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ORIGINAL PAPER

# Double Prevention of Cadmium Uptake by Iron and Zinc in Rice Seedling—A Hypotonic Study

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## Abstract

**Purpose** Rice can accumulate high levels of cadmium (Cd) from contaminated soils, leading to negative health effects when consumed. In the present study, we aim to test the hypothesis that pretreating rice seeds with essential metals, such as iron (Fe) and zinc (Zn), will reduce Cd absorption and transport during growth.

**Methods** Six metal ions, namely Fe, Zn, manganese (Mn), copper (Cu), calcium (Ca), and magnesium (Mg), were evaluated under a pretreatment condition at 10 times the regular dose. Based on their comprehensive effect on reducing Cd absorption and their potential as nutrient elements, Fe and Zn were selected for further studies. Rice seeds were placed in micronutrient growth media containing various concentrations of Fe, Zn, or a mixture of both for 30 days. Afterward, parts of the seedlings were collected for Fe and Zn analyses, while the remaining seedlings were transferred into media containing Cd for 5 days, during which Cd accumulation and membrane protein changes in the rice seedlings were monitored.

**Results** Regarding Cd inhibition, Fe was more effective in the shoots ($r = -0.879$, $P < 0.01$), resulting in an approximate reduction of 46.8%, while Zn was more effective in the roots ($r = -0.786$, $P < 0.01$), leading to a decrease of 26.9%. Simultaneously, putative glycine hydroxy methyltransferase, ferritin, and water stress inducible protein were detected in leaf membrane proteins of rice seedlings pretreated with Fe.

**Conclusions** Our study suggests that pretreating rice seeds with a certain concentration of a Fe and Zn mixture may provide double protection by Zn-mediated Cd absorption and by Fe-mediated Cd transport in the rice seedlings from Cd under the hydroponic conditions.

**Keywords** Mineral element · Cadmium · Metal transport · Accumulation · Interaction · Membrane protein

## 1 Introduction

Metal accumulation in plants has become an environmental concern due to their uptake from contaminated soils, which can enter the food chain. Transition metals are crucial for proper body functions through various biological processes (Menon et al. 2016). However, imbalanced metal homeostasis, either due to deficiency or overload, can be associated with organ dysfunction, leading to various disorders (Becker and Asch 2005). Among heavy metals, cadmium (Cd) is highly toxic to both plants and animals even at low concentrations due to its non-essentiality in living organisms (Huang et al. 2008). It also causes leaf chlorosis, necrotic lesions, wilting, inhibited root elongation, and reduced biomass in plants (Hussain et al. 2015; Tang et al. 2023). Cd can be accumulated in soil via natural processes and anthropogenic activities, such as atmospheric deposition, industrial activities, sewage sludge disposal, and fertilizer application (Liang et al. 2017; Dharma-Wardana 2018). Cd has an extremely long biological half-life (> 20 years), ranking 7th among the top 20 toxins declared by the US-EPA (Yang et al. 2004; Usman et al. 2022). Cereal crops such as rice, wheat, and maize fulfill the major food requirements worldwide. Among these cereals, rice has the ability to accumulate high levels of Cd, primarily through its roots, and can then translocate it to aerial parts, finally accumulating it in rice grains (Wiggenhauser et al. 2021). For humans,

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especially those whose staple food is rice, food is the most important source of non-occupational exposure to Cd. High levels of Cd in food can cause health problems.

Heavy metal entry into the food chain poses a significant threat to human health. Therefore, there is a global effort to find effective ways to remove heavy metals from the soil and other contaminated layers of the biosphere (Ghori et al. 2016). Previous studies have shown that soil heavy metal pollution remediation mainly involves two aspects: changing the form of Cd in the soil and reducing its reactivity, or directly decreasing the amount of Cd in the soil. The former effectively reduces the soil's effective cadmium content by adding soil-situ passivating agents, such as calcium oxide, silicate, magnesium oxide, and biochar, among others (Beesley et al. 2011; Sun et al. 2015; Abad-Valle et al. 2016). The latter reduces the total amount of heavy metals in the soil through methods like electrochemical leaching, the guest soil method, and phytoremediation (Liu et al. 2018). One common concern is whether employing chemical and physical methods for cleaning up contaminated areas will cause re-contamination in the treated regions (Ozyigit 2021). Additionally, due to the long restoration cycle of phytoremediation and the large amount of farmland that must be occupied simultaneously, its impact on agricultural production cannot be ignored. Therefore, as a green and sustainable strategy, the use of nutrient elements to regulate Cd accumulation and transport patterns in rice has been gaining attention. As a non-essential element in plants, Cd mainly enters plants through transport carriers of essential metal ions, such as the ZIP family or NRAMP family (Li et al. 2022; Himeno et al. 2019). The content of nutrient elements in plants is related to Cd absorption in crops, the same ion channels and transport carriers lead to antagonistic competitive absorption between essential and non-essential ions.

Cd accumulation in plants depends on root to shoot translocation, while accumulation in grains depends on both roots to shoot transfer and a direct pathway of Cd transport from roots to grains via xylem to phloem transfer in the stem (Harris and Taylor 2013). Essential micronutrients for plant metabolism, such as $Cu^{2+}$, $Zn^{2+}$, $Mn^{2+}$, and $Fe^{2+}$, are bivalent metal ions. However, non-essential metal ions like $Cd^{2+}$ can also enter plants using the same pathways, leading to accumulation and potential toxicity if consumed (Williams et al. 2000). Uncoupling the transport of Cd and beneficial micronutrients is an effective way for crops to reduce Cd accumulation. (Huang et al. 2020). Research has indicated that both Mn and Fe deficiency induce an up-regulation of HvIRT1 in plant, while overexpression of the transporter IRT1 could cause plants to exhibit higher levels of Cd accumulation (Pedas et al. 2008). Enrichment pairs of Zn and Mn in rice seedlings can effectively inhibit the expression of OsIRT1 and enhance the absorption and transport competition between Cd and nutrient elements in rice roots, thereby reducing Cd accumulation in roots (Huang et al. 2021). Zn has been regarded as an antagonistic element in limiting Cd entry into plants, with similar tendencies in uptake between Zn and Cd (Qin et al. 2020), and Fe supply inhibits Cd uptake in Arabidopsis, whereas Fe deficiency increases Cd accumulation in peanut (He et al. 2017). Foliar Fe application significantly reduces Cd accumulation by 15.9% in brown rice, decreases the translocation of Cd from roots to other plant tissues, and increases the net photosynthesis rate by 19.3% (Wang et al. 2021). Visibly, the uptake and transport of Cd in plants are closely related to the content of medium and micronutrient elements. Previous studies have shown cadmium accumulation in rice after application of nutrients such as Zn and Fe (Wang et al. 2021; Liu et al 2020; Huang et al. 2018). In the present study, we pretreated the rice seedling with Zn and Fe and then followed by growing the rice seedling in a Cd contaminated environment to see how the pretreatment affects Cd uptake and the expression of membrane proteins in stems and leaves of rice after pretreatment.

