Activation of Rice *nicotianamine synthase 2* (*OsNAS2*) Enhances Iron Availability for Biofortification

Sichul Lee¹, You-Sun Kim², Un Sil Jeon³, Yoon-Keun Kim², Jan K. Schjoerring⁴, and Gynheung An^{5,*}

Because micronutrients in human diets ultimately come from plant sources, malnutrition of essential minerals is a significant public health concern. By increasing the expression of *nicotianamine synthase* (*NAS*), we fortified the level of bioavailable iron in rice seeds. Activation of iron deficiency-inducible *OsNAS2* resulted in a rise in Fe content (3.0-fold) in mature seeds. Its ectopic expression also increased that content. Enhanced expression led to higher tolerance of Fe deficiency and better growth under elevated pH. Mice fed with *OsNAS2-D1* seeds recovered more rapidly from anemia, indicating that bioavailable Fe contents were improved by this increase in *OsNAS2* expression.

INTRODUCTION

Iron is a vital element for living organisms. Because Fe can form six coordinated links with electron donor atoms, it is associated to a wide range of metalloprotein active sites, under the form of various prosthetic groups including heme and [Fe-S] clusters (Hell and Stephan, 2003). The reversible redox reaction between Fe²⁺ and Fe³⁺ is required in production (photosynthesis) and consumption (respiration) of oxygen (Briat, 2008). Therefore, Fe is present in the proteins of the mitochondrial and chloroplastic electron transfer chains. Iron is also involved in enzymatic reactions required for nitrogen fixation and synthesis of DNA and plant hormones (Briat, 2008).

Because staple foods such as cereal grains are poor sources of key mineral nutrients, their consumption may cause micronutrient deficiencies (Gómez-Galera et al., 2010). For example, a low supply of iron (Fe) is a worldwide, prevalent problem that leads to poor health in general and impaired development in women and children in particular (Stein, 2010). Various strategies have been followed to tackle these mineral deficiencies, such as encouraging diversification in diet as well as supplementing and fortifying processed foods (Zhao and Shewry, 2011).

Biofortification, the delivery of minerals via micronutrient-dense crops, offers a long-term, sustainable, and food-based

solution to alleviate malnutrition (Mayer et al., 2008). Rice is the most important food source for more than half of the world's population (Jeon et al., 2011). Therefore, improving the nutritional quality of its grains is vital to human health.

Transgenic approaches have been used to elevate Fe accumulations in cereal grains. For example, expression of ferritin, an iron storage protein, in seeds can increase Fe contents in rice endosperm (Goto et al., 1999; Vasconcelos et al., 2003). However, contradictory results regarding iron bioavailability from ferritin have not yet been fully clarified (Hoppler et al., 2008). Manipulation of the transporters involved in absorption and translocation of metals has been suggested as another approach for enhancing mineral contents if the amount of the transporter is rate-limiting (Ramesh et al., 2004). Overexpression of rice Fe transporters *OsIRT1* and *OsYSL15* also has resulted in a slight rise in Fe concentration in seeds (Lee and An, 2009; Lee et al., 2009a). When *OsYSL2* is highly expressed in phloem cells by the *OsSUT1* promoter, the Fe concentration in polished rice is up to 4.4-fold higher compared with the wild type (Ishimaru et al., 2010).

Utilization of nicotianamine (NA), a chelator of metal cations, is another strategy for improving Fe concentrations (Douchkov et al., 2005). Nicotianamine is biosynthesized from three molecules of S-adenosyl methionine (SAM) via NA synthase (*NAS*; Higuchi et al., 1994). Specific expression of *HvNAS1* through the seed-specific *pGluB-1* promoter leads to transgenic plants containing 1.5-fold higher amounts of Fe in seeds (Usuda et al., 2009). Transgenic rice grains expressing that gene, driven by the rice *actin1* promoter, also have 1.3-fold more iron (Masuda et al., 2009). Targeted expression of *Pvferriitin* and *AtNAS1* improves Fe contents in rice endosperm by more than 6-fold (Wirth et al., 2009). Endosperm-specific expression of *OsNAS1* results in a significant rise in NA concentrations in both unpolished and polished grains (Zheng et al., 2010).

