using an isotope ratio mass spectrometer (*Delta V Advantage, Thermo Fisher Scientific*, Waltham, MA, USA) at the Graduate School of Tsinghua University (ShenZhen, China). Briefly, dried ground powder (2 mg) of each sample was calcined at a high temperature in the elemental analyzer. The ^{15}N concentration was calculated by comparison with the international nitrogen standard (Mariotti 1984).

Analysis of gene expression: Gene expression was analyzed using the RNA sequencing (RNA-Seq) method described by Wang *et al.* (2017b). The RNA-Seq procedure, including RNA isolation, library construction and sequencing, transcriptome assembly, gene functional annotation, and differential expression analysis, was conducted at the Novogene Bioinformatics Institute (Beijing, China). Read counts of each gene were converted into reads per kilobase per million values to normalize the gene expression (Mortazavi *et al.* 2008). Differentially expressed genes were determined using the DEseq method (Ander and Huber 2010). The threshold value of $\log_2$ fold change > 1 or < -1 with $P < 0.0005$ was employed to assess the significance of differential expression.

Statistical analysis: The significance of differences among means was statistically analyzed using Duncan's multiple range test implemented in the *IBM SPSS Statistics v. 20.0* software (*IBM Corporation*, Armonk, NY, USA) using the 5 % level of significance. All graphics were plotted using *Sigmaplot 12.0* software (*Systat Software*, San Jose, CA, USA).

Results

All plants look well during the experimental period, although root growth was differentially affected by the treatments (Fig. *1A*). Without Cd or Zn stress, the high concentration of Ca-K reduced root length. Under the low concentration of Ca-K, all Cd and Zn stresses reduced root length compared with that of the CK (Fig. *1A*). Under the

high concentration of Ca-K, Cd and Cd+Zn dramatically reduced root length, whereas Zn did not affect root length, compared with that of the CK (Fig. *1A*). All stress treatments did not change shoot length (Fig. *1B*), which might be resulted from the fact that various treatments were not severely stressful.

Compared with the low concentration of Ca-K, the high concentration of Ca-K reduced the Cd content in roots (Fig. *2A*) and increased the Cd content in shoots under Cd stress (Fig. *2B*). The Zn content in roots and shoots were even reduced under Zn stress (Fig. *2C,D*), and the Cd and Zn content was reduced in roots but remained unchanged in shoots under Cd+Zn stress due to high Ca-K concentration (Fig. *2A-D*). Under low concentration of Ca-K, Zn reduced the Cd content in roots by 65.0 % (Fig. *2A*), but increased the Cd content in shoots under Cd+Zn stress when compared with that under Cd stress (Fig. *2B*); however, Cd increased the Zn content in roots (Fig. *2C*), but reduced the Zn content in shoots under Cd+Zn stress when compared with that under Zn stress (Fig. *2D*). Under the high concentration of Ca-K, Zn reduced the Cd content in roots by 50.0 % (Fig. *2A*), but increased it in shoots under Cd+Zn stress when compared with that under Cd stress (Fig. *2B*); however, Cd increased the Zn content in roots (Fig. *2C*), but did not affect it in shoots under Cd+Zn stress when compared with Zn stress (Fig. *2D*).

Without Cd or Zn stress, the high concentration of Ca-K increased the Fe content in roots and shoots when compared with that under the low concentration of Ca-K (Fig. *2E,F*). Under the low concentration of Ca-K, Cd increased the Fe content in roots, but Zn and Cd+Zn reduced the Fe content when compared with that of the CK (Fig. *2E*); only Cd+Zn reduced the Fe content in shoots (Fig. *2F*). Under the high concentration of Ca-K, only Zn reduced the Fe content in roots and shoots when compared with that of the CK (Fig. *2E,F*). Compared with the low concentration of Ca-K, the high concentration of Ca-K reduced the Fe content in roots (Fig. *2E*), but increased that in shoots under Cd stress (Fig. *2F*); in addition, the Fe content in roots and shoots was increased under Cd+Zn stress (Fig. *2E,F*).
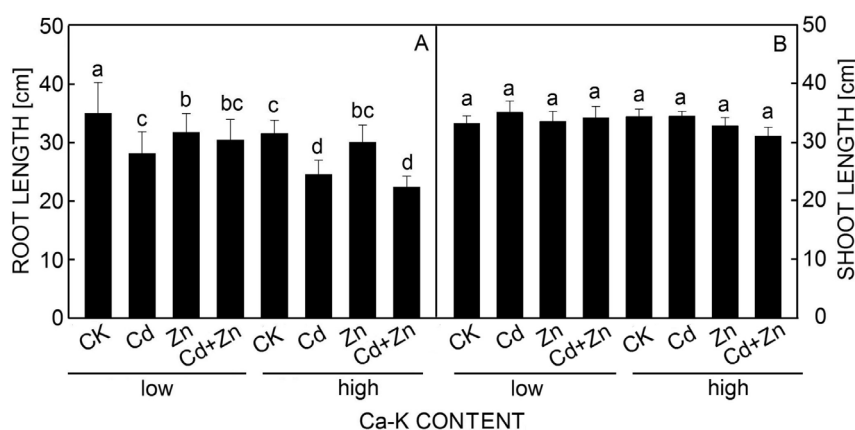