In the present study, we tested a hypothesis that Cd shares the same absorption and transport pathways with essential metal ions, and pretreatment of rice seeds with these metal ions alters the expression of Cd transport associated genes (e.g., *OsZIP*, *OsIRT*, etc.) and preoccupy the divalent metal transport channels, thereby, decreasing Cd absorption and transport during growth. We designed a series of hydroponic experiments to investigate the effects of six essential metal ions' enrichment on Cd accumulation in rice seedlings. Our method aims to inhibit Cd accumulation and transport in plants by regulating the content of nutrient elements in rice. The method is not restricted by the long half-life of Cd; it is environmentally friendly, has no risk of secondary pollution, and can be immediately applied to grow rice grains with Cd levels below permissible levels. We hope that our research results could provide a green way for the safe production of Cd-contaminated rice fields.

## 2 Materials and Methods

### 2.1 Chemical Reagents and Seed Germination

All chemicals were purchased from Sinopharm Chemical Reagent Co. Ltd® (Beijing, China). After an initial screening, the rice species Yuzhenxiang, which exhibited a higher capacity for absorbing Cd, was selected for this study (Zhang et al. 2017). These seeds were provided by the Hunan Academy of Agriculture Sciences. The rice seeds were germinated as previously described (Liu et al. 2007; Wei et al. 2021) and subsequently grown in a full nutrient solution containing the following compositions of the chemicals. The molar concentrations were expressed as μM of the chemicals: $(NH_4)_2SO_4$, 180 μM; $KNO_3$, 70; $KH_2PO_4$, 90; $MgSO_4 \cdot 7H_2O$, 270; Ca $(NO_3)_2 \cdot 4H_2O$, 180; NaEDTA-Fe·$3H_2O$, 20; $MnCl_2 \cdot 4H_2O$, 6.7; $CuSO_4 \cdot 5H_2O$, 0.16; $ZnSO_4 \cdot 7H_2O$, 0.15. Most of the metal solutions are in sulfate. According to the improved conventional nutrient solution of the International Rice Research Institute, manganese chloride is recommended as the source and was used in the present study. The pH of the nutrient solution under all treatments was maintained at about 5.5. The stock solution was 10-times (10x) concentrated and was kept at 4 °C until use.

### 2.2 Multiple Metals Absorption Experiments

All hydroponic experiments under laboratory conditions were performed as follows: rice seedlings were grown in plastic containers in the full nutrient solution. The average temperature throughout the test period was between 28 °C (daytime) and 20 °C (night), and the relative humidity was 70 ± 5%. Metal levels in rice plants grown in the full nutrient were considered as the base and used as controls. The plants were irrigated with distilled water as needed and the nutrient solution was replaced every three days. Each experimental condition was carried out at least in triplicate.

Two sets of experiments were conducted to investigate metal ion interactions and their pretreatment effects on Cd absorption. The first set examined the effects of each individual metal ion with 10 × concentrations of Fe, Mn, Cu, Zn, Ca, and Mg from the base nutrients on Cd absorption and transport. The experimental groups were as follows: 1. control or CK (base nutrient solution, Fe 20 μM, Mn 6.7 μM, Cu 0.16 μM, Zn 0.15 μM, Ca 180 μM, Mg 270 μM); 2. Fe (200 μM); 3. Mn (67 μM); 4. Cu (1.6 μM), 5. Zn (1.5 μM), 6. Ca (1800 μM), and 7. Mg (2700 μM). In brief, a 10 × dose of each individual metal ion was added to the full nutrient solution. We chose 10 × concentration to ensure a positive response that can be detected, if any. The pretreatment lasted 30 days to mimic the rice seeding growth of 26–30 days, which referred on the cultivation cycle of rice seedling in field. After 30 days, parts of the seedlings were collected for analyses of the enrichment of trace metal ions, and the rest were transferred into the full nutrient solution containing 10 μM Cd and were kept for 3 days. Following Cd treatments, seedlings were collected and analyzed for Cd concentrations. This 10 μM Cd concentration served as a screening testing for the inhibitory effects of each metal on Cd uptake.

Following the first set of experiments, Fe and Zn were found to have relatively high inhibition in Cd adsorption in both shoots and roots, and they were selected for the second set of experiments. The second set of experiments was carried out as follows: CK (base full nutrient solution), Fe (40 μM, 100 μM, and 200 μM), Zn (0.38 μM, 0.75 μM, 1.5 μM), Fe/Zn (Fe 100 μM + Zn 0.75 μM and Fe 200 μM + Zn 1.5 μM). After 30 days, parts of the seedlings were collected for analyses of the metal ions, and the remaining seedlings were transferred into a 50 μM Cd solution and kept for 5 days for Cd studies. A high concentration of 50 μM Cd was used to see how significant the inhibitory effects of Zn, Fe, and the combination of Zn and Fe would be on Cd uptake.

The collected seedlings were washed with running tap water and rinsed with ultrapure water to remove any metals attached to the plant surfaces. The roots and shoots were separated and oven dried for 30 min at 105 °C, then at 65 °C until they reached constant weight. The dried tissues were ground into a powder. The metal ion levels were measured using inductively coupled plasma mass spectrometry (iCap-Q, Thermo, USA) after digestion with mixed acid [$HNO_3 + H_2O_2$ (5:2 v/v)] and the results were expressed as mg of metals per kg of dried weight from the parts of rice plants specified.

The translocation factor (TF) and the bio-concentration factor (BCFs) were calculated as previously described by (Grispen et al. 2006):

$$TF = [Cd]shoot/[Cd]root$$

$$BCF = [Cd]shootorroot/[Cd]solution$$

### 2.3 Total and Membrane Protein Isolation and LC–MS/MS Analysis

Following the previous series of experiments, three treatments (Fe 200 μM; Zn 0.75 μM; Fe/Zn 100 /0.75 μM) were found to have a significant inhibition in Cd adsorption. Therefore, the seedlings of the above three treatments were selected for analyses of membrane proteins as follows.

Purification of the plasma membrane was processed as described with some modifications (Tamayo et al. 2017). The seedling leaves was homogenized and centrifuged under liquid nitrogen. After that, membrane proteins were incubated with 0.5% Triton X-100 in 20 mM Tris–HCl buffer (TBS, pH 7.0) containing 1 mM $MgCl_2$, 0.1 M NaCl, 1 mM DTT, and 0.5 mM EDTA for 2 h at 0℃. After ultracentrifugation (12,000 × g, 4℃, 10 min), the supernatants were collected for further column purification and used for analyses by SDS-PAGE and LC–MS/MS.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (15% SDS-PAGE) was utilized. The gels were stained using Coomassie Blue Staining (CBS) Solution. Because of the budget constraints and the dense band from Fe treatment which may cover both Zn and/or Fe treatment bands, only control and Fe treatment groups were used to perform LC–MS/MS analyses. The target bands of the CBS binding proteins were cut off from the 15% SDS-PAGE gel and digested with protease. The digested samples were analyzed by LC–MS/MS (Orbitrap Fusion, Thermo Fisher Scientific, USA). Based on the MS data, the candidate binding proteins were identified with the databases of UniProt, PubMed, and TMHMM.