Although the study by Zheng et al. (2010) did not focus on enhancing Fe concentrations, that research group did find the bioavailability of Fe to be twice as great as that of the control line, as measured by ferritin synthesis in an *in-vitro* Caco-2 cell model. We have previously shown that grains from activation-

¹Department of Biological Sciences, Dartmouth College, Hanover, New Hampshire, 03755, USA, ²Department of Life Science, Pohang University of Science and Technology, Pohang 790-784, Korea, ³Department of Internal Medicine, Korea University Guro Hospital, Seoul 152-703, Korea, ⁴Plant and Soil Science Laboratory, Department of Agriculture and Ecology, Faculty of Life Sciences, University of Copenhagen, Thorvaldsensvej 40, DK-1871 Frederiksberg C, Denmark, ⁵Department of Plant Molecular Systems Biotechnology and Crop Biotech Center, Kyung Hee University, Yongin 446-701, Korea

*Correspondence: genean@khu.ac.kr

tagged lines of *OsNAS3* have 2.9-fold more Fe (Lee et al., 2009b). Furthermore, the hemoglobin levels in anemic mice fed with seeds from those transgenic plants recover to normal readings when animals are placed on that diet for two weeks.

In this study, we used *OsNAS2* activation-tagged plants and *OsNAS2*-overexpressing transgenics to increase the amount of Fe in seeds and vegetative tissues. We demonstrated that bio-available Fe levels were significantly increased in the grains, as shown by the mouse feeding experiments.

MATERIALS AND METHODS

Plant materials and growth

An activation-tagged mutant (*OsNAS2-D1*) and transgenic plants over-expressing *OsNAS2* under the control of the maize *Ubiquitin* promoter have been described previously (Lee et al., 2011). Seeds from these plants were surface-sterilized and placed on an MS agar medium containing 30 μM ZnSO_4 , 100 μM Fe (III)-EDTA, 0.1 μM CuSO_4 , and 10 μM MnSO_4 as micro-nutrients. For deficiency tests, seeds were grown on MS media lacking ZnSO_4 (Zn-deficient), Fe (III)-EDTA (Fe-deficient), CuSO_4 (Cu-deficient), or MnSO_4 (Mn-deficient). To analyze *OsNAS2* under different iron concentrations, we grew seedlings for 7 days on MS media containing 0, 1, 10, 100, or 500 μM Fe (III)-EDTA. Other seedlings were transplanted into soil and grown to maturity in a greenhouse (14-h photoperiod).

RNA expression analysis

Shoots and roots from all treatment combinations were collected separately and frozen in liquid nitrogen. Total RNA was isolated with RNeasy Plus (Takara, Japan) and treated with RNase-free DNase I (Takara, Japan) to remove contaminating genomic DNA. First-strand cDNA was synthesized from 2 μg of total RNA in a 25- μl reaction mixture with M-MLV reverse transcriptase (Promega, USA). Synthesized cDNAs were used for RT-PCR and real-time PCR. Quantitative PCR analysis was performed on a Rotor-Gene 6000 real-time rotary analyzer (Corbett Life Science, Australia), using a SYBR premix Ex.Taq kit (Takara, Japan). The levels of *actin1* mRNA served to normalize the expression ratio for each gene. Changes in expression were calculated via the $\Delta\Delta\text{C}_t$ method. Primers for PCR are listed in Supplementary Table S1.

Measurement of chlorophyll concentrations

Seeds of WT and mutant plants were germinated and seedlings grown on MS agar plates with or without 100 μM Fe(III)-EDTA. 0.1 g of leaf samples was harvested and the chlorophyll was extracted with 1 ml of 80% acetone. After homogenization, the samples were incubated for 15 min and centrifuged at $15,000 \times g$ for 10 min. An aliquot of supernatant fraction was then taken to measure the A663 and A643 with a spectrophotometer. Chlorophyll concentrations, including chlorophyll a and b, were calculated according to the method of Arnon (1949).

