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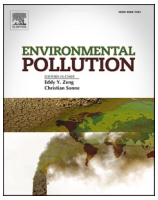

Zinc regulation of iron uptake and translocation in rice (*Oryza sativa* L.): Implication from stable iron isotopes and transporter genes☆

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ABSTRACT

Iron (Fe) is an essential nutrient for living organisms and Fe deficiency is a worldwide problem for the health of both rice and humans. Zinc (Zn) contamination in agricultural soils is frequently observed. Here, we studied Fe isotope compositions and transcript levels of Fe transporter genes in rice growing in nutrient solutions having a range of Zn concentrations. Our results show Zn stress reduces Fe uptake by rice and drives its δ^{56}Fe value to that of the nutrient solution. These observations can be explained by the weakened Fe(II) uptake through Strategy I but enhanced Fe(III) uptake through Strategy II due to the competition between Zn and Fe(II) combining with OsIRT1 (Fe(II) transporter) in root, which is supported by the downregulated expression of *OsIRT1* and upregulated expression of *OsYSL15* (Fe(III) transporter). Using a mass balance box model, we also show excess Zn reduces Fe(II) translocation in phloem and its remobilization from senescent leaf, indicating a competition of binding sites on nicotianamine between Zn and Fe(II). This study provides direct evidence that how Zn regulates Fe uptake and translocation in rice and is of practical significance to design strategies to treat Fe deficiency in rice grown in Zn-contaminated soils.

1. Introduction

Iron (Fe) is an essential nutrient and is involved in many physiological processes in plants (Kobayashi and Nishizawa, 2012). Despite the high abundance of Fe in Earth's crust, Fe is often not readily available to plants due to the low concentration of soluble Fe in soils (Marschner, 1995). Rice (*Oryza sativa* L.) is an important crop for global population, but insufficient Fe uptake by rice may lead to Fe deficiency in humans. Fe deficiency in rice is a worldwide problem and over one billion people are suffering from Fe-deficiency anemia (Clemens et al., 2002; Graham et al., 2012). Understanding the mechanisms of Fe uptake and translocation in rice is imperative for both plant growth and human health.

Zinc (Zn) is a vital micronutrient in most living organisms. It helps regulate redox reactions and protein synthesis (Sinclair and Krämer, 2012). Thus, it is often added to mineral fertilizers to enhance the growth of crop plants (Sadeghzadeh, 2013). However, a high level of Zn in soil is considered toxic which inhibits many metabolic functions in plants (Nagajyoti et al., 2010). Soils have been reported to be contaminated by Zn due to the expansion of industrial and agricultural activities (Nriagu and Pacynat, 1988; Nriagu, 1996). In response, plants have developed natural abilities to eliminate excess Zn out of cells or sequestrate Zn into subcellular compartments (Gupta et al., 2016). It has been shown that the successful maintenance of Zn homeostasis in plants greatly relies on the coordinated regulation of uptake-transporters (e.g., ZRT members and IRT-like proteins) and chelating compounds (e.g., nicotianamine or NA; Colangelo and Guerinot, 2006). For instance, Haydon et al. (2012) showed that the overexpression of *ZIF1* enhances the partition of metal chelator NA into vacuoles and leads to the pronounced NA accumulation in roots, accompanied by the vacuolar buildup of Zn.

According to the strategy used for Fe acquisition, higher plants have been traditionally classified into two groups: Strategy-I plants which

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acquire Fe(II) after reducing Fe(III) by reductases, and Strategy-II plants which secrete phytosiderophore (PS) that binds Fe(III) for subsequent acquisition of Fe(III)-PS (Marschner et al., 1986; Bughio et al., 2002; Ishimaru et al., 2006). However, rice and species of the Oryza genus closely related to domesticated rice employ the combined strategies for Fe uptake, which is composed by all features of Strategy II, common to all Poaceae species, and some features of Strategy I, common to non-Poaceae species (Wairich et al., 2019). Deoxymugineic acid (DMA) is the only type of PS molecule synthesized in rice plants, and rice exports DMA via OsTOM1 (Nozoye et al., 2011). Similar to Strategy-II plants, rice mainly absorbs Fe(III)-PS chelate carried by the *OsYSL15* (yellow stripe-like 15) transporter (Inoue et al., 2009; Lee et al., 2009). Rice can also absorb Fe(II) into roots carried by OsIRT1 (Fe-regulated transporter 1) as Strategy-I plants normally do (Vert et al., 2002; Lee, 2009). In Strategy-I plant, excess Zn was observed to induce significant deficiencies in Fe acquisition and upregulated the expression of Fe(II) uptake-transporter gene (*IRT1*) mostly due to non-selective transport of Zn and Fe(II) by IRT1 (Vert et al., 2002; Shanmugam et al., 2011). In cucumber (a Strategy-I plant), Fe deficiency in leaves was accompanied by a large accumulation of Zn (Vigani et al., 2018). However, in wheat (a Strategy-II plant), Zn and Fe help each other to translocate from root to shoot while competitive inhibition between Fe/Zn ratio was observed during the Fe-uptake by root (Hanjagi and Singh, 2017). Since Fe(III) is highly reactive and poorly soluble, it needs to be transported within the plant in a chelated form (Kobayashi and Nishizawa, 2012). In rice, three compounds are known to play major roles in Fe chelation during its long-distance transport: citrate, DMA and NA (Haydon and Cobbett, 2007; Kawakami and Bhullar, 2018). NA is the precursor of DMA in graminaceous species, and both of them contribute to Zn-acquisition (Rellán-Álvarez et al., 2008; Suzuki et al., 2008; Yoneyama et al., 2015). Thus, Fe and Zn homeostasis in plants may be closely-related due to their similar affinity to particular transporters and ligands that are responsible to their uptake and translocation (Lee, 2009; Haydon et al., 2012). Under various levels of Zn stress, Fe homeostasis could be complex that merit a detailed study to understand how Zn regulates Fe uptake and translocation in rice and is of practical significance to design strategies to treat Fe deficiency in rice grown in Zn-contaminated soils.