### 2.4 Statistical Analyses

Data were analyzed for each dose using one-way analysis of variance (ANOVA) to establish significant differences among the treatments. Means across all metal ions or various concentrations were compared by the least significant difference (LSD) test at the 0.05 level using SPSS version 17. The correlation between the Cd content and Zn or Fe content in roots and shoots was analyzed by using EXCEL (Microsoft Office 2019). All data are presented as means ± standard error (SE, $n = 3$).

## 3 Results

### 3.1 Effects of Metal Interactions on Their Absorption in Rice Toots and Shoots

Figure 1 shows the growth of rice seedlings under hydroponic conditions with 10 × enriched metal solutions for 10 days. Among the metals, Fe promoted the greatest growth, specifically in terms of leaf color and plant height, while there was no significant difference in plant height and leaf color in other treatments, and the growth of rice seedlings under Mn treatment was weaker. Figure 2A and B depict the levels of metal absorption in the roots and shoots, respectively, after 10 × enrichment of each metal. In roots, Mn, Fe, Ca, and to a lesser extent, Cu and Zn led to approximately 7×, 6×, 6×, 5×, and 4 × increases over the control, respectively (Fig. 2A-b, 2A-a, 2A-e, 2A-c, and 2A-d). Metals also interacted with each other, with notable effects including enhanced Mn absorption and decreased Fe absorption under the Cu treatment (Fig. 2A-c). Zn significantly inhibited the absorption of Fe, Cu, Ca, and Mg (Fig. 2A-d), while Mg also significantly inhibited Ca absorption (Fig. 2A-f). In shoots, the most noticeable increase over the control was observed for Mn absorption, which was 4 × higher (Fig. 2B-b), followed by Ca and Cu, which were 3 × and 2.5 × higher, respectively (Fig. 2B-e and 2B-c). The interactions between metal ions in shoots were consistent with roots, as Cu enhanced Mn content (Fig. 2B-c), Ca prevented the accumulation of Mg (Fig. 2B-e), and Mg inhibited Fe absorption (Fig. 2B-f). Additionally, Fe and Zn were found to inhibit each other (Fig. 2B-a and 2B-d).

When compared to the other nutrient elements, levels of Mn, Ca, and Mg in the shoots were approximately 3–5 × higher than those in the roots (Fig. 2A-b, e, f). These results suggest an active mechanism in the Yuzhenxiang rice seedlings for moving Mn, Mg, and Ca ions from the roots to the shoots. In contrast, levels of Zn, Cu, and Fe in the shoots were only about 50%, 40%, and 30% of those in the roots, respectively. This indicates that the uptake of Zn, Cu, and Fe may occur either passively through diffusion or be inhibited through controlled mechanisms. Furthermore, the observed

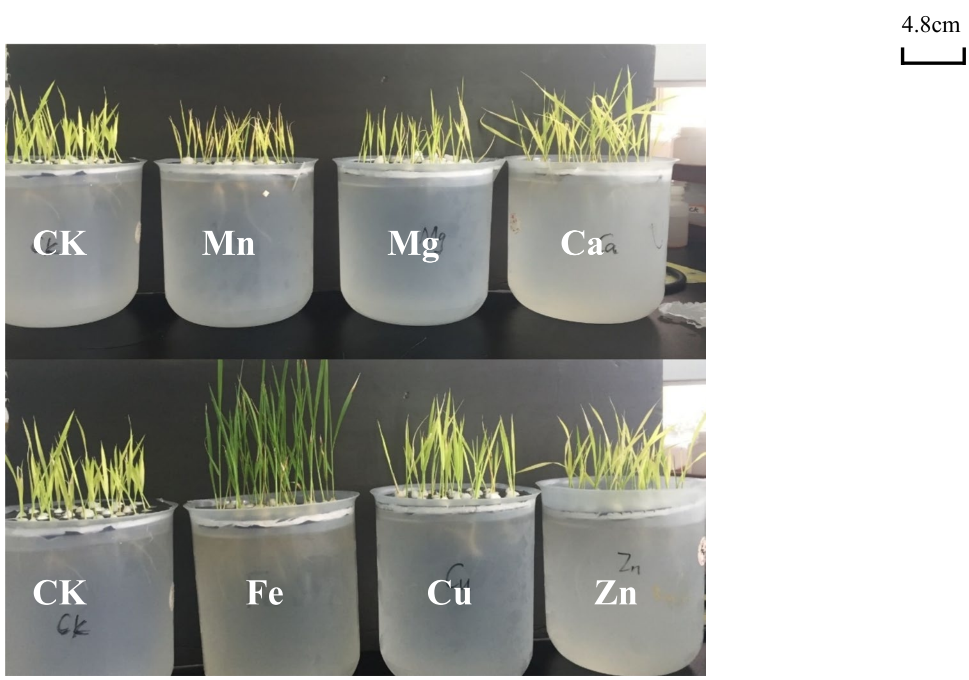



**Fig. 1** Growth diagram with rice seedlings of 10-time (10×) enrichment of each metal for 10 days. At the early stage of rice growth, the seedlings in all treatments (except for the Fe treatment) exhibit similar colors and growth. After 2 weeks and beyond, they all catch up as shown in Fig. 4A for 17 or 30-day treatment. This diagram is to show that there was a difference among groups at the beginning of the experiment, but the difference disappeared after two weeks. The final measurements were done on day 30, mimicking the 30-day growth period of the rice seedling stage

inhibition of Fe and Zn uptake and transport by each other suggests that they may share a common pathway for uptake and transport.

### 3.2 Effects of Metal Ion Pretreatment on Cd Accumulation

Following a 30-day pretreatment with each metal, rice seedlings were treated with 10 μM Cd for three days. Figure 3 shows that Cd contents in the roots and shoots of the rice seedlings ranged from 29.2 mg/kg (shoots) to 315 mg/kg (roots) across all samples. Cd levels in rice seedlings without Cd treatment were below detection limits (data not shown). In roots, pretreatment with Mg (Fig. 3a) and Zn inhibited Cd uptake with Zn being the most effective by inhibiting Cd uptake by 14% ($P<0.05$). Cd levels in shoots were 15% of those in roots (Fig. 3b), suggesting an active transport mechanism similar to Fe, Zn and Ca (Fig. 2a, d, and e), which conforms to the law of nutrient absorption and transportation in plants. All treatments inhibited Cd transport in the shoots, with Zn, Fe, and Cu showing inhibition rates of 38%, 30%, and 27%, respectively (Fig. 3b).

