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Correspondence and requests for materials should be addressed to N.K.N. (annaoko@mail.ecc.u-tokyo.ac.jp)

Iron biofortification in rice by the introduction of multiple genes involved in iron nutrition

Hiroshi Masuda[1,2], Yasuhiro Ishimaru[1,3], May Sann Aung[1], Takanori Kobayashi[1,2], Yusuke Kakei[1], Michiko Takahashi[1], Kyoko Higuchi[4], Hiromi Nakanishi[1] & Naoko K. Nishizawa[1,2]

[1]Global Agricultural Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan, [2]Research Institute for Bioresources and Biotechnology, Ishikawa Prefectural University, 1-308 Suematsu, Nonoichi, Ishikawa 921-8836, Japan, [3]Department of Faculty of Science and Graduate School of Science, Tohoku University, 6-3 Aramakiaza-aoba, Aoba-ku, Sendai, Miyagi 980-8578, Japan, [4]Department of Applied Biology and Chemistry, Tokyo University of Agriculture, 1-1-1, Sakuragaoka, Setagaya-ku, Tokyo 156-8502, Japan.

To address the problem of iron-deficiency anemia, one of the most prevalent human micronutrient deficiencies globally, iron-biofortified rice was produced using three transgenic approaches: by enhancing iron storage in grains via expression of the iron storage protein ferritin using endosperm-specific promoters, enhancing iron translocation through overproduction of the natural metal chelator nicotianamine, and enhancing iron flux into the endosperm by means of iron(II)-nicotianamine transporter *OsYSL2* expression under the control of an endosperm-specific promoter and sucrose transporter promoter. Our results indicate that the iron concentration in greenhouse-grown T_2 polished seeds was sixfold higher and that in paddy field-grown T_3 polished seeds was 4.4-fold higher than that in non-transgenic seeds, with no defect in yield. Moreover, the transgenic seeds accumulated zinc up to 1.6-times in the field. Our results demonstrate that introduction of multiple iron homeostasis genes is more effective for iron biofortification than the single introduction of individual genes.

Iron (Fe)-deficiency anemia is one of the most prevalent human micronutrient deficiencies in the world, affecting an estimated one-third of the world's population and causing 0.8 million deaths annually worldwide[1]. To address this problem, biofortification (i.e., the breeding of micronutrient-fortified crops) is advantageous for people who experience difficulty in changing their dietary habits because of financial, cultural, regional, or religious restrictions. Biofortification is also advantageous for governments because it is inexpensive and sustainable compared to nutritional supplement programs[2]. Rice is a particularly suitable target for biofortification because Fe-deficiency anemia is a serious problem in developing countries where rice is a major staple crop[1,3]. In addition, Fe translocation and Fe homeostasis in rice has begun to be understood at the molecular level[4].

Based on knowledge of Fe transportation and Fe homeostasis in rice, three approaches have been reported to produce Fe-biofortified rice. The first approach is enhancement of Fe accumulation in rice seeds by *ferritin* gene expression under the control of endosperm-specific promoters. Endosperm is the rice-seed tissue that accumulates a high concentration of starch and becomes the edible part of the seed after milling, at which point these seeds are known as polished or white seeds[3]. Ferritin is a ubiquitous protein for Fe storage and stores about 4,000 Fe atoms in a complex[5]. Goto *et al.* generated transgenic rice plants that expressed the soybean *ferritin* gene, *SoyferH1*, in the endosperm using the endosperm-specific 1.3-kb *GluB1* rice promoter; the transformants showed higher Fe accumulation in brown rice seeds[6]. A few reports have described the production of Fe biofortification rice by endosperm-specific expression of ferritin[7,8]. Qu *et al.* expressed *SoyferH1* under the control of both the *OsGlb1* promoter and 1.3-kb *GluB1* promoter to further increase seed Fe concentration[9]. However, enhancement of *ferritin* expression did not produce further increases in seed Fe content[9]. Therefore, in addition to increased Fe storage in seeds, enhanced Fe uptake from the soil and enhanced translocation within the plant body are thought to be required to further improve Fe biofortification in seeds.

The second approach involves increasing Fe transportation within the plant body by enhancing the expression of *NAS* genes. Nicotinamine (NA) is a chelator of metal cations such as Fe(II) and Zn(II), and it is biosynthesized from *S*-adenosyl methionine via NA synthase (NAS)[10]. All higher plants synthesize and utilize NA for the internal transport of Fe and other metals[11,12]. Takahashi *et al.* produced NA-deficient transgenic tobacco plants that