Fe isotopes are a well-developed tool to constrain the uptake and transport of Fe during plant growth (Von Blanckenburg et al., 2009; Dauphas et al., 2017; Caldelas and Weiss, 2017). Biogeochemical processes may trigger distinct Fe isotope fractionations (Guelke and Von Blanckenburg, 2007; Kiczka et al., 2010). Recent studies showed that Fe isotopes have the potential to distinguish strategies for Fe uptake and translocation in plants (Guelke and Von Blanckenburg, 2007; Von Blanckenburg et al., 2009; Kiczka et al., 2010; Guelke-Stelling and Von Blanckenburg, 2012; Moynier et al., 2013; Arnold et al., 2015; Liu et al., 2019). Guelke and Von Blanckenburg (2007) first discovered that some plants including soybean and lettuce are enriched in light Fe isotopes, while some other plants including wheat and maize are enriched in heavy Fe isotopes during Fe-uptake. The depletion of heavy Fe isotopes in gramineae was attributed to two processes during Fe-uptake – dissolution of Fe-bearing minerals and selective uptake of light Fe isotopes at the plasma membrane (Kiczka et al., 2010). Their subsequent work observed ~ −0.5‰ fractionation between oat and nutrient (i.e., $\Delta^{56}Fe_{oat\text{-}nutrient} = -0.5‰$), indicating plants may adapt to uptake mechanisms based on environmental conditions (Guelke-Stelling and Von Blanckenburg, 2012). Recent studies showed complex Fe isotope fractionations in rice. Rice grown in aerobic and anaerobic soils has smaller Fe isotope fractionation ($\Delta^{56}Fe_{shoot\text{-}soil}$ up to −0.5‰) than that grown hydroponically in Fe(III)-EDTA (with sufficient Fe supply, $\Delta^{56}Fe_{whole\ plant\text{-}nutrient} = -1.36‰$) or grown naturally in a paddy field (with deficient Fe supply, $\Delta^{56}Fe_{whole\ plant\text{-}soil\ solution} = 0.27‰$) (Arnold et al., 2015; Liu et al., 2019). In contrast, an Fe isotope fractionation factor of −0.9‰ was found between rice root to grain, which was explained as isotope fractionation in paddy soils during rice growth (Garnier et al., 2017). In addition, the significant enrichment of heavy Fe isotopes measured in root relative to soil porewater suggests the Fe uptake by root is more likely supplied by Fe plaque rather than the porewater (Garnier et al., 2017). Theoretical and experimental studies showed that significant Fe isotope fractionation may occur when Fe changes its oxidation state with Fe(III) enriching heavy Fe isotopes and Fe(II) enriching light Fe isotopes, and during chelating with some ligands (Fujii et al., 2014).

In this work, rice (*Oryza sativa* L. cv. youyou128) was grown in nutrient solutions with various Zn concentrations. Combining Fe isotope and Fe transporter gene analyses in the tillering and maturity stages, we explored whether and how Zn regulates Fe uptake and translocation strategies during rice growth. Our findings are expected to have important implications for understanding the physiological process of Fe uptake and translocation in rice grown in Zn-contaminated soils.

2. Materials and methods

2.1. Plant growth

Rice (*Oryza sativa* L. cv. youyou128) was hydroponically cultivated in an environmentally controlled greenhouse using Kimura B nutrient solution containing 370 μM $(NH_4)_2SO_4$, 550 μM $MgSO_4{\cdot}7H_2O$, 90 μM K_2SO_4, 180 μM KNO_3, 370 μM $Ca(NO_3)_2{\cdot}4H_2O$, 180 μM KH_2PO_4, 1 μM $CuSO_4{\cdot}5H_2O$, 5 μM $MnSO_4{\cdot}H_2O$, 10 μM H_3BO_3, 0.5 μM $Na_2MoO_4{\cdot}2H_2O$, 100 μM NaCl, 0.2 μM $CoSO_4{\cdot}7H_2O$, and 50 μM Fe(III)-EDTA (Guo et al., 2007). Additional $ZnSO_4{\cdot}7H_2O$ was added to nutrient solution to make the final concentration of 1 μM (CK, for control check), 10 μM (T1), 50 μM (T2), 100 μM (T3), and 400 μM (T4). The nutrient solution was adjusted to pH 5.6 with sodium bicarbonate and replaced every 3 days. The Fe speciation in the nutrient solution used in our experiments was modeled using MINTEQ 3.0 (Gustafsson, 2011) and the calculated results were shown in Table S1 of the Supporting Information (SI). Rice was collected in tillering (on day 30) and maturity (on day 120) stages. For gene-expression analyses, roots and leaves of rice (2–3 plants) cultured in nutrient solution with 1 μM (CK) and 100 μM (T3) of $ZnSO_4{\cdot}7H_2O$ were isolated quickly and frozen in liquid nitrogen for RNA extraction. Tissues of 10–20 plants were collected separately and cleaned for the measurements of dry weight (DW) and Fe isotope composition. More detailed information was given in Section S1 of the SI.

2.2. Measurements of Fe concentration and isotope composition

Samples were digested by a microwave digestion system following Xia et al. (2020). The detailed procedures can be found in Section S2 of the SI. The Fe concentrations were determined by an inductively-coupled-plasma optical-emission-spectrometry (ICP-OES by PerkinElmer, USA), while Zn concentrations were measured by an inductively-coupled-plasma mass-spectrometry (ICP-MS by PerkinElmer, USA). Separating pure Fe solution from other metals was performed using anion exchange chromatography, following a previous method by He et al. (2015). Fe isotope measurements were carried out on a MC-ICP-MS (Neptune Plus, Thermo Fisher, USA) in wet plasma mode at State Key Laboratory of Environmental Geochemistry, Institute of Geochemistry, Chinese Academy of Sciences. The standard-sample bracketing technique was used to correct the instrumental mass bias. Each sample was measured repeatedly for four times to assess the precision of the measurements. The Fe isotope composition is expressed as the deviation of isotopic ratio of the sample from that of standard IRMM-014 as:

$$\delta^{x}Fe\ (‰) = \left[\frac{\left({}^{x}Fe/{}^{54}Fe\right)_{sample}}{\left({}^{x}Fe/{}^{54}Fe\right)_{IRMM-014}} - 1\right] \times 1000 \quad (1)$$

where x refers to mass of 56 or 57. The USGS geological reference materials BHVO-2 and AGV-2 yielded $\delta^{56}Fe$ values of 0.13 ± 0.07‰ (2SD)

and 0.03 ± 0.05‰ (2SD), respectively, which agree with previously published results within analytical uncertainties (Dauphas and Rouxel, 2006; An et al., 2017).

2.3. Genome expression analysis of Fe transporters

The genome expressions of Fe transporters were analyzed by quantitative real-time PCR. The total RNA was extracted from frozen plant tissue using TRIzol reagent (Sigma, USA). The first-strand cDNA was synthesized from ~2 mg of total RNA using an All-In-One 5 × RT MasterMix Kit (abm, Canada). Technical replicates of gene-specific products were amplified in ~10 μL reactions using SYBR Green PCR Master Mix (Roche Diagnostics, Switzerland) and the primers were given in Table S2. The relative expression level for each sample was determined by quantifying the PCR efficiency and normalized to the geometric mean of genome expression of the reference, *α-tubulin* (Ishimaru et al., 2007). Relative gene expression was calculated using the $\Delta\Delta C_t$ method (ThermoFisher Scientific, USA).