Element analysis in plant tissues

Plant samples were dried for 2 days at 70°C before weighing. Afterward, they were digested in 1 ml of 11 N HNO_3 for 3 days in a 180°C oven. Following dilution, their metal concentrations were determined by atomic absorption spectrometry (AAS; SpectrAA-800, Varian, USA).

Mouse feeding experiments

Fe-bioavailability using anemic mice was tested as previously described (Lee et al., 2009b). After three weeks of weaning for pathogen-free female Balb/c mice, one group was fed with AIN-

93DIET (45 mg Fe kg^{-1}) as a control diet (CD) and the second group was given an iron-depleted (ID) diet (modified AIN-93G diet containing 3 mg Fe kg^{-1}). Two weeks later, mice fed with the ID diet were further divided into two groups. The first ($n = 10$) was given WT seeds; the second ($n = 10$), *OsNAS2-D1* seeds. After one, two, and four weeks of feeding, blood was collected from their orbital sinuses, and hemoglobin (Hb) and hematocrit (Hct) levels were analyzed by a commercial service (Green Cross Reference Lab, Korea).

RESULTS

Overexpression of *OsNAS2*

Rice has three *NAS* genes that are differentially regulated by iron (Inoue et al., 2003). Our analyses of *OsNAS2* under different metal statuses showed that this gene was strongly up-regulated in Fe-deficient shoots and roots (Fig. 1A), corresponding to results described previously by Inoue et al. (2003). By contrast, Zn-, Cu-, and Mn-deficiencies had no effect on this gene behavior (Fig. 1A). We also measured transcripts under different Fe concentrations (Fig. 1B). Increasing the iron level gradually reduced expression in the roots, whereas such expression was entirely diminished in the shoots even when the Fe supply was very low (Fig. 1B). These results indicated that *OsNAS2* functioning is sensitive to Fe deficiency.

To assess the roles of *OsNAS2* further *in planta*, we utilized our previously isolated tagging line (*OsNAS2-D1*) as well as overexpressing transgenic plants (Lee et al., 2011). Activation of the gene elevated the Fe level by 143% in flag leaves (Fig. 1C). The same results were noted from transgenic plants that over-expressed *OsNAS2* via the *Ubiquitin* promoter (Fig. 1D).

Increased expression of *OsNAS2* leads to enhanced tolerance of Fe deficiencies

Because *OsNAS2* was strongly induced under Fe deficiency, we tested whether seedling growth of the activation-tagging line *OsNAS2-D1* and the overexpressing lines varied from the WT under a limited Fe supply (Fig. 2). Compared with the WT, all transgenics showed enhanced growth on the Fe-deficient medium (Fig. 2A), differing from the control in their heights and total chlorophyll concentrations (Figs. 2B and 2C). For example, respective heights for *OsNAS2-D1*, OX-9 and OX-10 were increased to 121%, 120%, and 119%, and chlorophyll concentrations to 151%, 142%, and 143%, relative to the WT.

To evaluate whether activation of *OsNAS2* affected Fe distribution, we analyzed iron levels. When plants were grown in a metal-sufficient medium, Fe concentrations in shoots from *OsNAS2-D*, OX-9, and OX-10 were increased to 162%, 154%, and 159% compared with the WT (Fig. 2D), while respective root levels rose to 145%, 155%, and 200% (Fig. 2E). Under this deficiency, relative concentrations of Fe from *OsNAS2-D1*, OX-9, and OX-10 also were increased to 189%, 229%, and 156% in shoots (Fig. 2D), and to 183%, 163%, and 146% in roots (Fig. 2E). In Fe-limiting plants, Zn concentrations in both shoots and roots were increased, as compared with plants grown in a control MS medium (data not shown). Under Cu- or Mn-deficiencies, however, no visible phenotypic changes were observed, and Cu and Mn levels were unaltered (data not shown).

To examine the sub-cellular distribution of Fe, we analyzed concentrations in the chloroplasts, mitochondria, and mesophyll protoplasts (Supplementary Fig. S1) and found that levels at all tested sites were enhanced irrespective of the external Fe supply (Supplementary Fig. S1).