Fig. 1. Root and shoot lengths of seedlings treated with Cd, Zn, and Cd+Zn stresses under a low or high concentration of calcium and potassium. CK - control. Means ± SEs of 30 plants. The Duncan's multiple range test was used to test the significance of differences among the treatments at $\alpha = 0.05$. Different lower-case letters indicate statistically significant differences.
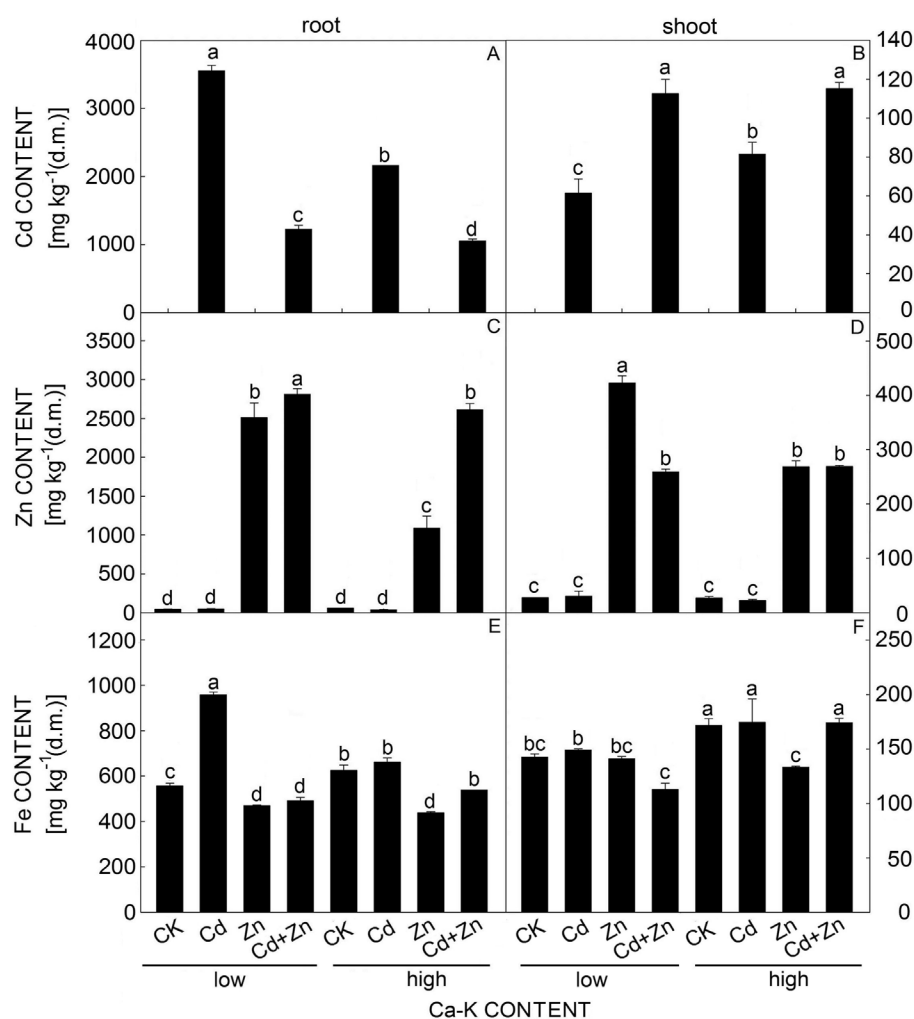
Fig. 2. Metal content in roots and shoots of seedlings treated with Cd, Zn, and Cd+Zn stresses under a low or high concentration calcium and potassium. CK - control. Means ± SEs of three biological replicates. The Duncan's multiple range test was used to test the significance of differences among the treatments at $\alpha = 0.05$. Different lower-case letters indicate statistically significant differences.

Under Cd and Cd+Zn stresses, the high concentration of Ca-K increased the Cd translocation factor compared with that under the low concentration of Ca-K (Fig. 3A). Compared with that under Cd stress, Zn dramatically increased the Cd TF under Cd+Zn stress; interestingly, the increase was greater under the low concentration of Ca-K (Fig. 3A). Under Zn stress, the high concentration of Ca-K increased the Zn TF compared with that under the low concentration of Ca-K (Fig. 3B). Under Cd+Zn stress, the high concentration of Ca-K did not affect the Zn TF (Fig. 3B). Compared with that under Zn stress, Cd reduced the Zn TF under Cd+Zn stress; the reduction in the Zn TF was greater under the high concentration of Ca-K (Fig. 3B).

