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Characterization of ^{68}Zn uptake, translocation, and accumulation into developing grains and young leaves of high Zn-density rice genotype*

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Abstract: Zinc (Zn) is an essential micronutrient for humans, but Zn deficiency has become serious as equally as iron (Fe) and vitamin A deficiencies nowadays. Selection and breeding of high Zn-density crops is a suitable, cost-effective, and sustainable way to improve human health. However, the mechanism of high Zn density in rice grain is not fully understood, especially how Zn transports from soil to grains. Hydroponics experiments were carried out to compare Zn uptake and distribution in two different Zn-density rice genotypes using stable isotope technique. At seedling stage, IR68144 showed higher ^{68}Zn uptake and transport rate to the shoot for the short-term, but no significant difference was observed in both genotypes for the long-term. Zn in xylem sap of IR68144 was consistently higher, and IR68144 exhibited higher Zn absorption ratio than IR64 at sufficient (2.0 μmol/L) or surplus (8.0 μmol/L) Zn supply level. IR64 and IR68144 showed similar patterns of ^{68}Zn accumulation in new leaves at seedling stage and in developing grains at ripening stage, whereas ^{68}Zn in new leaves and grains of IR68144 was consistently higher. These results suggested that a rapid root-to-shoot translocation and enhanced xylem loading capacity may be the crucial processes for high Zn density in rice grains.

Key words: Zinc, Stable isotope, High Zn-density rice genotype, Translocation, Remobilization
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1 Introduction

Rice (*Oryza sativa* L.) is a predominant staple food and a major source of dietary carbohydrate for more than half of the world's population (Zimmermann and Hurrell, 2002). Unfortunately, it is a poor source of essential micronutrients such as iron (Fe), zinc (Zn), and vitamin A. In plants, Zn plays an significant role as integral co-factor of over 300 enzymes which are involved in biosyntheses and turnovers of proteins, nucleic acids, carbohydrates, and lipids. Furthermore, Zn has a critical structural role in many proteins (Marschner, 1995). Recent data showed that nearly 50% of the world's population is at high risk of Zn deficiency (Welch and Graham, 2004), especially those are depend upon rice for their survival. Zn deficiency is serious as equally as iron (Fe) and vitamin A deficiencies. Zn deficiency causes a lot of health problems in humans, such as impairments of physical development, immune system, and brain function (Cakmak, 2008). Increasing the Zn content in rice grains through breeding, which provided sufficient genetic variation of high Zn-density rice, or through transgenic approaches, emerged as the times require

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and offered a suitable, cost-effective, and sustainable approach to solve this problem (Cakmak, 2008).

The root-soil interface is the first and most important barrier which affects Zn uptake (Welch and Graham, 2002). To increase Zn uptake by roots, the Zn availability in the rhizosphere must be increased (Welch, 1995), which can be performed by enhanced release rates of root-cell H^+, metal chelating compounds and/or reductants, by increasing root absorptive surface area (fine roots and root hairs), and by association with mycorrhizal fungi (Liu *et al.*, 2000; Ryan and Angus, 2003; Gao *et al.*, 2007). However, knowledge on Zn transport in rice plant is scant (Grusak *et al.*, 1999; Rengel, 1999). Zn transport in plants takes place through both the xylem and the phloem. Following absorption by the root, Zn is rapidly transported via the xylem to the shoot (Riceman and Jones, 1958). In rice plant, adequate Zn supply leads to a high proportion of Zn located in the shoots (especially stems), while with toxic level of Zn supply (150 μmol/L), a higher proportion of total Zn may accumulate in the roots (Jiang *et al.*, 2007). Zn appears to be most mobile in all micronutrients and its remobilization is closely related to leaf senescence (Marschner, 1995; Uauy *et al.*, 2006). It was shown that Zn can be transported in phloem of dicotyledonous plants, such as tobacco (*Nicotiana glauca*) (Hocking, 1980) and grape (Volschenk *et al.*, 1999). In monocotyledonous wheat it also showed good transport of Zn from stem and leaves to developing grains (Pearson *et al.*, 1995; 1996), as well as from older leaves to younger leaves (Page and Feller, 2005), indicating involvement of phloem transport. During grain filling stage, roots and stems are the largest Zn sources for translocation of Zn to the grains. However, grain could also accumulate Zn remobilized from leaves, as has been shown in soybean (Khan and Weaver, 1989) and wheat (Pearson and Rengel, 1995). Although the literature is limit for Zn transport from stems and leaves to rice grains, it is argued that it is different from wheat and barely (Stomph *et al.*, 2009). Jiang *et al*. (2007) have investigated the uptake and distribution of Zn during rice grain development and suggested that most of the Zn accumulated in the grains originates from uptake by root after flowering and not from Zn remobilization from leaves, both under sufficient or surplus Zn supply. However, previous researches showed that foliar Zn fertilizer still could increase Zn content in rice (Broadley *et al.*, 2007; Wissuwa *et al*., 2008), which suggested that Zn retranslocation in plant especially in late development stage may be also important for Zn density in rice. So far, manipulation of Zn transport in rice plant and its accumulation mechanism into grains are still unclear.

