Iron fortification of rice seeds through activation of the nicotianamine synthase gene


†Integrative Biosciences and Biotechnology, Pohang University of Science and Technology, Pohang 790-784, Republic of Korea; ‡Plant and Soil Science Laboratory, Department of Agriculture and Ecology, Faculty of Life Sciences, University of Copenhagen, DK-1871 Frederiksberg, Denmark; and ³Graduate School of Agricultural and Life Sciences, The University of Tokyo, Tokyo 113-8657, Japan

Communicated by Gurdev S. Khush, University of California, Davis, CA, October 12, 2009 (received for review November 15, 2008)

The most widespread dietary problem in the world is mineral deficiency. We used the nicotianamine synthase (NAS) gene to increase mineral contents in rice grains. Nicotianamine (NA) is a chelator of metals and a key component of metal homeostasis. We isolated activation-tagged mutant lines in which expression of a rice NAS gene, OsNAS3, was increased by introducing 35S enhancer elements. Shoots and roots of the OsNAS3 activation-tagged plants (OsNAS3-D1) accumulated more Fe and Zn. Seeds from our OsNAS3-D1 plants grown on a paddy field contained elevated amounts of Fe (2.9-fold), Zn (2.2-fold), and Cu (1.7-fold). The NA level was increased 9.6-fold in OsNAS3-D1 seeds. Analysis by size exclusion chromatography coupled with inductively coupled plasma mass spectroscopy showed that WT and OsNAS3-D1 seeds contained equal amounts of Fe bound to IP6, whereas OsNAS3-D1 had 7-fold more Fe bound to a low molecular mass, which was likely NA. Furthermore, this activation led to increased tolerance to Fe and Zn deficiencies and to excess metal (Zn, Cu, and Ni) toxicities. In contrast, disruption of OsNAS3 caused an opposite phenotype. To test the bioavailability of Fe, we fed anemic mice with either engineered or WT seeds for 4 weeks and measured their concentrations of hemoglobin and hematocrit. Mice fed with engineered seeds recovered to normal levels of hemoglobin and hematocrit within 2 weeks, whereas those that ate WT seeds remained anemic. Our results suggest that an increase in bioavailable mineral content in rice grains can be achieved by enhancing NAS expression.

M

icronutrient malnutrition affects more than half the world’s population, particularly in developing countries. Mineral deficiency results in poor health and higher rates of mortality, and it permanently impairs the cognitive abilities of infants (1). Although plant-derived foods contain a wide variety of micronutrients, their levels are usually too low to meet daily needs (2). Therefore, various means for micronutrient biofortification have been attempted through breeding and transgenic approaches (3).

Fe and Zn are essential micronutrients, with numerous cellular functions in plants and animals. Plants often exhibit Fe deficiency symptoms that are manifested by chlorosis and reduced crop yield and quality. In humans, Fe deficiency anemia is one of the most serious problems worldwide (4). Zn is another essential cofactor required for the structure and functioning of many proteins. Its deficiency is widespread in plants and animals (5).

Rice is a major crop and primary food source for >50% of the global population (6). Production of varieties containing high amounts of bioavailable Fe would improve Fe nutrition in regions where such a deficiency is rampant (7). Feing trials with 192 religious sisters in the Philippines have shown that eating high-Fe rice results in an increase in serum ferritin and total body Fe, therefore also demonstrating that consumption of biofortified rice, without any other changes in their diet, is efficacious in improving the Fe stores of women with Fe-poor diets in the developing world (8).

Increasing natural seed ferritin has been suggested as a way to elevate Fe content by genetic engineering. Goto et al. (9) have expressed the soybean ferritin gene in rice under the control of a rice endosperm-specific glutelin promoter, GluB-1. The Fe content of T1 seeds is as much as 3-fold greater than that of their untransformed counterparts (9). Introduction of a ferritin gene from Phaseolus vulgaris into rice grains also increases their Fe content up to 2-fold (10). However, a further rise in the Fe concentration cannot be achieved by enhancing ferritin expression (8).