2.4. Mass balance box model

To quantitatively describe Fe fluxes from nutrient solution to root and other rice tissues, we developed a mass balance box model using the biomass, Fe concentrations, and Fe isotope compositions of rice tissues (Fig. 1), while similar box-model has been widely used to describe carbon and hydrogen isotope fractionation in biosynthetic processes (Hayes, 2001). In this model, data from five major Fe reservoirs, including the nutrient solution, root, stem, leaf, and grain, in the tillering and maturity stages were used to calculate the Fe fluxes (φ_i) and apparent fractionation factors (ε_i) between these reservoirs and plant saps. In the maturity stage, the stems included nodes and internodes, and grains included peduncles since their δ^{56}Fe values are similar to each other within analytical uncertainties. Despite the large difference between δ^{56}Fe values of leaf and senescent leaf in the maturity stage, they were considered as the same reservoir since no accurate data was available for the starting time and rate of the development of senescent leaf. The detailed model calculation process is presented in Section S3 of the SI.

2.5. Calculation of Fe mass and isotope composition in plant tissues

The mass of Fe or Zn in each plant tissue (M_{tissue}) was calculated by multiplying the Fe or Zn concentration with the DW:

$$M_{tissue} = C_{tissue} \times W_{tissue} \quad (2)$$

where C_{tissue} and W_{tissue} are Fe or Zn concentration and DW of a tissue, respectively. To calculate the proportion of Fe absorbed into rice relative to the Fe mass added into nutrient, a simple mass balance calculation can be performed as follows:

$$w = \frac{Fe_{whole} \times n}{c \times v} \times 100\% \quad (3)$$

where Fe_{whole} and n are total Fe mass in each plant and number of plants in each pot, respectively; c is Fe concentration of nutrient solution, and v is total volume of nutrient added into each pot. Fe isotope compositions of shoot ($\delta^{56}Fe_{shoot}$) and whole plant ($\delta^{56}Fe_{whole\ plant}$) were calculated based on the mass balance equation as follows:

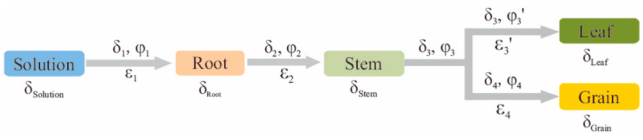

Fig. 1. Mass-balance box model of rice.

$$\delta^{56}Fe_{shoot\ or\ whole\ plant} = \frac{\sum \delta^{56}Fe_{tissue} \times M_{tissue}}{\sum M_{tissue}} \quad (4)$$

where $\delta^{56}Fe_{tissue}$ is δ^{56}Fe value of a tissue. The shoot in tillering stage includes stem and leaf, and that in maturity stage includes internodes, nodes, leaves, senescent leaves, and peduncles. The whole plant includes all tissues. The standard errors of these mean values are propagated from standard errors of DW of tissues and δ^{56}Fe values using Monte Carlo method.

During each three-day culturing experiment, the mass-balance calculation shows that the amount of Fe absorbed by rice is less than 5% of the total Fe in the nutrient solution (Table S3), which may result in an insignificant variation of $\delta^{56}Fe_{nutrient}$ value. For example, if the Fe uptake by rice follows a Rayleigh or batch process, the $\delta^{56}Fe_{nutrient}$ may increase by 0.10‰ (from 0.55‰ to 0.65‰) if the Fe isotope fractionation between nutrient solution and whole plant ($\Delta^{56}Fe_{nutrient\text{-}plant}$) is 2.0‰, by 0.05‰ if $\Delta^{56}Fe_{nutrient\text{-}plant} = 1.0$‰, or by 0.03‰ if $\Delta^{56}Fe_{nutriet\text{-}plant} = 0.5$‰.

3. Results

3.1. Biomass and Fe accumulation in rice

Fig. 2 depicts biomasses of root, shoot, grain, and whole plant, and Table S4 shows biomass of all plant tissues. Substantial declines in total DWs can be observed between the two stages in experiments T3 and T4. In particular, the DW of root systematically decreases with increasing Zn supply in both stages. But there is little difference in the DW of root, shoot, or whole plant among experiments CK, T1, and T2 in either stage.

Fe masses of rice tissues are shown in Fig. 3. In both stages, Fe masses of rice tissues aboveground generally decrease with increasing Zn supply (Fig. 3a and c). In particular, Fe masses of rice node and senescent leaf in the maturity stage show the biggest changes (Fig. 3c). In contrast, the Fe mass of root increase with increasing Zn supply. The data of Fe mass and Fe concentration are listed in Table S5 and Table S6. Zn concentration and mass of rice tissues in tillering and maturity stages increase with increasing Zn supply (Fig. S1). The data of Zn mass and Zn concentration are listed in Table S7 and Table S8.

3.2. Fe isotope fractionation in the tillering stage

Fig. 3b and Table S9 display Fe isotope compositions of rice tissues in the tillering stage. The calculated Fe isotope fractionation (ε_i) and transport efficiency (φ_i) between rice tissues and plant saps in the tillering stage are shown in Fig. 4 and Table S10. The nutrient solution enriches heavy Fe isotopes ($\varepsilon_1 = 0.45$‰) during Fe uptake in experiment CK. With increasing Zn supply, the ε_1 value generally decreases towards zero (0.01‰ in experiment T3). The plant saps from root to stem favors light Fe isotopes (ε_2 decreases from 2.31‰ to 0.71‰) with increasing Zn supply. The transport efficiency (φ_2/φ_1) from root to stem decreases dramatically with increasing Zn supply. During the Fe transport from stem to leaf, the stem favors heavy Fe isotopes, with the Fe isotope fractionation factor (ε_3) between stem and leaf varying slightly from 0.46‰ to 0.39‰ with increasing Zn concentrations in the growth solution. The transport efficiency (φ_3/φ_2) from stem to leaf decreases with increasing Zn supply.

3.3. Fe isotope fractionation in the maturity stage

Fig. 3d and Table S9 depict Fe isotope compositions of rice tissues in the maturity stage. Fig. 4 and Table S11 show the Fe isotope fractionation (ε_i) and transport efficiency (φ_i) in the maturity stage. Root absorbed Fe from the nutrient solution, lead to light Fe isotopes enrichment with ε_1 varying from 1.15‰ to 0.73‰. The CK experiment has the highest ε_1 value (1.15‰), and the other four experiments have