To resolve this unclearness, Zn stable isotope tracing method was employed to make a clear concept on Zn uptake and distribution in two different Zn-density rice genotypes, and xylem sap was also determined to compare xylem loading capacities of both genotypes. The aims of this present study were: (1) to compare ^{68}Zn uptake capacities by roots of two genotypes differing in grain Zn density; (2) to compare xylem loading capacity of Zn from root to shoot; and, (3) to follow long-distance transport of root-applied ^{68}Zn to new leaves at seedling stage and developing grains at ripening stage.

2 Materials and methods

2.1 Plant culture

Hydroponics experiments were carried out at Huajiachi campus, Zhejiang University, Hangzhou, China. Seeds of rice (*Oryza sativa* L.) cv. IR68144 (high Zn-density genotype) and IR64 (low Zn-density genotype) were obtained from the International Rice Research Institute, Manila, Philippines. It has been reported that Zn in the unpolished rice grains of IR68144 was about 37.0 mg/kg, whereas much lower Zn (25.5 mg/kg) was recorded in IR64 (Sellappan *et al.*, 2009). Seeds were surface-sterilized by washing with 70% ethanol for 1 min and soaking in 0.01 g/ml sodium hypochlorite for 5 min, rinsed thoroughly in deionized water (resistivity ≥18.2 MΩ·cm), and imbibed in deionized water for 48 h at 30 °C. Then seeds were germinated in quartz sand washed with 5% (v/v) HCl. For two weeks, only deionized water was supplied. After 14 d when seedlings grew onto two-leaf stage, they were transplanted into 2.5-L black plastic buckets which were covered with a polystyrol plate with seven evenly spaced holes (2 cm in diameter). The composition of nutrient solution was the same as described by Yang *et al.* (1994): 1.5 mmol/L NH_4NO_3, 1.0 mmol/L $CaCl_2$, 1.6 mmol/L $MgSO_4$, 1.0 mmol/L K_2SO_4, 0.3 mmol/L KH_2PO_4, 0.05 μmol/L H_3BO_3, 5.0 μmol/L $MnSO_4$, 0.2 μmol/L $CuSO_4$, and

0.05 μmol/L $(NH_4)_6Mo_7O_{24}$. Fe was supplied as ethylenediaminetetraacetic acid iron sodium ($Na_2FeEDTA$) at 20 μmol/L. The nutrient solution was changed weekly until the plants were eight weeks of age and every 3 d thereafter. The solution pH was adjusted to 5.5±0.1 every other day with NaOH or HCl. Buckets were placed in a growth chamber with a controlled atmospheric temperature (35/25 °C) and photoperiod (16-h light/8-h dark), with 75% relative humidity. Lights were made of fluorescence and incandescence, and intensity was 2000 lx.

For the different experiments described below, 1.0 μmol/L $ZnSO_4$ in nutrient solution was replaced by 1.0 μmol/L $^{68}ZnSO_4$ for stable isotope tracing during treatments. The nutrient solution containing 1.0 μmol/L $^{68}ZnSO_4$ was also replaced every 4 d. ^{68}Zn-enriched isotope was purchased as solid powder of ^{68}ZnO from the Cambridge Isotope Laboratory. The isotope abundances were 98.60% ^{68}Zn, 0.44% ^{64}Zn, 0.39% ^{66}Zn, 0.54% ^{67}Zn, and 0.03% ^{70}Zn. The solution of $^{68}ZnSO_4$ was prepared as follows: 21.0 mg ^{68}ZnO (powder) was dissolved in 5 ml 1.0 mol/L H_2SO_4, and then gently stirred for 48 h until it was completely dissolved. The solution was then diluted by using deionized water, and solution pH adjusted to 5.0 by adding 1.0 mol/L NaOH. The solution was then transferred to a 250-ml volumetric flask and made up to volume. The final concentration of $^{68}ZnSO_4$ in the solution was 1.0 mmol/L. The abundances of Zn isotope in non-enriched $ZnSO_4$ were 18.75% ^{68}Zn, 48.63% ^{64}Zn, 27.90% ^{66}Zn, 4.10% ^{67}Zn, and 0.62% ^{70}Zn.

2.2 Time-course of ^{68}Zn uptake by seedling stage rice plants

Rice plants were grown in normal nutrient solution up to 30 d (seedling stage) as described above. Before treatment, plants were cultured in normal nutrient solution except Zn supply for 7 d as a starvation treatment, and each pot had three individual plants as replication which were then transferred into a nutrient solution with 1.0 μmol/L ^{68}Zn supply from $^{68}ZnSO_4$ (isotopic abundance 98.60%). Different plant samples (new leaves, old leaves, stems, and roots) were collected for determinations of ^{68}Zn abundance and total Zn concentration at various time intervals of 0, 2, 4, 6, and 8 d after transferred to $^{68}ZnSO_4$ solution. At the onset of each sampling time, a 1.0 ml of nutrient solution was taken from each pot for ^{68}Zn concentration determination. Treatments were replicated three times.

