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Source and Strategy of Iron Uptake by Rice Grown in Flooded and Drained Soils: Insights from Fe Isotope Fractionation and Gene Expression

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ABSTRACT: Rice can simultaneously absorb Fe^{2+} via a strategy I-like system and Fe(III)-phytosiderophore via strategy II from soil. Still, it remains unclear which strategy and source of Fe dominate under distinct water conditions. An isotope signature combined with gene expression was employed to evaluate Fe uptake and transport in a soil−rice system under flooded and drained conditions. Rice of flooded treatment revealed a similar δ^{56}Fe value to that of soils ($\Delta^{56}Fe_{rice-soil}$ = 0.05‰), while that of drained treatment was lighter than that of the soils ($\Delta^{56}Fe_{rice-soil}$ = −0.41‰). Calculations indicated that 70.4% of Fe in rice was from Fe plaque under flooded conditions, while Fe was predominantly from soil solution under drained conditions. Up-regulated expression of *OsNAAT1*, *OsTOM2*, and *OsYSL15* was observed in the root of flooded treatment, while higher expression of *OsIRT1* was observed in the drained treatment. These isotopic and genetic results suggested that the Fe(III)−DMA uptake from Fe plaque and Fe^{2+} uptake from soil solution dominated under flooded and drained conditions, respectively.

KEYWORDS: *Fe plaque, OsYSL15, soil solution, OsIRT1, isotope fractionation, flooded conditions, uptake*

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INTRODUCTION

Iron (Fe) is abundant in the Earth's crust, and biological uptake of Fe is one of the important processes during Fe biogeochemical cycling in the environment.[1,2] Iron is an essential micronutrient for the growth of plants, but Fe in soils generally exists as Fe(III), which is sparingly soluble at the physiological pH range under oxic conditions.[1] Higher plants have evolved two distinct strategies to acquire iron from the rhizosphere. Non-graminaceous plants reduce Fe(III) to Fe^{2+} followed by an uptake through the Fe^{2+} transporter IRT1 (strategy I-like, Fe^{2+} uptake). Graminaceous plants secrete mugineic acid family phytosiderophores (MAs) through the TOM1/2 transporters (DMA secretion) to chelate Fe(III), which is then absorbed through the YS1/YSL transporters (strategy II).[3,4] Rice, as a strategy II plant, also possesses a ferrous transporter, OsIRT1, that allows it to absorb Fe^{2+}, in addition to the uptake of Fe(III) chelated with 2′-deoxymugineic acid [Fe(III)−DMA] by the OsYSL15 transporter [Fe(III)−DMA uptake].[5,6] Distinct water management practices, that is, flooding and drainage, are commonly applied during rice growth.[7] Since more Fe^{2+} is released into soil solution via dissimilatory Fe reduction during the flooding season,[8] direct uptake of Fe^{2+} from soil solution via a strategy I-like system may be more efficient than via strategy II. However, it was found that the expression of both strategy I-like gene *OsIRT1* and strategy II gene *OsTOM2*/*OsYSL15* was strongly induced by Fe deficiency or low Fe supply,[4−6] which is supposed to occur during the drainage season.[7] However, it remains difficult to determine the relative contribution of each strategy in the Fe uptake by rice under distinct water management practices.

Stable Fe isotope fractionation has been successfully used as a fingerprint to trace the sources and biogeochemical processes of Fe in soil−plant systems.[8−10] Generally, strategy I plants incorporate light Fe isotopes ($\Delta^{56}Fe_{plant-soil}$ = −0.15 to −1.6‰), while strategy II plants show no or slight enrichment of heavy Fe isotopes relative to soils ($\Delta^{56}Fe_{plant-soil}$ = 0.05 to 0.30‰).[9−11] Light Fe isotopes are enriched in strategy I plants because the Fe(III) reduction to Fe(II) can generate negative fractionation of Fe isotopes ($\Delta^{56}Fe_{Fe(II)-Fe(III)}$ = −1.44 to −3.0‰).[12,13] Garnier et al. (2017) reported that Fe isotopes in rice from a waterlogged paddy field were slightly lighter than those in the soil,[14] and similar negative fractionation was also observed in some strategy II plants.[15] The same direction of fractionation from soil to rice as in strategy I plants may be caused by the Fe^{2+} uptake from soil solution. However, Garnier et al. (2017) also found that the Fe isotopes in the root were much heavier than those in the soil solution ($\Delta^{56}Fe_{root-soil\ solution}$ = 1.74‰) but lighter than those in the Fe plaque ($\Delta^{56}Fe_{root-Fe\ plaque}$ = −0.51‰) and suggested that the Fe absorbed by roots was more likely supplied by Fe from plaque.[14] The formation of Fe plaque is mainly ascribed to the

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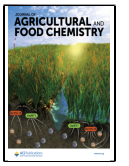

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re-oxidation of Fe^{2+} by O_2 from radial oxygen loss in the roots, and Fe(II) oxidation/precipitation favors an enrichment of heavy isotopes ($\Delta^{56}Fe_{Fe(III)-Fe(II)}$ = 2.9 to 3.2‰).[16−18] If both the soil solution and Fe plaque can serve as the direct source of Fe for rice,[14] their contributions to Fe accumulation in rice deserve investigation as different sources of Fe may alter the uptake strategy. Isotopic fractionation from soil solution/Fe plaque to rice is expected to provide a more comprehensive understanding of the pathways of Fe uptake under distinct water management practices.

Biomass and Fe concentration in grains have been reported to show no significant difference between rice grown in oxic and anoxic soils.[19] This may be caused by the Fe homeostasis maintained by rice, in which rice induces or represses expression of various genes related to Fe chelation, transport, and storage in response to Fe deficiency or excess.[3] Nicotianamine (NA), DMA, and citrate are the predominant Fe chelators in rice. *OsNAS* synthesizes NA, which is sequentially converted to DMA via reactions mediated by OsNAAT1 [2′-deoxymugineic acid (DMA) formation] and OsDMAS1 proteins.[20−22] *OsFRDL1* is expressed in the roots and nodes and exports citrate into the xylem, which is then complexed with Fe and favors Fe transport in the xylem.[23,24] Other genes encoding key Fe transporters in rice include *OsYSL2* [Fe(II)−NA export to phloem], *OsYSL18* [Fe(III)−DMA loading onto phloem], *OsVIT2* (vacuolar sequestration), and *OsFER2* (ferritin protein immobilization).[25−27] Fe is primarily transported to older leaves and husks from roots via xylem, with Fe(III)−citrate being the major form.[23,28] In the phloem, Fe can be transported in the form of Fe(III)−DMA and Fe(II)−NA to younger leaves and seeds from older leaves.[23,29] Regulation of such a complex network of genes strongly alters the Fe species accumulated in individual organs, which may also cause pronounced isotopic fractionation between them.[14] In addition to redox changes of Fe, different Fe-chelator complexes can induce Fe isotopic fractionation up to 1.5 to 3.0‰ according to quantum chemical calculations.[30] Within rice plants, Fe in stems has been found to be isotopically lighter than that in roots ($\Delta^{56}Fe_{stem-root}$ = −1.39 to −0.16‰).[10,11,14] However, Fe in stems can fractionate to leaves/husks/seeds in a negative ($\Delta^{56}Fe_{leaf/husk/seed-stem}$ = −0.67 to −0.32‰), positive (0.39 to 0.52‰), or non-resolvable (0 to 0.14‰) direction.[10,11,14] In particular, nodes can serve as a deposit of Fe and a relay point for Fe allocation to grains,[31,32] yet Fe isotope fractionation between nodes and connected organs has not been studied in detail.