The concentration of Ca-K influenced the uptake and translocation of Cd and Zn, which would result from changes in the subcellular distribution of Cd and Zn. In roots, the majority of Cd was bound in the cell wall fraction (Fig. 4A); in shoots, Cd was mainly bound in the cell wall and soluble fractions (Fig. 4B). Under Cd stress, the high concentration of Ca-K reduced the Cd content

in the root cell wall (Fig. 4A) and shoot soluble fraction (Fig. 4B). Under Cd+Zn stress, the high concentration of Ca-K reduced the Cd content of the root cell wall and soluble fractions (Fig. 4A) and the shoot soluble fraction (Fig. 4B). Compared with that under Cd stress, Zn remarkably decreased the Cd content of the root cell wall (Fig. 4A), and increased that of the root soluble fraction and shoot cell wall, soluble and organelle fractions under Cd+Zn stress in conjunction with the low concentration of Ca-K (Fig. 4B); in addition, Zn reduced the Cd content of the root cell wall (Fig. 4A), and increased that of the shoot soluble fraction under Cd+Zn stress in conjunction with the high concentration of Ca-K (Fig. 4B).

In roots and shoots, Zn was mainly bound in the cell wall fraction. Under Zn stress, the high concentration of Ca-K reduced the Zn content in cell wall and soluble fractions in roots and shoots compared with those under the low concentration of Ca-K (Fig. 4C,D). Under Cd+Zn stress, the high concentration of C-K reduced the Zn content in the root soluble fraction (Fig. 4C), and increased that of the shoot cell wall (Fig. 4D). Compared with Zn stress,
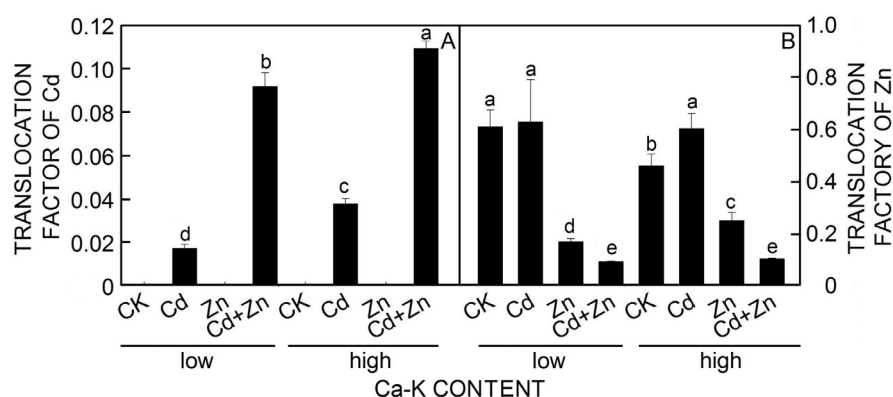
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Fig. 3. Translocation factors for Cd and Zn of seedlings treated with Cd, Zn, and Cd+Zn stresses under a low or high concentration of calcium and potassium. CK - control. Means ± SEs of three biological replicates. The Duncan's multiple range test was used to test the significance of differences among the treatments at $\alpha = 0.05$. Different lower-case letters indicate statistically significant differences.
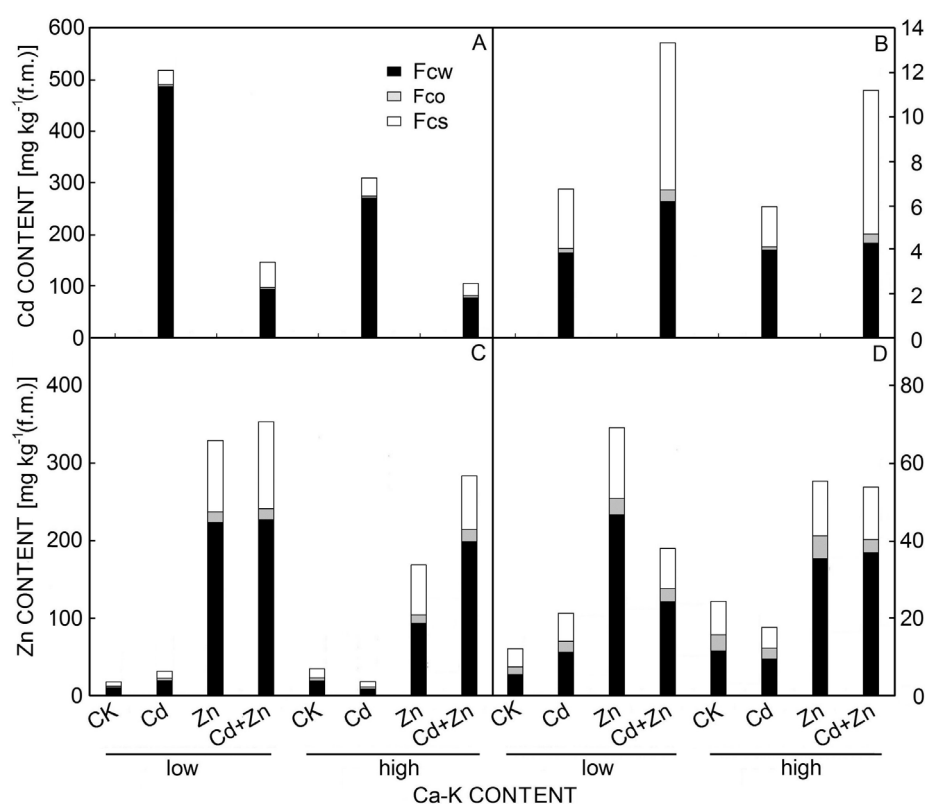