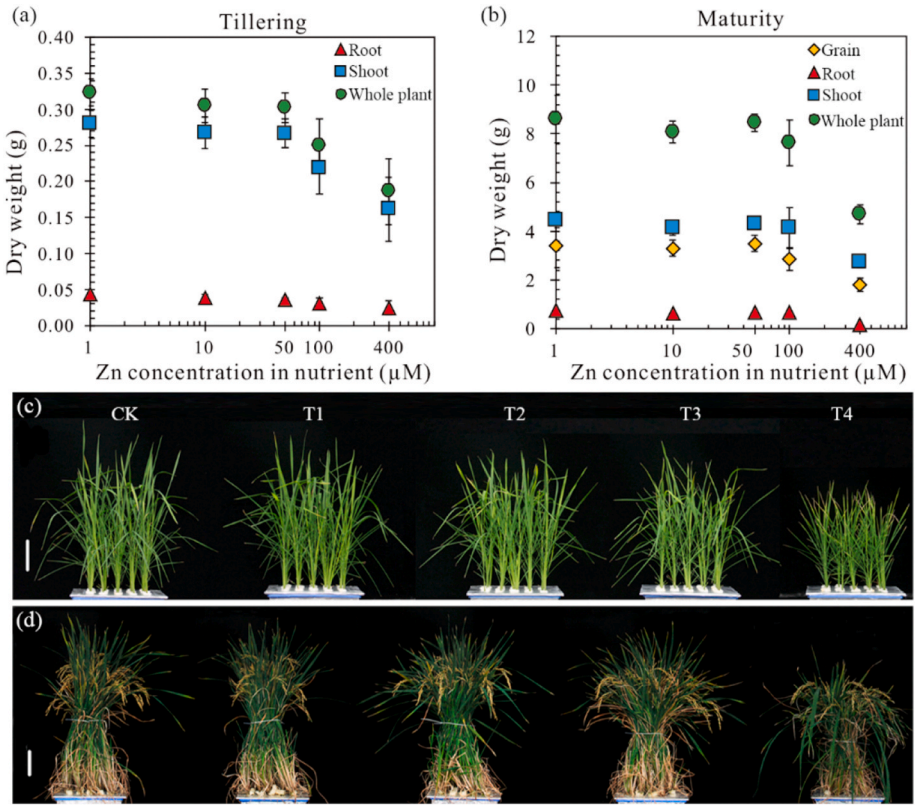

Fig. 2. Biomass (a–b) and phenotype (c–d) of rice in tillering and maturity stages under normal and Zn gradient excess conditions. The numbers in the treatment names (CK, T1, T2, T3, and T4) represent Zn concentrations in nutrient solutions (1, 10, 50, 100, and 400 μM). Photographs were taken 30 days and 120 days after seedlings were transferred to nutrient solution. Scale bars represent 10 cm.

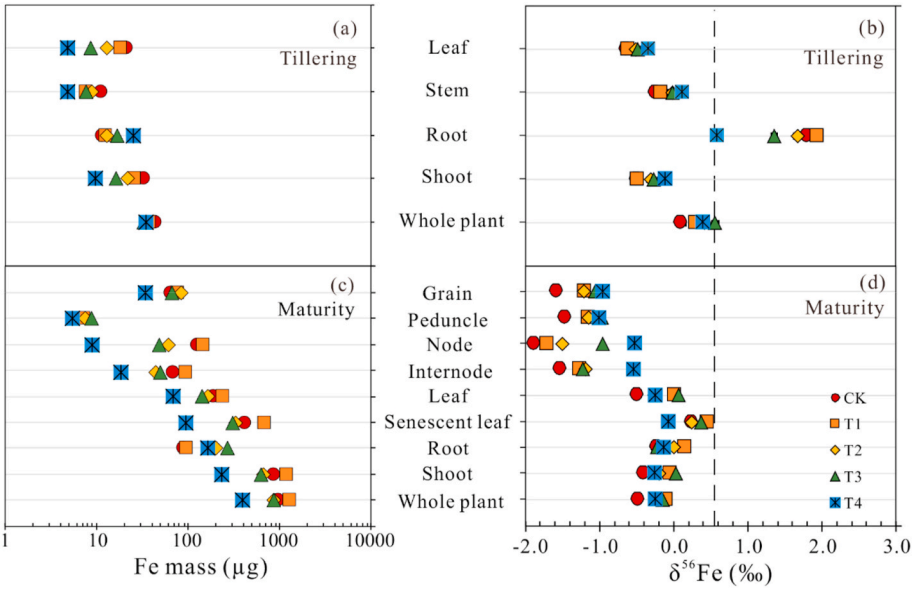

Fig. 3. Fe mass and Fe isotope compositions of tissues, shoot and whole plant in tillering (a, b) and maturity (c, d) stages. The numbers in the treatment names (CK, T1, T2, T3, and T4) represent Zn concentrations in nutrient solutions (1, 10, 50, 100, and 400 μM). The dotted lines refer to the Fe isotope composition of nutrient solution. Error bars show two times the standard deviation (2SD).

similar ε_1 values varying from 0.94‰ to 0.73‰. During the Fe transport from root to stem, except in experiment T3 which has a limited Fe isotope fractionation with an ε_2 value of 0.01‰, the Fe isotope fractionation between root and plant sap generally enriches light Fe isotopes, and ε_2 shows a small fluctuation among experiments with different Zn levels varying from 0.45‰ to 0.33‰. The transport efficiency (φ_2/φ_1) suggests that the Fe fraction transported from root to stem decreases with increasing Zn supply. The plant sap from stem to leaf favors heavy Fe isotopes, and ε_3 increases from −1.79‰ to −0.39‰ with increasing Zn supply. The transport efficiency (φ_3'/φ_2) during the process shows only a small fluctuation. But the transport efficiency (φ_4/φ_2) generally increases with increasing Zn supply during Fe transport

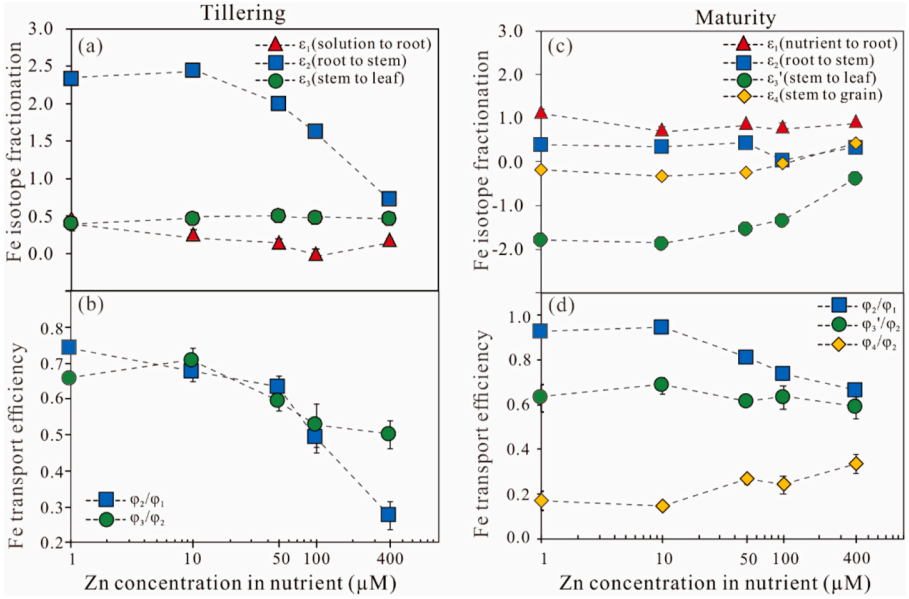

Fig. 4. Fe isotope fractionation and Fe transport efficiency in tillering (a–b) and maturity (c–d) stages. Error bars are two times the standard deviation (2SD).

from stem to grain.