In this study, the Fe isotope fractionation patterns in a soil−rice system were investigated when rice was grown under flooded and drained conditions. The relative expression of various genes responsible for Fe uptake and transport in rice was quantified in addition to the measurement of Fe concentrations in different soil pools and rice organs. In particular, the upper nodes and individual leaves were collected for analysis. Our objectives were to (1) determine the contribution of Fe sources (soil solution vs Fe plaque) and strategies [Fe^{2+} vs Fe(III)−DMA] to the Fe uptake by rice under distinct water management practices and (2) provide more comprehensive information to advance our understanding of the regulation of Fe transport to seeds during maintenance of Fe homeostasis in rice.

MATERIALS AND METHODS

Rice Growth Experiment. The rice (cv. *Oryza sativa* L. subsp. indica) variety Huanghuazhan was planted in pot experiments. The soil collected from a 0 to 20 cm depth in a paddy field in Shaoguan, Guangdong province, China (24.635485° N, 113.567534° E), was derived from sandstone with 23.9% clay, 49.6% sand, and 26.5% silt. The pH, HCl-extractable Fe, dithionite−citrate−bicarbonate-extractable Fe, and total Fe of the soil was 6.15, 0.62 g kg^{-1}, 24.6 g kg^{-1}, and 30.5 g kg^{-1}, respectively. The soil samples were air-dried, homogenized, and sieved to <5 mm before use. Five seedlings of rice were transplanted into a pot with 12 kg of the paddy soil. Three pots as individual experimental replicates were used for each water management practice, that is, flooded and drained, which were placed randomly in a greenhouse. Deionized water was added to full saturation with 5 cm of water above the soil surface for the flooded treatment and up to 75% of the soil moisture content for the drained treatment on a daily basis. The temperature during rice growth was in the range of 19−35 °C, and the relative humidity was 74.2−79.8%. Details of rice germination, cultivation processes, and determination of soil pH and Eh are provided in the Supporting Information.

Sample Preparation and Iron Measurement. During the time course of rice growth, the soil solution was sampled using a Rhizon sampler from Rhizosphere Research Products (Netherlands), which was inserted 10 cm below the soil surface. The soil and rice plants were collected at full maturity after 110 days of incubation. One aliquot of soil was extracted by 0.5 M HCl at a soil/extractant ratio of 1:10 (w/w), namely the HCl extract,[14] while another aliquot of soil was extracted using ammonium oxalate (0.2 mol L^{-1}, pH = 3.0), namely the $(NH_4)_2C_2O_4$ extract.[33] Soil remaining after the $(NH_4)_2C_2O_4$ extract was digested by aqua regia and reverse aqua regia to remove the organic matter in a proper sequence. Bulk soil was digested with a mixture of 0.8 mL HNO_3 and 2.4 mL HF in a beaker at 150 °C for 8 h prior to being dried at 160 °C to remove the remaining fluorine.

The rice plants were divided into roots, stems, leaves, rachises, and grains. Nodes I, II, and III were separated from the stems, while the flag, second, third, fourth, fifth, and bottom leaves were collected from top to bottom individually. A soaking−massaging treatment in distilled deionized water was carried out to remove the soil adhered to the root. Then, Fe plaque on the root surface was sequentially extracted by 0.5 M HCl and 0.1 M HCl with ultrasonic extraction for 15 min, twice for each molarity according to the previous method.[14] The HCl extraction and remaining roots were applied for the analyses of Fe concentrations and isotopes in Fe plaques and roots, respectively. Rice samples were dried at 55 °C for 48 h, and then, the grains were separated into husks and seeds. The rice samples were digested with the same mixture of HNO_3 and HF in a high-performance microwave digestion system (Milestone, Ethos Up, Italy). After evaporation to dryness, all the extracted samples were subjected to digestion with aqua regia to remove the organic matter.

Three experimental replicates from individual pots were collected and split into two parts: one for Fe concentration determination and the other for Fe isotope measurement. The average values and standard deviations of the Fe concentration were determined by measurements of the three experimental replicates, while samples from the three experimental replicates were combined and analyzed in triplicate for the measurement of Fe isotope composition. The concentrations of Fe in the soil solution, HCl extract, $(NH_4)_2C_2O_4$ extract, bulk soil, root Fe plaque, and rice samples were measured using the PerkinElmer Optical Emission Spectrometer (Optima 8000, USA) under the operational conditions described in a previous study.[34] The Fe recovery of the plant reference material was 98.3% for citrus leaf (GBW10020, 480 ± 30 mg Fe/kg).

Iron Isotope Analysis and Calculation. All the digested samples were purified using anion exchange chromatography in AG1-X8 resin (100−200 meshes, Bio-Rad, USA) following the procedure of Ding et al. (2019).[35] Once samples that dissolved in 0.2 mL of 6 N HCl were loaded, a series of 6 N HCl (4 mL) chromatographic separations were conducted to remove interfering elements and matrix components. Fe

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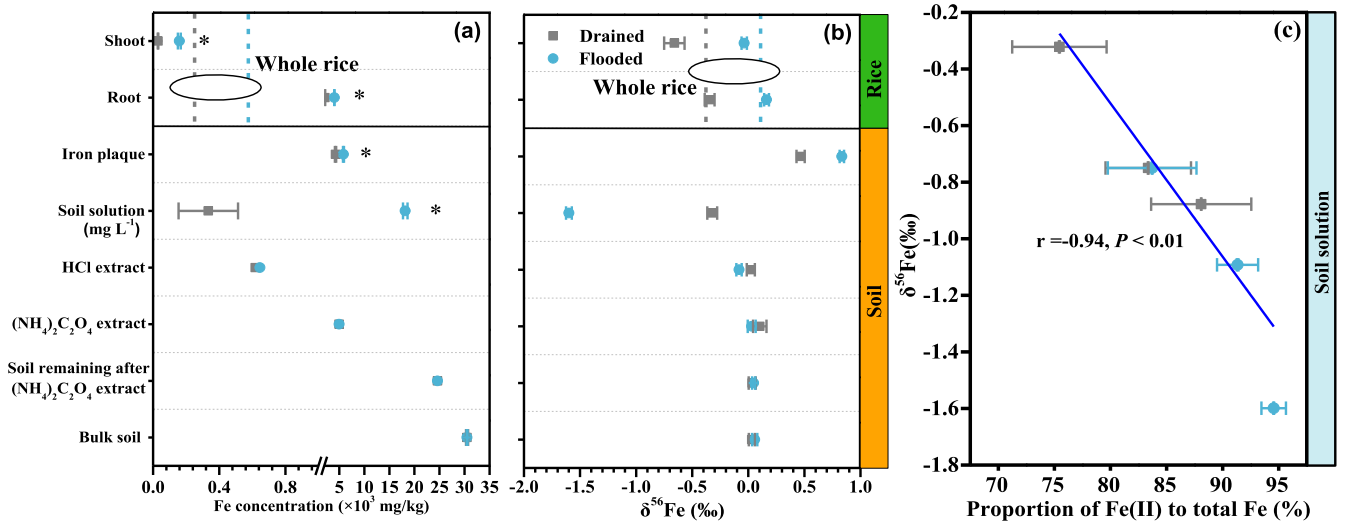