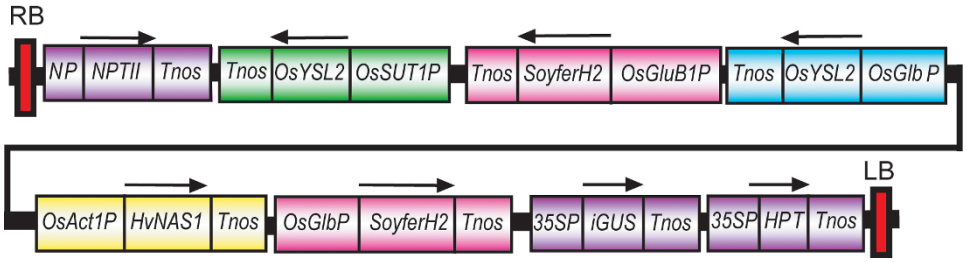

Figure 1 | The gene cassette introduced into rice to produce the Fer-NAS-YSL2 lines. Arrows show the direction of transcription. RB, right border; LB, left border; *NP*, *Agrobacterium tumefaciens* nopaline synthase gene (AF485783) promoter region; *NPTII*, neomycin phosphotransferase II gene (AF485783); *Tnos*, *A. tumefaciens* nopaline synthase gene terminator (AF485783); *OsSUT1P*, promoter region of the rice sucrose transporter gene *OsSUT1* (D87819); *OsYSL2*, rice nicotianamine–metal transporter gene (AB126253); *OsGluB1P*, 2.3-kb promoter region of the *OsGluB1* gene (AY427569)[41]; *SoyferH2*, soybean *Ferritin* gene (AB062754)[42]; *OsAct1P*, promoter region of the rice *OsActin1* gene (Os03g0718100); *HvNAS1*, barley nicotianamine synthase 1 gene (AB010086); *OsGlbP*, promoter region of the 26 kDa *OsGlb1* gene (AY427575)[41]; *iGUS*, *β*-glucuronidase gene with an intron (AF485783); *35SP*, cauliflower mosaic virus *35S* promoter (U28417); *HPT*, hygromycin phosphotransferase gene (K01193).

showed young leaves with serious chlorosis, and Fe and Zn concentrations in the leaves and flowers decreased as a result of disrupted internal metal transport. These reports suggest that NA plays an essential role in Fe translocation to seeds. In addition, overexpression of the barley *NAS* gene, *HvNAS1*, led to increased Fe and Zn concentrations in the leaves, flowers, and seeds of tobacco plants[12]. Likewise, overexpression of the NA synthase gene increased the Fe concentration in polished rice seeds threefold with greenhouse cultivation[13–15].

The third approach is enhancement of Fe flux into the endosperm by expression of the Fe(II)-NA transporter gene *OsYSL2*. Koike *et al.* identified the rice NA-Fe(II) transporter gene *OsYSL2*, which is preferentially expressed in leaf phloem cells, the vascular bundles of flowers, and developing seeds, suggesting a role in internal Fe transport[16]. *OsYSL2* knockdown mutant plants exhibit a 30% decrease in Fe concentration in the endosperm[17]. Simple overexpression of *OsYSL2* by the *35S* promoter did not increase Fe concentration in seeds. In contrast, enhancement of *OsYSL2* expression under the control of the rice sucrose transporter promoter *OsSUT1*, which drives high expression in the panicle and immature seeds during the seed maturation stage, increased Fe concentration in polished rice seeds by up to threefold[17].

Additionally, introduction of mugineic acid synthase gene was reported as another approach to increase Fe concentration in seeds. In graminaceous plants, NA is the precursor of mugineic acid family phytosiderophores (MAs), which are natural Fe(III) chelators used in Fe acquisition from the rhizosphere[18,19]. Graminaceous plants synthesize and secrete MAs into the rhizosphere by TOM1 transporter[20]. They form Fe(III)–MAs complexes and are taken up into the root via YS1 and YSL transporters[21,22]. Rice biosynthesizes 2′-deoxymugineic acid (DMA), which facilitates Fe uptake and internal transport[23,24]. Barley biosynthesizes not only DMA but also mugineic acid (MA) by MA synthase, IDS3[25,26]. However, rice lacks *IDS3* gene and does not produce MA. We previously reported that Fe concentration in polished rice seed increased up to 1.25 and 1.4 times in calcareous and normal soil cultivation in field, respectively, by introduction of barley *IDS3* genome fragment[27,28].

Each of these approaches could increase Fe concentration in polished rice seeds. However, a higher Fe concentration in seeds was required to reduce the human Fe deficiency anemia health problem. The target Fe concentration in polished rice seeds is over 15 ppm in field cultivation[29], but research has not yet achieved this yield. We hypothesized that a combination of these transgenic approaches would further increase the Fe concentration in seeds.

Here, we combined these three approaches and produced new transgenic rice lines with enhanced Fe accumulation in seeds using the Soybean *ferritin* gene under the control of two endosperm-specific promoters, the *OsGlb1* and 2.3-kb *OsGluB1*. Furthermore, these seeds exhibited enhanced Fe transportation within the plant body due to overexpression of *HvNAS1* and enhanced Fe translocation to seeds due to *OsYSL2* expression under the control of the *OsSUT1* promoter and *OsGlb1* promoter **(Supplementary Fig. 1)**. Gene insertion, ferritin accumulation in seeds, and higher expression of *OsYSL2* and *HvNAS1* were confirmed. The Fe concentration in polished T_2 seeds increased by up to sixfold in plants grown in soil in a greenhouse. In field cultivation, the Fe concentration in T_3 polished seeds increased up to 4.4-fold. This is the first report of the combination of three approaches to increase Fe accumulation in seeds.