Nicotianamine (NA), a chelator of metals, is ubiquitous in higher plants and is a key component of their metal assimilation and homeostasis (11). Therefore, manipulation of cellular NA concentrations seems to be another approach for improving Fe concentrations in plants (11). NA is synthesized from 3 molecules of S-adenosyl methionine by nicotianamine synthase (NAS). Overexpression of the HvNAS1 gene from barley doubles concentrations of Fe and Zn in young leaves and seeds of tobacco (12). Transgenic rice seeds expressing HvNAS1 driven by the seed-specific pgluB-1 promoter also show greater amounts of NA (4-fold) and Fe (1.5-fold) (13). Likewise, overexpression of AtNAS1 in tobacco leads to higher Fe contents in the leaves (11, 12). This increased NA also enables plants to tolerate toxic concentrations of nickel (11, 14). Disruption of the rice nicotianamine aminotransferase (NAAT1) enzyme, which uses NA as the substrate, results in a significant elevation of Fe concentrations in both seedlings (by 15.8% in shoots and 41.8% in roots) and seeds (1.8-fold) (15). Among the 3 OsNAS genes present in rice, OsNAS1 and OsNAS2 transcripts are markedly elevated in both roots and leaves in response to Fe deficiency, whereas OsNAS3 expression is induced in roots but suppressed in leaves when the Fe supply is inadequate (16).

In this study, we used activation and knockout mutants to examine the functioning of OsNAS3 in metal homeostasis within rice plants. Activation of that gene resulted in higher metal contents in the leaves and mature seeds. We also demonstrated that bioavailable Fe was significantly increased in the grains.

Results

Expression Patterns of OsNAS3 Under Micronutrient Deficiencies and Identification of Knockout Mutants. Previously, 3 rice NAS genes were isolated and their protein products shown to have enzy-


The authors declare no conflict of interest.

1Present address: Department of Internal medicine, Korea University Guro Hospital, Seoul, Korea.

2To whom correspondence should be addressed. E-mail: genean@postech.ac.kr.

This article contains supporting information online at www.pnas.org/cgi/content/full/0910950106/DCSupplemental.
munic activity (16). Here we focused on OsNAS53 because it behaves differently from the other 2. To examine whether it is regulated by metal status, we determined expression patterns under micronutrient deficiencies (Fig. 1A). Consistent with the previous data (16), OsNAS53 was upregulated ~2-fold in Fe-deficient roots but downregulated in shoots by that deficiency (Fig. 1A). As reported previously (17), Zn deficiency also enhanced expression of OsNAS53 in roots and shoots (Fig. 1A). Whereas insufficient Mn increased such expression only in the roots, Cu deficiency had no influence (Fig. 1A).

To investigate the roles of OsNAS53 further in planta, we identified a transferred DNA (T-DNA) insertional allele, osnas3-1, from the rice flanking sequence-tag database (18). In line 2D30228, T-DNA was inserted at the 3’ region of the OsNAS3 coding sequence (Fig. 1B). In homozygous plants, the OsNAS3 transcript was absent, indicating that the mutant is a null allele (Fig. 1C). Disruption of OsNAS3 resulted in a small reduction in Fe (16%) and Zn (21%) concentrations in flag leaves at the flowering stage [supporting information (SI) Fig. S1 A and B]. By contrast, Cu and Mn concentrations were not significantly altered (Fig. S1 C and D). In seeds, Fe, Zn, and Cu in osnas3-1 were slightly decreased (6%, 10%, and 23%, respectively) compared with WT, whereas the level of Mn was unchanged (Fig. 1 E–H). We next analyzed NA and 2’-deoxymugineic acid (DMA) in seeds and found that neither was significantly changed (Fig. 1 I and J).

To confirm that the Fe and Zn reduction was due to the mutation, we constructed pGA2973 by placing the 3’ gene-specific region of OsNAS3 in the antisense orientation under the control of the maize ubiquitin promoter (Fig. 1D). Among 14 independent hygromycin-resistant transgenic plants, 12 expressed the antisense transcript (Fig. 1D). As observed from the T-DNA insertional mutant, our antisense transgenic plants accumulated slightly less Fe and Zn in flag leaves (both 22%) and seeds (25% and 22%, respectively) (Fig. S1 A and B, Figs. 1 E and F).