2.3 Xylem sap collection and analysis

Plants of both genotypes (IR68144 and IR64) were grown hydroponically for ten weeks, and used for xylem sap collection. Plants of both genotypes were placed in the uptake solutions (2 mmol/L MES-Tris, 0.5 mmol/L $CaCl_2$; pH=5.8) with different Zn treatments including 0.5, 2.0, and 8.0 μmol/L Zn. Xylem sap collection procedure was according to Lu *et al.* (2009). Twelve plants from each treatment were de-topped using sharp blades at ~5.0 cm above the roots. Immediately after de-topping, each stem was rinsed with deionized water and blotted with absorbent paper to remove contaminants from cut cells. After discarding ~0.3 ml of sap, each cut surface was blotted again and 10-cm silicon tubing was fitted over the stem. Sap flowing from the tubing was collected in sterile vials at time-points of 4, 8, 12, and 24 h after treatment. At the onset of each xylem sap collection, a 1.0 ml aliquot of the uptake solution was taken from each pot for Zn determination. For xylem sap samples, a subsample of 0.5 ml was mixed with 4.5 ml of 2% (v/v) nitric acid. Zn concentrations in all samples were determined by inductively coupled plasma mass spectrometry (ICP-MS; Agilent 7500a, USA).

2.4 Kinetics of ^{68}Zn transport to developing grains

Plants were pre-cultured in normal nutrient solution until anthesis stage, and thereafter the procedure was analogous as in the preceding experiments: plants were transferred to nutrient solution with 1.0 μmol/L ^{68}Zn supply from $^{68}ZnSO_4$ (isotopic abundance being 98.60%). Whole rice grains were collected for determinations of ^{68}Zn abundances and total Zn concentrations at various time intervals of 0, 5, 10, 15, 20, and 25 d after transfer to the treated solution. Other elements (Fe, P, etc.) in rice grains were also determined by ICP-MS (Agilent 7500a, USA). At the onset of each sampling time, a 1.0 ml of nutrient solution was taken from each pot for ^{68}Zn concentration determination. Each treatment was replicated three times.

2.5 Sample preparation and digestion

After harvest, plant parts were separated and washed in running deionized water to remove

superficial nutrient solution. Roots were submerged in a 1-1 bath containing 1 mmol/L $LaCl_3$+0.05 mmol/L $CaCl_2$ for 10 min to remove Zn bounded in apoplast (Rengel, 1999).

Samples were oven-dried and milled by using MM301 (Retsch, Germany) with agate ball and internal wall. For digestion, 0.1 g (accuracy 0.1 mg) samples were mixed with 4 ml nitric acid (HNO_3, reagent grade) and 1 ml hydrogen peroxide (H_2O_2, analytical reagent; Beijing Chemical Works, China). Digestion was performed by using a hot block system (LabTech ED36, Germany). All samples were digested and analyzed in triplicates.

2.6 Determination of ^{68}Zn tracer concentration in rice plants by ICP-MS

Total Zn concentration ($c_{Zn,t}$, mg/kd dry weight (DW)) and final ratio of ^{68}Zn/^{66}Zn (R_{fin}) in rice plants were obtained directly by analysis of ICP-atomic emission spectroscopy (AES) (SHIMADZU ICPS-7510) and ICP-MS (Agilent 7500a), respectively. The ultimate goal of total Zn and Zn isotopic analyses was to determine the concentration of the newly-accumulated Zn into various parts of the rice plants during the tracing periods. Concentrations of the newly-accumulated Zn in different plant parts ($c_{Zn,a}$, mg/kg DW) were calculated from total Zn concentrations post exposure in the respective plant parts, the final ^{68}Zn/^{66}Zn ratio in the respective parts, and the relative fractions of ^{68}Zn and ^{66}Zn in both the exposure media ($f_{68\text{-enr}}$ and $f_{66\text{-enr}}$) and in the respective parts of control rice plants ($f_{68\text{-nat}}$ and $f_{66\text{-nat}}$), according to Eq. (1) (Todd *et al.*, 2009; Wolf *et al.*, 2009):

$$c_{Zn,a}=\frac{c_{Zn,t}(f_{68\text{-nat}}-R_{fin}f_{66\text{-nat}})}{R_{fin}(f_{66\text{-enr}}-f_{66\text{-nat}})+(f_{68\text{-nat}}-f_{68\text{-enr}})}, \quad (1)$$

where $f_{68\text{-nat}}$ and $f_{66\text{-nat}}$ are the natural abundances of ^{68}Zn and ^{66}Zn in normal nutrient solution (18.75% and 27.90%, respectively); $f_{68\text{-enr}}$ and $f_{66\text{-enr}}$ represent the abundance of ^{68}Zn and ^{66}Zn in ^{68}Zn-enriched $ZnSO_4$ bought from the Cambridge Isotope Laboratory (98.60% and 0.39%, respectively). Please note that all data of ^{68}Zn in the tables and figures of this paper refer to $c_{Zn,a}$ only. They do not include the ^{68}Zn accumulated with 'normal Zn' ($c_{Zn,t}$–$c_{Zn,a}$).

