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Running Title: Zn- OR Mn-RICH SEEDLINGS REDUCE Cd IN RICE

Rice (*Oryza sativa* L.) seedlings enriched with zinc or manganese: their impact on cadmium accumulation and the expression of related-genes

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ABSTRACT

Cadmium (Cd) contamination in paddy soils seriously threatens the safety of rice consumption. A hydroponic study was conducted to enrich rice seedlings with zinc (Zn) or manganese (Mn), and the uptake and transport characteristics of Cd in the Zn- and Mn-rich seedlings were subsequently investigated through a greenhouse pot trial. The results showed that hydroponic cultivation in 10–50 μmol L^{-1} Zn ($ZnSO_4 \cdot 7H_2O$) or 50–250 μmol L^{-1} Mn ($MnSO_4 \cdot H_2O$) for 30 days had no significant impact on rice growth, while the accumulation of Zn and Mn was 7.31–18.5 and 25.4–47.7 times higher, respectively, than that in the control. After transplantation to Cd-contaminated soil for 60 days, the accumulation of Cd in the Zn- and Mn-rich rice plants was 26.3–38.6% and 34.4–44.5% lower than that in the control, respectively, and the translocation factors of Cd from roots to shoots were also decreased by 23.3–41.3% and 25.3–37.0%, respectively. Furthermore, the relative expression level of *OsIRT1* (*Oryza sativa* iron-regulated transporter 1) was downregulated by 40.1–59.3% and 16.0–25.9%, respectively, in the Zn- and Mn-rich seedling roots. This downregulation may be one of the possible mechanisms contributing to the reductions in Cd absorption. Field experiments confirmed that the Zn- and Mn-rich seedlings produced brown rice (unpolished rice grains) with significantly decreased concentrations of Cd (34.2–44.4%). This study provides an innovative method for reducing the food safety risk from rice grown in slightly to moderately Cd-contaminated paddy soils.

Key Words: absorption enrichment, competition, field verification, gene expression, hydroponics, translocation factor

INTRODUCTION

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With the increasingly uncontrolled input of industrial hazardous wastes (e.g., wastes from the mining and smelting industry), sewage, phosphate fertilizers, and organophosphorus pesticides to soils (Liu *et al.*, 2009; Zhao *et al.*, 2015), large amounts of cadmium (Cd) are entering farmlands. According to the national survey report on soil pollution in China published in 2014, approximately 7% of the soil samples were contaminated with Cd, and Cd contamination was especially common in southern China (MEP and MLR, 2014). Consequently, the concentration of Cd in brown rice (unpolished rice grains) grown in some Cd-contaminated regions exceeds the food safety limit (0.2 mg kg^{-1}); this so-called "Cd-rice" is a matter of considerable public concern (Du *et al.*, 2013). Brown rice is the predominant crop food for the public in southern China, with an average consumption of 219 g $capita^{-1}$ d^{-1} (Hu *et al.*, 2016). The consumption of Cd-rice results in the overaccumulation of Cd in the human body and may subsequently cause certain incurable diseases, such as itai-itai disease and liver and kidney damage (Kim, 2003; Skroder *et al.*, 2015). To avoid health risks to humans in Cd-contaminated regions, it is imperative to reduce the absorption of Cd by rice from soil through some effective and low-cost methods.

In the past few decades, many methods have been explored to reduce the accumulation of Cd in rice, including soil- and plant-based methods. Soil-based methods mainly aim to reduce the content or bioavailability of Cd in the soil and include phytoextraction (Li *et al.*, 2014; Han *et al.*, 2019), electrokinetic remediation (Tang *et al.*, 2018), and in situ immobilization (Bian *et al.*, 2014; Hamid *et al.*, 2020). Plant-based methods mainly aim to weaken the absorption ability of rice by methods such as regulating the expression of Cd-related genes in rice roots by molecular technology, especially transgenesis (Yamaji *et al.*, 2013; Liu *et al.*, 2019). Gene regulation by transgenesis avoids the influences of soil variability; thus, Cd reduction performed by rice itself is always more stable than soil-based methods across different soils. However, transgenesis technology is still unacceptable to the public, and the knockout of Cd-related genes may decrease the uptake of some essential micronutrients that use the same transporters as Cd (Sasaki et al., 2012). Nevertheless, the expression of important Cd-related genes can also be regulated by the application of essential microelements, such as iron (Chen *et al.*, 2017). Moreover, zinc (Zn) and manganese (Mn) are also essential to rice, and they are actively absorbed through transporters. Iron-regulated transporter-like proteins (such as *OsIRT1*) and natural resistance-associated macrophage proteins (such as *OsNramp5*) are involved in the transport of Zn and Mn (Ishimaru *et al.*, 2011; Shao *et al.*, 2017). Interestingly, *OsNramp5* is a major transporter involved in the uptake of Cd and Mn by rice roots (Sasaki et al., 2012). *OsIRT1* has also been shown to be associated with Cd uptake in rice (Yang *et al.*, 2016). In addition, some transporters related to the upward transport of Zn or Mn in rice roots also function in the transport of Cd, such as heavy metal ATPases (*OsHMA2* and *OsHMA3*) (Yamaji *et al.*, 2013; Cai *et al.*, 2019). Therefore, the up- or downregulated expression of these transporters in response to the requirements of Zn or Mn for rice growth, would also change the absorption of Cd (Shao *et al.*, 2018; Lu *et al.*, 2019). Accordingly, we hypothesized that regulating the expression of some Cd-related genes is possible by enriching the absorption of Zn or Mn in rice and thus decreasing the ability of the rice to absorb Cd.