Results

Selection of Fer-NAS-YSL2 transgenic rice lines. We produced 45 independent transgenic rice lines (*Oryza sativa* cultivar Tsukinohikari; Fer-NAS-YSL2 line). These included the *OsGlb1* promoter–*SoyferH2*, *OsGluB1* promoter–*SoyferH2*, *OsActin1* promoter–*HvNAS1*, and *OsSUT1* promoter–*OsYSL2*, and the *OsGlb1* promoter–*OsYSL2* cassettes (**Fig. 1**). We used soybean *ferritin*, *SoyferH2*, as the *ferritin* gene. For comparison, single gene-introduced lines for *OsActin1* promoter–*HvNAS1* (AN; ref.13) and *OsSUT1* promoter–*OsYSL2* (SY; ref.17) were also used. T_0-regenerated plants were cultivated in a greenhouse, and those lines with a high Fe content in their T_1 polished seeds were selected (**Supplementary Fig. 2**). These lines carried all the introduced genes, as confirmed by genomic polymerase chain reaction (PCR) (**Fig. 2**). Ferritin accumulation in T_2 seeds was detected by Western blot analysis (**Fig. 3**). Enhanced expression of *OsYSL2* and *HvNAS1* in immature T_3 seeds of selected transgenic lines was confirmed by real-time RT-PCR analysis (**Fig. 4**). The Fe concentration in the T_2 seeds had increased up to sixfold in the Fer-NAS-YSL2 lines, threefold in the AN lines, and twofold in the SY lines compared to the NT line (**Fig. 5**). Thus, the introduction of multiple genes is a more effective method of increasing Fe concentrations in greenhouse-cultivated rice grains than single introduction of either *HvNAS1* or *OsYSL2*.

Field trial of Fer-NAS-YSL2 transgenic rice lines. In greenhouse cultivation, no environmental stress exists that reflects real cultivation in the paddy field. For practical usage, we therefore investigated whether Fer-NAS-YSL2 lines set seeds with increased Fe concentration under actual paddy field conditions. For this purpose, selected T_2 lines with high Fe content in their polished seeds (**Fig. 5**, arrows) were cultivated in an isolated paddy field in Jinju, Gyeongsang Province, Korea (**Supplementary Figs. 3 and 4**). The mean Fe concentrations in T_3 polished seeds from the NT, AN, and Fer-NAS-YSL2 lines were 0.9, 1.5, and 4.0 μg/g dry weight, respectively (**Fig. 6a**). Notably, the Fe concentration in the Fer-NAS-YSL2 seeds was 4.4-times higher compared to NT seeds and 2.7-times higher compared to AN seeds (**Fig. 6a**). Our results

demonstrate that multiple introductions of Fer-NAS-YSL2 are better than a single introduction of either *OsActin1* promoter–*HvNAS1* or *OsSUT1* promoter–*OsYSL2* under field conditions. In addition, the Fer-NAS-YSL2 seeds also accumulated zinc (Zn), another important micronutrient for humans, up to 1.6-fold more (**Fig. 6b**). Fer-NAS-YSL2 seeds did not accumulate the toxic heavy metal cadmium (Cd) more than seen in the NT line (**Fig. 6e**). In brown seeds, Fe and Zn concentrations in the Fer-NAS-YSL2 and AN lines were similar, having increased by up to 1.6- and 1.4-fold, respectively, compared to NT lines (**Table 1**). These increases in Fe and Zn concentration in brown rice might have been due to the *OsActin1* promoter–*HvNAS1* gene. In contrast, the Fe concentration in polished seeds increased to a greater degree in Fer-NAS-YSL2 lines than in the AN or SY lines. Moreover, in the field experiment, no negative effect on yield was found in terms of the panicle length, number of panicles per hill, number of total grains per panicle, number of total grains per hill, percentage of filled grain, total weight of grains per hill, or 1,000-grain weight in lines 19-2, 19-4, and 19-5, which had a high seed Fe concentration (**Supplementary Table 1**).

In addition to NA, DMA, which is synthesized from NA, is known as a natural chelator of metal cations in graminaceous plants[23,24]. The NA and DMA concentrations in T_3-brown seeds of Fer-NAS-YSL2 lines increased by up to six- and threefold, respectively, compared to NT brown seeds, and to a similar level in the AN line (**Fig. 7**).

Discussion

To produce Fe-biofortified rice, we combined three transgenic approaches. First, we enhanced Fe storage in grains via expression of the Fe storage protein ferritin using endosperm-specific promoters. Second, we enhanced Fe translocation through overproduction of the natural metal chelator nicotianamine. Third, we enhanced Fe flux into the endosperm through expression of the Fe(II)–NA transporter *OsYSL2* under the control of an endosperm-specific promoter and sucrose transporter promoter (**Supplementary Fig. 1**). As a result, Fe concentration of polished seeds increased up to sixfold in greenhouse cultivation and 4.4-fold in paddy field cultivation (**Figs. 5 and 6a**).

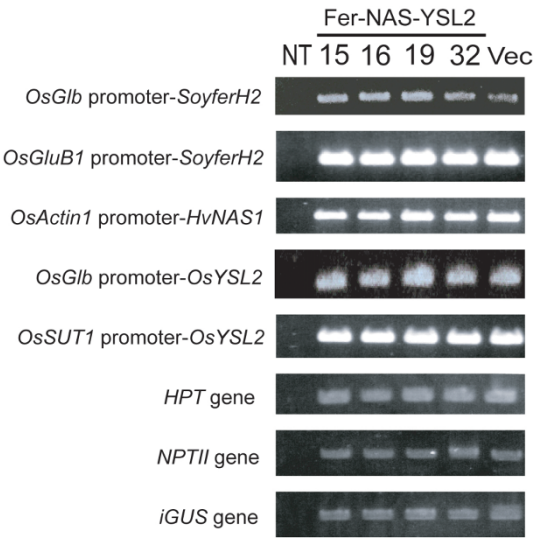

Figure 2 | Detection of transgene insertion in the transgenic lines. Rice genomic DNA was extracted from young leaves of selected T_1 plants cultivated in a greenhouse and used as the template for PCR. NT, non-transgenic rice; numerals, Fer-NAS-YSL2 transgenic lines No. 15, 16, 19, and 32, respectively; Vec, the vector containing the Fer-NAS-YSL2 cassette (vector o in **Supplementary Fig. 5**; positive control).

