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Rice HRZ ubiquitin ligases are crucial for the response to excess iron

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Iron is essential for virtually all organisms but is toxic when present in excess. To acquire the proper amount of iron, plants induce expression of various genes involved in iron uptake and translocation in response to low-iron availability. Two iron-binding ubiquitin ligases, OsHRZ1 and OsHRZ2, negatively regulate such iron deficiency responses in rice (*Oryza sativa*). Transgenic rice plants with repressed expression of *OsHRZ1* and *OsHRZ2* (*HRZ*-knockdown lines) are tolerant to low iron availability and accumulate iron in shoots and seeds under both iron-sufficient and iron-deficient conditions without a growth penalty. Although the expression of *OsHRZ1* and *OsHRZ2* is transcriptionally upregulated under iron-deficient conditions, the physiological relevance of this induction is not known. In the present study, we analyzed the response of *HRZ*-knockdown lines to excess iron. In the presence of severe excess iron, the *HRZ*-knockdown lines grew worse than non-transformants (NTs). The *HRZ*-knockdown lines showed stunted shoot and root growth and more severe leaf bronzing compared to NTs. Moreover, these lines accumulated more iron in shoots and exhibited severely elevated expression of various genes involved in iron uptake and translocation as well as jasmonate signaling compared to NTs. These results indicate that HRZ ubiquitin ligases are crucial for repressing iron deficiency responses and protecting cells from iron toxicity in the presence of excess iron. These results support the possibility that HRZs are intracellular Fe sensors and provide clues for developing plants tolerant of either iron deficiency or excess with higher iron contents in edible parts.

Introduction

Iron (Fe) is an essential element for virtually all organisms. Fe is utilized as an essential cofactor in numerous proteins in the form of the Fe–sulfur cluster, heme or free Fe, where it mediates various metabolic processes, including photosynthesis, respiration and chlorophyll biosynthesis (Marschner 1995). Although abundant in soils, Fe is sparingly soluble especially under high pH and aerobic conditions. Therefore, plants grown under low Fe availability, such as in calcareous soils, often fail to obtain sufficient Fe and suffer from Fe deficiency, which results in leaf yellowing called Fe chlorosis. This symptom typically appears on the newest leaves. Fe deficiency consequently reduces plant growth as well as crop yield and quality (Marschner 1995). Fe uptake from the soil into the plant not only is essential for plant growth and reproduction but it is also an essential source of Fe in humans and animals. Indeed, Fe and Zn deficiencies are among the most prevalent human micronutrient

Abbreviations – bHLH, basic helix-loop-helix; BTS, BRUTUS; EDTA, ethylenediaminetetraacetic acid; FBXL5, F-box leucine rich repeat protein 5; HRZ, hemerythrin motif-containing really interesting new gene- and zinc-finger proteins; IDEF, iron deficiency-responsive element-binding factor; IRO, iron-related transcription factor; JAs, jasmonates; MAs, mugineic acid family phytosiderophores; NT, non-transformant; RT-PCR, real-time polymerase chain reaction.

disorders. The former affects an estimated one-third of the world's population, causing about 800 000 deaths annually worldwide (WHO 2002, Mayer et al. 2008). Thus, the development of crops tolerant of low Fe availability with high Fe and Zn contents in edible parts has long been pursued for human nutrition.

Even though Fe is essential, excess Fe is deleterious because Fe^{2+} catalyzes the generation of reactive oxygen species in the Fenton reaction, promoting oxidative stress (Briat et al. 1995, Marschner 1995). Fe toxicity is a major nutrient disorder in plants grown under anaerobic conditions and in acidic soils, in which the solubility of Fe is increased because of both an increase in Fe^{3+} solubility and a reduction of Fe(III) to the more soluble Fe^{2+} (Becker and Asch 2005, Stein et al. 2009a). Fe toxicity inhibits root elongation and provokes the appearance of brown spots in leaves, resulting in reddish-colored or dried leaves; the most recognized symptom of Fe toxicity, called leaf bronzing (Becker and Asch 2005). In contrast to Fe deficiency-mediated chlorosis, leaf bronzing typically starts in older leaves.

Because of this toxicity, Fe uptake mechanisms are induced only under Fe-deficient conditions and repressed when Fe is sufficient. Fe uptake mechanisms in higher plants have been studied extensively and are categorized as Strategy I and Strategy II (Römheld and Marschner 1986). Strategy I, utilized by dicot and non-graminaceous monocot species, depends on ferric reduction and subsequent uptake of Fe^{2+} (Römheld and Marschner 1986). Strategy II is utilized by graminaceous species and relies on biosynthesis and secretion of mugineic acid family phytosiderophores (MAs), which are efficient Fe(III) chelators that solubilize rhizospheric Fe that is absorbed in the form of Fe(III)-MAs (Takagi 1976, Takagi et al. 1984). Rice is a graminaceous plant that utilizes Strategy II, but it also takes up Fe^{2+} as a partial Strategy I (Ishimaru et al. 2006).

Genes involved in both strategies, such as those encoding biosynthetic enzymes for MAs and transporter genes for MA efflux as well as Fe(III)-MAs and Fe^{2+} uptake, are strongly induced under Fe-deficient conditions and repressed under Fe-sufficient conditions at the transcript level (Kobayashi and Nishizawa 2012, Kobayashi et al. 2014). In rice, regulation is mediated by a transcriptional network of positive and negative regulators, including Iron Deficiency-responsive Element-binding Factor 1 (IDEF1), IDEF2, *Oryza sativa* Iron-related transcription factor 2 (OsIRO2), and OsIRO3 (Ogo et al. 2006, 2007, 2008, 2011, Kobayashi et al. 2007, 2009, 2014, Zheng et al. 2010). The expression of *OsIRO2* and *OsIRO3* is transcriptionally induced under Fe-deficient conditions similar to Fe uptake-related genes (Ogo et al. 2007, Zheng et al. 2010). *IDEF1* and *IDEF2* transcript levels remain unchanged according to Fe availability (Kobayashi et al. 2007, 2009, Ogo et al. 2008). The IDEF1 protein is subjected to 26S proteasome-mediated degradation, and its degradation is regulated by IDEF1-binding protein 1 (IBP1) belonging to the Bowman–Birk trypsin inhibitor family, and COP9 signalosome subunit 6 (CSN6) (Zhang et al. 2014, Tan et al. 2016).

Despite these findings, the identity of the signaling substances for Fe and the sensors that receive the signals and regulate the responses have not been identified in plants. IDEF1 binds directly to Fe^{2+} and other divalent metals, which suggests a role as an intracellular Fe sensor (Kobayashi et al. 2012). Furthermore, we previously identified another kind of potential Fe sensors in rice cells, designated *O. sativa* Hemerythrin motif-containing Really Interesting New Gene- and Zinc-finger protein 1 (OsHRZ1) and OsHRZ2, by searching for Fe-binding expressional regulators in rice (Ogo et al. 2006, Kobayashi et al. 2013). *OsHRZ1* and *OsHRZ2* are close homologs, and their transcripts are induced in roots and leaves under Fe-deficient conditions (Kobayashi et al. 2013). In vitro analyses have revealed that, both OsHRZ1 and OsHRZ2, as well as their ortholog in *Arabidopsis thaliana*, BRUTUS (BTS), bind to Fe and Zn and possess ubiquitination activity (Kobayashi et al. 2013, Selote et al. 2015). Transgenic rice lines with slightly decreased expression of *OsHRZ1* and moderately decreased expression of *OsHRZ2*, designated *HRZ*-knockdown lines, show substantial tolerance of low Fe availability in hydroponic culture and in calcareous soil (Kobayashi et al. 2013). Moreover, these lines accumulate about 2–4 times more Fe and about 1.3–1.5 times more Zn in seeds compared to non-transformants (NTs) under both sufficient and low Fe availability in soil, without any growth penalty (Kobayashi et al. 2013). These phenotypes are extremely promising for future applications of Fe- and Zn-fortified crops, which can be grown in calcareous soils. Gene expression analyses revealed that the expression of most known Fe deficiency-inducible genes involved in Fe uptake and/or translocation is markedly enhanced in *HRZ*-knockdown plants under Fe-sufficient conditions (Kobayashi et al. 2013). These results indicate that OsHRZ1 and OsHRZ2 are negative regulators of Fe deficiency-inducible genes for Fe uptake and translocation (Kobayashi et al. 2013). In addition, the expression of a subset of genes involved in the biosynthesis and signaling of jasmonates (JAs) is also enhanced in *HRZ*-knockdown roots, in which JA concentrations increase under Fe-sufficient conditions (Kobayashi et al. 2016). We also found that JA signaling negatively regulates the Fe deficiency response under Fe-sufficient conditions, but this negative regulation is

partially canceled at very early stages of Fe deficiency when JA biosynthesis is transiently activated in rice roots (Kobayashi et al. 2016). These results suggest that OsHRZ1 and OsHRZ2 regulate multiple Fe deficiency response pathways and that their function is dependent on Fe availability.