Previous studies have considered the application of Zn (Huang *et al.*, 2019) or Mn (Sun *et al.*, 2018) for the safe exploitation of Cd-contaminated soil. Basal, topdressing or foliar application of Zn at appropriate levels can reduce the accumulation of Cd in plants (Saifullah *et al.*, 2014; Qaswar *et al.*, 2017; Huang *et al.*, 2019). However, the long-term effects are restricted by the gradually decreased availability of exogenous Zn due to aging effects in the soil (Donner *et al.*, 2012). The continuous application of Zn or Mn in each season can maintain their efficiency. However, long-term Zn or Mn input will induce new ecological risks to soil and aquatic environments (Marks *et al.*, 2017; Baran *et al.*, 2018). Similarly, soil or foliar application of Mn fertilizer at high levels is also unsuitable for enriching rice with Mn. Therefore, an innovative Zn or Mn utilization method should be developed to enrich rice with Zn or Mn and avoid environmental risks. Rice planting often involves transplanting very young seedlings cultivated for approximately a month in a seedbed. Accordingly, it is possible to produce Zn- or Mn-rich seedlings by applying appropriate amounts of Zn or Mn during seedling growth. Subsequently, transplanting Zn- or Mn-rich seedlings into Cd-contaminated soil is a potential way to decrease the absorption of Cd by rice; this technique needs further study. In addition, Zn-Cd or Mn-Cd antagonism during the external uptake and internal transport processes in plant tissues was considered the main driving factor for Cd reduction (Huang *et al.*, 2017; Cai *et al.*, 2019). However, the effects of Zn or Mn on the expression of Cd-related genes are still unclear.

Therefore, the aim of this study was to obtain safe rice in Cd-contaminated soil by enriching seedlings with Zn or Mn. The study included three aspects as follows: 1) produce Zn- or Mn-rich rice seedlings by culturing seedlings in different amounts of exogenous Zn or Mn, 2) investigate the uptake and transport characteristics of Cd, Zn, and Mn in rice plants after transplanting Zn- and Mn-rich seedlings into Cd-contaminated soil, and 3) explore the possible mechanisms involved in the reduction of Cd in rice plants by studying the effects of Zn and Mn enrichment on the expression of Cd-related transporters in roots.

MATERIALS AND METHODS

Rice seedling cultivation and experimental design

Wuyunjing21, a japonica conventional rice (growth period of 151 days), was obtained from the Wujin District Institute of Agricultural Sciences in Changzhou City and used as the experimental cultivar. The rice seeds were sterilized in 30% (v/v) H_2O_2 for 15 min, soaked in deionized water at 25 ℃ in the dark for 24 h, and then germinated in moist gauze for another 24 h. Seeds with similar germination statuses were then selected to become the experimental seedlings. As a common nutrient solution for rice, Kimura B solution (pH 5.6) is frequently used to culture rice seedlings in hydroponics experiments (Lee *et al.*, 2013; Shao *et al.*, 2017) and was used in this work. The seedlings were grown hydroponically in Kimura B nutrient solution, which contained 0.36 mmol L^{-1} $(NH_4)_2SO_4$, 0.55 mmol L^{-1} $MgSO_4 \cdot 7H_2O$, 0.18 mmol L^{-1} KNO_3, 0.37 mmol L^{-1} $Ca(NO_3)_2 \cdot 4H_2O$, 0.18 mmol L^{-1} KH_2PO_4, 20 μmol L^{-1} $FeSO_4 \cdot 7H_2O$, 20 μmol L^{-1} EDTA-2Na, 0.5 μmol L^{-1} $MnCl_2 \cdot 4H_2O$, 3 μmol L^{-1} H_3BO_3, 1 μmol L^{-1} $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.4 μmol L^{-1} $ZnSO_4 \cdot 7H_2O$, and 0.2 μmol L^{-1} $CuSO_4 \cdot 4H_2O$. The seeds were cultured in 1/2 Kimura B solution (half of the concentration) for the first 3 days and then treated with Zn or Mn. According to the studies of Chen et al. (2013) and Chen et al. (2018), the addition of Zn^{2+} at levels below 211 μmol L^{-1} or Mn at levels below 500 μmol L^{-1} had no negative effect on the growth of rice seedlings. Therefore, two safe levels of Zn^{2+} (10, 50 μmol L^{-1}) and Mn^{2+} (50, 250 μmol L^{-1}) were used for this study. Five different treatments were applied in the hydroponic experiment and were labeled follows: 1) the control (CK), only Kimura B; 2) Zn1, Kimura B+10 μmol L^{-1} Zn ($ZnSO_4 \cdot 7H_2O$); 3) Zn2, Kimura B+50 μmol L^{-1} Zn ($ZnSO_4 \cdot 7H_2O$); 4) Mn1, Kimura B+50 μmol L^{-1} Mn ($MnSO_4 \cdot H_2O$); and 5) Mn2, Kimura B+250 μmol L^{-1} Mn ($MnSO_4 \cdot H_2O$). The nutrient solution and the Zn and Mn solutions were renewed every 4 days. After cultivation for 30 days, some of the seedlings (referred to as “rice seedlings”) were sampled to measure their Zn and Mn concentrations and the relative expression of Cd-related transporter genes. Other seedlings were transplanted to Cd-contaminated soil to participate in the pot trial.

Soil preparation and pot trial

An acidic paddy soil was collected from Guixi city, Jiangxi Province, China (N 28°01′, E 117°13′) to conduct the pot trial. The tested soil was air-dried and partly sieved through 2-mm mesh for the measurements of soil pH, cation exchange capacity, clay content, and available content of Cd, Mn, and Zn. Another part of the soil was sieved through a 0.15-mm mesh for the measurements of organic matter and the total contents of Cd, Mn, and Zn. The soil pH was 4.81, and the total Cd content was 0.68 mg kg^{-1}. Other principal properties of the topsoil (0–20 cm) are shown in Table S1 (see Supplementary Material for Table S1).