Isolation of Activation-Tagged Mutants of OsNAS3. To examine its roles further, we isolated the 2 independent activation-tagged alleles of OsNAS3 from our rice flanking sequence-tag database (19). In line 3A16471 (OsNAS3-D1), the 35S enhancer elements were inserted approximately 1.5 kb downstream of an ORF of OsNAS3. In another T-DNA activation-tagged line, 2D10007 (OsNAS3-D2), the enhancer elements were inserted approximately 1.9 kb downstream of the OsNAS3 ORF (Fig. 2A). T2 progeny of the 2 lines were obtained, and homozygous plants were selected by PCR (Fig. 2A). Subsequent quantitative real-time PCR analysis showed that OsNAS3 transcript levels were 60- and 30-fold higher in the seedling leaves from OsNAS3-D1 and OsNAS3-D2, respectively, compared with WT leaves (Fig. 2B). Furthermore, expression was increased in the flag leaves, flowers, and immature seeds of activation-tagged plants (Figs. S2 A–C). NA and DMA levels in OsNAS3-D1 seeds were increased 9.6- and 4.0-fold, respectively, over WT (Fig. 2 C and D).

Activation of OsNAS3 Leads to Increased Tolerance to Fe and Zn Deficiencies at the Seedling Stage. To examine the role of OsNAS3 under a limited metal supply, we grew seedlings from the activation-tagged lines on solid MS media with or without Fe and Zn. When metals were sufficient, phenotypes of the mutant plants did not differ from the WT segregants (Fig. 2E). Growth rates were identical, and chlorophyll contents were unaltered (Fig. 2 H and I). However, when Fe was limiting, the activation-tagged mutant plants had less chlorosis (i.e., 50% more chlorophyll) and taller shoots (by 126%) than the WT control (Fig. 2 F, H, and J). To evaluate the influence of OsNAS3 activation on Fe homeostasis, we examined expression of 2 ferritin genes. Transcripts were slightly increased when Fe was either sufficient or deficient in OsNAS3-D1 (Fig. S2 D and E). Under Zn deficiency, plant heights for OsNAS3-D1 were increased to 110% relative to WT (Fig. 2 G and H). Similarly, when Fe or Zn was deficient, plants of OsNAS3-D2 were taller, with less chlorosis.
Mn levels were unchanged (Fig. S5). NAS3-D1 were increased in all examined sites, whereas Cu and phyll protoplasts (Fig. S5). Fe and Zn concentrations in their concentrations in chloroplasts, mitochondria, and mesophyll protoplasts (Fig. S5). was also increased 1.4-fold in shoots and roots was 2.0-fold in shoots and 1.6-fold in roots) compared with the WT more Fe (by 1.7-fold in shoots and 1.6-fold in roots) and Zn Fe and Zn at the seedling stage. K J (Fig. 2. I1005 I11005 B R) indicate gene-specific primers for real-time PCR. (I) Real-time PCR analyses of WT and activation-tagged mutants, using RNA samples from seedling leaves. 4 OsNAS3-D2 OsNAS3-D1 plants exhibited improved tolerance to elevated levels of Zn, Cu, and Ni, being taller and less chlorotic (Fig. 3 A–D). In contrast, disruption of OsNAS3 resulted in greater sensitivity to excess Zn, Cu, and Ni (i.e., having the opposite phenotype of the OsNAS3-D1 plants) (Fig. 3 E–H). However, excess Cd was not associated with any clear differences between WT and mutant plants (Fig. S6 A–D).

We also compared metal concentrations between WT and mutant seedlings. Levels of Zn, Cu, and Ni in OsNAS3-D1 shoots were higher by 2.1-, 1.5-, and 1.3-fold, respectively, whereas the root Ni concentration was 2.1-fold greater (Fig. 3 I–K). Although clear distinctions were found when WT and osnas3-1 plants were exposed to excess Zn, Cu, and Ni, their metal concentrations were not different from each other (Fig. 3 I–K). 