The possible function of HRZs as Fe sensors has also been deduced from their domain structures (Kobayashi et al. 2013, Kobayashi and Nishizawa 2014). HRZs contain hemerythrin domains on the N-terminal side and three kinds of Zn-finger domains (CHY-, CTCHY- and RING-Zn-fingers) as well as a rubredoxin-type fold (also called Zn-ribbon) on the C-terminal side. These domain structures are conserved among plants and algae, including BTS in Arabidopsis (Long et al. 2010, Urzica et al. 2012, Kobayashi et al. 2013). Of these domains, the hemerythrin domain binds to Fe in animals and bacteria (Stenkamp 1994,Salahudeen et al. 2009 , Vashisht et al. 2009). We revealed previously that OsHRZ1, OsHRZ2 and BTS bind not only Fe but also Zn, and the major binding sites are situated on the N-terminal side, which contains the hemerythrin domains (Kobayashi et al. 2013). In addition, smaller portions of Fe and Zn are also bound to the C-terminal side containing three Zn-fingers and a rubredoxin-type fold (Kobayashi et al. 2013). The RING-Zn-finger domain mediates the enzymatic reactions of E3 ligase, which ubiquitinates specific proteins for 26S proteasome-mediated degradation or other functional modifications (Hua and Vierstra 2011). The mammalian Fe sensor protein F-box leucine rich repeat protein 5 (FBXL5) also contains a hemerythrin domain that binds to Fe and an F-box domain, which is another constituent of E3 ubiquitin ligases (Salahudeen et al. 2009, Vashisht et al. 2009). The FBXL5 protein is stabilized under Fe surplus conditions by Fe binding to the hemerythrin domain and ubiquitinates Iron Regulatory Protein 2 (IRP2) for degradation, which consequently derepresses the Fe deficiency response (Salahudeen et al. 2009, Vashisht et al. 2009). Moreover, receptors for various plant hormones are also composed of ligand-binding domains and constituents of E3 ubiquitin ligases (Hua and Vierstra 2011), which further supports the possibility of HRZs/BTS as Fe sensors that utilize Fe itself and/or Zn as the ligand(s) to sense Fe nutritional status.

Two basic helix-loop-helix (bHLH) transcription factors, AtbHLH105/IAA-LEUCINE RESISTANT 3 (ILR3), and AtbHLH115, are suggested as ubiquitination targets of Arabidopsis BTS (Selote et al. 2015). AtbHLH105 and AtbHLH115, together with AtbHLH034 and AtbHLH104, belong to the subgroup IVc bHLH transcription factors that positively regulate Fe deficiency responses in Arabidopsis (Selote et al. 2015, Zhang et al. 2015, Li et al. 2016, Liang et al. 2017). Similarly, a subgroup IVc bHLH transcription factor in rice, OsbHLH060/*O. sativa* Positive Regulator of Iron homeostasis 1 (OsPRI1), was recently suggested to be a ubiquitination target of OsHRZ1 (Zhang et al. 2017). OsbHLH060 positively regulates Fe deficiency responses possibly via the Fe deficiency-inducible bHLH transcription factors OsIRO2 and OsIRO3 (Zhang et al. 2017). However, this regulation does not fully explain the wide-ranging effects of *HRZ*-knockdown plants, which suggests the existence of other ubiquitination targets of HRZs. In addition, whether OsHRZ2 is involved in this regulatory pathway through OsbHLH060 is not known.

Although the aforementioned observations support the possibility of HRZs/BTS as Fe sensors, direct evidence remains limited. Selote et al. (2015) reported that BTS protein produced in vitro using a wheat germ extract system was less abundant when Fe was included in the reaction mixture. Mutations in the hemerythrin domain abolish this effect, which suggests that Fe binding to a hemerythrin domain might destabilize BTS. In addition to such protein-level regulation, the *BTS* transcript level also increases under Fe deficiency (Long et al. 2010), similarly to rice *HRZs*. Because of this regulation, BTS is thought to function mainly under Fe-limited conditions (Long et al. 2010, Selote et al. 2015). However, a complementation analysis using an Arabidopsis *bts* mutant indicated that deleting the hemerythrin domains did not dramatically affect the physiological function of BTS, in contrast to the essential function of the RING Zn-finger domain (Selote et al. 2015, Matthiadis and Long 2016), which suggests the limited importance of the hemerythrin domains in BTS function. Moreover, another study identified a *bts* mutant that disrupted expression of Fe-related genes more predominantly under Fe-sufficient than under Fe-deficient conditions (Hindt et al. 2017), similarly to our *HRZ*-knockdown rice (Kobayashi et al. 2013). These results suggest that HRZs/BTS function better under Fe-sufficient conditions than under Fe-deficient conditions, regardless of the Fe deficiency-induced expression of *HRZs*/*BTS* themselves.

The present study explored the possible role of HRZs under excess Fe to clarify the Fe dependence of HRZ function and provide clues for demonstrating HRZs as cellular Fe sensors. To this end, we analyzed the responses of *HRZ*-knockdown lines to various intensities of excess Fe. The results indicated that the *HRZ*-knockdown lines were hypersensitive to severe excess Fe conditions. These knockdown lines showed enhanced Fe accumulation in leaves and derepressed expression of Fe uptake and translocation-related genes to a pronounced degree under excess Fe. These results

indicate that HRZs are responsible for tolerance of excess Fe and suggest that HRZ alters their function in response to Fe levels.

Materials and methods

Plant materials and growth conditions

For severe excess Fe treatments, NT rice (*O. sativa* L. cultivar Tsukinohikari) was germinated on Murashige and Skoog medium (Murashige and Skoog 1962), whereas *HRZ*-knockdown lines 2i-1, 2i-2 and 2i-3 (Kobayashi et al. 2013) were germinated on Murashige and Skoog medium with hygromycin B (50 mg l^{-1}). After a 13-day culture followed by a 3-day acclimation, the plantlets were transferred to a hydroponic solution in a greenhouse at 28°C under natural light conditions. The hydroponic solution was a modified Kasugai's nutrient solution containing 0.35 mM $(NH_4)_2SO_4$, 0.18 mM Na_2HPO_4, 0.27 mM K_2SO_4, 0.36 mM $CaCl_2$, 0.46 mM $MgSO_4$, 18 μM H_3BO_3, 4.6 μM $MnSO_4$, 1.5 μM $ZnSO_4$, 1.5 μM $CuSO_4$, 1.0 μM Na_2MoO_4 and 35.7 μM $FeCl_2$ at pH 5.5. After 7 days, the NT and the *HRZ*-knockdown lines were exposed to excess ferrous Fe treatments of 1071 (×30), 1785 (×50) or 2499 (×70) μM $FeCl_2$ and the control solution of 35.7 (×1) μM $FeCl_2$ for 14 days. The solution pH was adjusted to pH 4.0 at preparation and every 2 days thereafter. The solution was renewed every 7 days. The newest and third newest leaves and the root system were harvested after 14 days.