The pot trial was conducted during the seedling growth period in a greenhouse at the Institute of Soil Science, Chinese Academy of Sciences, Nanjing, Jiangsu Province, China. PVC pots (diameter and height of 18 cm and 20 cm, respectively) were filled with 4 kg of dry soil, and basal fertilizers were evenly mixed with the soil at dosages of 0.20 g N kg^{-1} (CO $(NH_2)_2$), 0.15 g P_2O_5 kg^{-1} (CaH_2PO_4 H_2O), and 0.20 g K_2O kg^{-1} (KCl). The soil was kept moist for one week, and two uniform seedlings were then transplanted into each pot (each treatment was replicated three times). After cultivation in flooded conditions with a water layer of 2–3 cm for 60 days, all rice plants (referred to as "late-tillering rice") were collected, and the concentrations of Cd, Zn, and Mn were measured.

Field verification experiments

The effects of Zn or Mn enrichment in rice seedlings on reducing the accumulation of Cd in brown rice were verified under field conditions. Two field experiments were conducted in Guixi city, Jiangxi Province (Guixi site, N 28°01′, E 117°13′) and in Tongling city, Anhui Province (Tongling site, N 31°01′, E 117°53′), China. The field in Guixi had been contaminated due to the irrigation of wastewater from a copper smelting factory since 1990's. The area of Cd contamination was over 150 ha, with Cd levels ranging from 0.3 to 0.9 mg kg^{-1}. The Tongling field had been contaminated by sewerage from a copper mine since 1990's, and the contaminated area was over 200 ha. The Cd levels were similar to those in Guixi city. The soil pH of the experimental plot at the Guixi site was 4.76, and the total Cd content was 0.61 mg kg^{-1}. The soil pH of the experimental plot at the Tongling site was 5.41, and the total Cd content was 0.55 mg kg^{-1}. Other principal soil properties of the two sites are shown in Table S1 (see Supplementary Material for Table S1).

This experiment included four treatments at each site. Three of the treatments corresponded to the CK (blank control, seedlings in nutrient solution), Zn2 (seedlings in nutrient solution with 50 μmol L^{-1} Zn), and Mn2 (seedlings in nutrient solution with 250 μmol L^{-1} Mn) treatments employed in the above pot trial, and a conventional seedling cultivation practice (seedlings grown in paddy soil) was used as a contrast treatment (CT). Two local cultivars were used in the corresponding field experiments: Meixiangxingzhan (conventional indica rice, 123 days, obtained from the Jiangxi Xing'an Seed Industry Co., Ltd., China) in Guixi, and Jingliangyouhuazhan (hybrid indica rice, 138 days, obtained from Longping High-Tech, China) in Tongling. The rice seedlings used in each treatment were transplanted into a plot (4 × 5 m), and each treatment was replicated three times. The water and fertilizer management practices used were consistent with the local practices, briefly described as follows: basal application of compound fertilizers with a level of 450 kg ha^{-1} ($N:P_2O_5:K_2O$ = 15%:15%:15%) and then topdressing with 75.0 kg ha^{-1} of urea during the greening-up period and 50.0 kg ha^{-1} of compound fertilizers at the ear emergence stage; the water management was submergence until the late stage of filling (water layer with a depth of 3 cm), followed by wet irrigation and then field-drying for a week before the harvest. In the harvesting process, the rice grains were separated from the spike using a reaping machine equipped with a drying device. The yield of all rice in each plot was weighed and recorded using an electronic platform scale. Approximately 500 g grains from each plot were randomly collected for laboratory analysis. After rinsing, oven-drying, and decladding using a sheller (JLG-II, Institute of Grain Storage in Chengdu, China), brown rice was obtained. The concentrations of Cd, Zn, and Mn in the brown rice were determined.

Chemical analysis of samples

Some of the seedling samples from the hydroponic experiment and some of the rice plants from the pot trial were collected and divided into roots and shoots (both stems and leaves). The roots were first soaked in 5 mmol L^{-1} $CaCl_2$ solution for 20 min and then rinsed in both tap and deionized water. Thereafter, the roots and shoots were oven-dried to constant weights at 75 ℃ and then milled to powder using a blender (A11 basic, IKA, Germany). The brown rice collected from the field experiments was also pretreated similarly. The powder samples were digested using a DigiBlock ED54-Itouch digester system (LabTech, Beijing). The concentrations of Cd, Zn, and Mn in the digestion solution were measured using flame and graphite furnace atomic absorption spectrometry (SpectrAA 220FS and 220Z, Varian, USA). Detailed information on these methods is described in Huang et al. (2018).

The remaining seedling samples collected from the hydroponic experiments were quickly rinsed in tap water and then stored at -80 ℃. The relative expression level of *OsIRT1* and *OsNramp5* (associated with root Cd uptake) and *OsHMA3* and *OsHMA2* (associated with Cd transport in the root) were then determined. The entire root was separated from each seedling and stored in liquid nitrogen. Total RNA was isolated using a UNlQ-10 Column TRIzol Total RNA Isolation Kit (Sangon Biotech, China), and 1 μg of total RNA was used to synthesize first-strand cDNA using a RevertAid First Strand cDNA Synthesis Kit (Thermo, USA) following the manufacturer's instructions. The relative expression levels of *OsIRT1*, *OsNramp5*, *OsHMA3*, and *OsHMA2* were quantified using a quantitative RT-PCR system (BIO-RAD CFX96, Singapore), and *OsActin* was used to normalize the expression ratio for each gene. The primers for all genes are provided in Shao et al. (2017) and Chen et al.(2017).

Data analysis

The total amounts of Zn (T_{Zn}), Mn (T_{Mn}), and Cd (T_{Cd}) in the rice and the translocation factors (TFs) of Zn, Mn, and Cd from the root to the shoot were calculated using the following equation:

$$T = C_{root} \times DW_{root} + C_{shoot} \times DW_{shoot}$$

$$TF = C_{shoot}/C_{root}$$

where C_{root} and C_{shoot} represent the concentrations of Zn, Mn, or Cd in the root and shoot, respectively, and DW_{root} and DW_{shoot} represent the dry weights of the root and shoot, respectively.

Real-time fluorescence quantification data were analyzed using Bio-Rad CFX Manager software.