Figure 1. (a) Fe concentrations and (b) Fe isotope compositions in various soil pools, Fe plaque, roots, and shoots under distinct water conditions (details in Table S2). Dashed lines represent the Fe concentrations and δ^{56}Fe values of whole rice. (c) Relation between proportion of Fe(II) to total Fe and δ^{56}Fe values in soil solution. Three individual replicates of pots were analyzed for the Fe concentration with error bars referring to ±SD, and the significant differences determined by an independent-sample *t*-test are indicated by * ($P < 0.05$). Samples from three individual pots were combined and analyzed in triplicate for Fe isotope composition, and the δ^{56}Fe values are presented relative to the standard reference of IRMM-014 with the error bars denoting ±2SD.

was finally eluted with 9 mL of 0.4 N HCl, 1 mL of 8 N HCl, and 0.5 mL of high-purity water. After separation and purification, Fe isotope composition was determined using a *Neptune Plus* multi-collector inductively coupled plasma-mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) at the Institute of Geochemistry, Chinese Academy of Sciences (Guiyang, China). To correct the instrumental mass bias and time drifts, the standard−sample−standard bracketing approach was applied. The long-term monitoring of the in-house standards (GSB) revealed an external reproducibility of 0.05‰ (2SDs, $n = 37$) for δ^{56}Fe. The replicate monitoring of U.S. Geological Survey (USGS) rock standards such as BHVO-2 (0.08 ± 0.05‰, 2SD) and AGV-2 (0.12 ± 0.04‰, 2SD) concurred with previously reported results.[11,36] The Fe recovery rate during chemical purification was more than 98%. Each sample or standard was measured three times, and mass spectrometric reproducibility was monitored by running the IRMM-014 standard. All the acids used in this study were cleaned in Teflon distills under sub-boiling conditions and prepared with high-purity water (18.2 MΩ cm, Milli-Q, Millipore, USA) to ensure the lowest level of Fe in the procedure blank. The procedural blank in the whole experiment was less than 40 ng Fe, which was negligible compared to the Fe concentrations in all samples loaded into the resin (≥50 μg Fe). Samples were prepared on a class 10 laminar flow bench in a class 1000 clean room.

Fe isotope compositions were expressed using standard δ notation in units of per mil (‰) relative to the international Fe isotope standard IRMM-014 as follows

$$\delta^{56}\mathrm{Fe} = \left[\frac{(^{56}\mathrm{Fe}/^{54}\mathrm{Fe})_{\mathrm{sample}}}{(^{56}\mathrm{Fe}/^{56}\mathrm{Fe})_{\mathrm{standard}}} - 1\right] \times 1000 \quad (1)$$

The Fe isotope variation between two reservoirs, A and B, is expressed as

$$\Delta^{56}\mathrm{Fe}_{\mathrm{A-B}} = (\delta^{56}\mathrm{Fe})_{\mathrm{A}} - (\delta^{56}\mathrm{Fe})_{\mathrm{B}} \quad (2)$$

The standard error of such fractionation was estimated by propagating the standard errors measured on the $\delta^{56}\mathrm{Fe}_{\mathrm{A}}$ and $\delta^{56}\mathrm{Fe}_{\mathrm{B}}$ values.

The δ^{56}Fe values for whole rice, shoot, total leaf, and nodes were calculated according to the mass balance as follows

$$\delta^{56}\mathrm{Fe}_{\mathrm{plant/shoot/total\ leaf/node}} = \frac{\sum_i m_i c_i \delta^{56}\mathrm{Fe}_i}{\sum m_i c_i} \quad (3)$$

where m and c represent the dry weight (g) and Fe concentration (μg g^{-1}), respectively, and i refers to the plant tissues such as roots, stems, various leaves, various nodes, rachises, husks, and seeds.

Gene Expression Quantification. The samples of the root, stem, node, and flag leaf were frozen in liquid nitrogen for RNA extraction according to the manufacturer's protocols (Omega Bio-Tek, Norcross, USA). The purified RNA samples (2.0 μg) were converted into cDNA with a reverse transcriptase kit (Takara, Kyoto, Japan), and the cDNA was amplified as the template for real-time qPCR analysis using SYBR Premix Ex Taq II (Takara, Kyoto, Japan) and the gene primers in Table S1. The expression of genes was determined using iCycler iQ multi-color real-time PCR (Bio-Rad, Hercules, CA, USA). The rice gene *OsActin1* was applied as an internal control, and the relative expression of genes was estimated using the $2^{-\Delta\Delta Ct}$ method. Three experimental replicates from individual pots were carried out to evaluate the standard error of the average value of each gene expression.

Statistical Analysis. The normal distribution and homogeneity of variance were tested by normality plots with tests and the Levene test. An independent-sample *t*-test was conducted to evaluate the significant differences in the Fe concentration, dry weight, Fe mass, and relative expression of genes between the flooded and drained treatments at the $P < 0.05$ level.

RESULTS

Fe Concentration in the Soil−Rice System. During the whole period of rice growth, the concentration of dissolved Fe(II) in soil solution increased gradually to 17.2 ± 0.21 mg L^{-1} at the maturity stage under flooded conditions, while it remained stable at 0.25 ± 0.03 mg L^{-1} under drained conditions (Figure S1a). The dissolved Fe(II) accounted for 75−93% of the total Fe in soil solution under flooded and drained conditions (Figure S2a). Since the soil moisture content was maintained at 75% in the drained treatment, dissolution of Fe(II) that was adsorbed on the soil minerals and oxidation of FeS could contribute to high proportions of Fe(II) in soil solution under drained conditions.[12,37] In the