### 3.3 Effects of Fe and Zn on Rice Seedling Uptake and Transport

According to Pence et al. (2000), Fe and Zn transporter genes share similarities and can transport Cd. Our study found that Fe and Zn inhibited each other's uptake. To investigate this, we pretreated rice seedlings with various doses of Fe, Zn, or both. Figure 4a shows that the treatments did not affect the growth of the seedlings. Interestingly, the leaves of those treated with Fe or Zn appeared greener than the control. The levels of Fe and Zn in the roots increased in a dose-dependent manner (Fig. 4A and B).

Fe levels in the shoots of all treatments were higher than those of the control, and there was a clear dose-dependence (Fig. 4B-c). Similarly, Zn levels in the shoots increased with increasing doses of Zn (Fig. 4B-d). In contrast, the higher dose of the Fe-Zn mixture resulted in lower levels of these two metals in the rice shoots and roots compared to an equal application of single processing. For instance, in rice treated with Fe 200 μM + Zn 1.5 μM, the content of Fe and Zn in shoots was reduced by 7.1% and 31%, respectively. Conversely, under low dose mixed treatments (Fe 100 μM + Zn 0.75 μM), the accumulation of Fe and Zn in shoots and roots increased by different degrees (Fe increased by 26% to 44%, Zn increased by 1.3% to 26%). These results suggest an inhibition of metal transport from roots to shoots, which is consistent with previous observations that the transport of Fe and Zn from roots to shoots is inhibitory or passive at certain concentrations, providing a rationale for Cd uptake inhibition.

### 3.4 Effects of Fe, Zn, or a Mixture of Fe and Zn Pretreatment on Cd Accumulation

Figure 5 demonstrates that low doses of Fe inhibited Cd uptake by roots, but higher doses did not show significant inhibition. In general, Fe did not have obvious inhibitory effects on Cd uptake in the roots (Cd 50 μM in solution), whereas Zn alone and Zn + Fe mixture treatment inhibited Cd accumulation in roots ($P<0.05$), and the inhibition efficiency increased with the increase of zinc dose. Moreover, Fe inhibited Cd in shoots, and the inhibitory efficiency increased with the Fe dose. These results confirm that the Yuzhenxiang rice species has a high capacity for absorbing from liquids containing Cd. Notably, the mixture of Fe and Zn consistently decreased Cd levels in the shoots (Fig. 5).

As shown in Table 1, the translocation factors (TF) of 200 μM Fe, 0.75 μM Zn, and 100 μM Fe + 0.75 μM Zn were significantly lower than the control. Moreover, the bioconcentration factors (BCF) of all treatments in the roots and shoots were lower than the control ($P<0.05$, Table 1) except for1.5 μM Zn in shoots and 200 μM Fe in roots. It's worth noting that the BCF values of shoots under Fe treatments were significantly decreased by 27% ($P<0.05$), and the roots with Zn treatments were significantly decreased by 21% ($P<0.05$).

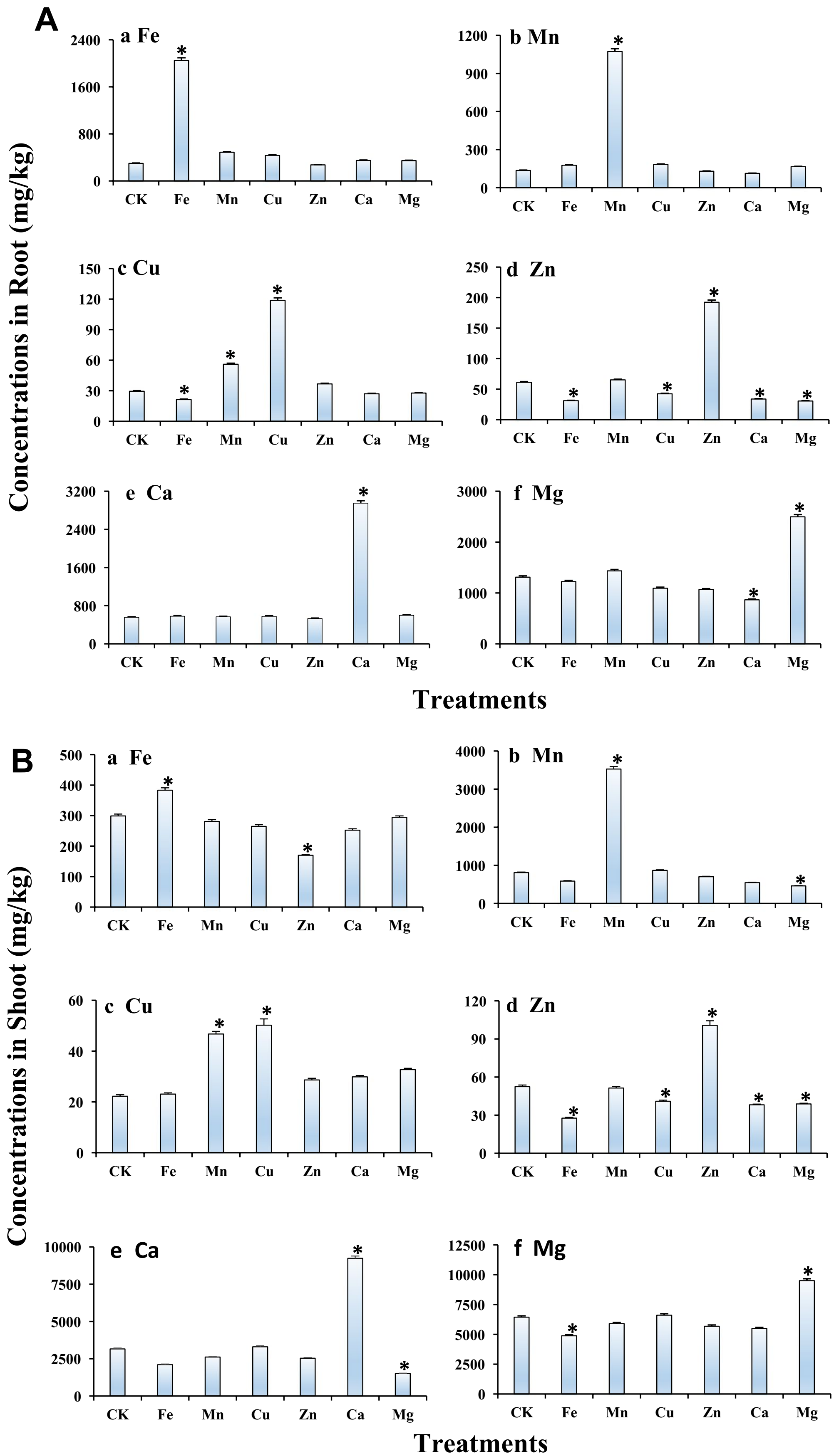
### 3.5 Correlation Between the Concentration of Cd and Zn or Fe

As shown in Fig. 6, there were negative correlations between Cd content and Zn in roots (Fig. 6b, $r$ = -0.786, $P<0.01$, data from CK, Zn alone treatment, and Fe&Zn mixed treatment) and Fe in shoots (Fig. 6c, $r$ = -0.879, $P<0.01$, data from CK, Fe alone treatment, and Fe&Zn mixed treatment). Based on the intensity of Cd in rice seedlings under single treatment of Fe or Zn and Fe&Zn coincidence treatment, these results suggest that the mixture of Fe and Zn may be the optimum decision to prevent Cd absorption and transport in the roots and shoots of rice seedlings under hydroponic conditions.