For a milder excess Fe treatment, NT, 2i-1 and 2i-2 lines were germinated as above. After 18-day culture followed by a 3-day acclimation, the plantlets were transferred to another modified Kasugai's nutrient solution containing 0.70 mM K_2SO_4, 0.10 mM KCl, 0.10 mM KH_2PO_4, 2.0 mM $Ca(NO_3)_2$, 0.50 mM $MgSO_4$, 10 μM H_3BO_3, 0.50 μM $MnSO_4$, 0.50 μM $ZnSO_4$, 0.20 μM $CuSO_4$, 0.01 μM $(NH_4)_6Mo_7O_{24}$ and 100 μM Fe(III)-ethylenediaminetetraacetic acid (EDTA) at pH 5.5 in a greenhouse at 28°C under natural light conditions. After 6 days, the plants were transferred to either excess Fe condition containing 500 μM Fe(III)-EDTA supplemented with 15.8 mg l^{-1} Tetsuriki-Type X fertilizer (containing approximately 19 μM Fe^{2+}; Aichi Steel, Aichi, Japan; Kobayashi et al. 2010) [×5 Fe(III) + Type X], or the control condition containing 100 μM Fe(III)-EDTA [×1 Fe(III)] at pH 5.5. The solution was renewed after 4 days. Roots were harvested after 7 days.

Measurement of bronzing scores and dry weights

After the 14-day exposure to excess Fe, the severity of Fe toxicity was measured in leaves using the bronzing score of the fully expanded newest leaf as well as the second, third and fourth newest leaves. The scoring system for Fe toxicity by Asch et al. (2005) adapted from IRRI–INGER (1996) was used as follows: [percent leaf area affected = score]: 0% = 0 [no symptoms], 1–9% = 1, 10–29% = 3, 30–49% = 5, 50–69% = 7, 70–89% = 9, 90–100% = 10 [dead leaf]. Shoot and root dry weights were measured after a 5-day incubation at 60°C.

Metal concentration measurements

Samples of the newest and third newest leaves and roots from control and Fe-treated plants were collected for metal concentration measurements according to Masuda et al. (2009) with a slight modification as follows: the roots of control plants were washed in distilled water whereas the roots of the plants exposed to excess Fe were washed in 50 mM Na-EDTA and Milli-Q water (Millipore, Bedford, MA). We measured Fe, Zn, copper (Cu) and manganese (Mn) concentrations in digested samples.

Gene expression analysis

Roots after the treatments were used for RNA extraction and quantitative real-time polymerase chain reaction (RT-PCR) analysis according to Kobayashi et al. (2016). Transcript abundance was normalized against the rice α-2 tubulin transcript level and was expressed as a ratio relative to the levels in ×1 NT roots. Primers used for quantitative RT-PCR were as follows: *OsNAS1* forward, 5′-GTCTAACAGCCGGACGATCGAAAGG-3′; *OsNAS1* reverse, 5′-TTTCTCACTGTCATACACAGATGGC-3′; *OsNAS2* forward, 5′-TGAGTGCGTGCATAGTAATCCTGGC-3′; *OsNAS2* reverse, 5′-CAGACGGTCACAAACACCTCTTGC-3′; *TOM1* forward, 5′-CACCAGTTGCAGATCGTATAGGGAGGAA-3′; *TOM1* reverse, 5′-TCGGAAAATACATTTGGATATTGCT-3′; *OsYSL15* forward, 5′-CACCCTGGTGAAGCAGCTGGTGCTC-3′; *OsYSL15* reverse, 5′-CGGCCATCGCCGTCGGCAGCGGCAC-3′; *OsIRO2* forward, 5′-CCGGCGGATCCCGCTCCCAC-3′; *OsIRO2* reverse, 5′-CGTCGTCGTCAGCTCCTTCT-3′; *OsIRT1* forward, 5′-CGTCTTCTTCTTCTCCACCACGAC-3′; *OsIRT1* reverse, 5′-GCAGCTGATGATCGAGTCTGACC-3′; *OsYSL2* forward, 5′-TCTGCTGGCTTCTTTGCATTTTCTG-3′; *OsYSL2* reverse, 5′-ACCATGTCGAACTCAGCATCCAGGA-3′; *OsLOX2;1* forward, 5′-AACGCTCCAAAACTACTTGC-3′; *OsLOX2;1* reverse, 5′-ACATTAAACATTGTGATACCTTGAG-3′; *OsLOX2;3* forward, 5′-TGGGAGGACATCTACTTGC-3′; *OsLOX2;3* reverse, 5′-AACATCAACAACAACCACTTC-3′; *OsJAZ1* forward, 5′-TTTGATTTCCACGTGTCTGTG-3′; *OsJAZ1* reverse, 5′-CCGTGTGC

ATGGATCCTTAC-3′; *OsFer1 + 2* forward, 5′-GTG AAGGGCAGTAGTAGGTTTCG-3′; *OsFer1 + 2* reverse, 5′-CGCGCGACATACACATGATTCTG-3′; *α-2 tubulin*, TaqMan Gene Expression Assays Os03562997_mH. *OsFer1 + 2* primers specifically amplify both *OsFer1* and *OsFer2* genes.

Statistical analysis

Statistical analysis was carried out using Microsoft Excel software. Comparisons were made between NT and each transgenic line for each condition, time point and plant part. For each set of comparisons, a two-sample Student's *t*-test for equal or unequal variance was carried out based on an *F*-test for equal variance (significance level = 0.05).

Results

The *HRZ*-knockdown lines are hypersensitive to severe excess Fe

We cultured the *HRZ*-knockdown lines (2i-1, 2i-2 and 2i-3; Kobayashi et al. 2013) and NT in a hydroponic solution at pH 4.0 supplied with 35.7 (×1) μM $FeCl_2$ as a control and 1071 (×30), 1785 (×50) or 2499 (×70) μM $FeCl_2$ as the excess ferrous Fe treatments for 14 days. Plants had similar appearances at the onset of the treatment (Fig. S1 in Appendix S1, Supporting Information). After 4 days, lines 2i-1 and 2i-3, particularly the latter, were stunted in growth and had a blasted leaf color compared to NT under the ×30, ×50 and ×70 Fe conditions (Fig. S1 in Appendix S1). This tendency became more pronounced after 7 days (Fig. S1 in Appendix S1) and even more pronounced after 14 days (Fig. 1A), when line 2i-2 also showed inferior growth compared to NT under the ×70 Fe condition. NT plants appeared rather healthy during the 14 days, except for a mild decrease in leaf growth under the ×50 and ×70 Fe conditions (Fig. 1A).

We also noticed leaf bronzing, a typical Fe toxicity symptom, in older leaves of all *HRZ*-knockdown lines grown under the ×30, ×50 and ×70 excess Fe conditions on day 4 of treatment but not in NT leaves or under the ×1 Fe condition (Fig. S1 in Appendix S1). This leaf bronzing was more pronounced at the end of the 14-day excess Fe treatment (Fig. 1B). Line 2i-3 exhibited the severest bronzing under the ×30 Fe condition, although all three *HRZ*-knockdown lines showed severe bronzing under the ×50 and ×70 Fe conditions.

Quantification of leaf bronzing with the bronzing score confirmed these results (Fig. 2). The bronzing score was always higher in older leaves than in new leaves. NT leaves had bronzing scores of near 0, which indicates scarce bronzing, except for a bronzing score of about 1 in older leaves under the ×70 Fe condition. The *HRZ*-knockdown lines had higher bronzing scores than NT in every leaf analyzed under the ×30, ×50 and ×70 excess Fe conditions. Line 2i-3 had the highest bronzing scores under any of these excess Fe conditions.

Measurement of plant growth during the Fe treatments also supported the susceptibility of the *HRZ*-knockdown lines to excess Fe (Fig. 3). Shoots of lines 2i-1 and 2i-3 were shorter compared to those of NT on day 4 of the ×30, ×50 and ×70 excess Fe treatments, and this difference continued thereafter. In addition, line 2i-2 also tended to have shorter shoots than NT on day 7 and thereafter under the ×70 excess Fe condition (Fig. 3A). NT had shorter shoots under the ×50 and ×70 Fe conditions, but not under ×30 Fe, compared to the ×1 Fe condition on day 7 and thereafter. By contrast, root growth was inhibited under the ×30, ×50 and ×70 excess Fe conditions compared to the ×1 Fe condition in all genotypes on day 7 and thereafter (Fig. 3B). The *HRZ*-knockdown lines, particularly line 2i-3, had shorter roots than NT under the ×50 and ×70 excess Fe conditions on day 4 and thereafter (Fig. 3B).