Statistical analysis

Data for each parameter are presented as the means ± standard deviations (SDs). The differences in each parameter between different experimental treatments in the same tissue were analyzed by one-way ANOVA (analysis of variance) after checking the homogeneity (Levene's test) and normality (Shapiro–Wilk test) of variances, with a significant *P* value of 0.05, followed by an LSD (least significant difference) post hoc test. Two-way ANOVA was applied to analyze the main effects of the treatment, the growth stage and their interaction on the biomass and elemental concentration of rice tissues. All statistical analyses were conducted using SPSS 19.0 software. All graphs were produced by OriginPro 2016 software.

RESULTS

Effects of Zn or Mn on rice growth

Compared with the control (CK), the Zn1, Zn2, Mn1, and Mn2 treatments had no significant impact on the dry weights of roots or shoots of rice seedlings or late-tillering rice. A two-way ANOVA showed that the dry weights of rice roots and shoots were mainly influenced by their growth stage (Table S2 (see Supplementary Material for Table S2)).

Fig. 1 Changes in the dry weights of roots and shoots of (A) rice seedlings and (B) late-tillering rice under different treatments. Shoot refers to both stem and leaf. CK: the control, Zn1 and Zn2: seedlings cultivated with 10 and 50 μmol L^{-1} Zn, Mn1 and Mn2: seedlings cultivated with 50 and 250 μmol L^{-1} Mn; in the same tissue, bars that have the same lowercase letter(s) indicate no significant differences between different treatments at $P < 0.05$.

Absorption of Zn and Mn in rice tissues

As shown in Fig. 2, the Zn concentrations in the roots and shoots of rice seedlings were greatly increased with an increase in the Zn addition level. Compared with those in CK, the root and shoot Zn were 15.6 and 6.09 times higher in the Zn1 treatment and 27.1 and 14.8 times higher in the Zn2 treatment (Fig. 2A), respectively. After the rice seedlings had been transplanted into soil for 60 days, the Zn concentrations in the roots and shoots of late-tillering rice under the Zn treatments were also higher than those of CK. However, the differences were significantly reduced. Compared with those under CK, root Zn was 51.4% higher in the Zn1 treatment, and root and shoot Zn was 93.3% and 45.9% higher under the Zn2 treatment (Fig. 2B), respectively. The two-way ANOVA showed that the concentrations of Zn in the root and shoot were also significantly influenced by the growth stage and the interaction of the treatment and growth stage (Table S2 (see Supplementary Material for Table S2)).

The changes in Mn in the roots and shoots under different treatments were similar to those of Zn (Fig. 2C and D). The concentrations of Mn in the roots and shoots of rice seedlings were 21.0 and 28.0 times those of the control under the Mn1 treatment and 32.0 and 51.1 times those of the control under the Mn2 treatment (Fig. 2C). However, the root and shoot Mn of late-tillering rice were only 1.48 and 1.03 times those of the control under the Mn1 treatment and 1.67 and 1.06 times those of the control under the Mn2 treatment (Fig. 2D). The two-way ANOVA showed that the concentrations of Mn in the root and shoot were also significantly influenced by the growth stage and the interaction of the treatment and growth stage (Table S2 (see Supplementary Material for Table S2)).

Fig. 2 Concentrations of Zn and Mn in rice roots and shoots. A and B represent seedlings and late-tillering rice under the Zn treatments, and C and D represent seedlings and late-tillering rice under the Mn treatments, respectively. Shoot refers to both stem and leaf. CK: the control, Zn1 and Zn2: seedlings cultivated with 10 and 50 μmol L^{-1} Zn, Mn1 and Mn2: seedlings cultivated with 50 and 250 μmol L^{-1} Mn; in the same tissue, bars with the same lowercase letter(s) indicate no significant differences between different treatments at $P < 0.05$.

As shown in Fig. 3A, during the early seedling growth period, the accumulation of Zn in rice seedlings was 7.31 and 18.5 times that of the control under the Zn1 and Zn2 treatments, respectively. However, during the growth period following seedling transplantation, the accumulation of Zn under the Zn1 and Zn2 treatments was only 80.4% and 45.5% that of the control. Throughout the growth period, the accumulation of Zn in rice under the Zn2 treatment was 53.1% higher than that of the control. Similar results were observed in the Mn treatments (Fig. 3B). Mn accumulation during the early seedling growth period was 25.4 and 47.7 times that of the control under the Mn1 and Mn2 treatments, respectively. However, the accumulation of Mn during the growth period following seedling transplantation was 89.2% and 85.8% that of the control under the Mn1 and Mn2 treatments, respectively. Throughout the growth period, the accumulation of Mn in rice under the Mn2 treatment was only 11.8% higher than that of the control.

Fig. 3 Total accumulation of (A) Zn and (B) Mn in rice during the early seedling growth period (30 d) and following transplantation (60 d). CK: the control, Zn1 and Zn2: seedlings cultivated with 10 and 50 μmol L^{-1} Zn, Mn1 and Mn2: seedlings cultivated with 50 and 250 μmol L^{-1} Mn; bars with the same lowercase letter(s) indicate no significant differences between different treatments during the same growth period at $P < 0.05$.

Absorption of Cd in Zn- or Mn-rich rice

In the late-tillering rice, the Zn and Mn treatments significantly decreased the concentrations of Cd in the roots and shoots compared with those in the control (Fig. 4A). Compared with those of CK, the concentrations of Cd in the roots decreased by 27.3%, 28.3%, 37.6%, and 37.0% under the Zn1, Zn2, Mn1, and Mn2 treatments, respectively. The Cd concentrations in the shoots decreased by 42.9%, 56.0%, 48.4%, and 54.7%, respectively. Similarly, the total accumulation of Cd in the late-tillering rice also decreased by 26.3%, 38.6%, 34.4%, and 44.5% under the Zn1, Zn2, Mn1, and Mn2 treatments, respectively, compared to that in the control (Fig. 4B).