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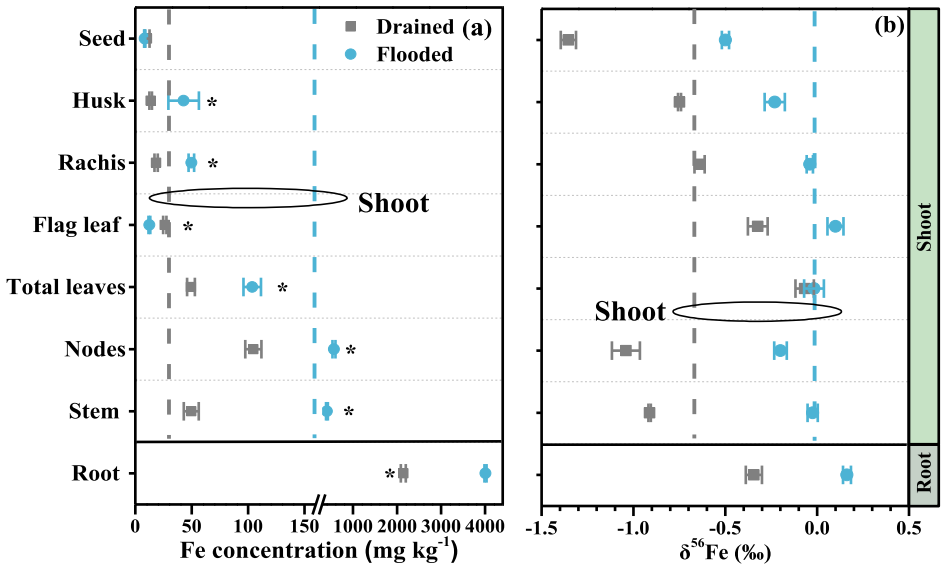

Figure 2. (a) Fe concentrations and (b) Fe isotope compositions of various rice organs under distinct water conditions. The δ^{56}Fe of nodes was calculated from node I, node II, and node III according to the mass-weighted mean. Dashed lines represent the δ^{56}Fe values of the shoots. Three individual replicates of pots were analyzed for the Fe concentration with the error bars referring to ±SD, and the significant differences determined by an independent-sample *t*-test are indicated by * ($P < 0.05$). Samples from three individual pots were combined and analyzed in triplicate for Fe isotope composition, and the δ^{56}Fe values are presented relative to the standard reference of IRMM-014 with the error bars denoting ±2SD.

flooded treatment, the soil pH increased gradually to as high as 7.21 ± 0.04, while the soil Eh was maintained at −201 ± 2.48 mV (Figure S1c,d). The drained treatment (pH = 7.0 ± 0.02, Eh = −106 ± 10.6 mV) showed a significantly lower soil pH but higher Eh than the flooded treatment at the maturity stage. Fe concentrations in the HCl extract (0.62 to 0.65 g kg^{-1}), $(NH_4)_2C_2O_4$ extract (4.96 to 4.99 g kg^{-1}), soil remaining after the $(NH_4)_2C_2O_4$ extract (24.6 g kg^{-1}), and bulk soil (30.4 to 30.5 g kg^{-1}) were similar between the flooded and drained treatments (Figure 1a). The Fe concentration in Fe plaques was significantly higher under flooded conditions than that under drained conditions (flooded: 5.84 ± 0.10 g kg^{-1} and drained: 4.26 ± 0.12 g kg^{-1}). The whole rice of flooded treatment showed an Fe concentration twofold higher than that of drained treatment (flooded: 576 ± 22.3 mg kg^{-1} and drained: 251 ± 18.5 mg kg^{-1}). The Fe concentrations in roots (flooded: 4010 ± 25.2 mg kg^{-1} and drained: 2140 ± 55.4 mg kg^{-1}) were substantially higher than those in shoots (flooded: 159 ± 8.54 mg kg^{-1} and drained: 29.9 ± 2.01 mg kg^{-1}) in both treatments. On the contrary, the dry weight of shoots (23.8 to 23.9 g) was higher than that of roots (2.8 to 2.9 g), which showed no significant difference between the flooded and drained treatments (Figure S3a). The majority of Fe mass was accumulated in roots under both conditions (flooded: 75.4% and drained: 89.4%) (Figure S3b). A lower proportion of Fe in roots of the flooded treatment than in the drained treatment suggested that Fe was preferentially transported to the shoots under flooded conditions.

Fe Isotope Fractionation from Soil to Rice. No apparent fractionation was observed among the HCl extract (−0.08 to 0.02‰), $(NH_4)_2C_2O_4$ extract (0.03 to 0.10‰), soil remaining after the $(NH_4)_2C_2O_4$ extract (0.03 to 0.05‰), and bulk soil (0.03 to 0.06‰) under both water management practices (Figure 1b). Fe in soil solution (flooded: δ^{56}Fe = −1.60 ± 0.03‰ and drained: δ^{56}Fe = −0.03 ± 0.04‰) was isotopically lighter relative to bulk soil, and the flooded treatment ($\Delta^{56}Fe_{\text{soil solution−bulk soil}}$ = −1.66 ± 0.03‰) showed a more pronounced fractionation than the drained treatment ($\Delta^{56}Fe_{\text{soil solution−bulk soil}}$ = −0.35 ± 0.05‰). The δ^{56}Fe values of the root Fe plaque were 0.83 ± 0.02 and 0.47 ± 0.04‰ under flooded and drained conditions, respectively. The fractionation from soil solution to Fe plaque was more pronounced under flooded conditions ($\Delta^{56}Fe_{\text{Fe plaque−soil solution}}$ = 2.43 ± 0.03‰) than under drained conditions ($\Delta^{56}Fe_{\text{Fe plaque−soil solution}}$ = 0.79 ± 0.06‰).

Fe isotope compositions (δ^{56}Fe) in the whole rice were 0.11 ± 0.02 and −0.38 ± 0.05‰ under flooded and drained conditions, respectively. The whole rice showed a similar isotope composition to that of bulk soil under flooded conditions ($\Delta^{56}Fe_{\text{whole rice−bulk soil}}$ = 0.06 ± 0.03‰), while it was close to the isotope composition of soil solution under drained conditions ($\Delta^{56}Fe_{\text{whole rice−soil solution}}$ = −0.06 ± 0.07‰). Fe isotopes in whole rice of flooded treatment were heavier than those in soil solution ($\Delta^{56}Fe_{\text{whole rice−soil solution}}$ = 1.71 ± 0.03‰) and lighter than those in Fe plaque ($\Delta^{56}Fe_{\text{whole rice−Fe plaque}}$ = −0.72 ± 0.03‰). Shoots were preferentially enriched in lighter isotopes than roots, and the drained treatment showed a slightly larger extent of fractionation between roots and shoots (flooded: $\Delta^{56}Fe_{\text{shoot−root}}$ = −0.20 ± 0.03‰ and drained: $\Delta^{56}Fe_{\text{shoot−root}}$ = −0.31 ± 0.10‰). The Fe isotope compositions of whole rice were close to those of roots, likely due to the fact that roots served as a larger reservoir than the shoots.