Fig. 1. Expression of *OsNAS2* and generation of transgenic plants. Real-time PCR analysis of *OsNAS2* under micronutrient deficiencies (A) and different Fe concentrations (B). Expression was quantified using real-time RT-PCR and normalized to *OsAct1*. Fe concentrations in flag leaves from WT, *OsNAS2-D1* (C), and *OsNAS2*-overexpressing transgenic plants (D). D1 indicates *OsNAS2-D1*. Error bars represent SE. Significant differences from WT were determined by Student's *t*-tests, **P* < 0.05.

Fig. 2. Characterization of *OsNAS2-D1* and *OsNAS2*-overexpressing plants under Fe deficiency. (A) Phenotypes of *OsNAS2-D1*, OX-9, OX-10, and WT plants grown on Fe-limiting (Fe-) media for 8 days. Scale bars = 2.5 cm. (B) Heights of *OsNAS2-D1*, OX-9, OX-10, and WT plants (n = 8 each) grown under Fe-deficiency. (C) Total chlorophyll concentrations in *OsNAS2-D1*, OX-9, OX-10, and WT plants (n = 4) grown under Fe-sufficient (MS) or Fe-deficient (Fe-) media. Fe concentrations in shoots (D) and roots (E) from *OsNAS2-D1*, OX-9, OX-10, and WT grown on standard MS media (MS), or Fe-free media. Error bars represent SE. Significant differences from WT were determined by Student's *t*-tests. **P* < 0.05.

Fig. 3. Growth test under conditions of low iron availability. *OsNAS2-D1* and OX-9 seeds were germinated and plants grown on solid alkaline media (pH 8.5) with (A) or without (B) Fe for 8 days. Bars = 2.5 cm. (C) Heights of *OsNAS2-D1*, OX-9, and WT plants ($n = 8$ each), and (D) chlorophyll concentrations of WT and mutant plants ($n = 4$) grown on solid alkaline media (pH 8.5) with or without Fe. Significant differences from WT were determined by Student's *t*-tests, * $P < 0.05$.

Transgenic plants have enhanced tolerance to low iron availability

In soil, pH influences the solubility and ionic form of several elements. For example, in an aerated solution with a pH > 8, ferric ion precipitates as the extremely insoluble ferric hydroxide. Here, we observed that *OsNAS2-D1* and OX-9 plants had improved tolerance to an alkaline (pH 8.5) environment (Figs. 3A, 3C, and 3D). Phenotypic differences were more pronounced when plants were grown in an Fe-free medium at the higher pH (Figs. 3B-3D).

Enhanced levels of *OsNAS2* regulate expression of Fe homeostasis-related genes

Because enhanced expression of *OsNAS2* increased the Fe levels in seedlings, we studied the behavior of representative genes involved in Fe homeostasis (Fig. 4). Transcript levels of *OsIRT1*, *OsIRT2* (Ishimaru et al., 2006), and *OsYSL15* (Inoue et al., 2009; Lee et al., 2009a) were significantly higher in transgenic roots (Figs. 4A-4C). These genes are membrane transporters responsible for Fe-uptake from soil (Inoue et al., 2009; Ishimaru et al., 2006; Lee et al., 2009a). Expression of *OsYSL2*, which functions as an Fe(II)-NA transporter important for Fe-translocation (Ishimaru et al., 2010), was also increased in transgenic roots relative to the WT (Fig. 4D). We have previously shown that expression by *OsNAAT1* and *OsDMAS1*,

which are related to phytosiderophore (PS) synthesis, is higher in the shoots and roots of transgenic plants under controlled growing conditions, thereby demonstrating the enhanced secretion of PS (Lee et al., 2011).

Ferritin is the principal iron storage protein in all living organisms (Harrison and Arosio, 1996). Therefore, we next examined the expression of two rice *ferritin* genes in seedling shoots (Figs. 4E and 4F). Transcripts were increased in transgenic tissues when compared with the WT, coinciding with the increased amounts of Fe measured in *OsNAS2-D1* and OX-9 plants.