Fig. 4. Subcellular distribution of Cd and Zn in roots (*A,C*) and shoots (*B,D*) of seedlings treated with Cd, Zn, and Cd+Zn stresses under a low or high concentration of calcium and potassium. CK - control, F_{CW} - cell wall fraction, F_{CO} - organelle fraction, F_{CS} - soluble fraction. Means of three biological replicates.

Cd did not change the Zn content of the root cell wall, but increased that of the root soluble fraction (Fig. *4C*) and reduced that of the shoot cell wall and soluble fractions under Cd+Zn stress under the low concentration of Ca-K (Fig. *4D*). Under the high concentration of Ca-K, Cd significantly increased the Zn content of the root cell wall (Fig. *4C*), and reduced that of the shoot cell organelles under Cd+Zn stress compared with that under Zn stress (Fig. *4D*).

Given that Ca-K and Zn differentially influenced the

uptake, translocation, and subcellular distribution of Cd, we investigated whether Ca-K and Zn differentially affected the Cd chemical forms. Under Cd stress, the high concentration of Ca-K enhanced the root wall fraction (Table 1) and the shoot F_W, F_{NaCl}, and F_R (Table 2), but reduced the root F_{HAC} (Table 1) and the shoot F_E and F_{HAC} (Table 2). Under Cd+Zn stress, the high concentration of Ca-K enhanced the root F_{NaCl}, F_{HAC}, and F_R (Table 1), but reduced the shoot F_E, F_W, F_{HAC}, and F_R (Table 2). Under the low concentration of Ca-K, Zn increased the root F_W and

Table 1. Chemical forms of Cd in roots. Cadmium content under control and Zn stress was below the detection limit.

| | Low concentration of Ca-K | | High concentration of Ca-K | |
	Cd [mg kg^{-1}]	Cd+Zn [mg kg^{-1}]	Cd [mg kg^{-1}]	Cd+Zn ([g kg^{-1}]
Fraction extracted by ethanol	9.30 ± 0.32	0.54 ± 0.08	8.74 ± 1.68	0.85 ± 0.11
Fraction extracted by water	7.97 ± 0.88	18.34 ± 1.82	14.82 ± 3.02	13.27 ± 2.22
Fraction extracted by NaCl	26.08 ± 1.32	13.33 ± 2.08	31.74 ± 4.14	22.74 ± 2.53
Fraction extracted by HAc	57.02 ± 0.32	6.11 ± 0.15	27.01 ± 2.90	12.70 ± 0.03
Fraction extracted by HCl	2.00 ± 0.06	0.67 ± 0.13	2.18 ± 0.66	0.59 ± 0.21
Fraction of residual Cd	0.46 ± 0.11	0.06 ± 0.00	0.46 ± 0.03	0.37 ± 0.02

Table 2. Chemical forms of Cd in shoots. Cadmium content under control and Zn stress was below the detection limit.