Fe Concentration and Isotope Fractionation within Shoots. Fe concentrations in stems, leaves, nodes, rachises, and husks were significantly higher under flooded conditions than those under drained conditions (Figure 2a). The flag leaf of the flooded treatment showed a significantly lower Fe concentration (12.5 ± 0.75 mg kg^{-1}) than that of drained treatment (26.1 ± 1.46 mg kg^{-1}), while the Fe concentrations in seeds revealed no significant difference between the flooded (8.34 ± 0.50 mg kg^{-1}) and drained (10.4 ± 2.18 mg kg^{-1}) treatments. Within the shoot, the majority of Fe was accumulated in stems under flooded conditions (80.4%),

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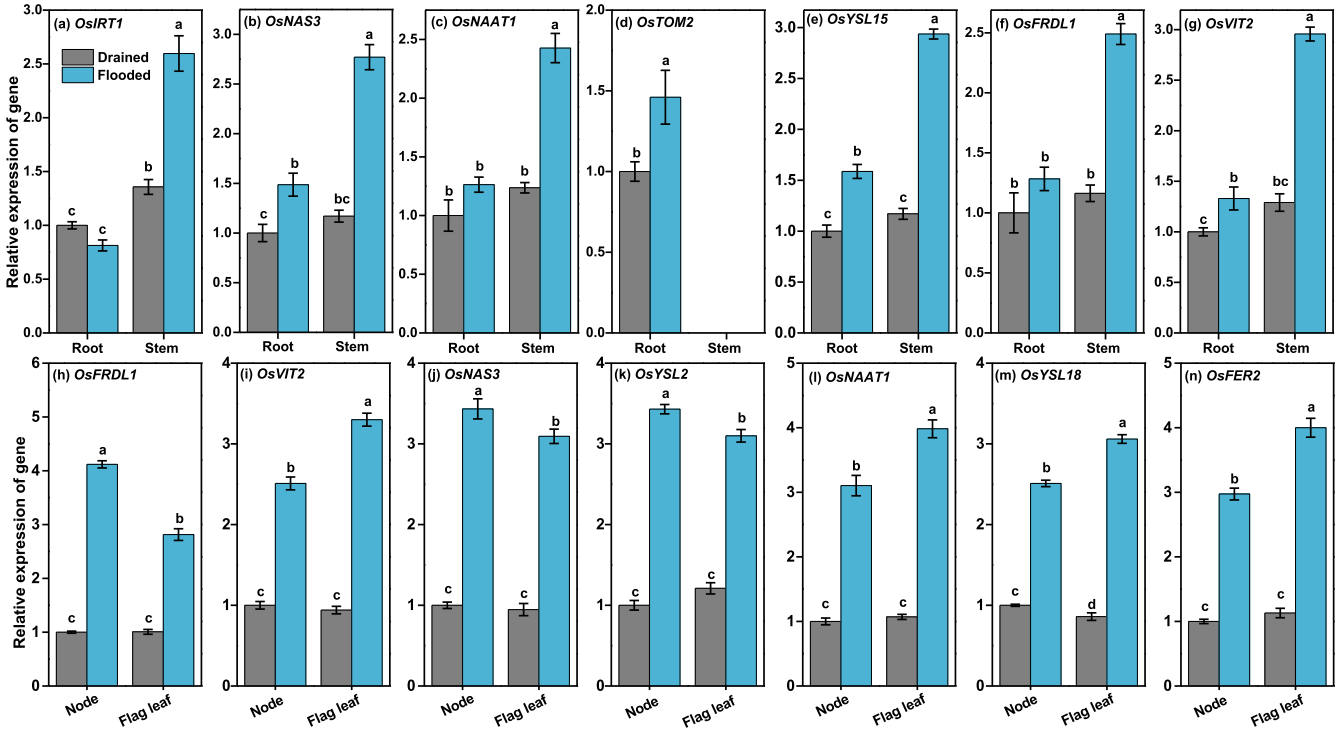

Figure 3. Relative expression of (a) *OsIRT1*, (b) *OsNAS3*, (c) *OsNAAT1*, (d) *OsTOM2*, (e) *OsYSL15*, (f) *OsFRDL1*, and (g) *OsVIT2* in roots and stems, with the expression in roots of drained treatment being used as the control for comparison. Relative expression of (h) *OsFRDL1*, (i) *OsVIT2*, (j) *OsNAS3*, (k) *OsYSL2*, (l) *OsNAAT1*, (m) *OsYSL18*, and (n) *OsFER2* in nodes and flag leaves, with the expression in nodes of drained treatment being used as the control for comparison. Three individual replicates of pots were analyzed, and the error bars denote the standard deviation. The letters indicate significant differences calculated with an independent-sample *t*-test at the $P < 0.05$ level.

while the stems and leaves accounted for 45.0 and 35.0% of the total Fe in shoots under drained conditions, respectively (Figure S3c). In addition, the drained treatment showed a higher proportion of Fe in rachises, husks, and seeds than the flooded treatment. These results implied that Fe was preferentially transported from stems to leaves, rachises, husks, and seeds from the stems under drained conditions.

Fe isotope compositions in different rice organs revealed similar directions of fractionation under flooded and drained conditions (Figure 2b). Stems were preferentially enriched in lighter isotopes relative to the roots (flooded: $\Delta^{56}Fe_{stem-root}$ = −0.19 ± 0.04‰ and drained: $\Delta^{56}Fe_{stem-root}$ = −0.57 ± 0.04‰), which was also observed in soil−rice systems reported previously.[10,11,14] Fe isotopes in the nodes were heavier than those in the stems ($\Delta^{56}Fe_{node-stem}$ = −0.18 to −0.13‰). Leaves favored an enrichment of heavier Fe isotopes relative to shoots/stems under drained conditions ($\Delta^{56}Fe_{leaf-stem}$ = 0.84 ± 0.05‰, $\Delta^{56}Fe_{leaf-shoot}$ = 0.59 ± 0.10‰), while the Fe isotope compositions of leaves, stems, and shoots were at a similar level under drained conditions (−0.04 to −0.02‰). Seeds were preferentially enriched in lighter isotopes relative to the leaves (flooded: $\Delta^{56}Fe_{seed-leaf}$ = −0.48 ± 0.06‰ and drained: $\Delta^{56}Fe_{seed-leaf}$ = −1.29 ± 0.07‰), and husks also favored an enrichment of lighter isotopes from the roots ($\Delta^{56}Fe_{husk-root}$ = −0.41 to −0.39‰). It appeared that Fe isotopic fractionation between various rice organs was more pronounced under drained conditions than under flooded conditions. Thus, distinct water management practices largely affected the extent of Fe isotope fractionation within the rice plant.