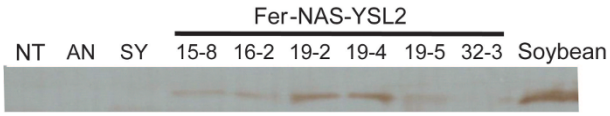

Figure 3 | Ferritin accumulation in T_2 brown seeds. Six mature T_2 brown seeds harvested from a greenhouse were subjected to Western blot analysis. NT, non-transgenic rice; AN, *OsActin1* promoter–*HvNAS1* line 8[13]; SY, *OsSUT1* promoter–*OsYSL2* line 18[17]; numerals, Fer-NAS-YSL2 transgenic lines No. 15-8, 16-2, 19-2, 19-4, 19-5, and 32-3, respectively; soybean, protein extracts from non-transformed soybean cotyledons (positive control).

In this article, three approaches were logically combined to enhance Fe(II)–NA translocation into seeds more effectively than previous methods. Overexpression of the *NAS* gene increases the NA concentration in the plant body. Abundant NA facilitates formation of Fe(II)–NA, which is stable under higher pH conditions, such as in phloem sap (pH 8.0)[30,31]. Consequently, Fe(II) transport in the plant body, including the phloem, is improved by *NAS* overexpression[13,14]. For effective translocation of enhanced Fe(II)–NA in phloem sap, we engineered the Fe(II)–NA transporter OsYSL2 to be under the control of the *OsSUT1* promoter, which expresses in the companion cell of phloem in flag leaves and rachis[32]. The rice antisense mutant of *OsSUT1* markedly reduces the sucrose uptake ability and filling rate of rice seeds[33]. Therefore, OsSUT1 is a key transporter of sucrose from the phloem to seeds and the *OsSUT1*

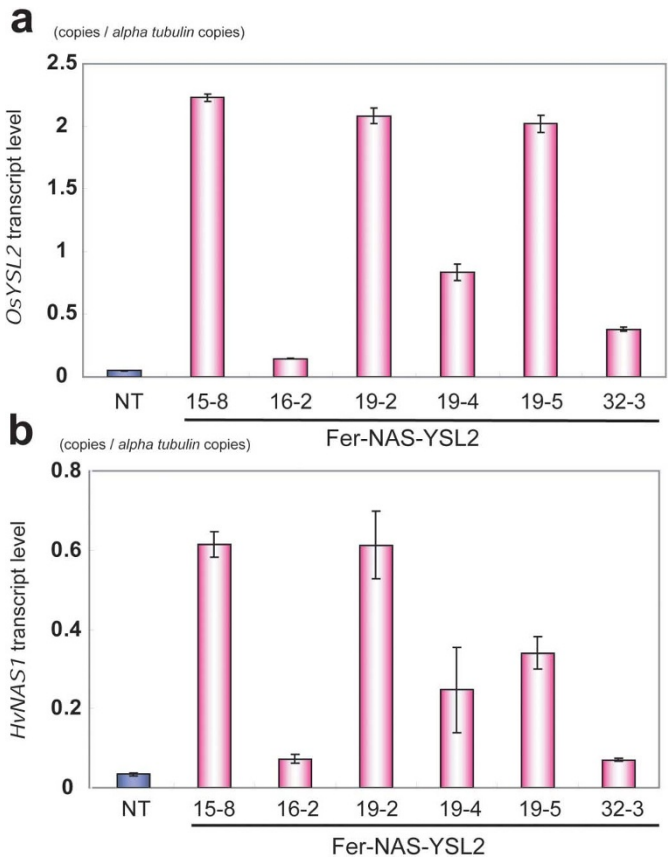

Figure 4 | Quantitative real-time RT-PCR analysis of *OsYSL2* and *HvNAS1*. (a) *OsYSL2* and (b) *HvNAS1* expression levels. T_2 plants were cultivated in commercially supplied soil (Bonsolichigou) in a greenhouse. Total RNA was extracted from immature T_3 seeds (seeds at an early milky stage, 10 days after fertilization) from each line (n = 3). Bars represent the means ± standard errors of three independent real-time RT-PCR reactions.

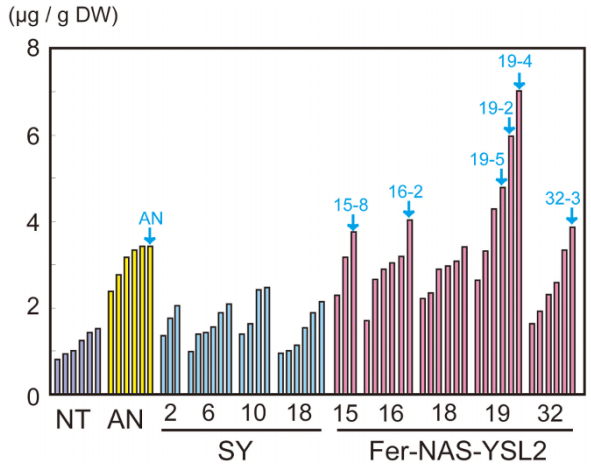

Figure 5 | Fe concentration of T_2 polished seeds. T_2 seeds from the ear of the main tiller were harvested in a greenhouse and polished using a Multi-beads Shocker® (Yasuikikai, Osaka, Japan), and the Fe concentration was measured by ICP-AES (SPS1200VR; Seiko Instruments Inc., Chiba, Japan), as described in ref.[13]. Bars represent the Fe concentration in polished seeds from independent transgenic or non-transgenic plants (n = 1). Numerals indicate the line numbers of independent T_1 lines. The arrows and numerals above the graphs show lines with a high Fe content selected for the field trial. NT, non-transgenic rice; AN, *OsActin1* promoter–*HvNAS1* transgenic rice line No. 8[13]; SY, *OsSUT1* promoter–*OsYSL2* transgenic rice lines[17]; Fer-NAS-YSL2, transgenic rice lines carrying the *OsGlb1* promoter–*Ferritin*, *OsGluB1* promoter–*Ferritin*, *OsSUT1* promoter–*OsYSL2*, *OsGlb1* promoter–*OsYSL2*, and *OsActin1* promoter–*HvNAS1*.