Enhanced Tolerance to Elevated Levels of Heavy Metals Is Confirmed by the Activation of OsNAS3. We examined whether increased expression of OsNAS3 alters tolerance to heavy-metal toxicity. OsNAS3-D1 plants exhibited improved tolerance to elevated levels of Zn, Cu, and Ni, being taller and less chlorotic (Fig. 3 A–D). In contrast, disruption of OsNAS3 resulted in greater sensitivity to excess Zn, Cu, and Ni (i.e., having the opposite phenotype of the OsNAS3-D1 plants) (Fig. 3 E–H). However, excess Cd was not associated with any clear differences between WT and mutant plants (Fig. S6 A–D).

We also compared metal concentrations between WT and mutant seedlings. Levels of Zn, Cu, and Ni in OsNAS3-D1 shoots were higher by 2.1-, 1.5-, and 1.3-fold, respectively, whereas the root Ni concentration was 2.1-fold greater (Fig. 3 I–K). Although clear distinctions were found when WT and osnas3-1 plants were exposed to excess Zn, Cu, and Ni, their metal concentrations were not different from each other (Fig. 3 I–K).
650 s), whereas OsNAS3D-1 had significantly more (7.0/11006 0.3 times) Fe bound in a low-molecular-weight complex (retention time, 990 s). As shown in the middle chromatogram, the Fe peak at 650 s coeluted with P. This peak has previously been identified as an Fe:IP6 oligomer complex (21). The amount of Fe bound to IP6 was similar in the 2 grain samples. However, OsNAS3-D1 had a higher concentration of the P peak eluting after 780 s, corresponding to the polymer of non-metal-binding IP6 and an increased level of P. The bottom chromatogram shows no difference in S peaks. The low-molecular-weight Fe peak did not coelute with either P or S. The mass calibration indicated an apparent molecular mass of \(1.300 \pm 400\) Da for the Fe complex, suggesting a cluster of Fe with several NA ligands. It should be noted that this low-molecular-weight Fe complex was not bound to IP6 or to any cysteine/methionine-containing ligands, strongly indicating binding to –NH\(^{-}\) and/or –COO\(^{-}\) functional groups, as found in NA.

Mice Fed with OsNAS3-D1 Seeds Recover More Rapidly from Anemia. Bioavailability of the increased Fe in OsNAS3-D1 seeds was examined through feeding experiments (Fig. 5A). We induced anemia by providing 3-week-old mice with an Fe-deficient (ID) diet containing 3 mg kg\(^{-1}\) Fe. During the 2-week diet period, their blood Hb level was reduced to 12.50 ± 0.48 g dL\(^{-1}\) vs. 16.10 ± 0.25 g dL\(^{-1}\) (i.e., the level from mice fed with a control diet containing 45 mg kg\(^{-1}\) Fe) (Fig. 5B Left). Similarly, hematocrit (Hct) levels declined to 42.60% ± 1.62% compared with the 54.00% ± 2.83% found in the control mice (Fig. 5B Right). These results indicate that the ID diet led to anemia. Subsequently, we divided those ID mice into 2 groups. The first was fed with...
OsNAS3-D1 seeds, the second with WT seeds. At the beginning of the experiment, blood Hb and Hct levels did not differ between the 2 groups (Fig. 5 C and D). After 2 weeks, however, those values were significantly higher in the first group. Mice fed with the mutant seeds had 20% more Hb and 13% more Hct than those values were significantly higher in the first group. Mice fed between the 2 groups (Fig. 5 C and D). These levels were also found in normal, nonanemic mice, indicating that recovery from anemia was more rapid in the first group. After 4 weeks, those values were maintained at higher levels in the first group, whereas the second group, fed with WT seeds, did not recover (Fig. 5 C and D). These results indicate that seeds from the activation-tagged lines contained significantly higher amounts of bioavailable Fe that were sufficient for recovery.