2.7 Statistical analysis

All data were statistically analyzed using SPSS Version 12.0. Differences between treatments were determined by the least significant difference (P<0.05) from the analysis of variance (ANOVA). Differences between two genotypes were tested by a paired t-test (P<0.05 or P<0.01). The figures were generated using the software SigmaPlot 10.0.

3 Results

3.1 ^{68}Zn absorption and transportation by root at seedling stage

Under adequate ^{68}Zn supply level, the high Zn-density genotype (IR68144) had a higher shoot dry matter yield, but a lower root dry matter yield, as compared with those in the low Zn-density genotype (IR64), and therefore, the root/shoot ratio of the former was significantly (P<0.01) lower regardless of the sampling time (Table 1). In the advanced growth stage, root/shoot ratios of both genotypes decreased due to higher shoot growth rate (Table 1).

Table 1 Dry weights (DWs) of roots and shoots of high (IR68144) and low (IR64) Zn-density rice genotypes during supplying $^{68}ZnSO_4$ for 8 d at seedling stage

Time (d)	Shoot DW (g/plant)		Root DW (g/plant)		Root/shoot ratio	
	IR68144	IR64	IR68144	IR64	IR68144	IR64
2	1.10*	0.84	0.28	0.33*	0.25	0.39*
4	1.33*	1.14	0.29	0.34*	0.21	0.30*
6	1.62*	1.34	0.33	0.39*	0.20	0.29*
8	1.78*	1.50	0.35	0.43*	0.19	0.29*

Plants were grown in normal nutrient solution up to 30 d (seedling stage), and then transferred into nutrient solution with 1.0 μmol/L ^{68}Zn. Data represent a mean of three plants. * Statistical significance at P<0.01 between the two genotypes

After treatments with $^{68}ZnSO_4$, the concentrations of ^{68}Zn in shoot parts (new leaves, older leaves, and stems) of the high Zn-density genotype (IR68144) were higher than those of the low Zn-density genotype (IR64), but lower in root parts (Table 2). The concentrations of ^{68}Zn in new leaves and stems of both genotypes increased by the time of supplying ^{68}Zn, while in older leaves it was continuously decreased (Table 2). In root, the concentration of ^{68}Zn

was almost stable, with exception of slight increase in IR64 and decrease in IR68144 after 2 d. The total amount of ^{68}Zn taken up by each plant increased with time, whereas higher uptake rate was observed in IR68144 (Fig. la). In a short time (first 2 d), more ^{68}Zn was absorbed in rice plant and translocated to the shoot in the high Zn-density genotype (IR68144), and became stable at 8 d (Fig. 1).

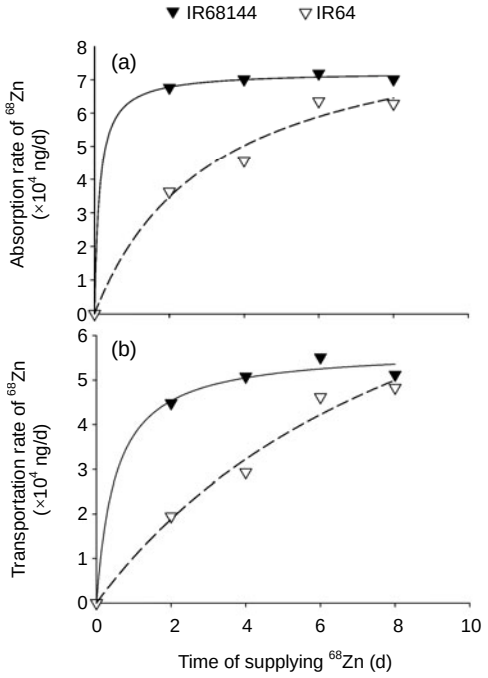

Fig. 1 Time-dependent kinetics of ^{68}Zn absorption and transportation of two rice genotypes after supplying ^{68}ZnSO$_4$

(a) Total amount of ^{68}Zn uptake by whole plant; (b) Total amount of ^{68}Zn translocated to shoot. Rice plants were grown in normal nutrient solution up to 30 d (seedling stage), and then transferred into nutrient solution with 1.0 μmol/L ^{68}Zn supply from ^{68}ZnSO$_4$. Samples were collected for determination of ^{68}Zn abundance and total Zn concentration at various time intervals of 0, 2, 4, 6, and 8 d after transfer

3.2 Kinetics of ^{68}Zn accumulation in new leaves and grains

The concentrations of ^{68}Zn in new leaves and grains of both rice genotypes increased rapidly by the time ^{68}ZnSO$_4$ was supplied (Fig. 2), indicating that ^{68}Zn in the nutrient solution was absorbed and accumulated into the new leaves and grains. The characteristics of ^{68}Zn accumulation in both genotypes were quite similar (Fig. 2). By comparison, ^{68}Zn concentration in IR68144 was higher than in IR64 (Fig. 2). In the developing grains, ^{68}Zn concentration reached the highest level at 15 d and then decreased; this is different from the new leaves and may be due to the growing complexion of rice grains. The 15th day after anthesis stage showed the greatest rate of grain filling, and large amounts of carbohydrate were transported into grains, increasing the biomass quite quickly (Fig. 3). Afterwards, ^{68}Zn concentration decreased due to dilution effects (Fig. 2).