### 3.6 Isolation and LC–MS/MS Analysis of Membrane Protein

The plasma membrane was extracted from the rice leaves, and the SDS-PAGE analysis (Fig. S1) showed that the target plasma membrane band was between 30 and 45 kDa. Notably, under the Fe treatment, the band appeared thicker than that of the control, and in the Fe/Zn 100/0.75 μM treatment, there were double bands.

To identify the proteins present, the bands between 35–45 kDa from the control and Fe treatment were subjected to further LC–MS/MS analysis. The MS data were analyzed using the Rice database of UniProt, and PubMed and proteins with


A
a Fe
b Mn
c Cu
d Zn
e Ca
f Mg
Concentrations in Root (mg/kg)
CK Fe Mn Cu Zn Ca Mg
Treatments
B
a Fe
b Mn
c Cu
d Zn
e Ca
f Mg
Concentrations in Shoot (mg/kg)
CK Fe Mn Cu Zn Ca Mg
Treatments

◂**Fig. 2** Effects of 10×enrichment of each metal on the absorption of the target metal as well as five other metals in the roots (A) and shoots (B) after 30 days. The full nutrient base solution contains the following compositions (μM of the chemicals): $(NH_4)_2SO_4$, 180 μM; $KNO_3$, 70; $KH_2PO_4$, 90; $MgSO_4·7H_2O$, 270; Ca $(NO_3)_2·4H_2O$, 180; NaEDTA-Fe·$3H_2O$, 20; $MnCl_2·4H_2O$, 6.7; $CuSO_4·5H_2O$, 0.16; $ZnSO_4·7H_2O$, 0.15, pH 5.5. For example, when Zn is 10×enriched, the final concentration of $ZnSO_4·7H_2O$ is 1.5 μM, and the concentration of other elements remains consistent with that of the original nutrient solution. The base solutions served as the control. Roots were collected for Zn as well as for Fe, Mn, Cu, Ca, and Mg analyses. Experiments were carried out in triplicate, and the results were expressed as mg of metal per kg of dried weight from the parts of the rice plants specifics (B). Data were presented as means ± SE with significant differences by LSD test, $P < 0.05$

scores over 50 were summarized in Table 2 and 3. In both the control and Fe treatment, similar proteins were identified, such as alanine aminotransferase and chlorophyll a/b-binding protein, which are related to energy transformation and photosynthesis (Table 2). However, only proteins induced by Fe were found in the following categories (Table 3): transmembrane protein, ferritin, water channel protein, chlorophyll a/b-binding protein precursor, acid phosphatase, rieske Fe sulfur protein, chloroplastic aldolase, etc. These specific proteins may be responsible for blocking Cd transport. These results await further investigation.

## 4 Discussion

Previous studies have shown that the mechanisms of Cd transport in plants may be similar to that of Zn (Sharifan and Ma 2021). Indeed, the Zn transporter, ZNT1, has been found to enhance the translocation of Cd in plants (Lasat et al. 2000). Sequence analysis of ZNT1 cDNA has indicated significant sequence homology with a putative Fe transporter, IRT1. In addition to $Fe^{2+}$, IRT1 may facilitate the transport of heavy metal divalent cations such as $Cd^{2+}$ and $Zn^{2+}$(Clemens 2006). In this study, several divalent metal cations were enriched and pretreated. The results showed mutual promotion or antagonistic competition between divalent cations. A large amount of Cu greatly promoted the accumulation of Mn in crops. However, Zn and Fe showed antagonism and blocked the absorption of each other (Fig. 2A, B), there was a strong competitive relationship between Fe and Zn, and the inhibiting effect of Zn on Fe absorption was more obvious, especially in root. While some studies indicated that Zn oxide nanoparticles also promoted Fe and Cu uptake in rice shoots (Sharifan and Ma 2021). Meanwhile, our research indicated that Cd accumulation in rice roots under Zn pretreatment and in rice seedlings under Fe pretreatment simultaneously decreased (Figs. 3 and 5). This phenomenon triggered our thinking on whether the accumulation of macronutrients in crops would break the chemical balance of absorption and transport of other ions with the same valence state.

Essential metals are distributed to different cells and organelles of plants depending on their required concentration through a variety of metal transporters (Shafiq et al. 2020). Among them, the Zn and Fe-regulated transporters, like the Protein (ZIP) family, have been reported in crops and are essential for the uptake of Zn (Tiong et al. 2015; Evens et al. 2017). However, the ZIP family members may also uptake Fe, Mn, and Cd (Li et al. 2013). Some studies indicated that the genes OsZIP5 and OsYSL15 are most likely responsible for the uptake and translocation of both Fe and Cd in rice seedlings (Ali et al. 2020). Furthermore, transgenic plants overexpressing OsZIP9 had significantly enhanced Zn/Cd levels in the aboveground tissues and brown rice (Tan et al. 2020). Studies have shown that transcripts of OsZIP11 were significantly induced under Fe deficiency but not under Zn, Cu, or Mn deficiency, and