Fig. 4 (A) Concentrations of Cd in roots and shoots and (B) total accumulation of Cd in late-tillering rice. Shoot refers to both stem and leaf. CK: the control, Zn1 and Zn2: seedlings cultivated with 10 and 50 μmol L^{-1} Zn, Mn1 and Mn2: seedlings cultivated with 50 and 250 μmol L^{-1} Mn; bars with the same lowercase letter(s) indicate no significant differences between different treatments in the same tissue at $P < 0.05$.

Transportation of Zn, Mn, and Cd in Zn- or Mn-rich rice

The Zn and Mn treatments also affected the transportation of Zn, Mn, and Cd within the rice (Fig. 5). In the late-tillering rice, compared with those of CK, the translocation factors from roots to shoots decreased by 31.9%–37.0% for Zn under the Zn treatments and 30.1%–33.8% for Mn under the Mn treatments. The translocation factors of Cd from roots to shoots were significantly decreased by 23.3%, 41.3%, 18.3%, and 30.0% under the Zn1, Zn2, Mn1, and Mn2 treatments, respectively, compared to those under the CK treatment.

Fig. 5 Translocation factors of Zn, Mn, and Cd from roots to shoots of late-tillering rice. CK: the control, Zn1 and Zn2: seedlings cultivated with 10 and 50 μmol L^{-1} Zn, Mn1 and Mn2: seedlings cultivated with 50 and 250 μmol L^{-1} Mn; bars with the same lowercase letter(s) indicate no significant differences between different treatments at $P < 0.05$.

Relative expression of Cd-related genes in seedling roots

After growth in a hydroponic solution containing high levels of Zn or Mn for 30 days, the relative expression levels of two genes (*OsNramp5* and *OsIRT1*) associated with Cd uptake by the root were downregulated to different degrees (Fig. 6). Compared with that under CK, the relative expression level of *OsNramp5* was slightly downregulated by 18.3% under the Mn2 treatment, but no significant difference was observed in the other treatments. For *OsIRT1*, the relative expression levels were downregulated by 40.1%, 59.3%, 16.0%, and 25.9% under the Zn1, Zn2, Mn1, and Mn2 treatments, respectively, compared to that under CK. No significant difference was observed in the expression of the two genes (*OsHMA3* and *OsHMA2*) associated with the transport of Cd in the root.

Fig. 6 Relative expression of *OsNramp5*, *OsIRT1*, *OsHMA3*, and *OsHMA2* in rice seedling roots. CK: the control, Zn1 and Zn2: seedlings cultivated with 10 and 50 μmol L^{-1} Zn, Mn1 and Mn2: seedlings cultivated with 50 and 250 μmol L^{-1} Mn; for the same gene, bars with the same lowercase letter(s) indicate no significant differences between different treatments at $P < 0.05$.

Yield and Cd concentration of field-grown brown rice in field experiments

In the two field experiments at the Guixi and Tongling sites, the addition of 50 μmol L^{-1} Zn or 250 μmol L^{-1} Mn to the rice seedlings had no significant impact on rice yield or Zn and Mn concentrations in the brown rice. However, the Cd concentrations in the brown rice were significantly decreased compared with those under CK and CT (Table I). Compared with the blank control (CK), the Cd concentrations in the brown rice from Guixi site were decreased by 39.0% and 31.7% under the Zn and Mn treatments, respectively, and by 32.4% and 26.5%, respectively, at the Tongling site. Compared with the contrast treatment (CT), the concentrations of Cd in brown rice at the Guixi site were decreased by 44.4% and 37.8% under the Zn and Mn treatments, respectively, and by 39.5% and 34.2%, respectively, at the Tongling site.

TABLE I
Yields (t ha^{-1}) and concentrations of Zn, Mn, and Cd (mg kg^{-1}) in brown rice (unpolished rice grains) in fields from two Chinese cities

Site	Treatment[a)]	Yield	Elemental concentration in brown rice		
			Mn	Zn	Cd
Guixi	CK	8.09±0.55a[b)]	15.6±2.07a	34.6±4.66a	0.41±0.03a
	Zn2	8.23±0.87a	15.3±1.96a	35.3±4.24a	0.25±0.01b
	Mn2	8.05±0.61a	16.0±3.39a	33.8±3.54a	0.28±0.01b
	CT	8.16±0.45a	14.9±1.38a	34.1±6.98a	0.45±0.04a
Tongling	CK	9.31±0.61a	10.5±1.48a	26.9±4.39a	0.34±0.04a
	Zn2	9.25±0.49a	10.3±2.24a	29.7±6.37a	0.23±0.01b
	Mn2	9.14±0.81a	12.2±2.74a	27.2±3.64a	0.25±0.02b
	CT	9.39±0.57a	11.1±1.24a	28.2±3.06a	0.38±0.03a

[a)]CK, Zn2, Mn2, and CT indicate the control, seedlings cultivated in 50 μmol L^{-1} Zn, seedlings cultivated in 250 μmol L^{-1} Mn, and conventional seedlings, respectively.

[b)]Each parameter was compared among the different treatments at the same site, and the same lowercase letter(s) in each column at the same site indicates no significant differences between the different treatments at $P < 0.05$.

DISCUSSION

Zn-rich seedlings inhibited the uptake and transport of Cd in rice

As an essential micronutrient, Zn is vital for plant growth, but a deficiency or an excess of Zn is harmful to plant physiological processes and inhibits photosynthesis or induces oxidative stress (Wang and Jin, 2005; Sagardoy *et al.*, 2010; Song *et al.*, 2011). In the present study, the Zn addition levels (10–50 μmol L^{-1}) were significantly lower than those associated with rice toxicity (Song *et al.*, 2011; Chen *et al.*, 2018); thus, no adverse influences occurred on the growth of rice seedlings. The Kimura B nutrient solution provided a normal level of Zn (0.4 μmol L^{-1}) to rice seedlings, so the control seedlings were not deficient in Zn.