We also measured the dry weights of shoots and roots (Fig. 4). NT plants did not show any differences in shoot dry weights but showed higher root dry weights in response to the ×30, ×50 and ×70 excess Fe conditions. The *HRZ*-knockdown lines showed lower shoot and root dry weights compared to NT under the ×30, ×50 and ×70 excess Fe conditions. The decrease in dry weight was greatest in line 2i-3 and smallest in line 2i-2.

Taken together, these results indicate that the *HRZ*-knockdown lines, particularly line 2i-3, were hypersensitive to severe excess Fe conditions of which NT rice was tolerant. Line 2i-2 showed the least sensitivity of the three lines but was more sensitive to excess Fe compared to NT.

The *HRZ*-knockdown lines hyperaccumulate Fe in leaves under excess Fe

We measured metal concentrations in the newest and third newest leaves as well as in whole roots after the 14-day ×1 and ×30 Fe treatments (Fig. 5; Fig. S2 in Appendix S1). The Fe concentration in leaves, particularly in older (third newest) leaves, was much higher in all genotypes under the ×30 Fe condition compared to the ×1 Fe condition (Fig. 5A). Notably, the *HRZ*-knockdown lines accumulated still higher concentrations of Fe compared to NT under the ×30 Fe condition but not under the ×1 Fe condition (Fig. 5A). The highest Fe concentration was observed in older leaves of line 2i-3 under the ×30 Fe condition. This line accumulated about six times more

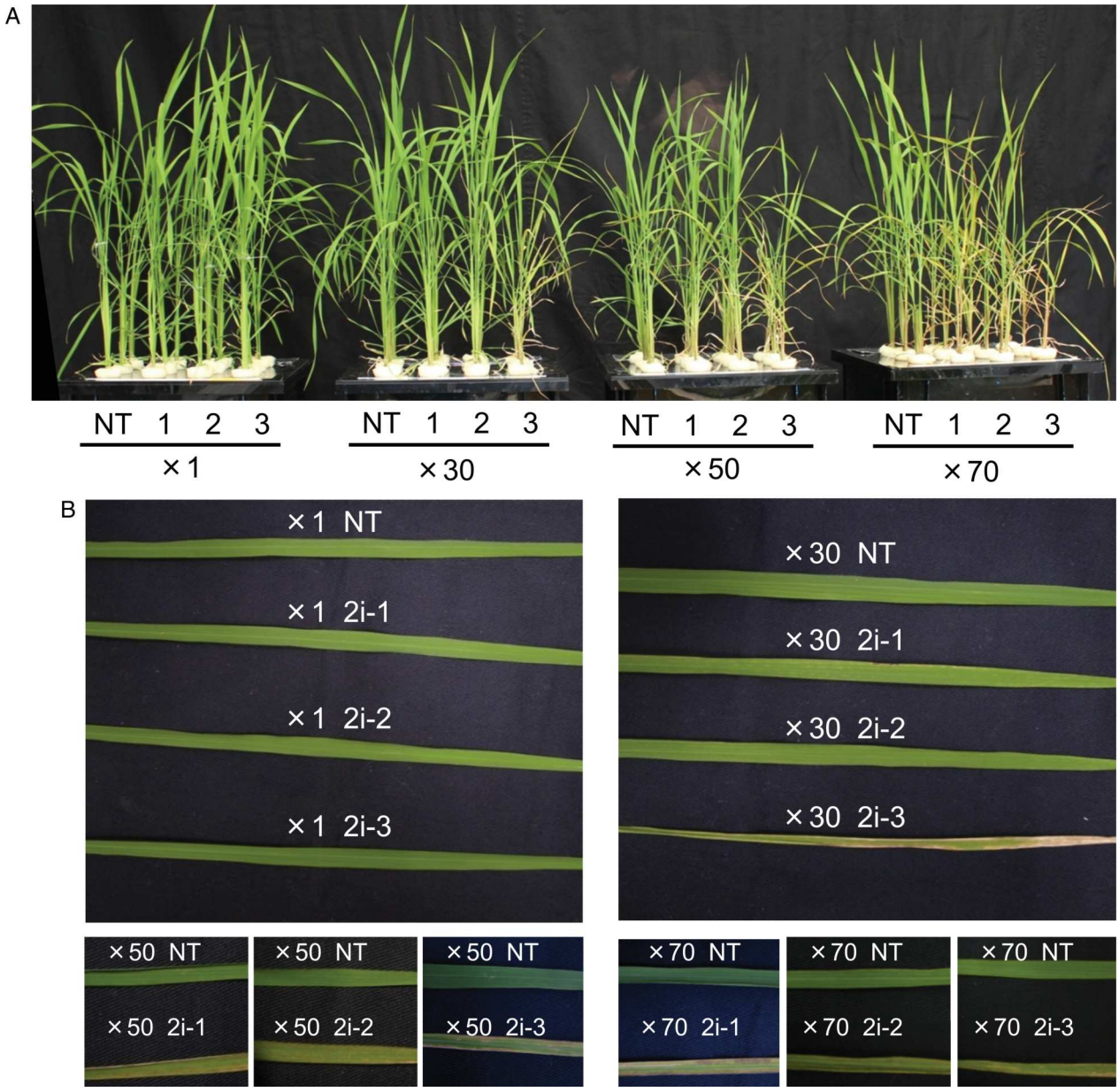

Fig. 1. Appearance of NT and *HRZ*-knockdown (lines 2i-1, 2 and 3; indicated as 1, 2 and 3, respectively) rice plants after 14 days Fe treatments. (A) Whole shoot appearance. (B) Representative leaf appearance. Plants were grown hydroponically under control (×1) and excess Fe (×30, ×50 and ×70) conditions at pH 4.0.

Fe in the third newest leaves compared to the newest leaves, whereas NT accumulated about three times more Fe in the third newest leaves compared to the newest leaves.

Root Fe concentrations showed a similar trend (Fig. 5B). However, the accumulation of Fe in the *HRZ*-knockdown lines compared to NT was relatively slight and significant only in line 2i-3 roots under the ×30 Fe condition (Fig. 5B).

Concentrations of Zn, Cu and Mn in leaves tended to decrease under the ×30 Fe condition compared to the ×1 Fe condition in all genotypes, particularly in older leaves (Fig. S2 in Appendix S1). The concentrations of these metals did not differ significantly between the *HRZ*-knockdown lines and NT, except for the higher concentrations of Zn, Cu and Mn in older leaves of line 2i-3; moderately lower concentrations of Zn in older leaves of lines 2i-1 and 2i-2; and moderately