3.4. Genome expression of Fe-transporters

Because experiments CK and T3 show pronounced ecological and chemical changes compared with other experiments, the transcript levels of Fe-transporter genes in these experiments were measured and compared to examine the effect of Zn on Fe-uptake. Our results show that only expression *OsIRT1* (a typical Fe(II)-transporter gene in rice, Bughio et al., 2002) in root is downregulated by elevated Zn level in both tillering and maturity stages (Fig. 5a and b). In contrast, the transcript abundances of *OsYSL15* (an Fe(III)-DMA transporter gene; Inoue et al. (2009); Fig. 5c and d) and *OsTOM1* (a PS efflux transporter gene; Nozoye et al., 2011, Fig. 5e and f) increase dramatically in root with increasing Zn supply in the nutrient solution. The rice leaf (Fig. 5g and h) shows elevated expression of *OsYSL2* (an Fe(II)-NA transporter gene; Koike et al., 2004; Ishimaru et al., 2010) with increasing Zn supply in both tillering and maturity stages. *OsYSL15* is expressed in leaves in the maturity stage and is upregulated under the increasing Zn stress (Fig. S2a). *OsYSL2* is expressed in grains in the maturity stage and is dramatically upregulated under increasing Zn stress (Fig. S2b).

4. Discussion

4.1. Uptake of Fe by root

In experiment CK with normal Zn supply in the nutrient solution (~1 μM Zn), the plant has a very positive ε_1 value in the tillering stage, indicating the preferential uptake of light Fe isotopes by root. This is in line with previous observations that significant enrichment of isotopically light Fe occurs in Strategy-I plants (Guelke-Stelling and Von Blanckenburg, 2012). As the Zn supply increases, the ε_1 value gradually decreases towards zero (e.g., −0.01‰ in experiment T3), suggesting that the elevated Zn stress leads to smaller Fe isotope fractionation between nutrient solution and plant. This is consistent with some previous studies which observed no or slightly positive fractionation during Fe uptake by Strategy-II plants (Guelke and Von Blanckenburg, 2007; Von Blanckenburg et al., 2009; Kiczka et al., 2010).

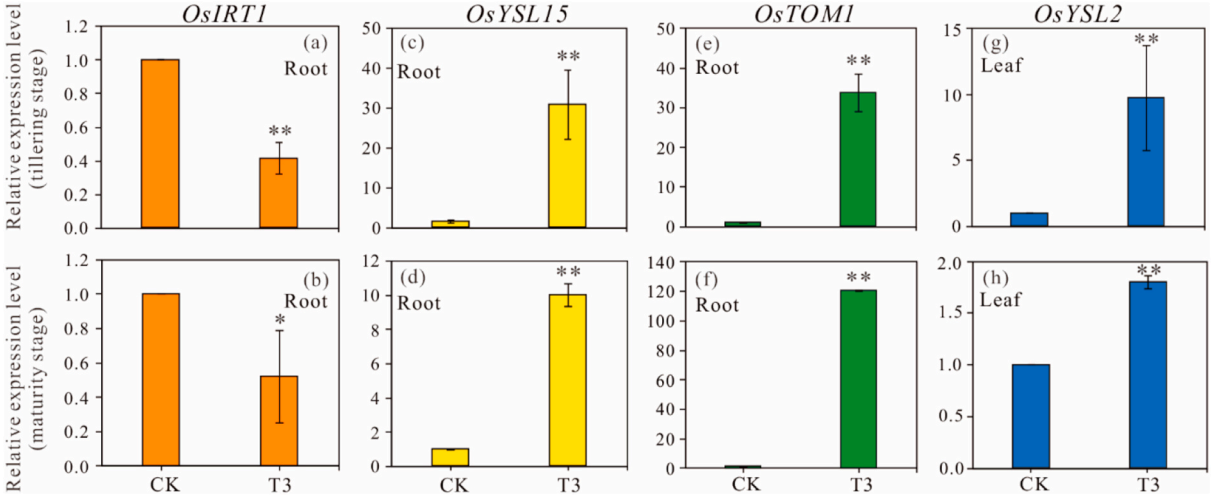

Fig. 5. Expression levels of *OsIRT1*, *OsYSL15*, *OsTOM1*, and *OsYSL2* in tillering and maturity stages. CK: control check with 1 μM Zn treatment; T3: 100 μM Zn treatment. Means ± SE of three technical replicates derived from three biological replicate are shown. Asterisks indicate significant differences from the CK value (two-sample *t*-test; $*P < 0.05$; $**P < 0.01$).

These systematic Fe isotope fractionations between nutrient solution and root are not due to the precipitation of Fe (hydr)oxides or Fe-uptake which may introduce changes in Fe isotope compositions of nutrient solution. The calculated Fe speciation in the nutrient solution used in our experiments shows that all Fe exists as Fe(III) in the nutrient solution (Table S1). No Fe precipitate is observed since 97%–99% of Fe(III) is bound to EDTA (forming Fe(III)-EDTA). Our mass-balance calculation (Section 2.5) showed that the total Fe mass absorbed by rice every three days only accounts for 0.9%–3.7% of Fe in the nutrient solution (Table S3), with more Fe absorption in the maturity stage, which may only increase δ^{56}Fe values of nutrient solution by < 0.1‰. Thus, these systematic changes of Fe masses and isotope compositions in root shall be affected by various amount of Zn in the nutrient solution, the only variable among the five sets of experiments.

Moynier et al. (2013) also observed significant Fe isotope fractionations among various organs from six species of higher plants and presented *Ab initio* calculations of Fe isotope fractionation factors of $[Fe(III)(H_2O)_6]^{3+}$, $[Fe(III)(cit)(H_2O)_3]^{0}$, $[Fe(III)H(cit)(H_2O)_4]^{+}$, $[Fe(III)(cit)_2]^{3-}$, and [Fe(III)-phytosiderophore]^{0}. These calculation results showed that fractionation factors between these species and $[Fe(III)(H_2O)_6]^{3+}$ vary between −0.44‰ and 1.06‰ at 25 °C, and suggested that some observed Fe isotope fractionations among organs of higher plants could be caused by changing organic ligands bound with Fe(III). In our study, most Fe(III) is complexed with EDTA in the nutrient solution and would be complexed with PS before entering root (Curie et al., 2001; Ishimaru et al., 2006; Inoue et al., 2009; Lee et al., 2009). Although there is no available Fe fractionation factor between Fe(III)-EDTA and Fe(III)-PS, this observed Fe isotope fractionation is unlikely caused by different organic ligands because Fe fractionation factor between Fe(III)-EDTA and Fe(III)-PS is unlikely to be affected by the variation of Zn concentration in the nutrient solution. Instead, we suggest that the Fe isotope fractionation between Fe in nutrient solution and that in the rhizosphere is caused by changing the redox state of Fe, and Zn may regulate Fe(II) uptake by root.