Besides, Table 3 shows that transportation of ^{68}Zn into rice grains continues up to maturity. The accumulation of ^{68}Zn in grains of both genotypes increased rapidly with time of supplying ^{68}Zn; however, before 15 d the accumulation of ^{68}Zn in IR64 was higher than that of IR68144 because of the earlier grain filling stage of IR64 (Table 3). After 15 d, ^{68}Zn accumulation of IR68144 was higher than that of IR64. The amount of Zn remobilized from vegetative tissues was increased even at late grain filling stage, with a great portion of total Zn allocated in grains, which was much higher than the level of Zn directly absorbed by the root after the anthesis stage.

Table 2 Concentration of ^{68}Zn in the dried tissues of two rice genotypes during supplying ^{68}ZnSO$_4$ for 8 d at seedling stage

Sampling time (d)	Concentration of ^{68}Zn (mg/kg)							
	IR64				IR68144			
	Old leaves	New leaves	Stem	Root	Old leaves	New leaves	Stem	Root
2	0.63^a	0.32^a	13.00^a	16.48^a	2.25^a	0.98^a	18.44^a	15.33^a
4	0.42^b	2.35^b	15.18^b	16.74^a	1.75^b	2.71^b	19.81^a	15.24^a
6	0.25^c	5.58^c	22.17^c	18.54^b	1.54^b	7.00^c	19.55^a	14.68^b
8	0.13^d	9.85^d	31.03^d	19.10^b	1.33^c	11.69^d	15.26^b	14.41^b

Plants were grown in normal nutrient solution up to 30 d (seedling stage), then transferred into nutrient solution with 1.0 μmol/L ^{68}ZnSO$_4$. Data represent a mean of three plants. Different superscript letters at the same column indicate statistical significance at P<0.05

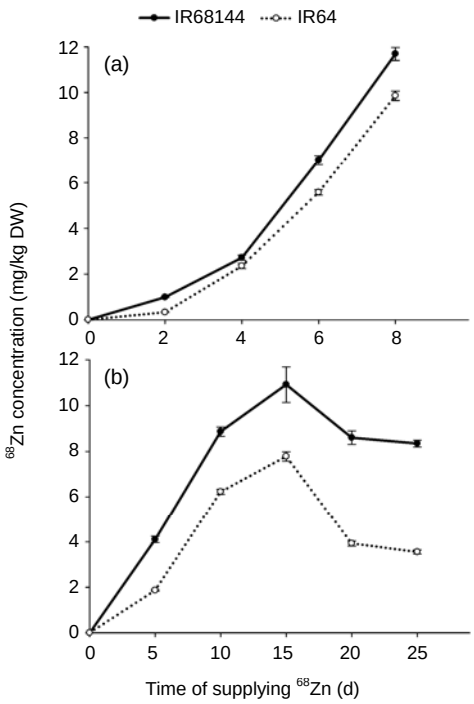

Fig. 2 Effect of $^{68}ZnSO_4$ treatment on the concentration of isotope tracer ^{68}Zn in new leaves and developing grains of two rice genotypes after supplying $^{68}ZnSO_4$

(a) New leaves were collected at various time intervals of 0, 2, 4, 6, and 8 d; (b) Grains were collected at various time intervals of 0, 5, 10, 15, 20, and 25 d after transfer. Rice plants were grown in normal nutrient solution for 30 d (seedling stage) and 100 d (anthesis stage), respectively, and then transferred into nutrient solution with 1.0 μmol/L ^{68}Zn supply from $^{68}ZnSO_4$. Data represent mean±SE of three replicates

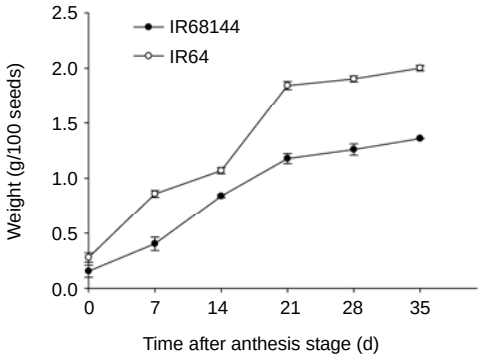

Fig. 3 Growth weight of grains in two rice genotypes under normal hydroponic condition

Rice plants were grown in normal nutrient solution for 100 d (anthesis stage), and then rice grains were collected at various time intervals of 0, 7, 14, 21, 28, and 35 d after that. Weight of 100 seeds per individual plants was determined. Data represent mean±SE of five replicates