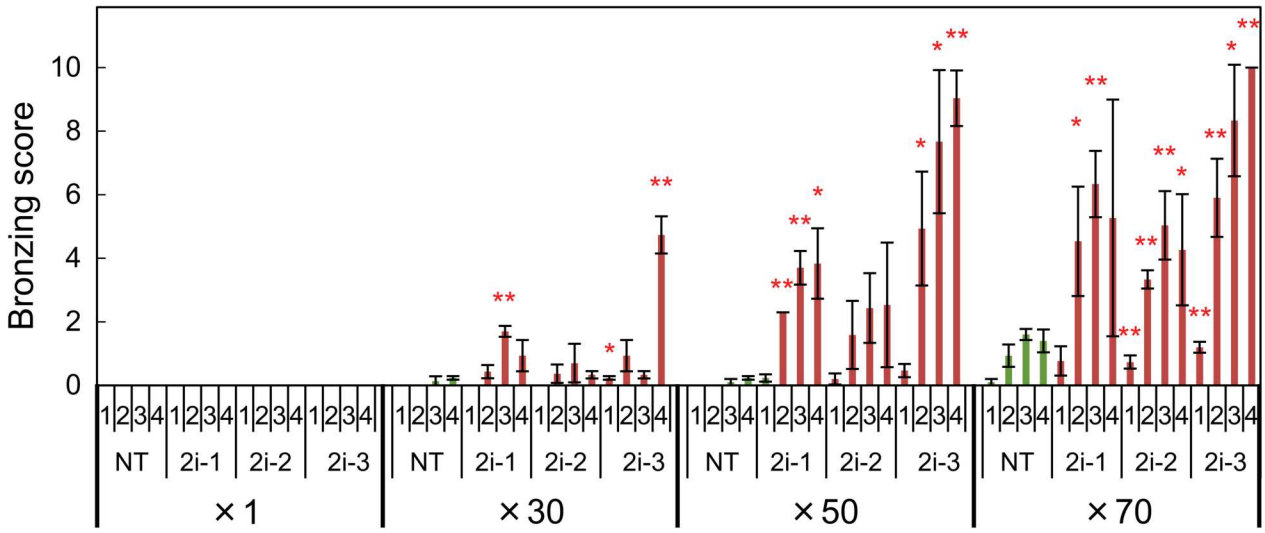

Fig. 2. Bronzing scores of NT and *HRZ*-knockdown (lines 2i-1, 2 and 3) rice after 14 days Fe treatments. Means ± SD (n = 6) are shown. The first, second, third and fourth newest leaves are indicated on the horizontal axis by 1, 2, 3 and 4, respectively. Plants were grown hydroponically under control (×1) and excess Fe (×30, ×50 and ×70) conditions at pH 4.0. Asterisks indicate significant differences compared to the NT level for each condition and plant part (*$P < 0.05$, **$P < 0.01$).

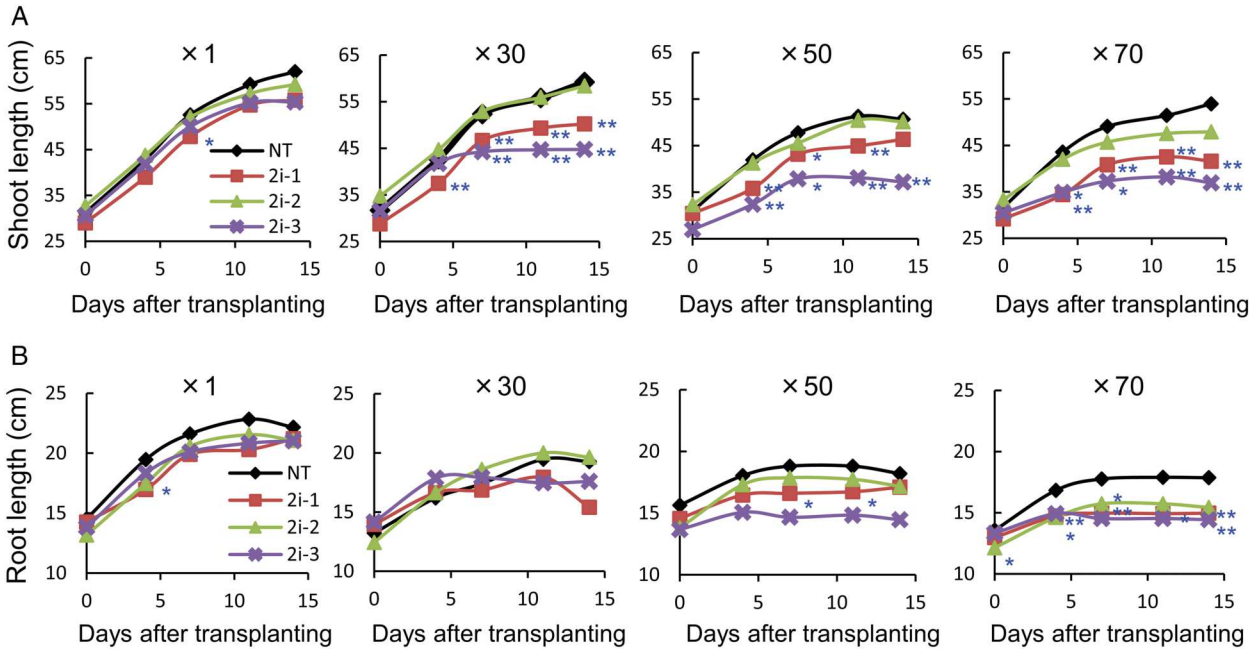

Fig. 3. Growth of NT and *HRZ*-knockdown (lines 2i-1, 2 and 3) rice during Fe treatments. (A) Shoot length. (B) Root length. Mean values (n = 3) are shown. Plants were grown hydroponically under control (×1) and excess Fe (×30, ×50 and ×70) conditions at pH 4.0. Asterisks indicate significant differences compared to the NT level at each time point (*$P < 0.05$, **$P < 0.01$).

lower concentrations of Cu in the newest leaves of all three *HRZ*-knockdown lines (Fig. S2 in Appendix S1). These results indicate that the *HRZ*-knockdown lines specifically hyperaccumulated Fe in leaves, particularly older leaves, under excess Fe conditions.

HRZ-knockdown roots tended to have slightly higher concentrations of Zn than those in NT under both the ×1 and ×30 Fe conditions (Fig. S2 in Appendix S1). Root Cu and Mn concentrations were similar between the *HRZ*-knockdown lines and NT

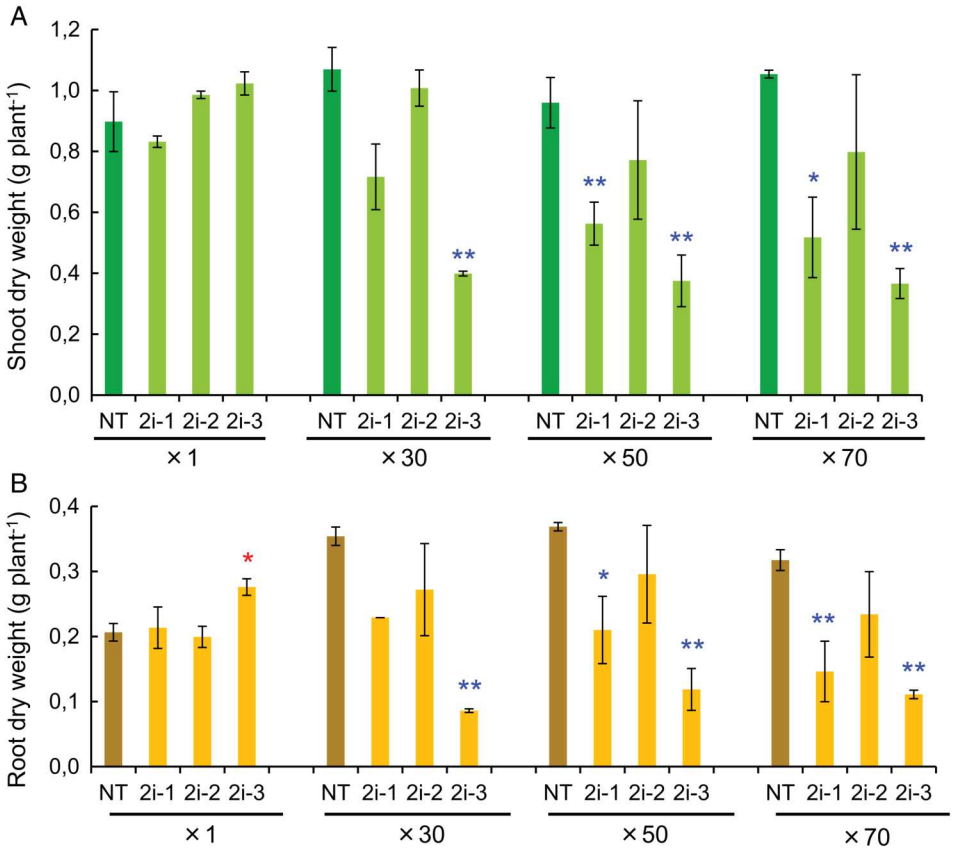

Fig. 4. Dry weight of NT and *HRZ*-knockdown (lines 2i-1, 2 and 3) rice after 14 days Fe treatments. (A) Shoot dry weight. (B) Root dry weight. Means ± SD (n = 2 for ×1 and ×30, n = 3 for ×50 and ×70) are shown. Plants were grown hydroponically under control (×1) and excess Fe (×30, ×50 and ×70) conditions at pH 4.0. Asterisks indicate significant differences compared to the NT level at each condition (*$P < 0.05$, **$P < 0.01$).

under the ×1 and ×30 Fe conditions (Fig. S2 in Appendix S1).