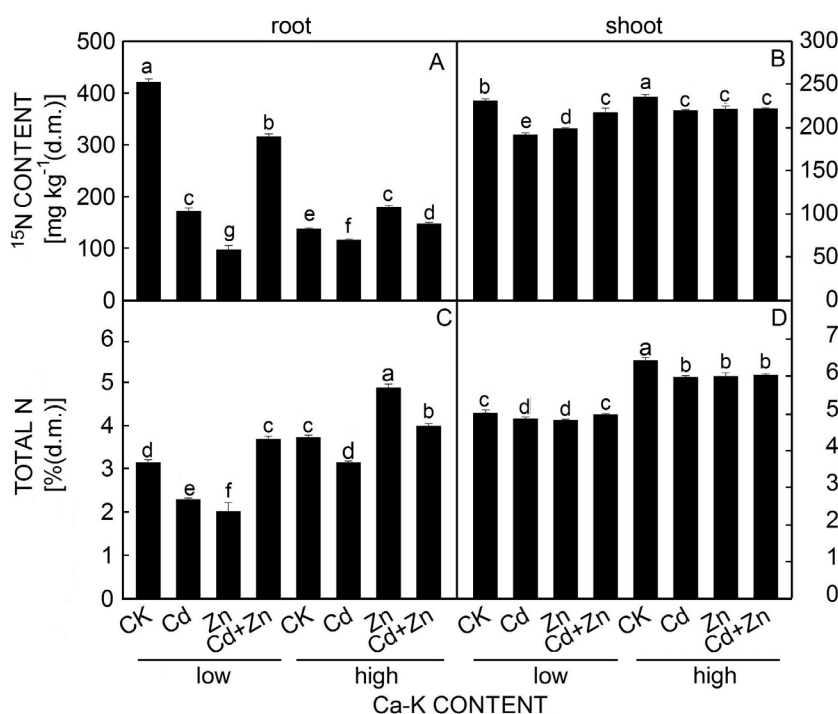| | Low concentration of Ca-K | | High concentration of Ca-K | |
	Cd [mg kg^{-1}]	Cd+Zn [mg kg^{-1}]	Cd [[g kg^{-1}]	Cd+Zn (mg kg^{-1})
Fraction extracted by ethanol	0.38 ± 0.02	0.41 ± 0.03	0.28 ± 0.02	0.26 ± 0.02
Fraction extracted by water	0.30 ± 0.04	0.90 ± 0.08	0.92 ± 0.13	0.63 ± 0.08
Fraction extracted by NaCl	0.15 ± 0.02	0.57 ± 0.06	0.25 ± 0.02	0.48 ± 0.10
Fraction extracted by HAc	0.41 ± 0.09	1.25 ± 0.17	0.22 ± 0.01	0.64 ± 0.06
Fraction extracted by HCl	0.20 ± 0.05	0.21 ± 0.05	0.28 ± 0.15	0.18 ± 0.03
Fraction of residual Cd	$0.00 \pm \pm0.03$	0.00 ± 0.01	0.20 ± 0.02	0.10 ± 0.01

Fig. 5. Nuclide ^{15}N and total N content in roots and shoots of seedlings treated with Cd, Zn, and Cd+Zn stresses under a low or high concentration of calcium and potassium. CK - control. Means ± SEs of three biological replicates. The Duncan's multiple range test was used to test the significance of differences among the treatments at $\alpha = 0.05$. Different lower-case letters indicate statistically significant differences.

reduced the root F_E, F_{NaCl}, F_{HAC}, F_{HCl}, and F_R (Table 1), and also enhanced the shoot F_W, F_{NaCl}, and F_{HAC} under Cd+Zn stress compared with that under Cd stress (Table 2). Under the high concentration of Ca-K, Zn notably reduced the root F_E, F_{NaCl}, F_{HAC}, and F_{HCl} (Table 1), and enhanced the shoot F_{NaCl} and F_{HAC} under Cd+Zn stress compared with

that under Cd stress (Table 2).

Without Cd or Zn stress, the high concentration of Ca-K significantly reduced ^{15}N uptake by roots (Fig. *5A*), but did not affect ^{15}N accumulation in shoots (Fig. *5B*). Under the low concentration of Ca-K, Cd, Zn, and Cd+Zn stresses inhibited ^{15}N uptake by roots (Fig. *5A*) and ^{15}N accumulation in shoots (Fig. *5B*). Under the high concentration of Ca-K, Cd reduced the ^{15}N content, whereas Zn and Cd+Zn enhanced ^{15}N accumulation, in roots compared with that of the CK (Fig. *5A*); all metal stresses reduced the ^{15}N content in shoots (Fig. *5B*). Under Cd and Cd+Zn stresses, the high concentration of Ca-K reduced ^{15}N uptake by roots (Fig. *5A*), but increased ^{15}N content in shoots only under Cd stress (Fig. *5B*). Under Zn stress, the high concentration of Ca-K increased the ^{15}N content in roots (Fig. *5A*) and shoots (Fig. *5B*).

In contrast to the results for ^{15}N uptake and accumulation, the high concentration of Ca-K significantly increased the total N content in roots (Fig. *5C*) and shoots (Fig. *5D*) under all stresses compared with those of the individual stresses in conjunction with low concentration of Ca-K. Compared with the CK, Cd and Zn significantly decreased the total N content in roots and shoots (Fig. *5C,D*); whereas Cd+Zn increased the total N content in roots under the low concentration of Ca-K (Fig. *5C*). Under the high concentration of Ca-K, all metal stresses reduced the total N content in shoots (Fig. *5D*); the total N content in roots was reduced by Cd, but increased by Zn and Cd+Zn compared with that of the CK (Fig. *5C*).