Gene Expression in Roots, Stems, Nodes, and Flag Leaves. The relative expression of the *OsIRT1* gene in roots for the Fe^{2+} uptake strategy was slightly higher under drained conditions than that under flooded conditions (Figure 3a). For the Fe(III)−DMA uptake strategy, however, the flooded treatment revealed significantly higher expression levels of *OsNAS3*, *OsTOM2*, and *OsYSL15* genes in roots relative to the drained treatment (Figure 3b−e). The *OsIRT1*, *OsNAS3*, *OsNAAT1*, and *OsYSL15* genes were also expressed in stems, and their expression levels were up-regulated under flooded conditions. The up-regulation of *OsNAS3* (NA synthesis) and *OsNAAT1* (DMA synthesis) genes in nodes and flag leaves was even higher than that in roots and stems under flooded conditions (Figure 3i,j). *OsYSL2* and *OsYSL18*, encoding transporters for phloem loading of Fe(II)−NA and Fe(III)−DMA, respectively, revealed significantly higher expression in nodes and flag leaves under flooded conditions than under drained conditions (Figure 3k,m). *OsFRDL1*, the encoding transporter for citrate export to the apoplast (xylem), and *OsVIT2*, responsible for vacuolar sequestration of Fe, were expressed in different organs of rice, including roots, stems, nodes, and flag leaves (Figure 3f−i). *OsFER2*, the encoding ferritin protein to immobilize Fe in the leaves, was not only expressed in the flag leaf but also in nodes (Figure 3n). In summary, most of the genes responsible for Fe uptake and transport in rice were up-regulated under flooded conditions relative to drained conditions, except for the *OsIRT1* gene in roots.

DISCUSSION

Fe Isotope Fractionation in Soil. The Fe isotopes in bulk soil were identical within error irrespective of the distinct water management practices (Figure 1b), which might be attributed to their large reservoir size and similar soil Fe concentrations

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under flooded and drained conditions.[38] The fractionation toward light Fe from bulk soil to soil solution could be expected because Fe released from soil into solution is mainly driven by reductive dissolution of Fe (oxyhydr)oxides, resulting in an enrichment of Fe(II) with light isotopes in soil solution.[7,39,40] The δ^{56}Fe values of soil solution decreased over time under flooded conditions but increased under drained conditions (Figure S2b), and a significantly negative correlation was found between the δ^{56}Fe values and Fe(II) proportions in soil solution (Figure 1c). These results suggest that the Fe isotope compositions of the soil solution are likely controlled by the Fe(II) proportions in it. A lower fractionation was observed between soil solution and bulk soil under drained conditions, which fits well with its less reductive iron dissolution at the soil−porewater interface compared with that under flooded conditions.

The flooding practice reduced the soil Eh and increased the pH values (Figure S1), providing an anoxic and reducing condition that facilitates dissimilatory Fe reduction.[7] Previous dissimilatory Fe reduction experiments performed on Fe(III) oxides with different bacteria revealed that δ^{56}Fe values for aqueous Fe(II) were 0.5 to 2.1‰ lighter than those for the initial Fe(III) oxide substrates, while fractionation between the aqueous Fe(II) and reactive Fe(III) pool on the Fe(III) oxide surface (extracted with 0.5 M HCl) could be up to −2.95‰.[13,41] These results indicated that Fe isotopes in the HCl extract from Fe(III) oxides were even isotopically heavier than those in the Fe(III) oxide substrates. In this study, however, Fe isotopes in the HCl extract were slightly lighter than those in bulk soil ($\Delta^{56}Fe_{HCl\ extract-bulk\ soil}$ = −0.14 to −0.01‰). HCl extraction from bulk soil has been reported to bring no significant Fe isotope fractionation or slightly negative fractionation (−0.32 to −0.18‰).[11,14,19] The slightly negative fractionation from bulk soil to the HCl extract may be caused by re-adsorption of Fe(II) and precipitation of Fe(III) onto other components/minerals with different isotopic signatures.[16,37,39] The current study also showed that no apparent fractionation was observed between the $(NH_4)_2C_2O_4$ extract and bulk soil ($\Delta^{56}Fe_{(NH_4)_2C_2O_4\ extract-bulk\ soil}$ = −0.03 to 0.07‰). Both $(NH_4)_2C_2O_4$ and HCl extracts are considered to be able to extract Fe from poorly crystalline Fe (oxyhydr)oxides in soils and are found to contain similar Fe concentrations.[39] This is different from our finding that the Fe concentrations in the HCl extract were one magnitude lower than those in the $(NH_4)_2C_2O_4$ extract. Since the HCl extract showed a lower Fe concentration and a slightly lighter isotope composition than the $(NH_4)_2C_2O_4$ extract, the HCl extract is probably a better indicator used to estimate the Fe readily available for reductive dissolution from soils.

The enrichment of heavy Fe isotopes in Fe plaque was mainly caused by Fe(II) re-oxidation in the rhizosphere due to the release of O_2 through aerenchyma channels in roots.[17,42] The Fe(III) products from abiotic and biological Fe(II) oxidation have been reported to be isotopically heavier than aqueous Fe(II) by about 1−3‰[16,18] due to the change in the redox state and the formation of stronger bonds in precipitates relative to aqueous phases. The formation of Fe plaque should be more pronounced under flooded conditions because it facilitates the release of Fe^{2+} into soil solution (Figure S1a) and the radial oxygen loss in roots.[43] This can be supported by the higher Fe concentration in Fe plaque (Figure 1a) and the higher percentage of Fe on the root surface of the flooded treatment (22.5%) than that of the drained treatment (6.32%) from the Fe mapping in TEM (Figure S4). A positive fractionation ($\Delta^{56}Fe_{iron\ plaque-Fe(II)aq}$ = 2.24‰) was found between the Fe plaque and porewater in a waterlogged paddy field,[14] which is similar to the fractionation obtained in our flooded treatment ($\Delta^{56}Fe_{Fe\ plaque-soil\ solution}$ = 2.43‰). The positive fractionation from soil solution to Fe plaque was less pronounced under drained conditions than under flooded conditions. This is probably due to the fact that the drained conditions generally favor faster rates of oxidation and precipitation, in which diffusion gradients at the solution−solid interface may limit the isotopic equilibrium ability between isotopically heavy $Fe(III)_{aq}$ in the liquid boundary layer and the remaining $Fe(III)_{aq}$ pool.[16]

Source and Strategy of Fe Uptake by Rice. Fe isotopes in whole rice showed no apparent fractionation relative to that in bulk soil under flooded conditions ($\Delta^{56}Fe_{whole\ rice-bulk\ soil}$ = 0.05‰), which is similar to that in a waterlogged paddy field.[14] In previous studies, Fe isotopes in plants were generally compared with those in bulk soils since most of the higher plants are generally grown in oxic soils where Fe is deficient in the soil solution.[9,15,19] The small fractionation between whole rice and bulk soil (0.16 to 0.36‰) is believed to be due to Fe(III) complexation via strategy II without any reduction of Fe(III) in the rhizosphere prior to Fe uptake.[10,11] However, the flooding regime makes rice a special graminaceous species because it can provide more Fe^{2+} in the soil solutions and enhance the formation of Fe plaque on the root surface (Figure 1a). In the flooded treatment, Fe isotopes in whole rice were between those in soil solution ($\Delta^{56}Fe_{whole\ rice-soil\ solution}$ = 1.71‰) and those in Fe plaque ($\Delta^{56}Fe_{whole\ rice-Fe\ plaque}$ = −0.72‰). It appeared that both soil solution and Fe plaque could be potential sources of Fe for rice, as they have a closer proximity to the root surface than soil.[17] In addition, the extent of fractionation between whole rice and Fe plaque was smaller than that between whole rice and soil solution. A similar phenomenon was also observed in the field samples harvested at different stages of rice growth,[11,14] and Fe plaque was suggested to be the main source of Fe taken up by rice.[14]