The *HRZ*-knockdown lines hyper-express Fe deficiency-inducible genes even more under excess Fe conditions

Next, we analyzed transcript levels of representative genes involved in Fe deficiency responses in the roots of the *HRZ*-knockdown lines and NT (Fig. 6). We used *HRZ*-knockdown lines 2i-1 and 2i-2 because the roots of line 2i-3 were severely damaged under ×30 and higher Fe conditions, and we were unable to extract proper RNA. Under the ×1 Fe condition, *O. sativa Nicotianamine Synthase 1* (*OsNAS1*), *OsNAS2*, *Transporter Of Mugineic acid 1* (*TOM1*), *O. sativa Yellow Stripe-Like 15* (*OsYSL15*), *OsIRO2*, *O. sativa Iron-Regulated Transporter 1* (*OsIRT1*) and *OsYSL2*, typical Fe deficiency-inducible genes involved in Fe uptake and translocation (Kobayashi et al. 2014 and references therein), showed higher expression in the *HRZ*-knockdown lines compared to NT except for decreased expression of *OsYSL2* in line 2i-2 (Fig. 6A), consistent with previous results (Kobayashi et al. 2013). In addition, expression of these genes in NT was similar or still lower under the ×30 Fe condition compared to the ×1 Fe condition (Fig. 6A), consistent with induction of these genes under Fe-deficient conditions (Kobayashi et al. 2014). Nevertheless, expression of these genes was not repressed at all under the ×30 Fe condition in the *HRZ*-knockdown lines but was much higher than under the ×1 Fe condition, particularly in line 2i-2, except for *OsIRT1* in lines 2i-1 and 2i-2 and *OsYSL2* in line 2i-2 (Fig. 6A). Similar expression patterns were also observed for *O. sativa Lipoxygenase 2;1* (*OsLOX2;1*), *OsLOX2;3* and *O. sativa Jasmonate ZIM-domain 1* (*OsJAZ1*), representative genes involved in JA biosynthesis and signaling (Fig. 6B). These results indicate that HRZs are crucial for repressing Fe deficiency-involved genes to a greater extent under excess Fe conditions.

We also analyzed the expression of Fe overload-inducible genes, *O. sativa Ferritin 1* (*OsFer1*) and *OsFer2*, which encode Fe storage proteins (Stein et al. 2009b). Summation of *OsFer1* and *OsFer2* expression

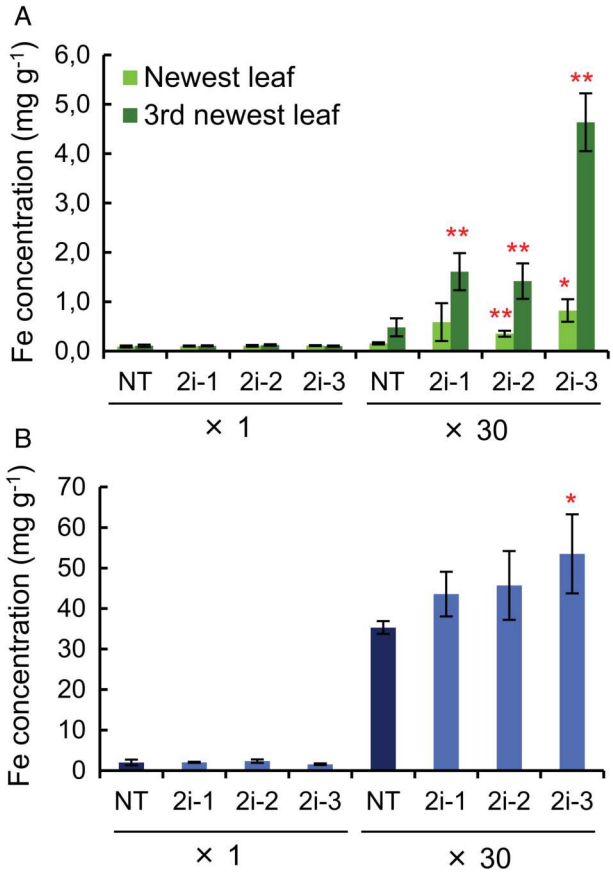

Fig. 5. Fe concentrations of NT and *HRZ*-knockdown (lines 2i-1, 2 and 3) rice after 14 days Fe treatments. (A) Leaf Fe concentrations. Pale and dark bars indicate concentrations in the newest and third newest leaves, respectively. (B) Root Fe concentrations. Means ± SD (n = 3) are shown. Plants were grown hydroponically under control (×1) and excess Fe (×30) conditions at pH 4.0. Asterisks indicate significant differences compared to the NT level for each condition and plant part (*$P < 0.05$, **$P < 0.01$).

was increased under the ×30 Fe condition compared to the ×1 Fe condition in NT (Fig. 6C), consistent with previous report (Stein et al. 2009b). Expression of *OsFer1* plus *OsFer2* was lower in the *HRZ*-knockdown lines under the ×1 Fe condition, but was higher under the ×30 Fe condition compared to NT (Fig. 6C), suggesting that the *OsFer* expression is regulated in a manner distinct from that of Fe deficiency-inducible genes, and is also misregulated in the *HRZ*-knockdown plants.

The *HRZ*-knockdown lines grow healthily but hyper-express Fe deficiency-inducible genes under milder excess Fe

We also tested a milder excess Fe condition which contained 500 μM Fe(III)-EDTA plus about 19 μM Fe^{2+} supplied by Tetsuriki-Type X fertilizer (Kobayashi et al. 2010) at pH 5.5 [×5 Fe(III) + Type X] for 7 days, in comparison with a standard control condition containing 100 μM Fe(III)-EDTA [×1 Fe(III)] (Fig. S3 in Appendix S1). The *HRZ*-knockdown lines did not show any Fe toxic symptoms or growth retardation under such condition. Expression analysis of typical Fe deficiency-inducible genes after 7-day treatment revealed that these genes are strongly repressed under the ×5 Fe(III) + Type X condition in NT. However, the *HRZ*-knockdown lines still hyper-expressed these genes under this condition, showing a greater difference in the expression ratios with the NT compared with the ×1 Fe(III) condition (Fig. S3 in Appendix S1). These results indicate that HRZs are functional under a wide range of excess Fe conditions, even though visible Fe toxicity symptoms appear only under severe excess Fe.

Discussion

In the present report, we provide evidence that *HRZ*-knockdown lines are hypersensitive to severe Fe-excess conditions, that is 1071 μM (×30) or more Fe^{2+} at pH 4.0 (Figs. 1–4; Fig. S1 in Appendix S1). These results indicate that HRZs are crucial for tolerance of excess Fe in rice. Of the three *HRZ*-knockdown lines tested, line 2i-3 showed the highest degree of susceptibility. This line corresponded to the most tolerant line under Fe-deficient conditions and also to the line with the strongest repression of *OsHRZ1* and *OsHRZ2* expression (Kobayashi et al. 2013). This observation suggests a possible negative correlation between *HRZ* transcript levels and susceptibility to excess Fe as well as tolerance of Fe deficiency. However, the second most hypersensitive line under excess Fe was line 2i-1, which did not correspond to the second most tolerant line under Fe-deficient conditions, which was line 2i-2 (Kobayashi et al. 2013). Unlike the other lines, line 2i-2 did not hyper-express *OsYSL2*, encoding an Fe(II)- and Mn-nicotianamine transporter responsible for internal Fe and Mn translocation (Koike et al. 2004, Ishimaru et al. 2010), in either Fe-sufficient, -deficient or -excess conditions either in the present study (Fig. 6A) or in our previous study (Kobayashi et al. 2013), for unknown reasons. This feature of *OsYSL2* expression might have resulted in less susceptibility to severe excess Fe conditions.