The differences in gene expressions were examined using RNA-Seq (Table 1 Suppl.). Under the high concentration of Ca-K, 18 genes involved in polysaccharide hydrolysis, two *nitrate transporters 1* (*NRT1*) *2.6* and *7.2*), and one *vacuolar iron transporter* (*VIT*) were up-regulated by Cd; the expression of these genes was not influenced by Cd under the low concentration of Ca-K. Under the low concentration of Ca-K, four genes involved in polysaccharide hydrolysis, three metal transporters, and two *NRT1/PTR* genes were up-regulated by Cd; however, the expression of these genes was not affected by Cd under the high concentration of Ca-K.

Under the high concentration of Ca-K, 11 genes involved in polysaccharide hydrolysis and Casparian strip establishment, three genes involved in four metal transporters *ATP-binding cassette subfamily B member 8* (*ABCB8*), *yellow stripe-like protein* (*YSL*) *6*, *Zn transporter* (*ZTP*), and *Cu transporter*), eight metal chelation synthesis genes [*nicotianamine synthase* (*NAS*) and *metallothionein* (*MT*)], and two *NRT1* genes (*NRT1 5.10* and *7.2*), were up-regulated by Zn. Under the low concentration of Ca-K, 27 genes (13 up- and 14 down-regulated) that participate in polysaccharide synthesis and hydrolysis, and seven metal transporters (*VIT*, *MATE*, *Ca* and *Cu transporters*) were regulated by Zn.

To investigate how Ca-K influenced Cd/Zn interactions, we classed the four subgroups including five subgroups. With regard to the Zn influence on responses to Cd, under the low concentration of Ca-K, 27 genes involved in polysaccharide synthesis and hydrolysis, 20 metal transporters, one *NAS* gene, and 11 *NRT* and *NH4$^+$-N transporter* genes were up-regulated by Cd, but not by Cd+Zn (inhibited by Zn). The Zn inhibition of the regulation of some genes was alleviated by the high concentration of Ca-K, which including six genes involved in polysaccharide synthesis and hydrolysis, and four metal transporters. Under the high concentration of Ca-K, eight genes that participated in polysaccharide and chitin hydrolysis, three metal transporters [*VIT* and two *ATP-binding cassette transporters* (*ABC transporters*)], and one *NH4$^+$-N transporter* were up-regulated by Cd, but not by Cd+Zn (up-regulation was inhibited by Zn).

With regard to the Cd influence on responses to Zn, under the low concentration of Ca-K, 22 genes involved in polysaccharide hydrolysis and source transport, 10 metal transporters (such as *HMA5* and *Ca transporter*), three metal chelation synthesis genes, and four *NRT1* genes were differentially expressed in response to Zn, but not to Cd+Zn (inhibited by Cd). The inhibition by Cd of some of these genes was alleviated by the high concentration of Ca-K, which included the up-regulation of *HMA5* and two *NRT1/PTR* genes. Under the high concentration of Ca-K, four genes that participated in polysaccharide hydrolysis and two *NRT1* genes were up-regulated by Zn, but not by Cd+Zn (inhibited by Cd). Three metal transporters, 16 *NAS* genes, and two *NRT1* genes were up-regulated by Zn and Cd+Zn, but not by Cd+Zn under the low concentration of Ca-K.

Discussion

Compared with the low concentration of Ca-K, the high concentration of Ca-K reduced the content of Cd and Zn in roots, suggesting that Ca-K reduced the uptake of Cd and Zn. Meanwhile, the TFs of Cd and Zn were both increased, which indicated that Ca-K promoted the translocation of Cd and Zn from roots to shoots. These results differed from the significant reduction in Cd content in shoots of faba bean and chickpea in response to Ca-K treatment (Siddiqui *et al.* 2012, Ahmad *et al.* 2016), and the increase in Cd content in roots and shoots of wheat in response to K treatment (Zhao *et al.* 2003). However, K and Ca reduce Cd uptake in soybean and wheat (Yang and Juang 2015). Application of NO_3^--N promotes the uptake and translocation of Cd compared with those in the absence of NO_3^--N (Wang *et al.* 2019). Thus, the reduced uptake and promoted translocation of Cd were caused by a high concentration of Ca-K.

In chickpea and faba bean, a high concentration of Ca-K restores the root and shoot growth retarded by Cd stress, and reduces the Cd content in roots and shoots (Siddiqui *et al.* 2012, Ahmad *et al.* 2016), which implies that the reduced Cd content was probably diluted by the increased biomass. In the present study, although the high concentration of Ca-K significantly reduced the uptake of Cd and Zn in roots, it also notably reduced the root length under Cd and Zn stresses. Thus, the reduced uptake of Cd and Zn in response to Ca-K was mediated through reduction in the capacity of the roots to acquire Cd and Zn, which would be supported by changes in gene expression,

Cd and Zn subcellular distribution, chemical forms of Cd, and N content.