Increased expression of *OsNAS2* causes an enhancement of Fe in mature seeds

We showed previously that NA and DMA levels in *OsNAS2-D1* seeds are increased to 19.8- and 3.5-fold, respectively, over the WT (Lee et al., 2011). Because NA can form stable complexes with Fe, we assessed here whether that greater amount of NA had any effect on the accumulation of Fe in sink tissues. Mature seeds from *OsNAS2-D1*, OX-9, and OX-10 plants contained more Fe (3.0-, 2.5-, and 2.3-fold higher, respectively; Fig. 5A). Because rice grains are usually milled prior to cooking, we also measured the status of iron remaining after that process. Polishing decreased the Fe level to 42% compared with the non-milled seed from segregated WT siblings of *OsNAS2-D1* (Figs. 5A and 5B). Iron concentrations were 2.9-, 2.5-, and 2.2-fold

Fig. 4. Expression of Fe homeostasis-related genes at seedling stage. Levels (black bars) of *OslRT1* (A), *OslRT2* (B), *OslYSL15* (C), and *OslYSL2* (D) in 8-day-old roots from *OsNAS2-D1* and OX-9, compared with WT plants (open bars) grown on standard MS media. Real-time PCR analysis of *OslFerritin1* (E) and *OslFerritin2* (F) from 8-day-old shoots of transgenic (black bars) and WT (open bars) plants.

Fig. 5. Measurement of Fe in mature seeds. (A) Fe concentration in whole seeds from WT, *OsNAS2-D1*, OX-9, and OX-10 plants. (B) Amount of Fe in milled seeds from WT, *OsNAS2-D1*, OX-9, and OX-10 plants. Data represent means $\pm$ SD of 4 replications. Significant differences from WT were determined by Student's *t*-tests, **P* < 0.05.

higher in milled seeds from *OsNAS2-D1*, OX-9, and OX-10, respectively, than in milled WT seeds (Fig. 5B). We previously reported that the content of Fe associated with the NA/DMA pool is increased 4.9-fold in mature *OsNAS2-D1* seeds (Lee et al., 2011). Altogether, these results suggested that increased amounts of NA in the transgenic plants form a stable complex with Fe.

Mice fed with *OsNAS2-D1* seeds recover more quickly from anemia

To evaluate the bioavailability of Fe when complexed with NA, we used mouse feeding experiments that applied our previ-

ously established system of tests with seeds from *OsNAS3*-activation-tagged plants (Lee et al., 2009b). In the current study, we assayed Fe-bioavailability in *OsNAS2-D1* seeds. After three weeks of weaning, we induced anemia by providing an Fe-deficient diet to some mice, which resulted in significant declines in blood hemoglobin and hematocrit levels compared with mice receiving a control diet (Figs. 6A and 6B). However, when those anemic mice were then fed with *OsNAS2-D1* seeds, their hemoglobin and hematocrit readings returned within two weeks to those recorded from control mice that had been fed a normal diet over the experimental period (Figs. 6A and 6B). In contrast, mice with induced anemia that were later fed with

Fig. 6. Mouse feeding experiments. Hemoglobin (A) and hematocrit (B) levels in mice fed for 4 weeks with control diet (CD), regular wild-type seeds (WT), or *OsNAS2-D1* seeds (D1).

normal rice did not recover from that condition, even after four weeks. This result indicated that the *OsNAS2-D1* seeds contain markedly higher amounts of bioavailable Fe.

DISCUSSION

In this study, we utilized *OsNAS2* to increase the amounts of both total Fe and bioavailable Fe in rice grains. Enhanced expression of that gene resulted in elevated amounts of NA, which then led to greater levels of Fe in the shoots, roots, and mature seeds. Transcripts were up-regulated by Fe-deficiency such that enhancing the expression of *OsNAS2* improved plant tolerance to that deficiency, as shown by their better growth and higher Fe contents. Because the NA level was increased in *OsNAS2*-overexpressing plants irrespective of metal status, we tested whether this might affect Fe-uptake and -translocation systems. Under normal growing conditions, transcript levels of Fe-uptake genes were increased in transgenic plants, resulting in higher Fe contents in roots, shoots, and seeds. We postulated that these elevated levels of NA might lead to increased secretion of PS, which stimulates Fe-uptake from the soil as well as translocation within a plant. When iron was lacking, the relative increase in transcripts for Fe deficiency-inducible genes was not as great in the transgenic plants as in the WT (data not shown). This indicated that those plants were less sensitive to the deficiency, probably because of their higher endogenous levels of Fe.