The metal concentration analysis revealed that all three *HRZ*-knockdown lines accumulated much higher concentrations of Fe in shoots compared to NT under the ×30 Fe condition, but Fe accumulation was only moderately higher than that of NT in roots (Fig. 5). These results suggest that enhanced Fe translocation from roots to shoots might be the main reason for enhanced Fe

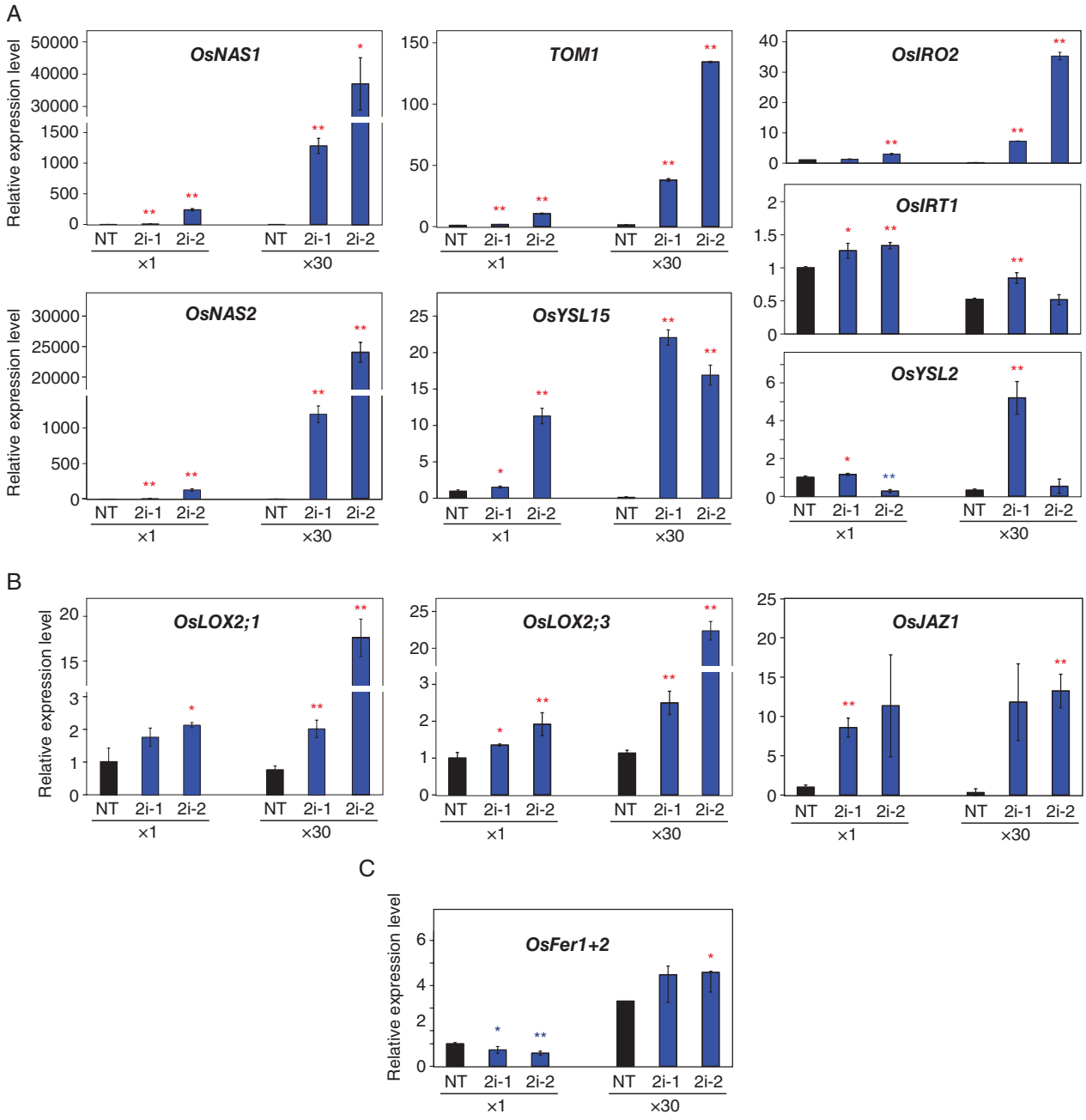

Fig. 6. Transcript levels of representative genes involved in Fe deficiency responses in NT and *HRZ*-knockdown (lines 2i-1, 2 and 3) rice roots after 14 days Fe treatments. (A) Genes involved in Fe uptake and translocation. (B) Genes involved in the JA biosynthesis and signaling. (C) Genes involved in Fe storage. Plants were grown hydroponically under control (×1) and excess Fe (×30) conditions at pH 4.0. Roots were harvested and used for quantitative RT-PCR analysis. Transcript abundance was normalized against the rice α-2 tubulin transcript level and expressed as a ratio relative to the levels in NT under the ×1 Fe condition (means ± SD, n = 3). Asterisks indicate significant differences compared to the NT level at each condition (*$P < 0.05$, **$P < 0.01$). *OsFer1 + 2* indicates the summation of *OsFer1* and *OsFer2* expression.

toxicity in the *HRZ*-knockdown lines. A previous study revealed enhanced Fe accumulation in *HRZ*-knockdown lines in both leaves and seeds under both normal and low Fe availability in soil and hydroponic cultures (Kobayashi et al. 2013). In the present study, enhanced accumulation of Fe was observed under the ×30 but not the ×1 Fe condition. The ×1 Fe condition in our present experiment was quite different from the previous control condition: the latter contained Fe(III)-EDTA at pH 5.5 instead of Fe^{2+} at pH 4.0. The *HRZ*-knockdown lines hyper-express the genes involved in Strategy II-based Fe(III) uptake more strongly than the Fe^{2+} uptake transporter gene *OsIRT1* either in the present study (Fig. 6A) or in our previous study (Kobayashi et al. 2013), which might explain the differences in the Fe concentration trend in leaves under control Fe condition.

We previously showed that the Zn concentration consistently increases in *HRZ*-knockdown seeds compared to those of NT under both normal and low Fe availability in soil, whereas it increases less consistently in leaves (Kobayashi et al. 2013). In the present study, Zn concentrations in leaves and roots were similar or slightly increased in the *HRZ*-knockdown lines compared to NT (Fig. S2 in Appendix S1). Because an increase in Fe and Zn concentrations in the edible parts of plants in a wide range of growth conditions is an extremely important trait for future applications of Fe- and Zn-fortified crops, our results provide baseline data for further examinations of Fe and Zn accumulation traits under various growth conditions. In contrast to Fe concentrations in roots and leaves varying dependent on growth conditions, Fe concentration in rice seeds is strictly controlled and is similar under Fe-sufficient and -deficient conditions (Kobayashi et al. 2013), highlighting superiority of certain genotypes such as the *HRZ*-knockdown lines which accumulate high Fe in seeds. Further analysis will be needed regarding Fe concentrations in the seeds of the *HRZ*-knockdown lines grown under excess Fe conditions to understand the traits of these lines.

In addition to mineral fortification, tolerance of low Fe availability is another important trait of the *HRZ*-knockdown lines. We revealed that these lines, particularly the most tolerant line under low Fe conditions (i.e. line 2i-3), were hypersensitive to excess Fe. However, our growth conditions, 1071 μM (×30) or more Fe^{2+} at pH 4.0, represent a very severe Fe excess and the NT rice used in the present study (Tsukinohikari cultivar) is one cultivar that is highly tolerant of excess Fe (data not shown). Furthermore, the low pH used in this study is also an important factor. In fact, the toxic effects of Fe occur under low pH conditions because Fe in soil solution rarely precipitates as various oxides, hydroxides or carbonate at low pH (Nozoe et al. 2008). In comparison, the *HRZ*-knockdown lines grew healthily without any symptoms under a milder excess Fe condition at pH 5.5 (Fig. S3 in Appendix S1). These observations suggest that future application of *HRZ* knockdown might not be limited by Fe toxicity problems except in severely acidic soils.