3.3 Zn transport in xylem

Time-dependent variation of Zn concentration in xylem sap of the plants and the uptake solution is shown in Fig. 4 for both IR68144 and IR64. Zn concentrations in the xylem sap of both genotypes increased with increasing Zn level in the uptake solution at each time point (Fig. 4a), but showed different rate patterns. For high Zn-density genotype (IR68144), Zn concentration increased rapidly up to 12 h and then decreased, whereas for IR64, it increased to 8 h and then decreased. Regardless of treatments, Zn concentration in the xylem sap of

Table 3 Accumulation of ^{68}Zn into rice grains of each individual plant at various time when $^{68}ZnSO_4$ supply was initiated at anthesis stage

Genotype	Time (d)	^{68}Zn[a] (μg/plant)	Zn[b] (μg/plant)	Total Zn[c] (μg/plant)	^{68}Zn content (%)
IR64	0	0	10.95±0.32	10.95	0
	5	0.77±0.02	31.05±0.59	31.82	2.41
	10	10.81±0.19	46.32±0.73	57.13	18.92
	15	14.86±0.41	59.29±0.91	74.15	20.01
	20	15.62±0.29	74.81±1.03	90.43	17.27
	25	17.74±0.48	75.39±0.89	93.13	19.05
IR68144	0	0	10.68±0.47	10.68	0
	5	0.55±0.02	32.67±0.53	33.22	1.66
	10	5.74±0.14	54.09±0.87	59.83	9.60
	15	19.88±1.42	66.21±0.71	86.09	23.09
	20	22.73±0.77	76.50±1.32	99.23	22.90
	25	27.07±0.48	79.43±1.03	106.50	25.42

Plants were grown in normal nutrient solution for 100 d (anthesis stage), and then transferred into nutrient solution with 1.0 μmol/L ^{68}Zn. [a] ^{68}Zn refers to Zn absorbed from nutrient solution after anthesis stage; [b] Zn refers to Zn remobilized from vegetative tissues; [c] Total Zn means the sum of both. Data represent mean±SE for three replications

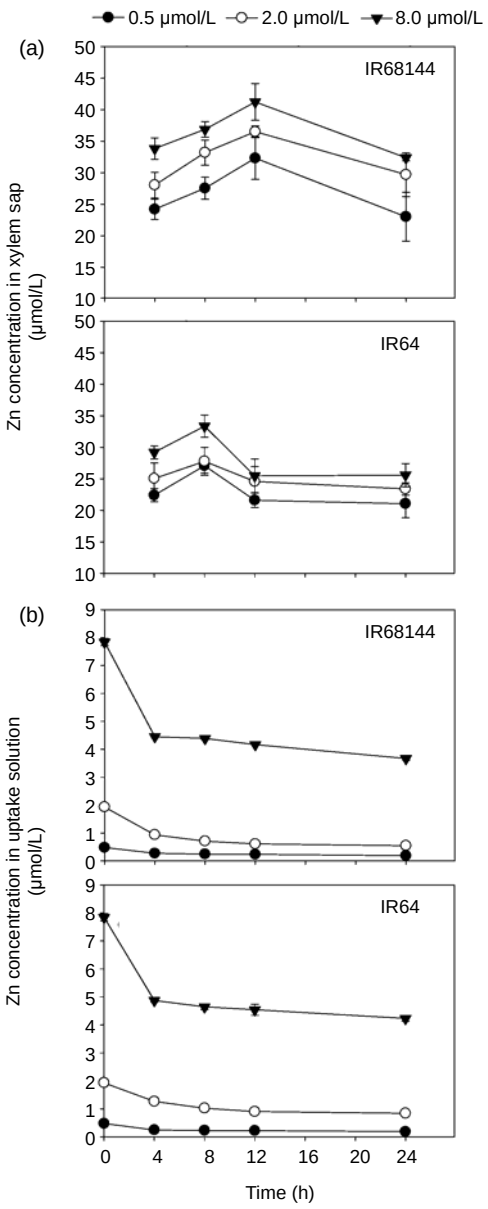

Fig. 4 Time-course Zn concentrations in the xylem sap (a) and uptake solution (b) of IR68144 and IR64 with treatments of 0.5, 2.0 and 8.0 μmol/L Zn

Twelve plants in the same pot were treated as one replicate. Data represent mean±SE of three replicates

IR68144 was consistently higher than that of IR64, especially with 8.0 μmol/L Zn supply level (Fig. 4a). In contrast to Zn concentration in xylem sap, Zn concentration in the root of IR64 was much higher than that of IR68144 (data not shown). Zinc concentration in the uptake solution decreased gradually, especially during first 4 h (Fig. 4b). Zn concentration was consistently higher in the xylem sap of both rice genotypes than in the uptake solution (Fig. 4). At the Zn-deficient level (0.5 μmol/L Zn supply), Zn in the external solution deceased by 50% within 4 h, and no significant difference was observed for the two genotypes (Fig. 4b), but the decreasing ratio of Zn in solution was approximately 50% for IR68144, but less than 40% for IR64, at sufficient (2.0 μmol/L) or surplus (8.0 μmol/L) Zn supply. After 24 h, IR68144 also had higher Zn absorption ratio than IR64 at sufficient (2.0 μmol/L) or surplus (8.0 μmol/L) Zn supply, showing lower Zn concentration in uptake solution (Fig. 4b).