promoter is suitable for the expression of *OsYSL2* to enhance Fe(II)–NA translocation in seeds[17]. Additionally, *OsYSL2* expressed in endosperm cells under the control of the *OsGlb1* promoter may enhance transport of Fe(II)–NA into endosperm cells. Moreover, *ferritin* expressed under the control of the *OsGlb1* promoter and the *OsGluB1* promoter accumulates Fe in seed endosperm cells. Fe incorporation into ferritin requires it to be in the Fe(II) form[5]. Therefore, enhancement of Fe(II)–NA transportation to seeds is important for Fe accumulation in seed ferritin, rather than Fe(III)–citrate, Fe(III)–DMA, or other Fe(III)–chelator complexes. As a result, Fe concentration in the seeds of Fer-NAS-YSL2 rice was markedly elevated compared to the result of a single application of each approach **(Figs. 5 and 6a)**.

The Japanese rice variety used in this research, Tsukinohikari, contained a low Fe concentration, 0.9 μg/g in field-cultivated polished seeds **(Fig. 6a)**. In some rice varieties, the Fe concentration of polished rice is higher than that of this variety[34]. Thus application of this transgenic approach to higher Fe concentration varieties has the potential to achieve the target Fe concentration for human health.

In addition to the increased Fe concentration in seeds, many advantages exist in using Fer-NAS-YSL2 rice. Fe-biofortification of polished rice by overexpression of *NAS* mitigated Fe-deficiency anemia in mice to a greater degree than the seeds of NT rice[14]. Increasing the NA concentration by enhancing *NAS* expression may improve the bioavailable mineral content of rice grains. Additionally, Fe is well absorbed by the human gastrointestinal tract from soybean ferritin[35]. These results support the notion that Fe-fortification of rice by endosperm expression of *ferritin* and overexpression of *NAS* represents an effective human dietary Fe source. Moreover, increased NA in rice will likely reduce the rates of high-blood-pressure disease[36].

Note that the Zn concentration also increased in Fer-NAS-YSL2 lines **(Fig. 6b)**. Some reports show that higher NA production increases the Zn concentration in seeds of rice plants[13,15,37]. Endosperm-specific *ferritin* expression also contributes to the increased Zn concentration in rice seeds[8]. Zn deficiency is one of the most critical micronutrient deficiency problems in human health[1], therefore representing an advantage of Fer-NAS-YSL2 rice in terms of practical applications.

We conclude that the introduction of multiple genes, including *ferritin*, under the control of endosperm-specific promoters, *NAS* overexpression, and *OsSUT1* and *OsGlb1* promoter-driven *OsYSL2* expression led to an increased concentration of bioavailable Fe in rice and will assist in mitigation of Fe-deficiency anemia globally.

Methods

Plant materials. *japonica* rice (*O. sativa* L.) cultivar Tsukinohikari was used as the non-transgenic control and for transformation. The *OsActin1* promoter–*HvNAS1* and *OsSUT1* promoter–*OsYSL2* transgenic lines were originally from refs.13 and 17, respectively.

Vector construction and rice transformation. Construction of the Fer-NAS-YSL2 transformation vector **(Fig. 1)** is shown in **Supplementary Fig. 5**. *Agrobacterium tumefaciens* (strain C58) was used to introduce the construct into *O. sativa* L. cv. Tsukinohikari using the method outlined in ref.38. Forty-five independent Fer-NAS-YSL2 lines were obtained as T_0 transgenic plants.

Greenhouse cultivation. T_0 transgenic plants, T_1 selected plants, and T_2 selected plants were cultivated in commercially supplied soil used for cultivation of rice nurseries in Japan (Bonsolichigou; Sumitomo Chemicals, Tokyo, Japan) with slow-release fertilizers (LongTotal-70 and -140; JCAM AGRI. Co. Ltd., Tokyo, Japan) in a greenhouse. Using the seeds obtained, high-Fe lines were selected based on the Fe concentration in T_1- **(Supplementary Fig. 2)** and T_2- **(Fig. 5)** polished seeds. T_3-immature seeds were used for gene expression analysis by real-time RT-PCR.

Detection of ferritin accumulation in T_2 brown seeds. Six mature T_2 brown seeds harvested from a greenhouse were homogenized with a mortar and pestle, soaked in extraction buffer [4% sodium dodecyl sulfate (SDS), 5% 2-mercaptethanol, 20% glycerol, 20 mM Tris–HCl, 8 M urea, and 0.1% bromophenol blue, pH 6.8], and shaken for 30 min. The resulting extracts were centrifuged at 13,000 rpm for 20 min and supernatant fractions were collected. Protein separation by SDS-polyacrylamide gel electrophoresis, transfer to polyvinylidene fluoride membranes, and detection with antibodies were performed as described in ref.6.