Discussion

In higher plants, NA plays a key role in metal homeostasis as a ubiquitous metal chelator (11). In rice, NA is synthesized by 3 genes that are differentially regulated by Fe (16). Similar expression patterns and the very close genomic location of OsNAS1 and OsNAS2 on the same chromosome suggest their functional redundancy and recent duplication (16). In contrast, OsNAS3 expression is induced in roots but suppressed in leaves when Fe is lacking.

In this study, we demonstrated that the NA level was significantly increased by activation of OsNAS3, resulting in elevated Fe and Zn contents in both leaves and seeds. Under Fe-deficient conditions, this improved Fe content in the OsNAS3-D1 plants was associated with better growth and greener leaves. These visual differences were supported by increased chlorophyll and Fe concentrations. OsNAS3 activation also resulted in greater tolerance to Zn deficiency. The opposite phenotypes observed in the osnas3-1 knockout or antisense plants also demonstrate the involvement of OsNAS3 in metal homeostasis. Therefore, our findings indicate that NAS genes could be candidates for the improvement of metal concentrations in rice.

Another positive outcome of OsNAS3 activation is that it protects plants against toxic heavy metals. The importance of NA for heavy-metal metabolism has been previously reported (11, 12, 14). For example, overexpression of AtnNAS1 leads to improved tolerance against higher amounts of Ni (11). Here, we showed that OsNAS3 activation enhanced tolerance to elevated levels of Zn, Cu, and Ni, but not to Cd. These suggest that the chelation of Zn, Cu, and Ni in the xylem or phloem may be an important factor in providing tolerance to these metals. Our study also demonstrated that NA is not a chelator of Cd.

Constitutive overexpression of NAS genes results in elevated levels of Fe and Zn in transgenic tobacco plants (11, 14). We also generated transgenic rice plants overexpressing OsNAS3 under the control of the maize ubiquitin promoter. However, the increase in metal concentration was not significant compared with WT. This discrepancy was likely due to differences in expression patterns. OsNAS3 has been reported to be expressed in the pericycle and companion cells (16), whereas ectopic expression of OsNAS3 by the ubiquitin promoter caused a general increase in transcripts. Therefore, maintaining the endogenous expression pattern may be critical for efficiently elevating metal contents.

Grain Fe and Zn contents were significantly increased in OsNAS3-D1. This is a desirable characteristic for biofortification—the delivery of micronutrients via micronutrient-dense crops—and offers a cost-effective and sustainable approach to solving micronutrient deficiencies (3). In our OsNAS3-D1 seeds, Fe and Zn amounts were increased not only in nonmilled seeds but also in the milled grains. However, we needed to examine bioavailability because the higher levels of micronutrients did not always lead to a proportionate increase in the amount of bioavailable micronutrients.

The main strategy for Fe fortification in rice grains by genetic engineering has been to use the soybean ferritin gene, which encodes an Fe-rich storage protein, under the control of an endosperm-specific promoter (9, 10). To produce grains with more bioavailable Fe in the endosperm, Drakakati et al. (22) have coexpressed soybean ferritin and Aspergillus phytase genes in the maize endosperm and have described 20–70% increases in seed levels of Fe and phytase (22). The in vitro digestion/Caco-2 cell system also demonstrates that improvements are associated with greater uptake of cellular Fe (22). However, the bioavailability of Fe from ferritin has been a matter of controversy (23). Nonanemic women who consume reconstituted soybean ferritin as an Fe source show improvements in Fe status (23), but other studies on soybean ferritin have suggested poor availability (24).

Recently, Masuda et al. (25) have shown that Fe and Zn concentrations in rice grains can be increased via the introduction of barley genes involved in phytosiderophore synthesis. Polished rice seeds with IDS3 inserts have up to 1.40-fold and 1.35-fold higher Fe and Zn concentrations, respectively, than measured in nontransgenic rice seeds (25). Rice nat1 mutant seeds have a significantly higher concentration of Fe in both unpolished (1.8-fold) and polished (3.8-fold) grains than in WT (15). However, it has not been proven whether these changes accompany any rise in bioavailable Fe.