When rice absorbs Fe using Strategy I, Fe(III) has to be reduced to Fe(II) in the root cell membrane by ferric chelate reductase before Fe can be carried by transporter protein (Aung and Masuda, 2020). The reduction of Fe(III) to Fe(II) is known to be associated with significant Fe isotope fractionation which enriches light Fe isotopes in Fe(II) (Johnson et al., 2002; Welch et al., 2003), thereby resulting in lower δ^{56}Fe values of whole plants. However, the OsIRT1 protein can also transport Zn from the rhizosphere to root (Lee, 2009). Thus, Zn may compete with Fe(II) to bind with the OsIRT1 protein, and excess Zn may weaken Fe(II) acquisition by rice using Strategy I. To avoid Zn toxicity due to excess Zn, rice could lower the expression of *OsIRT1* to reduce Zn uptake (Fig. 5a). Zn stress might also trigger the plant self-regulation mechanism to increase Fe uptake, thus enhancing the Fe acquisition using Strategy II (Fig. 5b and c). This explanation is also supported by our Fe isotope fractionation data. In the case without excess Zn treatment (CK) in the tillering stage, the Fe isotope fractionation between nutrient solution (ε_1) and rice is positive, indicating Strategy I is the prevailing mechanism for Fe uptake (Fig. 4a). With increasing Zn supply, the uptake of Fe through Strategy I decreases, but uptake through Strategy II increases, leading to lower ε_1 values (Fig. 4a). The rice treated with ~100 μM Zn (T3) has a near-zero ε_1 value in the tillering stage. Because Fe uptake using Strategy II does not require the reduction of Fe(III), our results suggest that the processes of changing Fe(III)-EDTA to Fe(III)-PS and transporting Fe(III)-PS across the root cell membrane are unlikely to result in significant Fe isotope fractionation, and excess Zn dramatically disrupts Strategy-I Fe uptake pathway.

In the maturity stage, however, the ε_1 value of rice without excess Zn is much higher than in the tillering stage (Fig. 4c), and ε_1 shows small fluctuations at Zn levels of ~100 μM (T3) to ~400 μM (T4). This complication of increased Fe(II) uptake under Zn stress in the maturity stage can be explained by some processes in response to the increased Fe demand over the course of rice growth, particularly in the reproductive growth stage. Although rice can use Strategy II for Fe uptake, the amount of PS secreted by rice for Fe(III) uptake is relatively small (Garnica et al., 2018). Hence, rice may enhance the Strategy-I Fe uptake mechanism by increasing total transcript level of Fe(II) transporter genes, thereby increasing the uptake ratio of Fe(II) to Fe(III) and leading to the enrichment of light Fe isotopes in the whole plant, despite that excess Zn may suppress Strategy-I Fe uptake by OsIRT1 (Fig. 5).

4.2. Transport of Fe from root to stem

Our results show that the total Fe mass reduces in shoot but increases in root with increasing Zn supply in both tillering and maturity stages (Fig. 3), indicating that Zn stress dramatically restrains Fe upward-translocation from root to stem. In the tillering stage, the Fe isotope fractionation factor between root and stem (ε_2) is very positive in experiments CK and T1 (average ε_2 ~2.37‰), suggesting the stem is enriched in light Fe isotopes, and decreases with increasing Zn concentration from experiments T1 through T4 to ~0.71‰ (Fig. 4b). In the maturity stage, however, ε_2 is 0.37 ± 0.04‰ (excluding experiment T3) and shows no significant variation with increasing Zn concentration (Fig. 4d). Because our box-model calculates whole Fe fluxes from root to stem, these ε_2 values include isotopic effects due to Fe-translocation in both phloem and xylem and may provide some constraints on the translocation process.

Ecological and biochemical studies suggested that Fe can enter both phloem and xylem, and waits to be translocated to aboveground parts after entering the symplasm of rice root (Kobayashi and Nishizawa, 2012). In the phloem and xylem, Fe is commonly bound to ligands (including citrate, NA, and DMA) and then transported within plant in Fe-ligand forms because free metal ions are toxic in the saps of xylem and phloem (Haydon and Cobbett, 2007). Previous studies suggested that most Fe in rice is bound to citrate in xylem sap, forming Fe(III)-citrate or Fe(II)-citrate (Ariga et al., 2014). But some observations also demonstrated that Fe(III)-DMA should play, at least, auxiliary roles during Fe-transport via the xylem (Kakei et al., 2009; Nozoye et al., 2011). Phloem sap, contrary to xylem sap, has a slightly alkaline pH of 8.0 (Fukumorita and Sugar, 1982). Under such condition, not citrate but NA and/or DMA are proposed to be the predominant chelator(s) for Fe-transport, forming Fe(II)-NA and/or Fe(III)-DMA complexes, respectively (Von Wiren et al., 1999; Rellán-Álvarez et al., 2008), and NA is often considered the main ligand in the cytosol and plays a key role in transporting Fe(II) through the phloem (Kumar et al., 2017; Caldelas and Weiss, 2017).

Because Fe has been translocated from nutrient to root as Fe(II) and Fe(III)-DMA (as discussed in the last section), translocating from root to stem may involve changing organic ligands which can introduce Fe isotope fractionations. In fact, Moynier et al. (2013) attributed some Fe isotope fractionations between root and shoot of some higher plants to the change of organic ligand. However, this Fe isotope fractionation is not expected to be affected by Zn stress. Thus, in this work we propose an additional mechanism to explain the Fe isotope fractionation between root and stem under various Zn stress.

In plants including rice, Zn in the xylem sap exists both in a free ion form and in a form bound to NA (Marschner, 1995; Yoneyama et al., 2015). In fact, based on *in silico* and *in vitro* analyses, NA is proposed to be more important for Zn-transport via xylem than for Fe-transport (Von Wiren et al., 1999; Rellán-Álvarez et al., 2008). Zn in phloem sap has been suggested to bind primarily to NA or DMA in rice (Kato et al., 2010; Haydon et al., 2012; Clemens et al., 2013), and ESI-TOF analysis of the rice phloem sap suggested that NA is the only chelator for Zn in the phloem sap (Nishiyama et al., 2012). When Zn competes against Fe(II) for binding sites on NA, it inhibits the transport of Fe(II) from root to stem, thereby reducing Fe in shoot and increasing Fe in root with increasing Zn supply. Moreover, in addition to Fe(II), Fe(III) can be transported in the forms of Fe(III)-DMA and Fe(III)-citrate through the xylem (Palmer and Guerinot, 2009). Moynier et al. (2013) calculated the