Quantitative real-time RT-PCR analysis. Total RNA was extracted from immature T_3 seeds (seeds at an early milky stage, 10 days after fertilization) of each line harvested from a greenhouse. Seeds were crushed using a Multi-beads Shocker® (Yasuikikai, Osaka, Japan). Next, RNA was extracted using an RNeasy Plant Mini Kit (Qiagen KK, Tokyo, Japan). First-strand cDNA was synthesized using ReverTra Ace (Toyobo, Osaka, Japan) with oligo-d$(T)_{30}$. Real-time RT-PCR was carried out using the 7300 Real-Time PCR System (Applied Biosystems, Tokyo, Japan) with SYBR Green I (Takara, Shiga, Japan) and ExTaq™ Real Time-PCR Version (Takara, Tokyo, Japan). The primers used were as follows: *OsYSL2* forward (5'-GAG GGA CAA CGG TGT CAT TGC TGG T-3') and *OsYSL2* reverse (5'-TGC AGA AAA GCC CTC GAC GCC AAG A-3') for *OsYSL2* expression, *HvNAS1* forward (5'-GGA CGT CGC CGA CCT CAC CCA G-3') and *HvNAS1* reverse (5'-CAG GGA CGC CCC CTC CAC C-3') for *HvNAS1* expression. Transcript levels were normalized to the expression levels of alpha-*Tubulin*, as determined using the primers alpha-*Tubulin* forward (5'-TCT TCC ACC CTG AGC AGC TC-3') and alpha-*Tubulin* reverse (5'-AAC CTT GGA GAC CAG TGC AG-3'). The sizes of the amplified fragments were confirmed by agarose gel electrophoresis.

Detection of transgene insertion in the transgenic lines. Total DNA was prepared from leaves of T_2-transgenic lines and the non-transgenic line, according to the method described in ref.39. The *OsGlb1* promoter–*SoyferH2* cassette was detected using the *OsGlb1* promoter forward primer (5'-GAG CTA AGA GTT ATC CCT AGG-3') and *SoyferH2* reverse primer (5'-CCA CAT TGA TCT GCT CGT TGA TTG C-3'). The 2.3-kb *OsGluB1* promoter–*SoyferH2* cassette was detected using the 2.3-kb *OsGluB1* promoter forward primer (5'-GCT TGC TAC GCA AAA TGA CAA CAT GC-3') and *SoyferH2* reverse primer. The *OsActin1* promoter–*HvNAS1* cassette was detected using the *OsActin1* promoter forward primer (5'-GCA GCG CAT GGG TAT TTT TTC TAG-3') and *HvNAS1* reverse primer (5'-GCA CTA GTA TTC CCG ATC TAG TAA CAT AGA TG-3'). The *OsGlb1* promoter–*OsYSL2* cassette was detected using the *OsGlb1* promoter forward primer and *OsYSL2* reverse primer (5'-GCC CAA CGA AGC TAA TCG CAA AGA GG-3'). The *OsSUT1* promoter–*OsYSL2* cassette was detected using the *OsSUT1* promoter forward primer (5'-CCC GTG AAA TAA TCG CAC CGT CTA G-3') and *OsYSL2* reverse primer. *HPT* was detected using the *HPT* forward primer (5'-CGG CAT CTA CTC TAT TCC TTT GC-3') and *HPT* reverse primer (5'-GTC TCC GAC CTG ATG CAG CTC-3'). *NPTII* was detected using the *NPTII* forward primer (5'-GAT GGA TTG CAC GCA GGT TCT C-3') and *NPTII* reverse primer (5'-GCC AAC GCT ATG TCC TGA TAG C-3'). *iGUS* was detected using the *iGUS* forward primer (5'-CTG TGG AAT TGA TCA