During the Zn uptake processes within the rice root, zinc-regulated transporters and iron-regulated transporter-like proteins such as *OsZIP1*, *OsZIP3*, *OsZIP4*, *OsZIP5*, *OsIRT1* and *OsIRT2* play vital roles (Ramesh, 2003; Ishimaru *et al.*, 2006; Yang *et al.*, 2009; Lee *et al.*, 2010). By regulating the expression of these transporters, the uptake and transport of Zn in rice can be regulated. Unfortunately, several Zn transporters, such as *OsIRT1* and *OsIRT2*, also function in the uptake of Cd (Nakanishi *et al.*, 2006; Lee and An, 2009). The upregulated expression of *OsIRT1*, which is induced by iron deficiency, also enhances the uptake of Cd (Nakanishi *et al.*, 2006). In the current study, the expression level of *OsIRT1* was downregulated by cultivation with Zn at a high level. This result indicates that a sufficient supply of Zn may inhibit the expression of Zn-related transporters. Subsequently, the uptake of Zn was greatly decreased after Zn-rich seedlings were transplanted into the soil. Cd is not essential for rice growth, and the inhibition of the expression of *OsIRT1* may have weakened the uptake of Cd by rice roots after the Zn-rich seedlings were transplanted (Fig. S1 (see Supplementary Material for Fig. S1)). In addition, the Zn absorption ability was also weakened in Zn-rich rice, indicating that the high accumulation of Zn in seedlings may weaken the rice tissue's requirements for Zn following transplantation. As a result, the absorption of Cd was subsequently reduced.

Once Cd is taken up into rice roots, its upward transport is vital for its allocation to aerial parts of the plant, and certain transporters, such as *OsHMA3* and *OsHMA2*, play an important role in this process (Ueno *et al.*, 2010; Yamaji *et al.*, 2013). *OsHMA3* is a tonoplast-localized transporter that functions in the sequestration of Cd to the tonoplast; therefore, the expression of *OsHMA3* inhibits the transport of Cd in root cells (Ueno *et al.*, 2010). Perhaps because *OsHMA3* is a Cd-specialized transporter that is insensitive to other metals (Ueno *et al.*, 2010), enrichment with Zn did not influence the expression of *OsHMA3* in the seedling roots. Xylem loading is important for the transport of Cd to aerial plant parts; *OsHMA2* is a key transporter in this process and also has a function in the xylem loading of Zn (Takahashi *et al.*, 2012). Although Zn enrichment had no effect on the expression of *OsHMA2* in seedling roots, the translocation factors of Cd from the root to the shoot were significantly decreased. This could be because Zn is an essential nutrient for rice tissue growth, and *OsHMA2* preferentially transports Zn to meet rice growth needs (Yamaji *et al.*, 2013). Therefore, Zn enrichment could enhance the competition between Zn and Cd for adsorption sites on *OsHMA2* and thus result in inhibiting the upward transport of Cd (Fig. S1 (see Supplementary Material for Fig. S1)).

Mn-rich seedlings inhibited the uptake and transport of Cd in rice

Similar to Zn, Mn also participates in many plant metabolic processes, such as photosynthesis and the redox process (Shenker *et al.*, 2004). Both a deficiency in Mn and its overaccumulation are detrimental to plant growth. Previous studies have demonstrated that a Mn level ranging from 0.5 to 500 μmol L^{-1} is safe for rice growth (Sasaki *et al.*, 2011; Chen *et al.*, 2013). Therefore, the Mn addition level in this study (50 and 250 μmol L^{-1}) had no negative impact on rice growth.

Rice has a strong tolerance for Mn, and most of the Mn in roots is transported to the aerial parts of the plant (Tsunemitsu *et al.*, 2018). Therefore, the Mn-rich seedlings accumulated large amounts of Mn from the soil. Nevertheless, their Mn accumulation ability was slightly weakened. The uptake of Mn by rice roots is also controlled by several transporters, such as *OsNramp5* and *OsMTP9*. *OsNramp5* mainly functions in the transport of Mn from the rhizosphere to the parenchymal root cells (Sasaki *et al.*, 2012; Ueno *et al.*, 2015). A large amount of Mn accumulation in rice roots following Mn cultivation could thus induce the degradation of the transcribed or translated *OsNramp5* and result in the inhibition of Cd uptake (Tsunemitsu *et al.*, 2018). The slightly downregulated expression level of *OsNramp5* under the Mn2 treatment in this study may also have contributed to the decrease in Cd accumulation in rice (Fig. S1 (see Supplementary Material for Fig. S1)). In addition, Mn cultivation inhibited the expression of *OsIRT1*, which is involved in the uptake of Cd by roots (Nakanishi *et al.*, 2006); this could be another reason for the decreased amounts of Cd in the Mn-rich rice.

Although the high accumulation of Mn in seedling roots did not affect the expression of *OsHMA3* or *OsHMA2*, the upward transport of Cd in rice plants was also inhibited by Mn enrichment. In addition to *OsHMA3* and *OsHMA*2, the transport of Cd and Mn in the root is also associated with *OsNramp5*, which functions in transporting Cd and Mn from the endodermis to the stele and then moving them upwards through phloem transport and xylem loading (Shao *et al.*, 2017). This same transporter (*OsNramp5*) in rice roots can induce strong competition between Cd and Mn (Fig. S1 (see Supplementary Material for Fig. S1)). The high accumulation of Mn in seedling roots could occupy adsorption sites on *OsNramp5* in the endodermis, thereby inhibiting the transport of Cd.