In rice, three *NAS* genes are responsible for the trimerization of S-adenosylmethionine to NA (Inoue et al., 2003). Among them, expression of *OsNAS1* and *OsNAS2* is similar to each other, which are strongly up-regulated by Fe deficiency. They are located at the same chromosome about 2 kb away, indicating recent gene duplication and functional redundancy. Although we did not examine *OsNAS2* knockout lines due to the lack of available mutants, we speculate that disruption of this gene will not change the sensitivity to Fe deficiency because *OsNAS1* could complement the loss of *OsNAS2*.

Iron deficiency is a major cause of reduced crop yields, particularly in calcareous soils, which account for about 30% of the world's cultivated soils (Kobayashi et al., 2008). When barley nicotianamine aminotransferase genes are expressed in rice, the transformants show enhanced tolerance to low Fe-availability (Takahashi et al., 2001). Furthermore, transgenic rice plants transformed with an *OsIRT1* promoter, *refre1/372*, encoding ferric-chelate reductase, exhibit enhanced tolerance to low Fe-availability in both hydroponic cultures and calcareous soils (Ishimaru et al., 2007). When our *OsNAS2*-overexpressing transgenic plants were grown under high pH, transfor-

mant was tolerant to low-Fe conditions, as manifested by their greener and larger shoots.

Although the total amount of Fe in seeds is an important determinant of nutritional quality, what really matters is the amount of bioavailable Fe, and how well it is absorbed by the human gut (Zhu et al., 2007). With our previously established mouse-feeding system, we examined Fe-bioavailability and found rapid restoration from anemia in the *OsNAS2-D1* feeding group. Anemic mice given those seeds started to recover after only one week and reached normal hemoglobin and hematocrit levels within two weeks, indicating that the *OsNAS2-D1* seeds contain an increased amount of bioavailable Fe that is successfully absorbed into the mouse gut. Although the effect of these seeds has not yet been examined in humans, the results obtained with this mouse system strongly suggest that the NA-Fe present in *OsNAS2-D1* seeds would also be bioavailable to humans.

Although we observed several positive outcomes of *OsNAS2* activation, there were some unfavorable aspects. For example, transgenic plants were shorter and produced less grain, thus negatively influencing productivity (Supplementary Fig. S2). Because they contained higher amounts of iron during all growth stages, that reduced productivity might have been due to Fe-mediated oxidative stress. Excess amounts of iron can catalyze the generation of reactive oxygen species via the Fenton reaction (Arosio et al., 2009), thereby indicating that its homeostasis must be tightly regulated to promote proper plant growth. It is also possible the defect was due to over accumulation of other metals such as Zn, Cu, and Co. However, it is unlikely due to accumulation of other toxic metals since NA preferentially binds to a few metals listed above. This yield reduction seen in our *OsNAS2-D1* crop was much less critical compared with plants that over-express Fe-transporter genes (Lee and An, 2009; Lee et al., 2009a). Thus, considering the increase in bioavailable Fe content in those seeds, this small number of negative effects is tolerable.

In addition to its roles in metal homeostasis, NA serves as an anti-hypertensive substance in humans (Usuda et al., 2009). It preferentially inhibits the circulatory and tissue angiotensin I-converting enzyme (ACE), which plays an important role in the rennin-angiotensin system to regulate both arterial blood pressure and the salt-water balance (Hayashi and Kimoto, 2007). Because NA levels were significantly improved in our transgenic seeds, it will be worthwhile to examine those anti-hypertensive properties in rats.

In conclusion, we have demonstrated that the technology for enhancing the supply of bioavailable Fe in rice grains is now in place. Moreover, this approach is potentially transferable to

other plant species for attaining similar alterations in micronutrient contents.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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