Fe isotope fractionation factors are ~1.43‰ between Fe(III)-PS and Fe (III)-citrate, and ~1.10‰ between Fe(II)-NA and Fe(II)-citrate at 25 °C. Under an extreme Zn stress when all Fe(II) transport is blocked, ε_2 can be no more than 1.43‰ which cannot explain very positive ε_2 values in experiments CK, T1, T2 and T3 during the tillering stage. These high ε_2 values require converting Fe(II) into Fe(III) in root which produces much bigger Fe isotope fractionation compared with changing ligands. For example, the Fe isotope fractionation factor between $[Fe(III)(cit)_2]^{3-}$ and $[Fe(II)(cit)_2]^{4-}$ is ~2.9‰, and between Fe(III)-PS and Fe(II)-NA is ~3.24‰ at 25 °C (Moynier et al., 2013). At low Zn level (e.g., experiment CK), if some Fe(III)-DMA is converted to Fe(II)-NA in root which is transported to stem via phloem, the observed high ε_2 values can be explained. In experiment T4 with highest Zn level, the Fe(II)-NA transport in phloem is significantly disrupted, most Fe(II) could turn into Fe (III)-DMA, part of which is transported to stem via xylem as Fe (III)-citrate/Fe(III)-DMA/Fe(III)-NA. If we assume all Fe isotope fractionation in this case is caused by changing organic ligand from Fe (III)-DMA to Fe(III)-citrate with the calculate fractionation factor of ~1.43‰ at 25 °C (Moynier et al., 2013), it can be calculated, based on isotope balance, that ε_2 = 0.71‰ corresponds to ~50% of Fe being translocated to stem and ~50% being stored in root, which is higher than the total Fe transport efficiency (φ_2/φ_1) of 28% calculated using box-model. This suggests that, other than the Fe(III)-citrate, part of Fe (III) is transported to stem via xylem as Fe(III)-DMA and/or Fe(III)-NA.

As rice grows, particularly in the period entering the maturity stage, the demand for Fe increases significantly due to the rapid growth of rice. The transport of Fe(II)-NA via the phloem may be insufficient to meet the increased demand for Fe for plant growth, and the transport of Fe (III)-DMA through the xylem likely increases in the maturity stage, leading to reduced and nearly constant ε_2 values compared to those in the tillering stage. In experiments CK and T1, ε_2 is ~0.37‰, which may be explained by the high transport efficiency φ_2/φ_1 (92–94%) and small amount of Fe(III) is transported by Fe(III)-DMA/citrate. In experiments T2 through T4, ε_2 changes slightly but φ_2/φ_1 decreases significantly with increasing Zn stress (Fig. 4c and d), which can be explained by the disruption of Fe(II)-NA transporting path, and decreasing amount of Fe (III) transformed in the form of Fe(III)-DMA/citrate through the xylem.

Physiologically rice stem consists of node and internode in the maturity stage. Zn concentration increases the most and the Fe concentration decreases the most in node than in internode and other tissues, which appears to suggest that nodes are more sensitive to excess Zn stress. Under low Zn stress (experiments CK, T1 and T2), δ^{56}Fe values of internode are generally higher than that of node by 0.33–0.43‰ (Fig. 3d). But under high Zn stress (experiments T3 and T4), δ^{56}Fe values of internode are equal to or slightly lower than that of node (Fig. 3d). Previous studies suggested that translocation of Fe and Zn in stem is mainly mediated in node, which has highly developed and fully organized vascular systems (Yamaji and Ma, 2014; Yamaji and Ma, 2017). But the molecular mechanism controlling Fe transportation and Fe isotope fractionation between node and internode in the maturity stage remains poorly understood and should be explored further in the future. However, our results show that both node and internode vary in similar fashion with increasing Zn stress.

4.3. Transport of Fe from stem to leaf and grain

In the tillering stage, the positive ε_3 value (0.45 ± 0.03‰) indicates stem sap (including both phloem and xylem) is enriched in heavy Fe isotopes compared with leaf. This apparent fractionation factor (ε_3) varies little with increasing Zn stress in the nutrient solution, suggesting Fe(III)/Fe(II) ratio in the sap stays constant, and more Fe may have been transported in the form of Fe(III)-citrate which is not affected by Zn stress. Moreover, because φ_3/φ_2 decreases significantly with increasing Zn stress from experiments T1 through T4 (Fig. 4a and b), transporting Fe in the form of Fe(II)-NA may have been reduced, which might also affect Fe(III) transportation (e.g., affecting Fe(III)-NA) to maintain the Fe(III)/Fe(II) ratio, as well as maintaining the proportion of each organic ligand in the sap, although it is not clear to us how this delicate balance is kept.

In the maturity stage, the apparent Fe fractionation factor (ε_3) is very negative (between −1.89‰ and −0.39‰), indicating leaf is concentrated in heavy Fe isotopes compared with stem, which is unlikely caused by changing organic ligands, since Fe-citrate in the sap is typically enriched in light Fe isotopes compared with Fe-NA/Fe-PS (Moynier et al., 2013). This more likely involves changing redox state from Fe(II) to Fe(III). For example, converting Fe(II)-NA to Fe(III)-DMA will yield a maximum ε_3 of −3.24‰ and converting Fe(II)-NA to Fe(III)-citrate will yield a maximum of ε_3 of −1.81‰ at 25 °C (Moynier et al., 2013). As the Zn-stress increases, the Fe(II)-NA translocation is disrupted and more Fe (II)-NA could be converted to Fe(III)-citrate/Fe(III)-DMA, which could have resulted in increasing ε_3 values with increasing Zn stress (e.g., if all Fe(II)-NA was converted Fe(III)-DMA, ε_3' would be zero). Moreover, Fe in stem may be translocated to leaf, senescent leaf and grain which have dramatically different Fe isotope compositions (Fig. 3): $\delta^{56}Fe_{grain}$ (between −1.57‰ and −0.96‰) < $\delta^{56}Fe_{leaf}$ (between −0.48‰ and 0.08‰) < $\delta^{56}Fe_{senescent\ leaf}$ (between −0.08‰ and 0.46‰). Compared with the δ^{56}Fe value of stem (0.13 ± 0.20‰), more Fe has been translocated from stem to grain as Fe(II) under low Zn stress (e.g., experiment CK). As Zn stress increases, $\delta^{56}Fe_{grain}$ value increases, suggesting Fe(III) increases in the sap from stem to grain. This increase may be more than enough to compensate the decrease of Fe(II)-transport, which can explain the increase of φ_4/φ_2 with increasing Zn stress. In contrast, the higher $\delta^{56}Fe_{senescent\ leaf}$ value suggests that a significant amount of Fe from stem to senescent leaf is Fe(III). As Zn stress increases, $\delta^{56}Fe_{senescent\ leaf}$ value decreases, which suggests that more Fe(III) has been transported to rice grain. Previous studies suggested that the reduction of Fe(III)-DMA/Fe (III)-NA may occur during Fe transportation from stem to leaf/grain, following the transfer of Fe(II) across the cell membrane with OsYSL2 in the phloem (Koike et al., 2004; Ishimaru et al., 2010). Older leaves can act as Fe sources in phloem that participate in Fe unloading to rice stem and/or grain (Sperotto, 2013; Stomph et al., 2014; Schippers et al., 2015). Due to the limitation of our modeling, this process is not able to be confirmed.