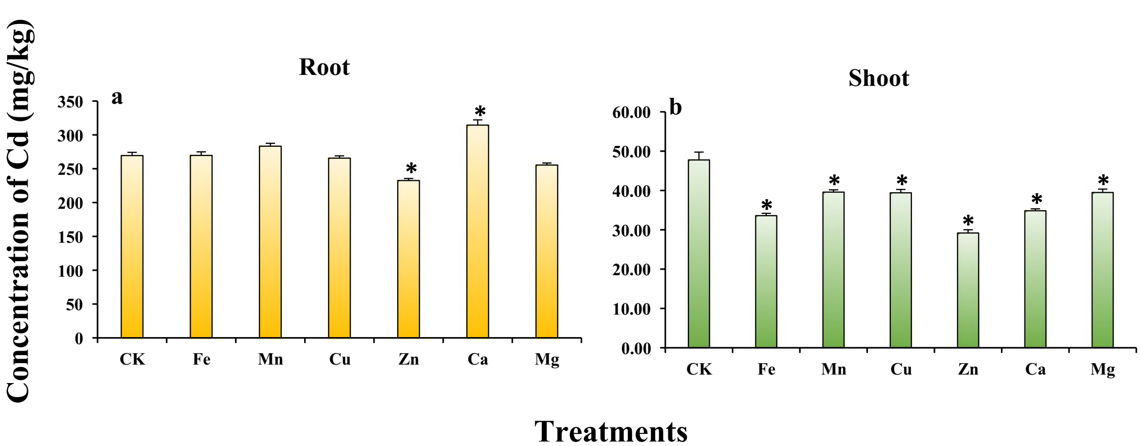
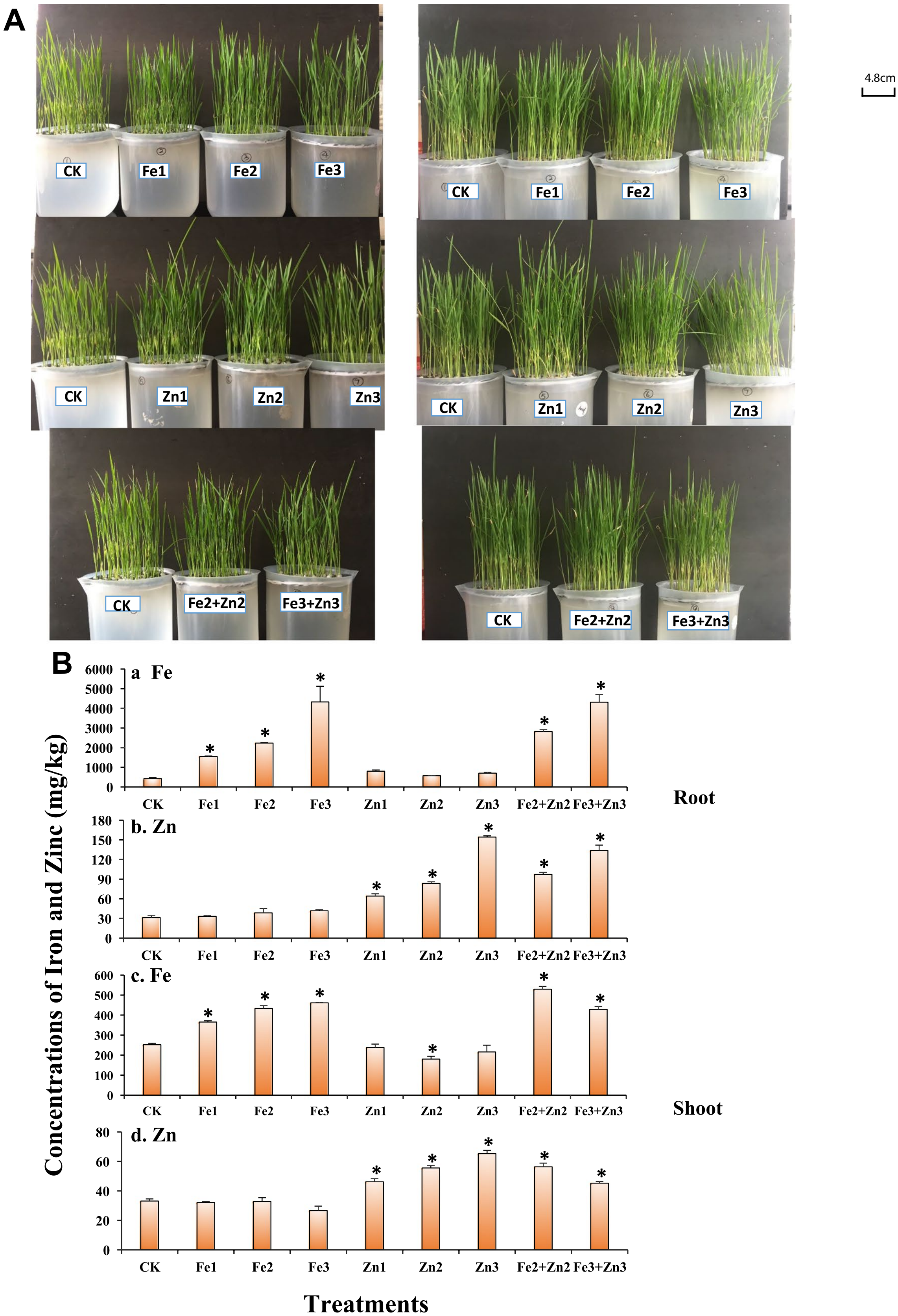



**Fig. 3** Effects of 10×enrichment of each metal on the absorption of Cd in the roots and shoots. Rice seedlings grown under the base or enriched solutions for 30 days were further exposed to 10 μM Cd solution for 3 days. Rice roots and shoots were collected and analyzed for Cd content. Significant differences were detected by LSD test, $P < 0.05$


A
CK
Fe1
Fe2
Fe3
4.8cm
Zn1
Zn2
Zn3
Fe2+Zn2
Fe3+Zn3
B
a Fe
b. Zn
c. Fe
d. Zn
Root
Shoot
Concentrations of Iron and Zinc (mg/kg)
Treatments
6000
5000
4000
3000
2000
1000
0
180
150
120
90
60
30
600
500
400
300
200
100
80
60
40
20
*

◂**Fig. 4** Effects of various concentrations of Fe, Zn, and a mixture of Fe and Zn on the growth of rice seedlings for 17 and 30 days (A) and the absorption of Fe and Zn in the roots and shoots (B). Experimental details were described in the legend of Fig. 1. Significant differences were detected by LSD test, $P<0.05$. They were carried out as follows: CK (base full nutrient solution), Fe1-Fe3 (40 μM, 100 μM, and 200 μM), Zn1-Zn3 (0.38 μM, 0.75 μM, 1.5 μM), Fe1 + Zn1 and Fe2 + Zn2 (Fe 100 μM + Zn 0.75 μM or Fe 200 μM + Zn 1.5 μM), the same labels below

OsZIP11 played an important role in Fe acquisition during rice growth and development (Zhao et al. 2022). It follows that Cd, Zn, and Fe may share the same uptake and transport genes. To test this hypothesis, rice seedlings were pretreated with Fe, Zn, or a mixture of Fe and Zn. Our belief is that pretreatment of rice seedlings with these metal mixtures will downregulate genes responsible for Fe and Zn uptake and transport, thereby achieving a dual effect in reducing Cd accumulation and transport in rice.

Indeed, we had several important findings in the present study that not only supported our hypothesis, but also provided a mechanistic view of having two essential metals together to prevent Cd uptake. Firstly, our results suggest that a mixture of Fe and Zn pretreatments may present a better strategy than a single metal treatment. Regression analyses showed negative correlations between Zn and Cd in roots and between Fe and Cd in shoots (Fig. 6). These results indicate that double inhibition of the pathway by two different metals on two different parts of the rice seedling may be more effective in preventing Cd uptake under extreme environmental stress than by single metal treatment. Secondly, the appropriate ratio of Fe and Zn is very important to prevent the accumulation and transport of Cd. High levels of Fe significantly inhibited Zn levels in the shoots (Fig. 2B-a) and, to some extent, in the roots (Fig. 2A-a). Fe-enrichment promoted the development of Fe plaque on the surface of rice roots, which decreased the migration activity of Cd in roots, and reduced the transport of cadmium to the aboveground part (Yang et al. 2020; Yin et al. 2020). The research indicated that Fe prevents Cd from migrating from roots to shoots, and Zn high levels of Zn weaken the effect of Fe (Table 1). The inhibitory effects of Zn and Fe were reciprocal. High levels of Zn significantly inhibited Fe levels not only in the roots (Fig. 2A-d) but also in the shoots (Fig. 2B-d), the development of Fe plaque was weak, which decreased the fixation effect of Cd. Meanwhile, Zn and Cd shared transport channels, and the zinc absorption demand of rice is reduced under high zinc environment, thus reducing the accumulation of Cd by roots. Due to the competitive relationship between Fe and Zn, the effect of high concentration of Fe/Zn treatment on Cd inhibition is weaker than that of low concentration mixed treatment (Table 1). These results suggest that 1) the Zn pathway in Yuzhenxiang rice,