Advantages and disadvantages of Zn or Mn-rich seedlings with respect to reducing Cd

Soil and foliar applications are the most commonly used methods of applying Zn and Mn in order to effectively reduce Cd accumulation in plants (Li *et al.*, 2014; Saifullah *et al.*, 2016). The present study innovatively proposes that Zn- or Mn-rich seedlings have the potential to reduce Cd accumulation in rice, with a reduction of approximately 40%. Furthermore, Zn- and Mn-rich seedlings had no negative impact on the subsequent accumulation of Zn or Mn in brown rice or on the rice yield. Enriching seedlings with Zn or Mn is easy to achieve at a low cost; this method involves little risk to the environment and minimizes the potential for large amounts of Zn or Mn to accumulate in soils. For Cd-contaminated paddy fields, cultivating Zn- or Mn-rich seedlings during the early seedling stage, combined with other technologies (such as lime immobilization) during the period of transplantation, could be an effective method for ensuring the production of safe rice. However, the Zn- or Mn-rich seedlings in the present study were hydroponically cultured; it would be necessary to convert them to a growth matrix or seedbed when applying this method in the field. It is thus important to investigate a suitable method for cultivating Zn- or Mn-rich seedlings during the early seedling growth stage and to identify appropriate amounts of Zn or Mn to be applied in this method. In addition, the effects of Zn- and Mn-rich seedlings on reducing rice Cd in different rice cultivars or soil types are still unclear and need to be further studied.

CONCLUSIONS

The present study found that enriching rice seedlings with Zn or Mn inhibited the expression of *OsIRT1* and enhanced competition for Cd absorption and transport in rice roots. Therefore, enriching rice seedlings with Zn or Mn greatly reduced the accumulation of Cd in brown rice; moreover, no negative impact on rice yields was observed. The results indicate that enriching seedlings with Zn or Mn is an effective, low-risk method for safe rice cultivation in slightly to moderately Cd-contaminated soils.

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SUPPLEMENTARY MATERIAL

Supplementary material for this article can be found in the online version.

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Fig. 1

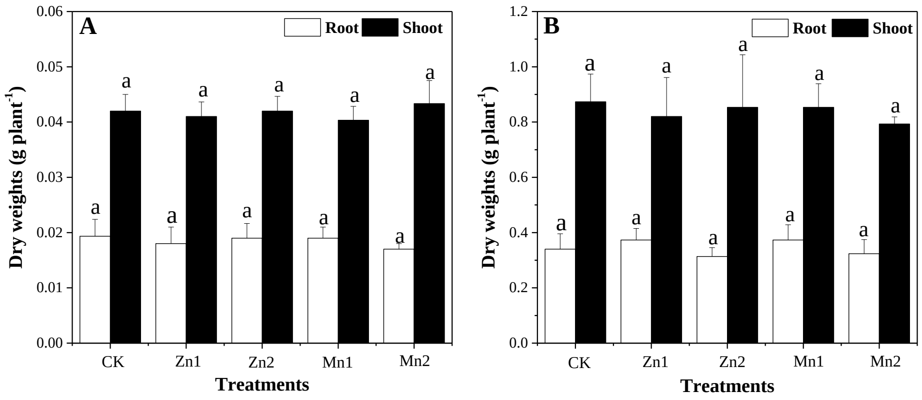


Fig. 2

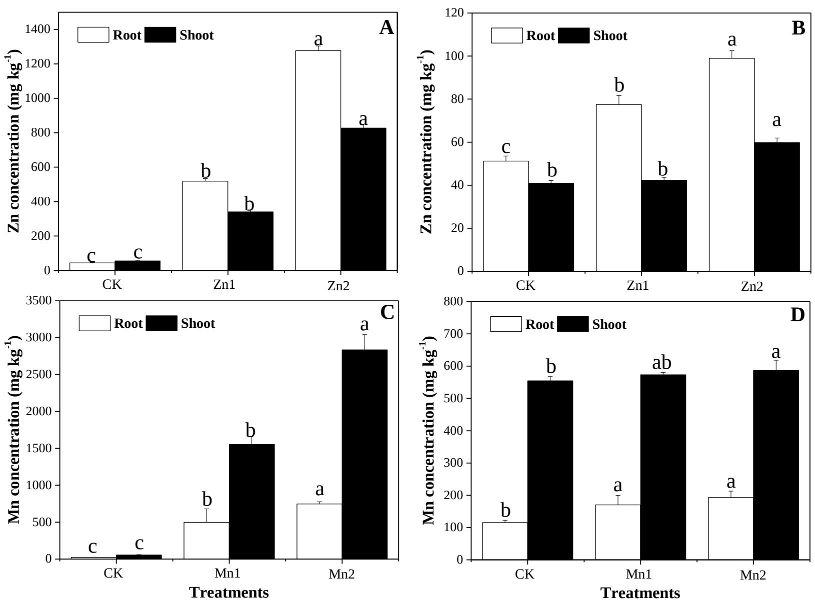


Fig. 3

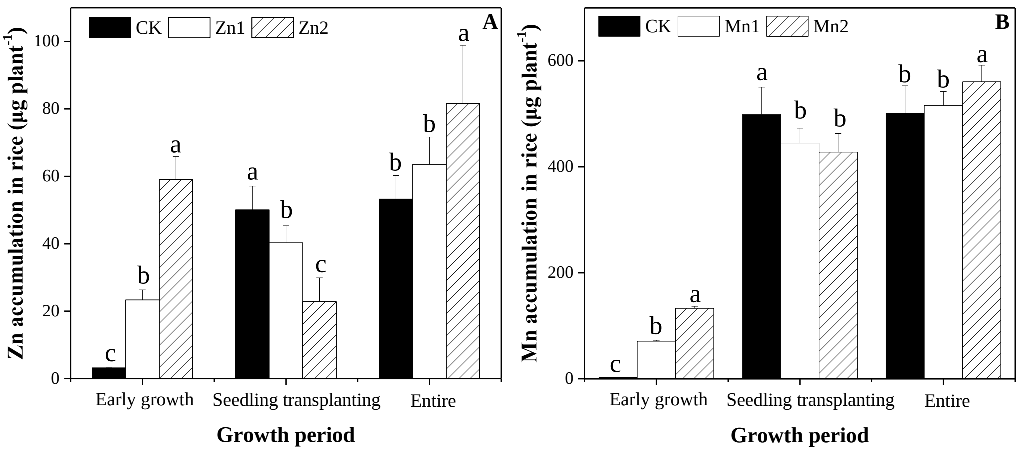

A
CK
Zn1
Zn2
Zn accumulation in rice (μg plant-1)
Early growth
Seedling transplanting
Entire
Growth period
B
CK
Mn1
Mn2
Mn accumulation in rice (μg plant-1)
Early growth
Seedling transplanting
Entire
Growth period