In this study, soil solution and Fe plaque were supposed to be the direct sources of Fe for uptake, although the Fe in these two pools was actually from bulk soils and the Fe concentrations in them may vary over time during the growth of rice. Assuming that the Fe concentrations in soil solution and Fe plaque were constant, their relative contributions to the Fe accumulated in whole rice, that is, $f_{soil\ solution}$ and $f_{Fe\ plaque}$, could be calculated using the following equations.

$$\delta^{56}Fe_{whole\ rice} = f_{soil\ solution} \times (\delta^{56}Fe)_{soil\ solution} + f_{Fe\ plaque} \times (\delta^{56}Fe)_{Fe\ plaque} \quad (4)$$

$$f_{soil\ solution} + f_{Fe\ plaque} = 1 \quad (5)$$

Calculations revealed that the relative contributions of Fe plaque and soil solution were 70.4 and 29.6%, respectively, under flooded conditions, indicating that Fe plaque acted as the main source of Fe absorbed by rice. Given that Fe plaque mainly consists of Fe(III) (oxyhydr)oxides,[44] the uptake of Fe(III) from Fe plaque should be mediated via strategy II. The up-regulation of *OsNAS3*, *OsNAAT1*, *OsTOM2*, and *OsYSL15* genes in roots of flooded treatment (Figure 3b−e) also suggested that the DMA formation via OsNAS3 and

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OsNAAT1, DMA secretion via OsTOM2, and uptake of Fe(III)−DMA via OsYSL15 could be facilitated under flooded conditions.[6,20−22] Fe released abiotically by strong chelators from Fe-bearing minerals has been found to preferentially extract lighter Fe isotopes.[12] As such, the release of Fe(III) from Fe plaque via DMA chelation is expected to be preferentially enriched in lighter isotopes, which can partially explain the negative fractionation from Fe plaque to whole rice under flooded conditions.

On the other hand, rice has developed the strategy I-like system to directly absorb Fe^{2+} via the OsIRT1 transporter, probably as a result of its adaptation to the anoxic and reductive conditions where Fe^{2+} is abundant.[45] However, excessive accumulation of Fe^{2+} is toxic for cells as it mediates formation of reactive oxygen species, leading to oxidative stress responses and physiological disorders.[46] A previous hydroponic experiment of rice supplied with both Fe^{2+} and Fe^{3+}−DMA revealed that the rate of translocation of Fe absorbed as Fe^{3+}−DMA was greater than that for Fe^{2+}.[47] When more DMA was secreted into the rhizosphere by rice in our flooded treatment, the uptake of Fe(III)−DMA would be more efficient than that of Fe^{2+}. The flooded treatment had higher Fe(II) concentrations in soil solution and relatively lower expression of the *OsIRT1* gene in roots than the drained treatment, which could result in the lower contribution of soil solution as the Fe source for uptake under flooded conditions. Therefore, the Fe isotopes in the whole rice should be from both Fe plaque and soil solution, and rice mainly absorbed the Fe(III) form Fe plaque via the Fe(III)−DMA complexation strategy under flooded conditions.

Under drained conditions, Fe isotopes in whole rice were lighter than those in bulk soil ($\Delta^{56}Fe_{\text{whole rice−bulk soil}}$ = −0.41‰), suggesting that Fe uptake via the strategy I-like system may dominate. When compared with soil solution and Fe plaque, the whole rice showed no fractionation relative to soil solution ($\Delta^{56}Fe_{\text{whole rice−soil solution}}$ = −0.06‰) but fractionated negatively from Fe plaque ($\Delta^{56}Fe_{\text{whole rice−Fe plaque}}$ = −0.84‰). The calculations of these two sources indicated that the Fe in rice was predominantly from soil solution under drained conditions. The low supply of Fe in soil solution (Figure 1a) should have induced an up-regulated expression of both strategy I-like and II genes to facilitate the uptake of Fe^{2+} and Fe(III)−DMA.[4−6] All the genes for strategy I-like and II were also expressed in the roots of drained treatment (Figure 3a,d,e), indicating that rice simultaneously absorbed Fe^{2+} and Fe(III)−DMA.

However, the drained treatment revealed a relatively higher expression of the *OsIRT1* gene (strategy I-like) but a significantly lower expression of *OsTOM2* and *OsYSL15* genes (strategy II) compared with the flooded treatment. This suggests that DMA secretion may be limited under drained conditions. In a previous study, the rate of translocation of Fe absorbed as Fe^{2+} was greater than that of Fe^{3+} when the hydroponic solution was supplied with Fe^{2+} and Fe^{3+}.[47] Fe^{2+} was suggested to be the major form of Fe taken up by rice when the secretion of DMA was very low.[47] If soil solution acted as the main source of Fe for rice under drained conditions, Fe^{2+} uptake via OsIRT1 would be more efficient than via strategy II since the concentrations of dissolved Fe(III) were significantly lower than those of dissolved Fe(II) (Figures S1a,b and S2a). The aforementioned results suggested that once Fe(II) was released from soils into soil solution, rice was more liable to absorb the Fe(II) in the soil solution under drained conditions.

It should be noted that any isotope fractionation potentially caused by membrane transporters for Fe uptake has not been considered in the calculation of relative contributions of the two sources in this study. Recently, the isotope fractionation during the biological uptake of metal elements such as cadmium has been reported using yeast cells that were transformed to express the genes of specific transporters from plants.[48] If the transporters involved in the two strategies favor different Fe isotope fractionations, they may also explain the different fractionation directions of whole rice−Fe plaque and whole rice−soil solution. Yet, it remains poorly understood how Fe isotopes fractionate during the Fe(III)−DMA uptake via OsYSL15 or the Fe^{2+} uptake via OsIRT1, which deserves further study in the future.

Fe Isotope Fractionation within Rice. Rice in both flooded and drained treatments revealed similar directions of Fe isotope fractionation: stems/shoots < roots, husks < roots, and seeds < stems < leaves (Figure 2b). Fe isotope fractionation in rice is strongly associated with the Fe species that are transported and stored in different organs and the oxidized Fe species that are usually enriched in heavy Fe isotopes relative to their reduced counterparts.[37] Quantum chemical calculations indicated that Fe(III)−phytosiderophore can be 1.5% heavier than Fe(III)−citrate and up to ∼3% heavier than Fe(II)−NA.[30] Fe(III)−DMA as one of the Fe(III)−phytosiderophore complexes is supposed to be enriched in heavier isotopes than Fe(III)−citrate and Fe(II)−NA as well. Previous pot experiments with rice grown in oxic and anoxic soils showed lighter Fe isotopes in shoots than in bulk soils, although the isotope signature of roots was not provided.[20] A negative fractionation from roots to stems was observed in field samples of rice ($\Delta^{56}Fe_{\text{stem−root}}$ = −1.39 to −0.16‰),[10,11,14] which is similar to our findings. The lighter isotopes in stems than in roots are likely ascribed to the presence of Fe(III)−citrate in xylem and a mixture of Fe(II)−NA and Fe(III)−DMA in phloem of stems, while Fe(III)−DMA with the heaviest isotopes is considered to be the main form of Fe accumulated in root cells.[1,11] Since the roots accumulated the highest concentration and mass of Fe (Figures 1a and S3b), the excessive Fe in roots could be sequestered in vacuoles and/or deposited in apoplast probably in the form of Fe(III)−phosphate or Fe(III)−hydroxides.[15,49] These oxidized Fe species also likely contributed to the enrichment of heavy isotopes in roots. Fe in husks is primarily transported from roots via xylem,[28] which would have favored an enrichment of Fe(III)−citrate in the husks with lighter isotopes relative to the roots ($\Delta^{56}Fe_{\text{husk−root}}$ = −0.41 to −0.39‰).