We analyzed the transcript expression levels of typical Fe uptake/translocation-related genes induced by Fe deficiency (Fig. 6A). Notably, repression of these genes was severely disrupted in the *HRZ*-knockdown lines and their expression levels were rather increased under ×30 Fe compared with under ×1 Fe (Fig. 6A), whereas strong repression of these genes was observed under higher Fe availability in NT roots. This expressional feature might account for the enhanced Fe translocation from roots to shoots (Fig. 5) and ultimately more severe Fe toxicity. Tolerance of Fe toxicity can also be affected by other factors. For example, rhizospheric oxidization of Fe^{2+} by oxygen transport from shoots to roots through the aerenchyma causes precipitation of Fe on the root surface (Asch et al. 2005, Deng et al. 2010, Abiko et al. 2012). Some tolerant cultivars have larger diameter pith cavities in shoots and the primary root that increase the absolute volume of aerenchyma and the number of lateral roots, increasing root oxidation power and Fe exclusion ability (Wu et al. 2014). We analyzed the expression of genes involved in the formation of lysigenous aerenchyma in rice roots (Yamauchi et al. 2017), but these genes were not repressed by *HRZ* knockdown of roots under normal conditions (GEO Series accession number GSE39906, Kobayashi et al. 2013), which suggests that the *HRZ*-knockdown lines might not be defective in the formation of aerenchyma. We observed enhanced expression of ferritin genes in *HRZ*-knockdown roots under the ×30 Fe condition (Fig. 6C), suggesting a Fe overload in root symplast because ferritin genes are induced in response to intracellular Fe overload (Briat et al. 1995, Stein et al. 2009b). The *HRZ*-knockdown lines showed more pronounced Fe hyperaccumulation and severe bronzing in older (third newest) leaves than in the newest leaves (Figs. 2 and 5A). This suggests that the older leaves are the main tissues of Fe susceptibility of the *HRZ*-knockdown lines, where bronzing might be caused either by enhanced formation of an Fe oxide plaque or different mechanisms involving Fe entry into the cells and/or aberrant distribution. Further analysis on Fe localization in tissues or organelles will shed light on precise mechanisms of Fe susceptibility of the *HRZ*-knockdown lines.

Our results indicate that HRZs repress the expression of genes involved in Fe uptake/translocation more actively under excess Fe (Fig. 6A). Genes

involved in JA biosynthesis and signaling are also regulated similarly (Fig. 6B), suggesting a conserved pathway of HRZ-mediated regulation among Fe uptake/translocation and JA-related genes. Less pronounced enhancement of HRZ-mediated repression was also observed under a milder excess Fe (Fig. S3 in Appendix S1). Considering these results, along with the previous observation that such HRZ function is more evident under Fe-sufficient conditions than under Fe-deficient conditions (Kobayashi et al. 2013), HRZs are thought to be activated by an abundance of Fe. This notion is also compatible with the possible function of HRZs as intracellular Fe sensors that might alter or modify their own activity or stability by binding directly to either Fe, Zn or both (Kobayashi and Nishizawa 2014, 2015). Further biochemical analyses including determination of affinities/dissociation constants of the HRZ-metal bindings will be important for clarifying the underlying molecular mechanisms.

Given our evidence that HRZs are functional and physiologically crucial under excess Fe conditions, transcriptional induction of *HRZ* genes under Fe-deficient conditions appears somewhat counterintuitive. Whether the expression level of HRZ proteins is also dependent on Fe nutritional status is unknown, although HRZ proteins are susceptible to 26S proteasome-mediated degradation in vitro under both Fe-sufficient and -deficient conditions to similar degrees (Kobayashi et al. 2013). BTS is thought to be a functional ortholog of HRZ in Arabidopsis because of the high similarity in both the amino acid sequence and phenotypes of knockdown or loss-of-function mutants (Long et al. 2010, Kobayashi et al. 2013, Selote et al. 2015, Hindt et al. 2017). In vitro results show less abundant production of the BTS protein in the presence of Fe, which suggests a preferred function under Fe-deficient conditions (Selote et al. 2015). Nevertheless, Hindt et al. (2017) reported a novel BTS mutant, *bts-3*, in which the expression of many Fe deficiency-inducible genes are derepressed under Fe-sufficient but not Fe-deficient conditions. This mutant accumulates high levels of Fe in roots, leaves and seeds and exhibits Fe toxicity symptoms when grown under Fe-sufficient conditions (Hindt et al. 2017). These results suggest that BTS is more functional under higher Fe concentrations, like HRZs, opposing a previous hypothesis by Selote et al. (2015) of a preferred function of BTS under Fe-deficient conditions. Hindt et al. (2017) proposed that *BTS* induction under Fe-deficient conditions might allow for quick turning off of the Fe deficiency response upon a sudden increase in Fe availability. This scenario might also be compatible with rice growing under semi-submerged conditions, in which seasonal variation in precipitation, flooding and drainage can cause sudden fluctuations in soil Fe availability for plants. For example, Fe^{2+} concentration increases sharply in reduced soil/solution with a low pH, because the Fe^{2+} oxidation rate decreases (Elec et al. 2013). In this scenario, induction of *HRZs/BTS* under Fe-deficient conditions could make sense even if their main function is to repress Fe deficiency responses to prevent excessive Fe uptake under excess Fe conditions. Further examinations will be needed to clarify the precise function of HRZs in Fe nutrition and to uncover the nature of Fe sensors and signals in plant cells.

Conclusions

We provide evidence that HRZ ubiquitin ligases are functional not only under Fe-deficient and Fe-sufficient conditions but even more so under excess Fe conditions, when they repress Fe deficiency responses. HRZs are crucial for tolerating severe excess Fe conditions. Our results support the possible function of HRZs as intracellular Fe sensors and provide information for future applications of *HRZs* to mineral-fortified crops with consistent growth under unfavorable Fe conditions.

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Author contributions

M. S. A., T. K., H. M. and N. K. N. designed the research. M. S. A., with assistance from H. M., performed most of the experiments. T. K. performed the gene expression analysis and milder excess Fe treatments. M. S. A. and T. K. analyzed the data. T. K. wrote the manuscript with assistance from M. S. A. and discussion with all the authors.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Appendix S1. Supporting Information.

Fig. S1. Plant appearance of NT and *HRZ*-knockdown rice during Fe treatments. (A) Whole shoot appearance at the onset of the Fe treatments. (B) Whole shoot appearance 4 days after the onset of the Fe treatments. (C) Whole shoot appearance 7 days after the onset of the Fe treatments. (D) Representative leaf appearance 4 days after the onset of the Fe treatments. Plants were grown hydroponically under control (×1) and Fe excess (×30, ×50, and ×70) conditions at pH 4.0.

Fig. S2. Metal concentrations of NT and *HRZ*-knockdown rice after 14-day Fe treatments. (A) Leaf Zn concentrations. (B) Leaf Cu concentrations. (C) Leaf Mn concentrations (A–C) Pale and dark green bars indicate concentrations in the newest and third newest leaves, respectively. (D) Root Zn concentrations. (E) Root Cu concentrations. (F) Root Mn concentrations. Means ± SD (n = 3) are shown. Asterisks indicate significant differences compared to the NT level for each condition and plant part (*$P < 0.05$; **$P < 0.01$).

Fig. S3. Growth feature and gene expression of NT and *HRZ*-knockdown rice during milder excess Fe treatments. (A) Shoot length. Plants were grown hydroponically under control [×1 Fe(III)] and excess Fe [×5 Fe(III) + Type X] conditions at pH 5.5. Mean values (n = 3–9) are shown. No significant differences were observed between the *HRZ*-knockdown and the NT levels at each time point. (B) Transcript levels of representative genes involved in Fe uptake and translocation. Roots were harvested after 7-day treatment and used for quantitative real-time-polymerase chain reaction analysis. Transcript abundance was normalized against the rice α-2 tubulin transcript level and expressed as a ratio relative to the levels in NT under the ×1 Fe(III) condition (means ± SD, n = 3). Numerals above the bars indicate expression ratios to the NT level at each condition. Asterisks indicate significant differences compared to the NT level at each condition (*$P < 0.05$, **$P < 0.01$).