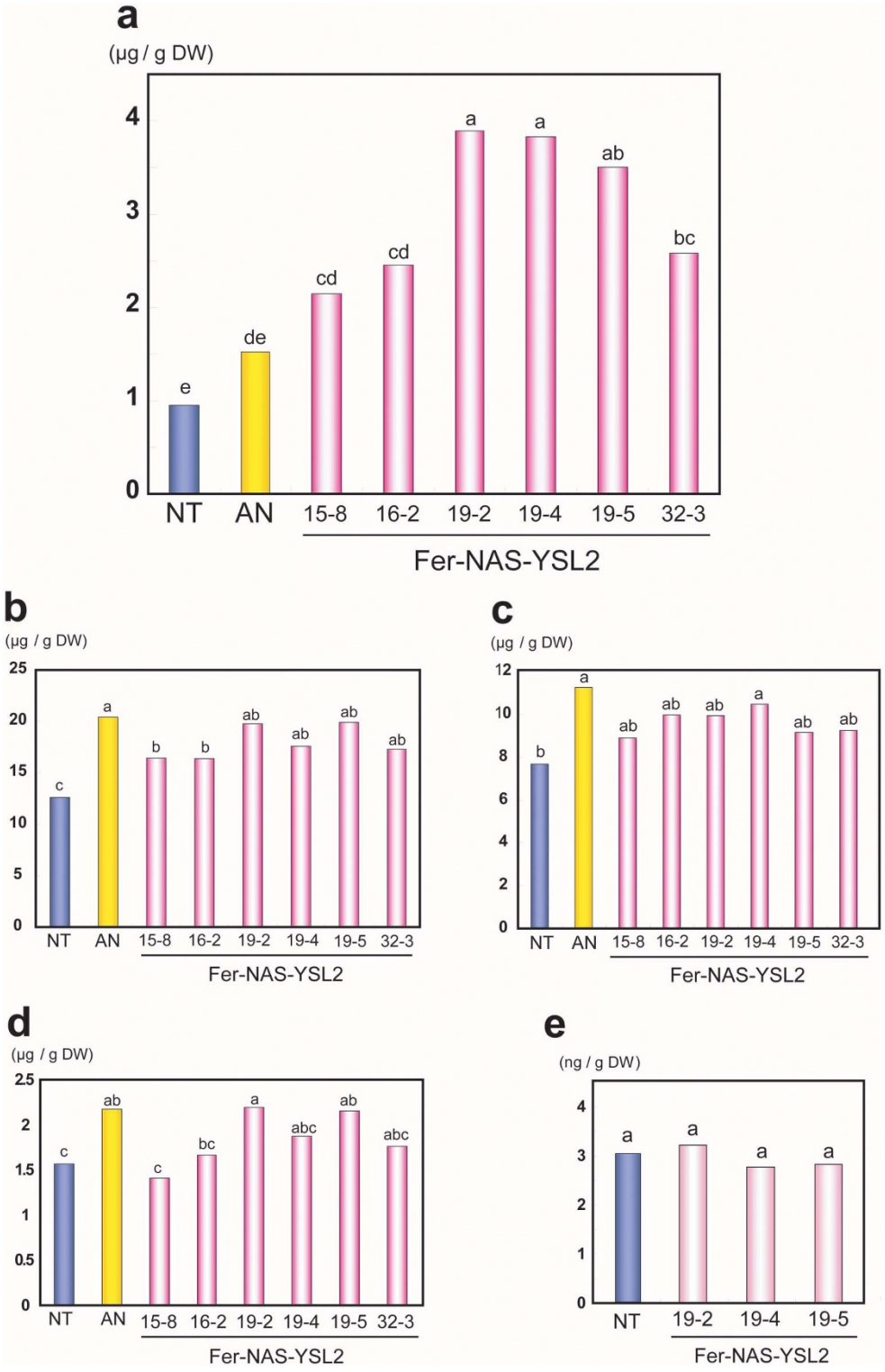

Figure 6 | Metal concentration in T_3 polished seeds obtained from the paddy field. (a) Fe concentration; (b) Zn concentration; (c) manganese (Mn) concentration; (d) copper (Cu) concentration; (e) cadmium (Cd) concentration. T_3 seeds from an ear of the main tiller were harvested from the field and polished, and the metal concentrations were measured (indicated as the average of the middle four hills in each block). ANOVA with Tukey–Kramer HSD test was used for each four-block data set (n = 4). Letters above the bars indicate significant differences ($P < 0.05$); (a, $P < 0.0204$; b, $P < 0.0428$; c, $P < 0.0314$; d, $P < 0.0396$). NT, non-transgenic rice; AN, *OsActin1* promoter–*HvNAS1* line 8[13]; numerals, Fer-NAS-YSL2 transgenic lines.

GCG TTG G-3') and *iGUS* reverse primer (5'-CGC AAG TCC GCA TCT TCA TGA C-3').

Cultivation in an isolated paddy field. Selected T_2 lines with a high Fe content in their polished seeds (**Fig. 5**, arrows) were used for field experiments. Seeds were germinated on 20 May 2009, as follows. First, seeds were surface-sterilized with 70% ethanol and then with 10% sodium hypochlorite and 0.1% Triton X-100 with shaking at 37°C for 30 min. Sterilized seeds were sown on half-strength Murashige and Skoog medium (sucrose 30 g/l, NH_4NO_3 1.65 g/l, KNO_3 1.9 g/l, $CaCl_2 \cdot 2H_2O$ 440 mg/l, $MgSO_4 \cdot 7H_2O$ 370 mg/l, KH_2PO_4 170 mg/l, Fe–EDTA 42.1 mg/l, H_3BO_3 6.2 mg/l, $MnSO_4 \cdot 4H_2O$ 22.3 mg/l, $ZnSO_4 \cdot 7H_2O$, 8.6 mg/l, KI 0.83 mg/l, $Na_2MoO_4 \cdot 2H_2O$ 250 ng/l, $CuSO_4 \cdot 5H_2O$ 25 ng/l, $CoCl_2 \cdot 6H_2O$ 25 ng/l, thiamine–HCl 100 ng/l,

Table 1 | Metal concentration in T_3 brown seeds obtained from the paddy field T_3 seeds from the ear of the main tiller were harvested from the middle four hills in each plot, and the Fe, Zn, Mn, and Cu concentrations in brown seeds were measured. Values are μg/g dry weight (DW). The data represent the means ± standard errors of four block replications for each line (n = 4) (Supplementary Fig. 3). NT, non-transgenic rice; AN, *OsActin1* promoter–*HvNAS1* line; 15-8, 16-2, 19-2, 19-4, 19-5, and 32-3, Fer-NAS-YSL2 transgenic lines