4.4. Zn regulation of Fe uptake and translocation in rice

Based on previous studies (Briat et al., 2007; Connorton et al., 2017; Kawakami and Bhullar, 2018; Clemens, 2019), the Fe transport pathways in rice are summarized in Fig. 6. Fe in rhizosphere enters the root through epidermal cell membrane with the help of transporter proteins: OsIRT1 for Fe(II) and OsYSL15 for Fe(III)-DMA. From root to stem, Fe can be transported as Fe(II)-NA by OsYSL2 in phloem and as Fe(III)-DMA in xylem. The amount of Fe(III)-DMA present in root depends on the amounts of DMA secreted from root and transporter protein OsTOM1 present at the interface between nutrient solution and root. Fe(II)-NA can be unloaded from phloem as Fe(II)-NA and Fe(III) in xylem can be unloaded as Fe(III)-DMA/Fe(III)-citrate from stem to young leaf where Fe is used predominantly in the production of enzyme cofactors (heme-Fe, Fe–S) or components of electron transport chains. Fe(II)-NA in phloem can also be unloaded from stem to grain or senescent leaf, and Fe (III) in xylem can be unloaded as Fe(III)-DMA/Fe(III)-citrate/Fe(III)-NA from stem to grain or senescent leaf. Inside senescent leaf, some Fe(III) can be reduced to Fe(II) which reloads back to phloem.

Under this framework, Zn regulation of Fe uptake and translocation in rice can occur in the following places based on our Fe stable isotope tracer and gene expressions. First, at the interface between nutrient solution and root, Zn competes with Fe(II) for the transporter protein OsIRT1, affecting the Fe(III)/Fe(II) ratio in the transport media. Second, Zn regulates the protein OsYSL2 which helps to transport the Fe(II) (reduced from Fe(III)-DMA/Fe(III)-citrate/Fe(III)-NA) from xylem into phloem (Fig. S2b). Finally, Zn competes against Fe(II) for binding sites on NA and inhibits Fe(II)-NA loading in phloem since Zn in phloem sap

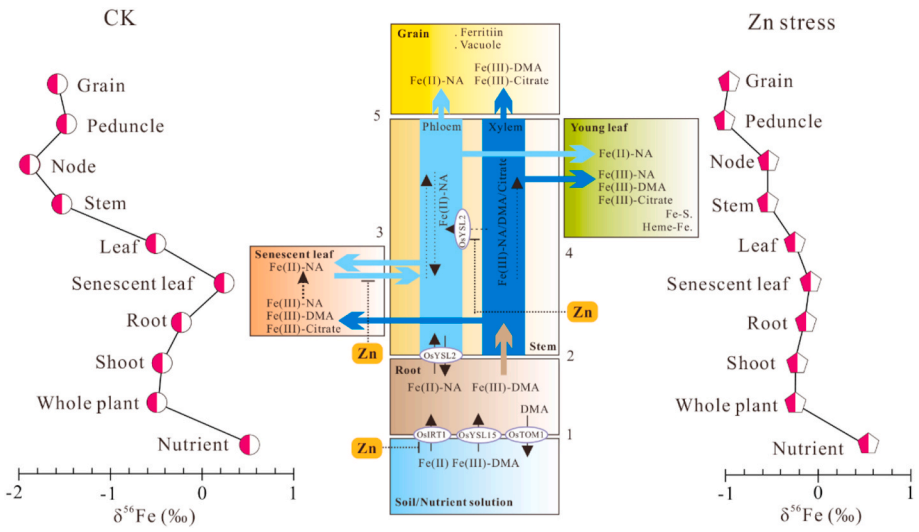

Fig. 6. Scheme of Fe uptake and transport regulated by excess Zn in rice. Arrows represent the long-distance circulation of Fe via different transporters. This figure is modified based on Briat et al. (2007), Kawakami and Bhullar (2018), and Clemens (2019). 1. Fe uptake from the rhizosphere into the root epidermal cells: Strategy I (via OsIRT1) and Strategy II (via OsYSL15); 2. Fe xylem loading: transport in the xylem is as an Fe(III)-citrate/Fe(III)-NA complex, and in rice plants, also as an Fe(III)-DMA complex. Fe phloem loading and unloading: transport in the phloem is as an Fe(II)-NA complex; 3. Fe phloem and xylem unloading and remobilization in senescent leaves (source); 4. Fe phloem and xylem unloading into young leaves (sink): transport as an Fe(II)-NA and Fe(III)-NA/Fe(III)-DMA/Fe(III)-citrate, respectively. Fe is used predominantly in the production of enzyme cofactors (heme-Fe, Fe–S) or components of electron transport chains; 5. Fe phloem and xylem unloading into grains (sink): transport as an Fe(II)-NA and Fe(III)-DMA, respectively. Fe is stored as ferritin or in vacuoles.

has also been suggested to bind primarily to NA in rice (Lee et al., 2012; Nishiyama et al., 2012; Clemens et al., 2013).

5. Conclusions and implications

Based on Fe isotope ratios and transporter genes, this study clearly demonstrates that Zn levels in growth media determine the strategies of Fe uptake and translocation in rice. In this study, the rice in tillering stage preferentially uses Strategy II to absorb and transports Fe(III)-DMA under excess Zn stress, which is supported by the downregulated expression of *OsIRT1* and upregulated expression of *OsYSL15*. During Fe translocation, excess Zn decreases magnitude of Fe isotope fractionation among aboveground tissues and inhibits Fe(II) transport and remobilization in rice, indicating a competition of binding sites on nicotianamine between Zn and Fe(II).

Once further studies on precise Fe isotope fractionation factors involved in Fe uptake as well as Fe translocation within plants are available, stable Fe isotope signatures of rice tissues can provide quantitative information about the efficiency of Fe uptake and the internal distribution of Fe in plants, which may be applied to facilitate Fe-enrichment in rice grain and improve human health. The expansion of industrial and agricultural activities (e.g., mining, smelting, and use of fertilizers) has led to increasingly severe soil Zn pollution that poses a potential risk to crops (Nagajyoti et al., 2010). Moreover, our findings could be of significance for rice cultivation in Zn-contaminated paddy soils that exhibit Fe deficiency. We propose that isotope composition analysis combined with transporter gene expression analysis could thereby become useful and complementary tools for studying plant physiological processes, especially those related to multi-metal homeostasis (e.g., Zn with Fe/Ni/Cd).

Author statement

Qiqi Wu, Ting Gao, and Chengshuai Liu conceived and designed the research. Qiqi Wu, Yuhui Liu, Yafei Xia, and Meng Qi performed the experiments. Qiqi Wu, Ting Gao, Chengshuai Liu, and Runsheng Yin analyzed the data. Qiqi Wu, Ting Gao, and Zhengrong Wang wrote the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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