After Fe is transported to leaves via xylem, it is usually stored in Fe(III)−ferritin proteins in the plastids and/or sequestered in the vacuoles, in addition to functioning in the chloroplasts and mitochondria.[3,26,27] Although the isotope signature of Fe in these subcellular organelles of leaves has not been identified, the positive fractionation from stems to leaves ($\Delta^{56}Fe_{\text{leaf−stem}}$ = 0.01 to 0.84‰) suggests that Fe species stored in leaves are preferentially enriched in heavy isotopes relative to the mixture in stems. The Fe in older leaves can be re-mobilized into Fe(III)−DMA or Fe(II)−NA, which is then transported to younger leaves and seeds via phloem.[27] The negative fractionation from leaves to seeds ($\Delta^{56}Fe_{\text{seed−leaf}}$ = −1.28 to −0.48‰) was also observed in previous studies of soil−rice

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systems.[10,14] It is noteworthy that Fe isotopes in the nodes were relatively lighter than those in the stems/shoots ($\Delta^{56}Fe_{node-stem/shoot}$ = −0.38 to −0.13‰) and heavier than those in the seeds ($\Delta^{56}Fe_{node-seed}$ = 0.30 to 0.31‰). Fe is mainly localized at the parenchyma cells in the nodes of rice, with Fe being strongly co-localized with phosphorus in the vacuoles.[32,50] If the Fe stored in nodes is mainly associated with phosphorus in insoluble forms, Fe species with light isotopes such as Fe(II)−NA from roots and older leaves could be preferentially re-distributed to the seeds due to the kinetic fractionation effect.[37]

Within shoots, the expression levels of the tested genes in stems, nodes, and flag leaves were all up-regulated under flooded conditions as compared with the drained conditions, including *OsVIT2* (vacuolar sequestration), *OsFER2* (ferritin immobilization), *OsFRDL1* (citrate export into xylem), *OsNAS3* and *OsNAAT1* (NA and DMA synthesis), and *OsYSL18* and *OsYSL2* [phloem loading of Fe(III)−DMA and Fe(II)-NA] (Figure 3). Since much more Fe has been absorbed by rice under flooded conditions, such an up-regulation could be expected, which not only enhanced Fe immobilization in individual organs but also facilitated Fe distribution within shoots.[1,45] As a result, the Fe accumulation in most of the organs was substantially increased under flooded conditions (Figure S3a); however, no increase in Fe accumulation was observed in the seeds (Figures 2a and S3b). A similar phenomenon was also reported in a previous study when rice was grown in oxic and anoxic soils.[20] Fe transport to the seeds appeared to be restricted regardless of the amounts of Fe taken up by rice roots.[3,45] The up-regulation of gene expression did not cause apparent changes in the direction of isotope fractionation within rice either (Figure 2b). However, the extent of fractionation among different organs within shoots appeared to be larger under drained conditions than under flooded conditions. The excess Fe transported from roots to shoots was preferentially immobilized in stems under flooded conditions (Figure S3b). When limited Fe was transported to the shoots under drained conditions, the proportions of Fe mass distributed from stems to leaves, husks, and seeds were significantly higher than those under flooded conditions (Figure S3c). This may explain the larger extent of Fe isotope fractionation among these organs within shoots under drained conditions than under flooded conditions, given that the Fe species transported and stored in various organs were similar under both conditions.

Implications. Two strategies and a number of transporters responsible for Fe uptake by rice have been identified so far,[1] and the behavior of Fe isotopes has also been used to trace the biogeochemical transport of Fe in soil−rice systems.[10,11,14] However, the preference of sources and strategies during Fe uptake by rice is complicated and poorly understood under the flooding and drainage regime. To the best of our knowledge, the current study is the first to determine the relative contribution of Fe sources (soil solution vs Fe plaque) and dominant strategies of Fe uptake [Fe(II) vs Fe(III)−DMA] under distinct water management practices by linking the Fe isotope signature with gene expression levels. Our results demonstrated that distinct water management practices not only altered the Fe isotope fractionation between soils and rice but also regulated the gene expression of transporters involved in the two strategies of Fe uptake. Rice principally absorbed Fe(III) from Fe plaque via strategy II under flooded conditions, while the Fe(II) in soil solution was preferentially absorbed via strategy I under drained conditions. These findings provided new insights into the regulation of Fe uptake by rice in response to distinct water management practices. Recent studies have highlighted that the combined strategies for iron uptake are not only exclusive to the domesticated rice previously proposed but also present in other species of graminaceous plants.[51] The strategies of Fe uptake in other higher plants may vary particularly in response to changes in the environment, such as drought and deluge. In addition, Fe isotopic compositions and gene expression levels in plants may also vary during the whole growth period, which deserves further study.

Fe deficiency in humans is a widespread problem worldwide, and Fe biofortification of staple crops like rice is a promising approach to address human Fe deficiency. However, our results confirmed that the flooding management practice could not increase the Fe concentration in rice seeds, although it had substantially increased the Fe availability in soils and the Fe uptake by rice. Since excess Fe in shoots was mainly accumulated in the stems, likely in insoluble forms, regulation strategies that promote the chelation of Fe and its transport from stems to seeds deserve further investigation. This study, together with previous studies, suggests that NA is the most important chelator responsible for Fe loading into the seeds via phloem.[14,52] It would be practical to plant the rice cultivars or transgenic rice lines with high capability of NA synthesis and Fe(II)−NA loading into the phloem in the nodes during rice production in the paddy field.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.1c08034.

Additional experimental methods; Fe(II) and Fe(III) concentrations in soil solution, pH and Eh in soil; proportions of Fe(II) to total Fe and δ^{56}Fe values in soil solution; dry weight and proportions of Fe mass in various organs to whole rice and to the shoots; TEM image, distribution maps of iron element, and percentages of associated elements of Fe plaque on the root surface; primers for qPCR analysis; and Fe concentrations and δ^{56}Fe values of various soil pools and rice organs (PDF)

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Notes

The authors declare no competing financial interest.

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