Uptake and transport of Cd and Zn are mediated by a series of metal transporters in plants, such as *ABC*, natural resistance-associated macrophage protein (NRAMP), Zn transporter, VIT, and YSL (Curie *et al.* 2009, Wang *et al.* 2010, Park *et al.* 2012, Xu *et al.* 2015, Connorton *et al.* 2017). Overexpression of *AtABCC1* enhances the Cd content in roots of *Arabidopsis* (Park *et al.* 2012). In the present study, two *ABC transporters* and one *Cu transporter* were up-regulated by Cd under the low concentration of Ca-K, but not by Cd under the high concentration of Ca-K, which might result in a higher Cd content in roots under Cd stress in conjunction with the low concentration of Ca-K than that under Cd stress with the high concentration of Ca-K. Overexpression of *TaVIT1* increases the Zn content in barley (Connorton *et al.* 2017), which implies that Zn is sequestrated into the vacuoles (Li *et al.* 2001). Thus, the up-regulation of three *VIT* genes in DPW increased the Zn content in roots and shoots and in the soluble fraction (including vacuoles) under Zn stress under the low concentration of Ca-K, when compared with Zn stress and the high concentration of Ca-K. Zinc transporter, ABC transporter, and YSL transport Zn into the cytoplasm (Wang *et al.* 2010, Xu *et al.* 2015), which facilitates translocation of Zn to shoots. Thus, up-regulation of these transporters enhanced the Zn TF under Zn stress under the high concentration of Ca-K. Increase in shoot NO_3^--N uptake enhances Cd xylem loading and therefore promotes Cd translocation (Li *et al.* 2010). Under Cd and Zn stresses, a high concentration of Ca-K would significantly increase the NO_3^--N content in shoots, which would also account for the enhanced TFs for Cd and Zn under the high concentration of Ca-K in the present study.

Previous studies indicate that plant cell wall binding and/or vacuole sequestration crucially determines the acquisition capacity for Cd and Zn in roots and shoots, and the translocation of Cd and Zn from roots to shoots (Xin *et al.* 2014, Lai 2015, Wang *et al.* 2015, Cheng *et al.* 2018). Cell wall polysaccharides, including pectin and hemicellulose, are major binding sites for Cd and Zn; therefore, degradation of polysaccharides in the cell wall can reduce Cd and Zn uptake by roots and promote their translocation to shoots (Han *et al.* 2014, Li *et al.* 2015). Compared with the low concentration of Ca-K, the high concentration of Ca-K induced up-regulation of a greater number of genes that participate in polysaccharide hydrolysis in DPW under Cd stress. In contrast, compared with the high concentration of Ca-K, the low concentration of Ca-K resulted in down-regulation of a greater number of genes involved in polysaccharide hydrolysis and up-regulation of several genes involved in polysaccharide synthesis under Zn stress. Thus, the high concentration of Ca-K reduced polysaccharide content under Cd and Zn stresses compared with that under the low concentration of Ca-K, thus reducing the Cd and Zn content in the root cell wall fractions, and ultimately promoting Cd and Zn translocation to shoots. Interestingly, under Zn stress, the high concentration of Ca-K resulted in up-regulation of three genes encoding Casparian strip proteins, which

implied that development of the Casparian strips was promoted to reduce Zn entry into roots (Lux *et al.* 2011).

The Ca-K concentration also affects the Cd chemical forms, which would influence Cd migration (Lai 2015, Wang *et al.* 2015). The F_W fraction shows a greater capability to migrate, which facilitates xylem loading to transport Cd to shoots (Wang *et al.* 2015). In contrast, the F_{HAc} fraction shows a lower migration than F_W, which is mainly bound to the cell wall to limit Cd translocation (Lai 2015, Wang *et al.* 2015). Compared with the low concentration of Ca-K, application of Ca/K enhanced root F_W and reduced root F_{HAc}, which, therefore, enhanced the Cd TF, in the present study.

Our present study indicated that Cd and Zn uptakes share the same pathways (Cheng *et al.* 2018), because Zn significantly reduced Cd uptake, whereas Cd dramatically increased Zn uptake. Meanwhile, the high concentration of Ca-K alleviated the reduction in Cd uptake caused by Zn, and reinforced the promotion of Zn uptake caused by Cd. Compared with Cd stress, Cd+Zn also significantly reduced Fe uptake under the low concentration of Ca-K, but did not affect Fe uptake under the high concentration of Ca-K, suggesting that the reduced Cd uptake did not promote Fe uptake under Cd+Zn stress. Thus, Cd uptake does not share the transporters required for Fe uptake. Our previous studies also indicated that two natural resistance-associated macrophage proteins from DPW may be involved in Cd uptake, but not in Fe transport (Peng *et al.* 2018a,b). In contrast, Zn dramatically promoted Cd translocation, whereas Cd dramatically inhibited Zn translocation. Furthermore, the high concentration of Ca-K alleviated the promotion of Cd translocation caused by Zn, and reinforced the inhibition of Zn translocation caused by Cd. These results implied that reduced Zn translocation promoted Cd translocation. Thus, Cd translocation may share the transporters that participate in Zn translocation.