Fig. 4

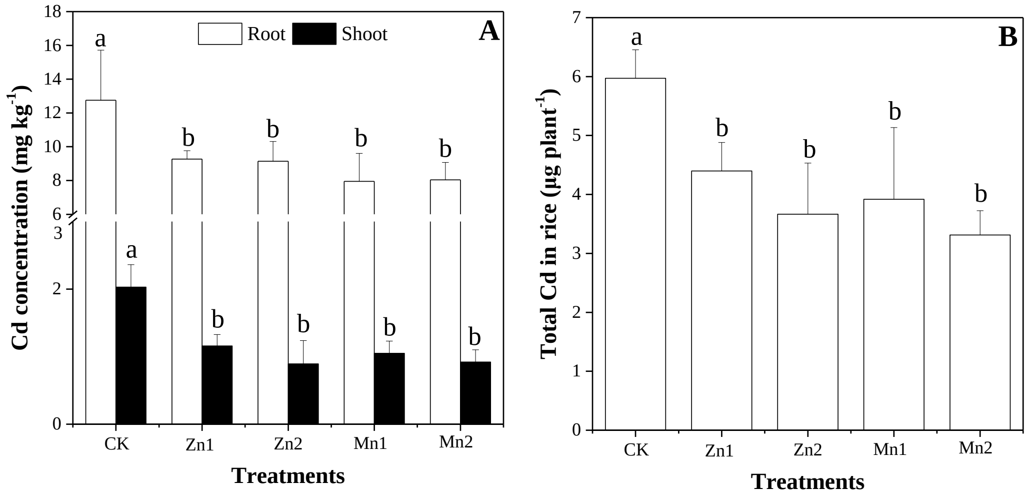
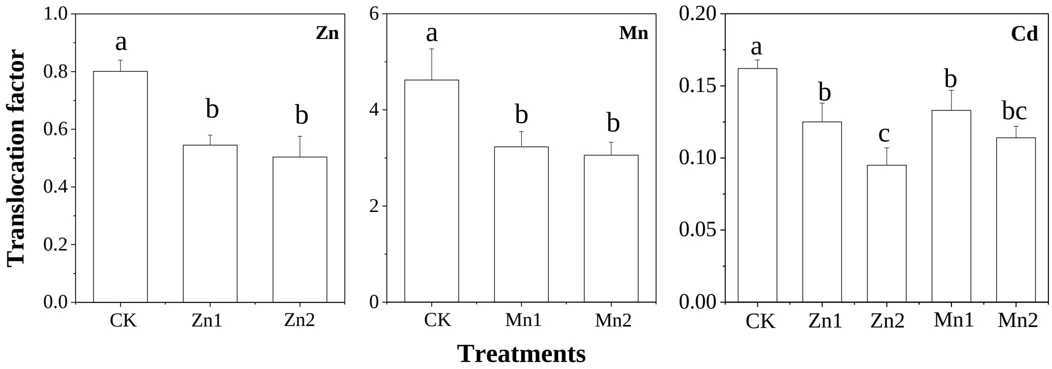
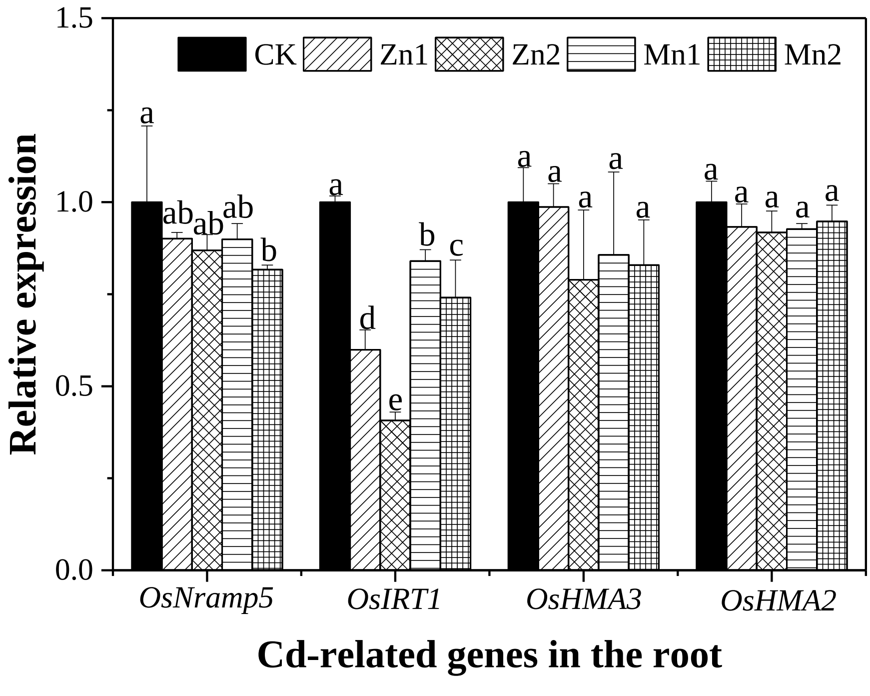

A
Root
Shoot
Cd concentration (mg kg-1)
CK
Zn1
Zn2
Mn1
Mn2
Treatments
B
Total Cd in rice (μg plant-1)
CK
Zn1
Zn2
Mn1
Mn2
Treatments

Fig. 5



Translocation factor
Zn
Mn
Cd
CK
Zn1
Zn2
Mn1
Mn2
Treatments

Fig. 6



CK
Zn1
Zn2
Mn1
Mn2
Relative expression
OsNramp5
OsIRT1
OsHMA3
OsHMA2
Cd-related genes in the root