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

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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 T2 polished seeds was sixfold higher and that in paddy field-grown T3 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.
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. Likewise, overexpression of the NA synthesize gene increased the Fe concentration in polished rice seeds threefold with greenhouse cultivation.

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. OsYSL2 knockdown mutant plants exhibit a 30% decrease in Fe concentration in the endosperm. 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.

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. In rice, the rice nicotianamine–metal transporter gene (AB126253) and the soybean nopaline synthase gene (AF485783) promoter region; OsSUT1P, hygromycin phosphotransferase gene (K01193).

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 OsGlbl promoter–SoyferH2, OsGlub1 promoter–SoyferH2, OsActin1 promoter–HvNAS1, and OsSUT1 promoter–OsYSL2, and the OsGlbl 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. T2-regenerated plants were cultivated in a greenhouse, and those lines with a high Fe content in their T3 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 T2 seeds was detected by Western blot analysis (Fig. 3). Enhanced expression of OsYSL2 and HvNAS1 in immature T2 seeds of selected transgenic lines was confirmed by real-time RT-PCR analysis (Fig. 4). The Fe concentration in the T2 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 paddie field. For practical usage, we therefore investigated whether Fer-NAS-YSL2 lines set seeds with increased Fe concentration under actual paddie field conditions. For this purpose, selected T2 lines with high Fe content in their polished seeds (Fig. 5, arrows) were cultivated in an isolated paddie field in Jinju, Gyeongsang Province, Korea (Supplementary Figs. 3 and 4). The mean Fe concentrations in T3 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 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 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 overexpression13,14. 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 rachis32. The rice antisense mutant of OsSUT1 markedly reduces the sucrose uptake ability and filling rate of rice seeds33. Therefore, OsSUT1 is a key transporter of sucrose from the phloem to seeds and the OsSUT1...
Endosperm-specific ferritin expression also contributes to the increased Zn concentration in rice seeds\(^5\). Zn deficiency is one of the most critical micronutrient deficiency problems in human health\(^5\), 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.** *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 CS8) 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 T2 transgenic plants.

**Greenhouse cultivation.** T2 transgenic plants, T2 selected plants, and T2 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 (LAMTotal-70 and -140; LAM AGRI Co. Ltd., Tokyo, Japan) in a greenhouse. Using the seeds obtained, high-Fe lines were selected based on the Fe concentration in T1 (Supplementary Fig. 2) and T2 (Fig. 5) polished seeds.

**Detection of ferritin accumulation in T2 brown seeds.** Six mature T2 brown seeds harvested from a greenhouse were homogenized with a mortar and pestle, soaked in extraction buffer [4% sodium dodecyl sulfate (SDS), 5% 2-mercaptoethanol, 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 T1 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), and RNA was extracted using an RNeasy Plant Mimi Kit (Qiagen KK, Tokyo, Japan). First-strand cDNA was synthesized using ReverTra Ace (Toyobo, Osaka, Japan) with oligo-d(T)\(_\text{30}\). 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 Ex Taq™ Real-Time PCR- versión (Takara, Tokyo, Japan). The primers used were as follows: OsYSL2 forward (5'-GAA CGT CGC CGA CAA CCG GTG CAT TGG TCG TGG T-3') and OsYSL2 reverse (5'-TGC AGA AAA ACA CCT CTC GAC AAC AAG A-3') for OsYSL2 expression, HvNAS1 forward (5'-GGA CGT CGG CCT CCT CAC CCA G-3') and HvNAS1 reverse (5'-CAG GGA GCC CCT 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 TTC-3') and alpha-Tubulin reverse (5'-AAC CTT GAC GAC TCG 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 T2-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 GGA CAA CGG CCT CAC CCT CAC CCA G-3') and SoyferH2 reverse primer (5'-CAG GGA GCC CCT CTC CAC C-3') and SoyferH2 reverse primer (5'-CAG GGA GCC CCT CTC CAC C-3'). The OsActin1 promoter–HvNAS1 cassette was detected using the OsActin1 promoter forward primer (5'-GCA GGC GGG CAT CGT TAT TTT TTC TAG-3') and HvNAS1 reverse primer (5'-G TAAT ATC GAC CTC GCA CTC CCA G-3'). The OsActin1 promoter–OsYSL2 cassette was detected using the OsActin1 promoter forward primer (5'-GAC AAG CAA CCA AGA GAT CTT TAA CGA CAC CGC TCA C-3') and OsYSL2 reverse primer (5'-GCA GGC GGG CAT CGT TAT TTT TTC TAG-3') and OsYSL2 reverse primer (5'-GTA GAG GAG GAG TTG TAA CTA CGA CTC CCA G-3'). The OsSUT1 promoter–OsYSL2 cassette was detected using the OsSUT1 promoter forward primer (5'-CAG GGA GCC CCT CTC CAC C-3') and OsYSL2 reverse primer (5'-GTA GAG GAG GAG TTG TAA CTA CGA CTC CCA G-3'). The OsGlcB1 reverse primer (5'-GCA GGC GGG CAT CGT TAT TTT TTC TAG-3') and OsSUT1 reverse primer (5'-GTA GAG GAG GAG TTG TAA CTA CGA CTC CCA G-3'). The iGUS was detected using the iGUS forward primer (5'-CTG GAT TAA TGA TGA-3') and iGUS reverse primer (5'-GTA GAG GAG GAG TTG TAA CTA CGA CTC CCA G-3').
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 T2 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, NH4NO3 1.65 g/l, KNO3 1.9 g/l, CaCl2.N2H2O 440 mg/l, MgSO4.N7H2O 370 mg/l, KH2PO4 170 mg/l, Fe–EDTA 24.2 mg/l, H3BO3 6.2 mg/l, MnSO4.4H2O 22.3 mg/l, ZnSO4.7H2O 8.6 mg/l, KI 0.83 mg/l, Na2MoO4.2H2O 250 mg/l, CuSO4.5H2O 25 mg/l, CoCl2.6H2O 25 mg/l, thiamine–HCl 100 ng/l, KI 0.83 mg/l, Na2MoO4.2H2O 250 mg/l, CuSO4.5H2O 25 mg/l, CoCl2.6H2O 25 mg/l, thiamine–HCl 100 ng/l, KI 0.83 mg/l, Na2MoO4.2H2O 250 mg/l, CuSO4.5H2O 25 mg/l, CoCl2.6H2O 25 mg/l, thiamine–HCl 100 ng/l).

Figure 6 | Metal concentration in T3 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. T3 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 815; numerals, Fer-NAS-YSL2 transgenic lines.
Table 1 | Metal concentration in T3 brown seeds obtained from the paddy field T3 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

| 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±5.2 | 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/cm³ gravity). The number of total grains per panicle was counted based on the ear of the main tiller.

Metals concentrations of seeds. T1 and T2 seeds from the ear of the main tiller were harvested from a greenhouse and polished using a Multi-beads Shocker (Sanyo, Japan), as described in ref.13. T3 seeds from an ear of the main tiller were harvested from the middle four hills in each plot and 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 ICP8S100 (Shimadzu Co., Kyoto, Japan).

Figure 7 | NA and DMA concentrations in T3 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 T3 seeds. T3 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 the project. M. T. discussed the data, advised rice transgenic and metal concentration analysis. H. N. discussed 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/srep.

**Competing financial interests:** The authors declare no competing financial interests.

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