Here, Fe specification by SEC-ICP-MS analyses indicated that the NA-bound Fe level was increased 7-fold in OsNAS3-D1 seeds compared with WT, whereas phytic acid-bound Fe was unchanged. Because the NA level was increased 9-fold in the seeds, this suggests that most of the additional NA was bound to Fe, such that the amount of Fe transferred to the seeds was not a limiting factor. This implies improved Fe bioavailability in OsNAS3-D1 seeds. Therefore, we tested Fe-deficient mice to demonstrate further that the bioavailable Fe concentration in OsNAS3-D1 grains actually was increased.

Testing the nutritional qualities of genetically modified foods is a rigorous process because any initial study of mineral nutrition requires one to label foods with either radioactive isotopes for animal studies or stable isotopes for human trials (26). Here, we established a simple mouse model system to assess Fe bioavailability. We observed that feeding mice with a low-Fe diet for 2 weeks resulted in a significant reduction in blood Hb and Hct levels. These anemic mice were then used for comparing bioavailability by feeding regular or engineered grains. By measuring Hb and Hct levels in their blood, a rise in Fe content can be monitored indirectly. We observed a rapid recovery from anemia in the OsNAS3-D1 seed-feeding group compared with those that ate WT seeds. Within 2 weeks, the anemic mice were fully recovered when fed engineered grains. However, they did not recover from the Fe deficiency when regular grains were given. This study in mouse demonstrates that transgenic rice seeds containing high Fe could alleviate Fe-deficiency anemia. Furthermore, our results suggest that it may be feasible to produce Fe-rich rice as an Fe supplement for animals, including humans. The first human feeding trial, conducted by Haas et al. (8), has shown that consumption of biofortified rice that is selected by breeding improves the Fe nutritional status of Philippina women. Therefore, combining conventional breeding with transgenic approaches would further increase mineral contents in rice grains.

It will be worthwhile to examine whether combining OsNAS3-D1 and a high-mineral germplasm would further raise seed mineral contents. Phytic acid in cereal grains is considered an antinutrient (27). Therefore, generating a rice hybrid between OsNAS3-D1 and a low-phytate variety would provide great potential in improving diets and human health.
Materials and Methods

Plant Growth Conditions. WT and transgenic rice seeds were germinated on a standard M5 agar medium supplemented with 0.1 μM CuSO4, 100 μM Fe (III)-EDTA, 30 μM ZnSO4, and 10 μM MnSO4 as micronutrients. Seedlings were grown for 10 days at 28 °C under continuous light, then transplanted to soil in the greenhouse and raised to maturity. For our micronutrient deficiency tests, seeds were germinated and grown on an MS medium lacking CuSO4, Fe (III)-EDTA, ZnSO4, or MnSO4.

RNA Expression Analysis. For RT-PCR, the RNA preparation, cDNA synthesis, and real-time PCRs were performed as previously described (28). Primers for PCR are listed in Table S1.

Isolation of OsNAS3 Mutant Plants. The sequences flanking the T-DNA insertion sites were determined by inverse PCR analyses (18, 19). Putative OsNAS3 mutant lines were isolated from our rice flanking sequence-tag database (www.postech.ac.kr/life/pfg). T2 progeny of the primary mutants were grown to maturity for seed multiplication. Progeny were then genotyped by PCR, with 3 primers. For the OsNAS3 knockout mutant line 2D30228 (asna3-1), we used 2 gene-specific primers (F1 and R1) and a T-DNA-specific primer (RB). For OsNAS3 activation mutants, 3 primers were included: for OsNAS3-D1 (line 3A16471), 2 gene-specific primers (F2 and R2) and an RB primer; and for OsNAS3-D2 (line 1001007), 2 gene-specific primers (F2 and R3) and an RB primer. After genotyping, OsNAS3 transcript levels were evaluated by RT-PCR or real-time PCR, using cDNA prepared from 8-day-old seedling leaves, flag leaves, −10 cm panicles, and immature seeds collected at 5 days after pollination. Primers for genotyping are listed in Table S1.