4 Discussion

The physiological basis of Zn transport in rice plants and the controlling process of Zn accumulation in edible portion of seeds are not understood with any certainty (Welch and Graham, 1999; Graham *et al.*, 2001). The first and most important barrier of rice plants to accumulate more Zn in edible tissues resides at the root-soil interface (i.e., the rhizosphere) for Zn absorption (Welch, 1995). In this study, $^{68}ZnSO_4$ was used as the source of Zn in hydroponics experiments. The results show that more ^{68}Zn was absorbed in rice plants and translocated to the shoot by roots of high Zn-density rice genotype (IR68144) in early 2 d (Fig. 1), with higher ^{68}Zn concentration in new leaves (Table 2), when compared with those of IR64. Fibrous root system of IR68144 had more root tips and total surface area than that of IR64 (Table 4), so ^{68}Zn could be absorbed more quickly from nutrient solution in the short-term.

Similar results were also obtained in barley and rice (Genc *et al.*, 2007; Chen *et al.*, 2009). Genc *et al.* (2007) showed that the greater Zn uptake and better growth in Pallas (wild-type barley) compared with *brb* (root-hairless mutant) in Zn-deficient soil, which could be attributed primarily to greater root surface area due to root hairs in Pallas, rather than other root morphological differences. Interestingly, ^{68}Zn concentration in stems of IR68144 decreased after 6 d when ^{68}Zn was deficient in the solution; however, it was not seen in IR64 (Table 2). This suggested that ^{68}Zn deposited in stems of IR68144 might be retranslocated into new growing tissues under Zn-deficient condition.

Table 4 Root morphologies of two rice genotypes under normal hydroponics condition

Genotype	Total length (cm/plant)	Total surface area (cm^2/plant)	Total volume (cm^3/plant)	Length of root with diameter <0.4 mm (cm)	Number of root tips (tip/plant)
IR64	478.39 ± 51.71^{Aa}	56.11 ± 12.18^{Bb}	0.53 ± 0.17^{Aa}	313.88 ± 10.56^{Aa}	2046.11 ± 356.36^{Bb}
IR68144	559.32 ± 62.46^{Aa}	65.71 ± 18.23^{Aa}	0.58 ± 0.22^{Aa}	355.91 ± 25.41^{Aa}	3707.67 ± 339.64^{Aa}

Plants were grown in normal nutrient solution for 30 d (seedling stage). Data represent mean±SE of six independent replicates. Different upper and lower superscript letters indicate statistical significance at $P<0.01$ and $P<0.05$, respectively, between the two rice genotypes

The efficiency of root-to-shoot translocation is theoretically dependent on four processes (Lasat *et al.*, 1996; Palmgren *et al.*, 2008): (1) Zn sequestration in the root; (2) efficiency of the radial symplastic passage; (3) xylem loading capacity; and, (4) Zn movement efficiency in the xylem vessels. It has been suggested that decreased root cell sequestration may facilitate enhancing Zn root-to-shoot translocation in the hyperaccumulators (Yang *et al.*, 2006); however, very limited literature was available on Zn sequestration in the root of rice. Root uptake of divalent cations typically exhibits two phases: apoplastic binding and symplastic uptake (Hart *et al.*, 1998; Zhao *et al.*, 2002). Passive (apoplastic) uptake involves diffusion of ions in the soil solution into the root endodermis along a chemical potential (concentration) gradient, while active ion uptake occurs against the concentration gradient with high selectivity of ions and energy-consuming mechanism (Marschner, 1995). In this study, a higher Zn concentration in xylem sap was observed compared to the uptake solution, and still increased within 4–8 h while Zn concentration in the uptake solution was decreasing (Fig. 4). We believe that root to shoot translocation of Zn in both rice genotypes is through symplastic passage. Additionally, Zn concentration in the xylem sap of IR68144 was consistently higher than that of IR64, especially with 8.0 μmol/L Zn supply level (Fig. 4a), but lower Zn concentration in root in contrast to IR64 (Table 2), showing enhanced transport capacity of Zn to shoot. Thus, we suggest that efficient transport of Zn into root symplasm and efflux into xylem vessels may play an important role in Zn accumulation into grains.