Under the low and high concentrations of Ca-K, different *ABC transporters* were up-regulated by Cd, which indicated that Cd was taken up by ABC transporters in roots (Park *et al.* 2012). However, up-regulation of these genes was inhibited by Zn under Cd+Zn stress, which suggested that Zn reduced Cd uptake. A number of *ABC transporters* up-regulated by Cd were inhibited by Cd+Zn under the low concentration of Ca-K, but were up-regulated by Cd+Zn under the high concentration of Ca-K. Thus, the high concentration of Ca-K alleviated the reduction of Cd uptake caused by Zn. In rice, overexpression of *OsHMA2* increased Cd uptake by roots and reduced Cd accumulation in shoots (Takahashi *et al.* 2012). Under the low concentration of Ca-K in DPW, a *HMA2* gene was up-regulated by Cd, but the up-regulation was inhibited by Zn under Cd+Zn stress, suggesting one mechanism by which Zn reduced Cd uptake and promoted Cd translocation. In addition, Zn and Fe transporters play crucial roles in Fe uptake and transport (Evens *et al.* 2017). Under the low concentration of Ca-K, in DPW several *Zn and Fe transporters* were up-regulated by Cd, but not by Cd+Zn, which indicated that Cd promoted Fe uptake to alleviate Cd toxicity (Wu *et al.* 2012).

In addition, Ca-K and Zn also influenced polysaccharide

metabolism of root and shoot cell walls and the chemical forms of Cd, which determines the capacity to acquire Cd as well as its removability (Xin *et al.* 2014, Lai, 2015, Wang *et al.* 2015, Cheng *et al.* 2018). Under the low and high concentrations of Ca-K, numerous genes involved in polysaccharide synthesis and hydrolysis were regulated by Cd; however, expression of these genes was not inhibited by Zn. Compared with that under Cd stress, Zn significantly reduced the Cd content in the cell wall fraction under Cd+Zn stress. In addition, Zn significantly reduced the Cd content in root F_{NaCl} and F_{HAc}, which show lower migration and are mainly bound in the cell wall unclear (Lai 2015, Wang *et al.* 2015). These results indicated that Zn reduced the Cd acquisition capacity of roots and thereby inhibited Cd uptake. In addition, Zn increased the Cd content in the cell wall fraction and soluble fraction (including vacuoles) in shoots, regardless of the supply of Ca-K. Thus, Zn increased the acquisition capacity for Cd in shoots, and ultimately enhanced Cd translocation to shoots and the Cd content in shoots (Cheng *et al.* 2018). Under Cd+Zn stress, although some inhibitory effects caused by Zn under the low concentration of Ca-K were overcome under the high concentration of Ca-K, the high concentration of Ca-K reduced the Cd content in the root cell wall fraction and soluble fraction, thus reducing the capacity of roots to inhibit Cd uptake. Interestingly, the high concentration of Ca-K increased the Cd content of root F_{NaCl} and F_{HAc}, which implies that the high concentration of Ca-K reduced Cd translocation. In contrast, it enhanced Cd translocation under Cd+Zn stress compared with that under Cd+Zn stress with the low concentration of Ca-K, which may indicate that passive transport through the apoplast (*i.e.*, the cell wall) is involved in the transport of Cd from the root to the shoot (Zhao *et al.* 2010, Li *et al.* 2017).

Ca-K and Cd also regulated expression of a number of metal transporters, and influenced cell wall metabolism and the subcellular distribution of Zn to mediate Zn uptake and transport. Under the low concentration of Ca-K, several *VIT* genes were up-regulated by Zn in roots but not by Cd+Zn. In rice, OsVITs sequestrate Zn into vacuoles to reduce Zn translocation (Zhang *et al.* 2012). Up-regulation of these genes decreased the Zn content in roots and in root cell vacuoles, and enhanced Zn translocation, which is consistent with the up-regulation of *HMA5* that promotes metal translocation to shoots (Wang *et al.* 2018). Since Cd did not influence the Zn content in the root cell wall fraction, the reduction in Zn translocation under Cd+Zn stress resulted from the increased Zn content in the root soluble fraction. Under the high concentration of Ca-K, several genes that participate in polysaccharide hydrolysis were up-regulated by Zn but not by Cd+Zn. Thus, Cd increased the availability of binding sites for Zn under Cd+Zn stress compared with that under Zn stress, and consequently enhanced the Zn content in the root cell wall to increase Zn uptake and to reduce Zn translocation. In addition, under Cd+Zn stress, supply of exogenous Cd/K reduced the Zn content in the root cell wall fraction and soluble fraction, which reduced the Zn acquisition capacity of roots and Zn uptake.

In summary, our results reveal that application of Ca-K changed gene expression, Cd and Zn subcellular distribution, chemical forms of Cd, and then influenced the Cd and Zn uptakes and translocations.

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