Vector Construction and Plant Transformation. To generate the OsNAS3-overexpression construct, we amplified the full-length cDNA sequence by a primer pair (OxF and OxR). For the OsNAS3 antisense construct, the 3’ gene-specific region of OsNAS3 was amplified by another primer pair (ANF and AnR). The PCR products were inserted into pGA1611, generating gene-specific region of primer pair (OxF and OxR). The PCR products were inserted into pGA1611, generating gene-specific region of primer pair (OxF and OxR).

Mouse Feeding Experiments. Pathogen-free female BALB/c mice (purchased from Orient Bio) were maintained under a 12 h:12 h light/dark cycle. After 3 weeks of weaning, some mice were fed an AIN-93DIET (45 mg Fe kg−1) as a control diet. Others were given an ID diet (modified AIN-93G diet containing 3 mg Fe kg−1). Two weeks later, we measured the blood Hb and Hct levels for each group. Afterward, mice exposed to the ID diet were divided into 2 groups. The first group (n = 10) was fed WT seeds; the second group (n = 9), OsNAS3-D1 seeds. At 2 and 4 weeks after the start of this feeding experiment, blood was collected from their orbital sinuses, and Hb and Hct levels were analyzed by a commercial service (Green Cross Reference Lab).

Acknowledgments. We thank In-Soon Park and Kyungsook An for plant transformation, Jongdae Kyung for technical assistance with the AAS measurements, and Priscilla Licht for English editing. ICP-MS measurements were conducted at the Korea Basic Science Institute in Busan, Korea. This work was supported, in part, by Grant CG1111 from the Crop Functional Genomic Center, the 21st Century Frontier Program; Grant 20070401–034-001–007–03–00 from the BioGreen 21 Program, Rural Development Administration; Basic Research Promotion Fund KRF-2007–341-C00028 from the Korea Research Foundation, Grant funded by the Korean Government (MOERD); Grant FOOD-CT-2006–03622 from the Sixth Framework Programme for Research and Technological Development by the European Union (EU-FP6) project Metabolomics for Plants, Health and OutReach (META-PHOR); and Grant FOOD-CT-2006–016253 from PHIME (public health impact of long-term, low-level mixed element exposure in susceptible population strata).
Supporting Information