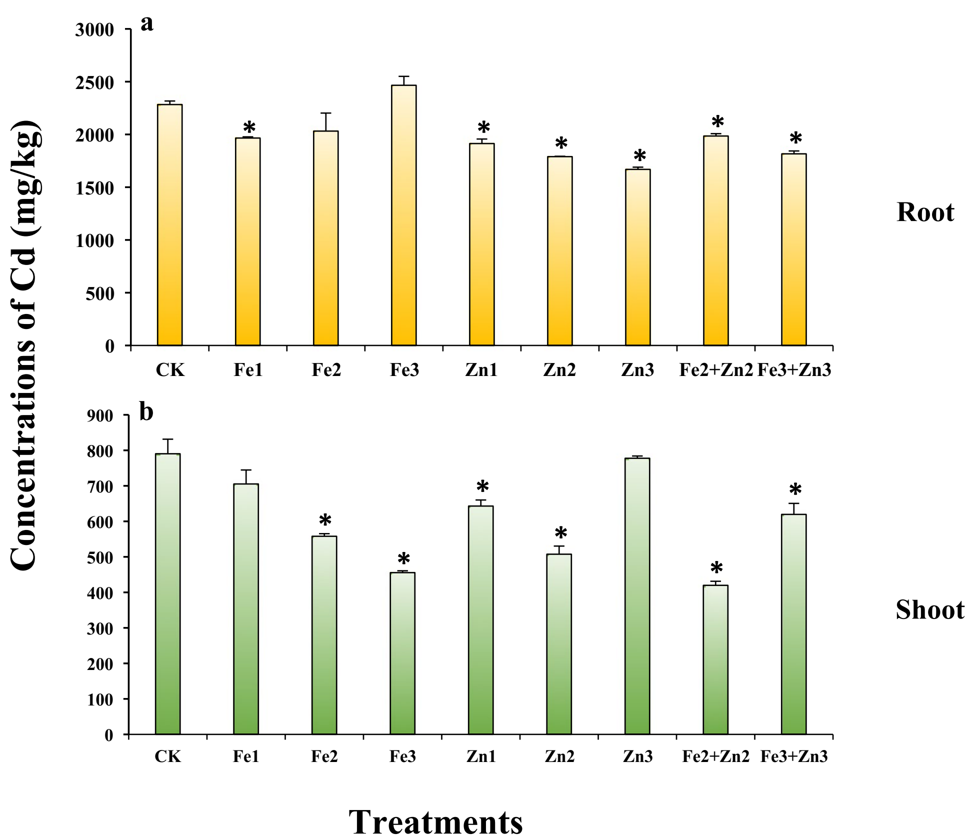



**Fig. 5** Effects of pretreatments of Fe, Zn, and the mixture on the absorption of Cd in the roots and shoots. Rice seedlings grown under the base or enriched solutions for 30 days were further exposed to 50 μM Cd for 5 days. Significant differences were detected by LSD test, $P<0.05$

**Table 1** Translocation factor (TF) and bioconcentration factors (BCF) of rice seedlings after nine pretreatments followed by 50 μM Cd solution for 5 days

| No | TF (%) | BCF | |
|---|---|---|---|
| | | Shoot | Root |
| CK | 34.6 ± 1.63 | 142.4 ± 5.68 | 411.3 ± 4.62 |
| Fe 40 μM | 35.9 ± 1.33 | 127.1 ± 4.73* | 354.1 ± 1.34* |
| Fe 100 μM | 27.9 ± 2.21* | 100.5 ± 0.88* | 366.1 ± 23.36* |
| Fe 200 μM | 18.5 ± 0.55* | 82.1 ± 0.61* | 444.28 ± 10.15 |
| Zn 0.38 μM | 33.7 ± 1.01 | 115.8 ± 2.32* | 344.7 ± 5.89* |
| Zn 0.75 μM | 28.4 ± 0.82* | 91.4 ± 2.76* | 322.4 ± 0.41* |
| Zn 1.5 μM | 46.6 ± 0.40* | 140.1 ± 0.76 | 300.6 ± 2.55* |
| Fe/Zn 100/0.75 μM | 21.1 ± 0.26* | 75.6 ± 1.53* | 357.6 ± 2.70* |
| Fe/Zn 200/1.5 μM | 34.2 ± 1.55 | 111.6 ± 3.72* | 327.2 ± 3.74* |

* means significant correlation at $P < 0.05$ level

if controlled by ZNT1, may be shared by Fe, Cu, Cd, and even Ca and Mg. 2) the Fe pathway if regulated by IRT-1, can be mainly shared by Zn and Cd.

It is important to note that the involvement of Fe ions can effectively interfere with the upward transport mechanism of Cd. A protein membrane was purified and identified from Fe-rich rice leaves, revealing the presence of a 35–45 kDa polypeptide. Guerinot et al. (2000) identified ZIP proteins with sizes ranging from 36 to 39 kDa, thus the rice protein may be a member of the ZIP family. This polypeptide was found to contain the structure of chain A, which is involved in carbon fixation and the single-cell single-carbon pathway in rice rubisco complexed with Nadp(h). Additionally, transmembrane protein, ferritin, and water stress-inducible protein were detected in this membrane protein under Fe treatment. Compared to CK, the effective expression of these proteins may be involved in Fe-mediated blockade of upward Cd transport.

The weaknesses of the present study were as follows: 1) These experiments were carried out under hydroponic conditions with an aqueous Cd solution. Whether the same treatment under low Cd-contaminated soil conditions remains unknown and awaits further investigation. 2) Some specific proteins of leaf membrane proteins in Fe-rich rice were