Results of the present study show similar patterns of ^{68}Zn accumulation into new leaves at seedling stage and into developing grains at ripening stage by root supply (Fig. 2). The concentrations of ^{68}Zn in new leaves and grains of IR68144 were higher than those of IR64 at the same developing stage. It is hypothesis that rice plants with high Zn concentration in new leaves at seedling stage will have high Zn density in grains, but it still needs further investigation. If so, it will become more convenient for the plant breeder to choose high Zn-density rice genotypes. After anthesis stage, both of the rice genotypes continued to uptake the root-supplied Zn with high accumulation occurring in the rice grains (Table 3). This was consistent with the findings of cotton (Constable *et al.*, 1988), red spring wheat (Miller *et al.*, 1994), and aerobic rice (Jiang *et al.*, 2007). Of the total plant Zn, 50% (cotton), 10% (wheat), or 20% (rice) was taken up between anthesis and maturity. Here, in the high Zn-density genotype (IR68144) it was 25%, higher than that in the low Zn-density genotype IR64 (20%), indicating that IR68144 had a more efficient transport system for Zn bypass from root to grain. It was reported that Zn was a mobile micronutrient and could remobilize from vegetative tissues (such as stems, roots, and senescence leaves) into grains, which was confirmed in wheat (Pearson *et al.*, 1995; 1996). However, in rice, there is little evidence to show the capacity of phloem remobilization of Zn from vegetative tissues to grains. A large proportion of Zn in the grains was remobilized from vegetative tissues during grain developing stage (Table 3), which amounts to three to four times greater than that directly absorbed by roots after anthesis stage in IR68144 and IR64. Therefore, we suggest that the phloem remobilization capacity could be an important factor which is responsible for high Zn density in rice grains.

In the cereal grains, Zn is preferentially stored together with phytate, which is a strong chelator of divalent cations and significantly reduces mineral bioavailability (Bohn *et al.*, 2008). Phytate is the primary storage form of phosphate and inositol in cereal seeds (Bouis, 2000). It accumulates rapidly during seed development and can account for up to several percent of the seed DW (Lott, 1984). In this study, P accumulation in the grains of IR64 was higher than that of IR68144 during the grain filling

stage, which was consistent with the result of Liu *et al*. (2004). The content of Fe in developing grains was also analyzed (Fig. 5b) and had the same tendency as Zn accumulation in grains. Hao *et al*. (2005) found that Zn and Fe were distributed through the whole transverse section of IR68144 grains, whereas the elements were not detected in the center of IR64 grains. Although there was no significant difference between the protein contents of polished rice (approximately 10%) with the two genotypes (data not shown), the grains of IR68144 had significantly higher methionine content and lower tyrosine content than those of IR64 (P<0.01). Those results suggest that high Zn density in rice grains is also related with the chemical compositions of grains, such as P and other elements.

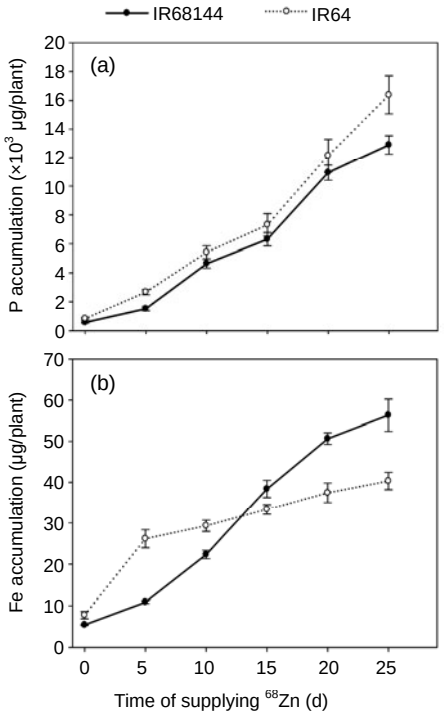

Fig. 5 Accumulations of P (a) and Fe (b) during the rice grain developments of two rice genotypes
Rice plants were grown in normal nutrient solution for 100 d (anthesis stage), and then rice grains were collected at various time intervals of 0, 5, 10, 15, 20, and 25 d after that. Accumulations of P and Fe were determined by ICP-MS. Data represent mean±SE of three replicates

Our findings suggest that for Zn density in rice grains, the mobility of Zn within the plants seems to be more important than the root uptake ability. In the low Zn-density genotype, a great amount of Zn absorbed by the root was deposited in the roots and stems, and less was remobilized into developing grains compared to high Zn-density genotype. Efforts on promoting this portion of Zn to grains could be the key point for enhancing Zn density in seeds. Root-shoot distribution of Zn has been suggested to be controlled mainly by heavy metal transporting ATPases (Hussain *et al*., 2004), which were thought to transport Zn across the plasma membrane of root vascular cells into the xylem for transport to the shoot. Increasing the expression of *HMA4* or a closely related gene is therefore likely to enhance the rates of Zn translocation from root to shoot for biofortification purposes (Hanikenne *et al*., 2008). The yellow stripe-like transporter (YSL) proteins transport metal-nicotianamine complexes and have mainly been studied with respect to Fe homeostasis (Koike *et al*., 2004; Schaaf *et al*., 2005). However, YSL transporters could also have roles in Zn mobilization. Waters and Grusak (2008) found that these proteins were required for efficient mobilization of Zn from sentence leaves and fruit hulls into seeds. However, little literature was available on the effect of these transporters in rice, and further investigation is necessary to understand the mechanisms of manipulating Zn transport in rice plants.

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