	(μg/g DW)			
Line	Fe	Zn	Mn	Cu
NT	10.8 ±0.5	26.8 ±1.3	43.4 ±3.2	2.80 ±0.31
AN	17.8 ±1.5	38.4 ±1.6	55.6 ±9.5	4.10 ±0.35
15-8	12.2 ±0.4	29.5 ±1.4	37.2 ±1.3	2.61 ±0.28
16-2	13.8 ±1.1	31.1 ±3.8	47.1 ±7.0	3.04 ±0.51
19-2	16.5 ±0.7	37.1 ±1.1	54.2 ±5.0	3.88 ±0.29
19-4	15.5 ±0.5	34.1 ±1.7	52.4 ±5.9	3.42 ±0.42
19-5	17.0 ±1.0	38.6 ±1.0	46.7 ±3.7	3.69 ±0.13
32-3	14.3 ±0.6	31.0 ±1.0	46.6 ±6.7	3.19 ±0.25

nicotinic acid 500 ng/l, pyridoxine-HCl 500 ng/l, glycine 2 mg/l, *myo*-inositol 100 mg/l, agar 8 g/l, pH 5.8) with 30 mg/l hygromycin (for transgenic lines) or without hygromycin (for the NT line). Germinated nurseries were grown in soil in a greenhouse until transplantation. The paddy field was established in an isolated field (6.0×10.65 m) at Gyeongsang University, Jinju, Korea (35°N, 128°E) (**Supplemental Fig. 3**). The soil type was SiL (silt loam) with a soil pH of 6.45 (1:5 soil–water extract) and soil EC 0.47 (sd/m). The Zn, Mn, and Cu concentrations of the soil were 1.27, 46.9, and 1.91 (mg/kg), respectively (0.1 N HCl extraction). Cd was not detected. Twenty-seven-day-old seedlings were transplanted to the paddy field on 16 June 2009. A commercial fertilizer (Toruzou-kun, N:P:K = 14:20:14; Zennou, Tokyo, Japan) was applied at 60 kgN/ha. The paddy field was submerged in water until it was drained 2 weeks before harvest. The plants were harvested on 29 October 2009 (135 days after transplantation). The plants entered the heading stage around 18–23 August 2009 (65–70 days after transplantation). After harvest, the inner four hills from each population were measured to determine the grain yield and concentrations of metals, NA, and DMA.

Paddy field plant height and yield analysis. Plant height was measured on the harvest date. The yield in each block was calculated as the average of the middle four hills in each plot. The data represent the means ± standard errors of four block replications for each line (**Supplemental Fig. 3**). Filled grains were selected by soaking in NaCl solution (1.06 g/cm^3 gravity). The number of total grains per panicle was counted based on the ear of the main tiller.

Metals concentrations of seeds. T_1 and T_2 seeds from the ear of the main tiller were harvested from a greenhouse and polished using a Multi-beads Shocker® (Yasuikikai), as described previously[13]. The Fe concentration in the polished seeds was measured by inductively coupled plasma atomic emission spectroscopy (ICP-AES) (SPS1200VR; Seiko Instruments Inc., Chiba, Japan), as described in ref.13. T_3 seeds from an ear of the main tiller were harvested from the middle four hills in each plot in the field and used for metal concentration analysis. Seeds were polished using a Multi-beads Shocker®. The Fe, Zn, Mn, and Cu concentrations of polished seeds were measured as described in ref.13. The Cd concentration of polished seeds was measured using a Thermo Fisher X series2 ICP-MS (Thermo Fisher Scientific Inc., Waltham, MA, U.S.A.). Fe, Zn, Mn, and Cu in brown seeds were digested as described in ref.13 and measured with an ICPS8100 (Shimadzu Co., Kyoto, Japan).

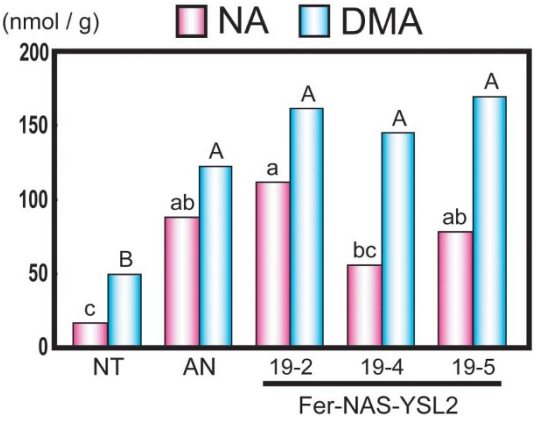

Figure 7 | NA and DMA concentrations in T_3 brown seeds harvested from the paddy field. Letters indicate significant differences ($P < 0.05$) by ANOVA with Tukey–Kramer HSD test used for each four-block data set (NA, $P < 0.0406$; DMA, $P < 0.0042$); n = 4.

Determination of the NA and DMA concentrations in T_3 seeds. T_3 seeds from the ear of the main tiller were harvested. The NA and DMA concentrations in brown seeds were analyzed as described in refs.13 and 40. The NA and DMA concentrations were calculated as the average of the middle four hills in each block.

Statistics. Analysis of variance (ANOVA) with the Tukey–Kramer HSD test was used to compare data with JMP8 (SAS Institute, Cary, NC, U.S.A.). The level of significance was set at $P < 0.05$.

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Author contributions

H. M. designed the research, performed all experiments, analyzed data and wrote the paper. Y. I. advised real time RT-PCR, designed the research, discussed the data and improved the paper. M. S. A. advised field experiment design, also analyzed metal concentration in rice seeds and improved the paper. T. K. advised making vector, western blotting analysis and improved the paper. Y. K. also performed nicotianamine and deoxymugineic acid concentration measurement in rice seeds. K. H. grew and observed transgenic plants in green house. M. T. disccussed the data, advised rice transgenic and metal concentration analysis. H. N. disccussed the data, designed the research and supervised the project. N. K. N. discussed the data, designed the research, improved the paper and supervised the project.

Additional information

Supplementary information accompanies this paper at http://www.nature.com/scientificreports

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