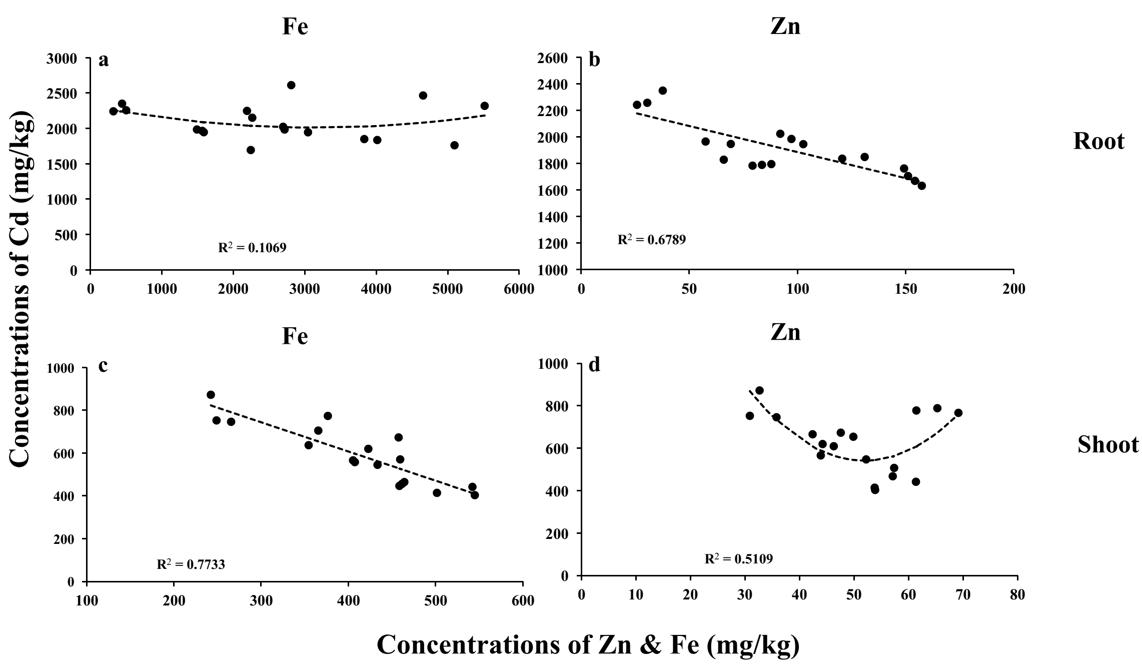



**Fig. 6** Correlation between the concentrations of Cd and Fe or Zn in shoots and roots. Data were obtained from Figs. 4 and 5

**Table 2** Information of proteins related to energy transformation and photosynthesis in both the CK and Fe treatments

| No | Protein name | Accession No | Mass(kDa) | species |
|---|---|---|---|---|
| 1 | Putative ADP-glucose Pyrophosphorylase | gi\|50,582,723\|gb\|AAT78793.1\| | 55.791 | Oryza sativa Japonica Group |
| 2 | Alanine aminotransferase | gi\|29,569,153\|gb\|AAO84040.1\| | 53.947 | Oryza sativa Indica Group |
| 3 | Alanine aminotransferase | gi\|4,730,884\|dbj\|BAA77260.1\| | 53.130 | Oryza sativa |
| 4 | Chloroplast chlorophyll a-b binding protein 8 | gi\|149,392,115\|gb\|ABR25924.1\| | 20.889 | Oryza sativa Indica Group |
| 5 | Chlorophyll a/b-binding protein | gi\|3,075,488\|gb\|AAC14566.1\| | 31.387 | Oryza sativa Japonica Group |

**Table 3** Information of specific proteins related to energy transformation and photosynthesis under the Fe treatment

| No | Protein name | Accession No | Mass(kDa) | species |
|---|---|---|---|---|
| 1 | PSII 47 kDa protein | gi\|12,013\|emb\|CAA33973.1\| | 56.134 | Oryza sativa Japonica Group |
| 2 | Putative glycine Hydroxy methyltransferase | gi\|31,126,793\|gb\|AAP44712.1\| | 61.487 | Oryza sativa Japonica Group |
| 3 | Rubisco activase large isoform precursor | gi\|8,918,359\|dbj\|BAA97583.1\| | 51.764 | Oryza sativa (japonica cultivar-group) |
| 4 | Transmembrane protein | gi\|1,632,822\|emb\|CAA70156.1\| | 31.578 | Oryza sativa |
| 5 | Putative alanine aminotransferase | gi\|33,146,868\|dbj\|BAC79866.1\| | 53.772 | [Oryza sativa Japonica Group] |
| 6 | Water channel protein | gi\|5,381,216\|dbj\|BAA82258.1\| | 19.445 | [Oryza sativa Indica Group] |
| 7 | Chlorophyll a/b-binding protein precursor | gi\|3,789,954\|gb\|AAC67558.1\| | 26.397 | [Oryza sativa Japonica Group] |
| 8 | Chlorophyll a/b-binding protein CP26 precursor—maize | gi\|62,733,869\|gb\|AAX95978.1\| | 30.379 | [Oryza sativa Japonica Group] |
| 9 | Chloroplast photosystem I reaction center subunit II precursor-like protein | gi\|29,367,391\|gb\|AAO72568.1\| | 22.134 | [Oryza sativa Japonica Group] |
| 10 | Putative Rieske Fe-sulfur protein Tic55 | gi\|47,497,136\|dbj\|BAD19185.1\| | 60.715 | Oryza sativa Japonica Group |
| 11 | Aldehyde dehydrogenase | gi\|8,163,730\|gb\|AAF73828.1 \|AF162665_1 | 59.626 | Oryza sativa |
| 12 | Argininosuccinate synthase, chloroplast precursor, | gi\|77,554,103\|gb\|ABA96899.1\| | 52.502 | Oryza sativa Japonica Group |
| 13 | Ferritin | gi\|14,091,661\|gb\|AAK53812.1 \|AF370029_1 | 28.335 | Oryza sativa |
| 14 | Chloroplastic aldolase | gi\|218,155\|dbj\|BAA02730.1\| | 42.350 | Oryza sativa Japonica Group |
| 15 | Retrotransposon protein, | gi\|108,708,286\|gb\|ABF96081.1\| | 130.895 | Oryza sativa Japonica Group |

possibly identified, but not verified by antibodies or other research methods. Therefore, the exact mechanism of Cd inhibition by Fe and Zn is unclear. 3) Because this study was preliminary and did not reach the stage of collecting rice grains, further tests are needed to determine whether pretreatment can effectively reduce the Cd level in rice grains.

## 5 Conclusions

Our study suggests that pretreatment of rice seeds with Fe and Zn could effectively inhibit Cd uptake and transport in rice seedlings under hydroponic conditions. The Zn inhibits Cd uptake in the roots, while Fe inhibits Cd transport from the root to shoots, providing double protection. The appropriate dose mixture of Fe (100 μM) and Zn (0.75 μM) pretreatments may present a more effective approach than a single metal treatment in terms of reducing Cd accumulation and enhancing crop vegetative growth. Furthermore, the polypeptide with a molecular weight of 35–45 kDa obtained from iron-rich rice is presumed to be member of the ZIP family, and iron-rich rice enhances the expression of this protein. The effective expression of transmembrane protein, ferritin, and water stress-inducible protein in this polypeptide may be related to the blockade of iron mediated upward cadmium transport. Our method only treats rice seeds or seedlings and no chemical passivating agents were added to the farmland, which is of significant importance to the safe production of rice, and also leaves additional time to develop more environmentally-friendly solutions for polluted soil.

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**Data Availability** All data related to this manuscript are incorporated in the manuscript.

## Declarations

**Conflict of Interest** The authors declare no competing interests.

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