Lee et al. 10.1073/pnas.0910950106

Fig. S1. Fe (A), Zn (B), Cu (C), and Mn (D) concentrations in flag leaves from WT, osnas3-1, and OsNAS3-antisense (AN-2) plants. Each measurement was from four leaves at flowering stage. Error bars represent SE. Significant differences from WT were determined by Student’s t test; *, P < 0.05.
Fig. S2. Real-time PCR analyses of WT and OsNAS3-D1 mutants, using RNA samples from flag leaves (A), flowers (B), and immature seeds (C). Gene expression was relative transcript level between OsNAS3 and actin1 transcripts. Real-time PCR analysis of OsFerritin1 (D) and OsFerritin2 (E). RNA was prepared from 8-day-old shoots grown on standard MS (Fe⁺) or Fe-deficient (Fe⁻) media. Transcript levels are represented by ratio between mRNA level for OsFerritin genes and that for rice actin1. Vertical bars indicate standard deviation. Significant differences from WT were determined by Student’s t test; *, P < 0.05.
Fig. S3. Enhanced tolerance of OsNAS3-D2 plants under Fe and Zn deficiencies. WT and OsNAS3-D2 seeds were germinated and seedlings grown on solid MS medium in presence of 100 μM Fe and 30 μM Zn (A) or in absence of Fe (B) or Zn (C). Lower panels are enlargements of second leaves. Pictures were taken 8 days after germination. (Scale bars, 5 cm.) Heights (D) and total chlorophyll contents (E) of WT and OsNAS3-D2 plants. Data are means ± SD (n = 8 for height; n = 4 for measurements of chlorophyll contents). Significant differences from WT were determined by Student’s t test; *, P < 0.05.
Fig. S4. Phenotypes of osnas3-1 and antisense transgenics under Fe and Zn deficiencies. Plants of WT, osnas3-1, and OsNAS3-antisense (AN-2) were grown on solid MS medium in presence of 100 μM Fe and 30 μM Zn (A and D) or in absence of Fe (B and E) or Zn (C and F). Lower panels are enlargements of second leaves. Pictures were taken 8 days after germination. (Scale bars, 5 cm.) Quantification of phenotypes for osnas3-1 and antisense transgenic plants. Heights (G) and total chlorophyll contents (H) in WT, osnas3-1, and OsNAS3-antisense (AN-2) plants. Data are means ± SD (n = 8 for height, n = 4 for chlorophyll content). Significant differences from WT were determined by Student’s t test; *, P < 0.05.
Fig. S5. Fe (A), Zn (B), Cu (C), and Mn (D) concentrations in chloroplasts, mitochondria, and protoplasts from WT and OsNAS3-1 plants grown on standard MS (Fe+) or Fe-deficient (Fe−) media. Error bars represent SE. Significant differences from WT were determined by Student t tests; *, P < 0.05.
Fig. S6. Phenotypes of OsNAS3-D1 and osnas3–1 plants exposed to elevated levels of Cd. WT, OsNAS3-D1 (A), and osnas3–1 (B) plants were grown for 10 days on solid agar containing half-strength MS medium supplemented with 0.3 mM CdCl₂ (A and B). (Scale bars, 2.5 cm.) (C) Average heights (n = 8) of WT and mutant plants grown on media containing excess metals. (D) Cd from shoots and roots of WT, OsNAS3-D1, and osnas3-1.
Fig. S7. Fe (A), Zn (B), Cu (C), and Mn (D) contents in flag leaves of WT, OsNAS3-D1, and OsNAS3-D2. Each measurement was from 4 leaves at flowering stage. Error bars represent SE. Significant differences from WT were determined by Student’s t test; *, P < 0.05.
Fig. S8. Ni (A), Cd (B), Se (C), Mg (D), Ca (E), and P (F) contents in mature seeds of WT and OsNAS3-D1. Error bars represent SE. Significant differences from WT were determined by Student’s t test; *, P < 0.05.
<table>
<thead>
<tr>
<th>Primer</th>
<th>Nucleotide sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>OsNAS3-forward</td>
<td>5'-GTGATCAACTCCGTATCATC-3'</td>
</tr>
<tr>
<td>OsNAS3-reverse</td>
<td>5'-TCACTCTCATATGGGAAAAA-3'</td>
</tr>
<tr>
<td>OsAct1-forward</td>
<td>5'-TGGAAGCTGCGGTATCCAT-3'</td>
</tr>
<tr>
<td>OsAct1-reverse</td>
<td>5'-TACTCAAGCCTGGCAATCCACA-3'</td>
</tr>
<tr>
<td>F1</td>
<td>5'-GTGATCAACTCCGTATCATC-3'</td>
</tr>
<tr>
<td>R1</td>
<td>5'-TCCCCCTGTTTAGATCATTTT-3'</td>
</tr>
<tr>
<td>F2</td>
<td>5'-TTTAGGGGAAAATGGAGTTACT-3'</td>
</tr>
<tr>
<td>R2</td>
<td>5'-CTGTAACACTTTAACGACCAA-3'</td>
</tr>
<tr>
<td>F3</td>
<td>5'-GGCTTTGCTTTGTCATAGGC-3'</td>
</tr>
<tr>
<td>RB</td>
<td>5'-CAAGTTAGTCAATGTAATTAGCCAC-3'</td>
</tr>
<tr>
<td>Anf</td>
<td>5'-AAGGTACCATCCCTCCTCCAACTACGACA-3'</td>
</tr>
<tr>
<td>AnR</td>
<td>5'-AAAAGCTTAGTTACCTGCTCATATGGGAAAA-3'</td>
</tr>
<tr>
<td>OX-F</td>
<td>5'-AAAAGCTTAGTTACCTGCTCATATGGGAAAA-3'</td>
</tr>
<tr>
<td>OX-R</td>
<td>5'-AAGGTACCATCCCTCCTCAGATCATGGGAAAA-3'</td>
</tr>
</tbody>
</table>