Plant Biotechnology Journal

Plant Biotechnology Journal (2011) **9**, pp. 865–873 doi: 10.1111/j.1467-7652.2011.00606.x

Bio-available zinc in rice seeds is increased by activation tagging of *nicotianamine synthase*

Sichul Lee[1,2], Daniel P. Persson[3], Thomas H. Hansen[3], Søren Husted[3], Jan K. Schjoerring[3], You-Sun Kim[4], Un Sil Jeon[5], Yoon-Keun Kim[4], Yusuke Kakei[6], Hiroshi Masuda[6], Naoko K. Nishizawa[6] and Gynheung An[1,*]

[1]*Department of Plant Molecular Systems Biotechnology and Crop Biotech Center, Kyung Hee University, Yongin, Republic of Korea*

[2]*Department of Biological Sciences, Dartmouth College, Hanover, NH, USA*

[3]*Plant and Soil Science Laboratory, Department of Agriculture and Ecology, Faculty of Life Sciences, University of Copenhagen, Frederiksberg C, Denmark*

[4]*Department of Life Science, Pohang University of Science and Technology (POSTECH), Pohang, Republic of Korea*

[5]*Department of Internal Medicine, Korea University Guro Hospital, Seoul, Republic of Korea*

[6]*Graduate School of Agricultural and Life Sciences, The University of Tokyo, Tokyo, Japan*

Received 21 September 2010;
revised 30 December 2010;
accepted 5 January 2011.
**Correspondence* (Tel +82-31-201-3470; fax +82-31-204-3178; email genean@khu.ac.kr)

Keywords: activation tagging, bio-availability, mouse, nicotianamine, rice, zinc.

Summary

We generated rice lines with increased content of nicotianamine (NA), a key ligand for metal transport and homeostasis. This was accomplished by activation tagging of rice *nicotianamine synthase 2* (*OsNAS2*). Enhanced expression of the gene resulted in elevated NA levels, greater Zn accumulations and improved plant tolerance to a Zn deficiency. Expression of Zn-uptake genes and those for the biosynthesis of phytosiderophores (PS) were increased in transgenic plants. This suggests that the higher amount of NA led to greater exudation of PS from the roots, as well as stimulated Zn uptake, translocation and seed-loading. In the endosperm, the *OsNAS2* activation-tagged line contained up to 20-fold more NA and 2.7-fold more zinc. Liquid chromatography combined with inductively coupled plasma mass spectrometry revealed that the total content of zinc complexed with NA and 2′-deoxymugineic acid was increased 16-fold. Mice fed with *OsNAS2-D1* seeds recovered more rapidly from a zinc deficiency than did control mice receiving WT seeds. These results demonstrate that the level of bio-available zinc in rice grains can be enhanced significantly by activation tagging of *OsNAS2*.

Introduction

Rice is one of the most important food crops in the world, providing calories and micronutrients for more than 3 billion people (Fitzgerald *et al.*, 2009). Opportunities have now arisen for improving the nutritional value of those grains. Higher micronutrient contents from staple crops, such as rice, could solve mineral malnutrition, which is a significant public health issue in most developing countries (Zhu *et al.*, 2007). Unfortunately, this valuable food material is a poor source of zinc (Zn), which is essential for all organisms. It is a cofactor in more than 300 enzymes and has important structural roles in many metalloproteins (Palmgren *et al.*, 2008). Zn deficiency is prevalent among children and pregnant women, especially in developing countries. It affects more than 25% of the world's population, causing growth retardation, skin diseases, hypogonadism, immune dysfunction and cognitive impairment (Cakmak, 2008; Gómez-Galera *et al.*, 2010). Thus, improving its bioavailability in cereal grains will generate major health benefits for a large number of susceptible persons (Zhu *et al.*, 2007).

Strategies to increase Zn levels involve either agronomic fortification by application of Zn fertilizer or biofortification via plant breeding or genetic engineering (Cakmak, 2009; Gómez-Galera *et al.*, 2010). Only a few reports have described such engineering approaches with rice grains. One method utilizes nicotianamine (NA), a key ligand involved in metal transport and homeostasis in plants (Douchkov *et al.*, 2005). NA is synthesized from S-adenosyl methionine in a process catalyzed by NA synthase (NAS; Inoue *et al.*, 2003). Transgenic rice grains expressing barley *NAS* under control of the rice *Actin1* promoter contain 30% more Zn (Masuda *et al.*, 2009). Coexpression of *NAS* and *ferritin* led to a 50% increase of the Zn concentration in rice endosperms (Wirth *et al.*, 2009). Endosperm overexpression of *OsNAS1* results in a rise in Zn content of up to 1.6-fold (Zheng *et al.*, 2010). Recently, a more significant increase in the Zn level, i.e. 2.2-fold, has been obtained by activation-tagging of *OsNAS3* (Lee *et al.*, 2009b).

None of the above studies has addressed the bioavailability of the elevated Zn content, which is fundamental to the successful implementation of any biofortification strategy (Hoppler *et al.*, 2008). The bioavailability of a given element is determined by both its total concentration and its distribution among various chemical species (Zhu *et al.*, 2007). The phosphorus-rich compound phytic acid is considered the dominating ligand controlling Zn levels in seeds (Raboy, 2003). However, very limited analytical evidence is available for the actual Zn-binding forms in cereal grains. Here, we show that elevated expression of *OsNAS2* leads to a marked increase in Zn content in the endosperm complexed with NA and deoxymugineic acid (DMA). We further demonstrate, through mice feeding experiments, that Zn bioavailability is substantially improved in seeds from genetically engineered rice.

Results

Isolation of *OsNAS2* activation-tagged mutants and generation of overexpressing plants

An activation-tagged allele of *OsNAS2* was identified from our rice flanking sequence-tag database (Jeon *et al.*, 2000; Jeong *et al.*, 2002, 2006). In Line 1B04309 (*OsNAS2-D1*), 35S enhancer elements were inserted approximately 2.0 kb downstream of the *OsNAS2* ORF (Figure 1a). Quantitative real-time (qRT) PCR analysis indicated that, compared with the wild type (WT), *OsNAS2* transcript levels were higher in the shoots of the mutant (Figure 1b). Expression of *OsNAS2* was also increased in flag leaves, flowers and immature seeds of activation-tagged line (Figure 1c–e). At the flowering stage, activation of *OsNAS2* was associated with elevated concentrations of Zn (42%) in flag leaves (Figure 1f).

To confirm that these observations were due to increased *NAS* expression, we generated 13 transgenic plants with ectopic expression of full-length *OsNAS2* cDNA (Figure 1g). Transcript levels were determined by qRT-PCR, using RNA samples from flag leaves of independent transgenics (Figure 1h). Several plants with elevated foliar Zn levels were identified (Figure 1i); plants #9 (OX-9) and #10 (OX-10) were selected for further investigations.

Increased expression of *OsNAS2* confers enhanced tolerance to a Zn deficiency

To test whether the higher level of *OsNAS2* transcripts affects plant development and metal distribution, we treated plants of

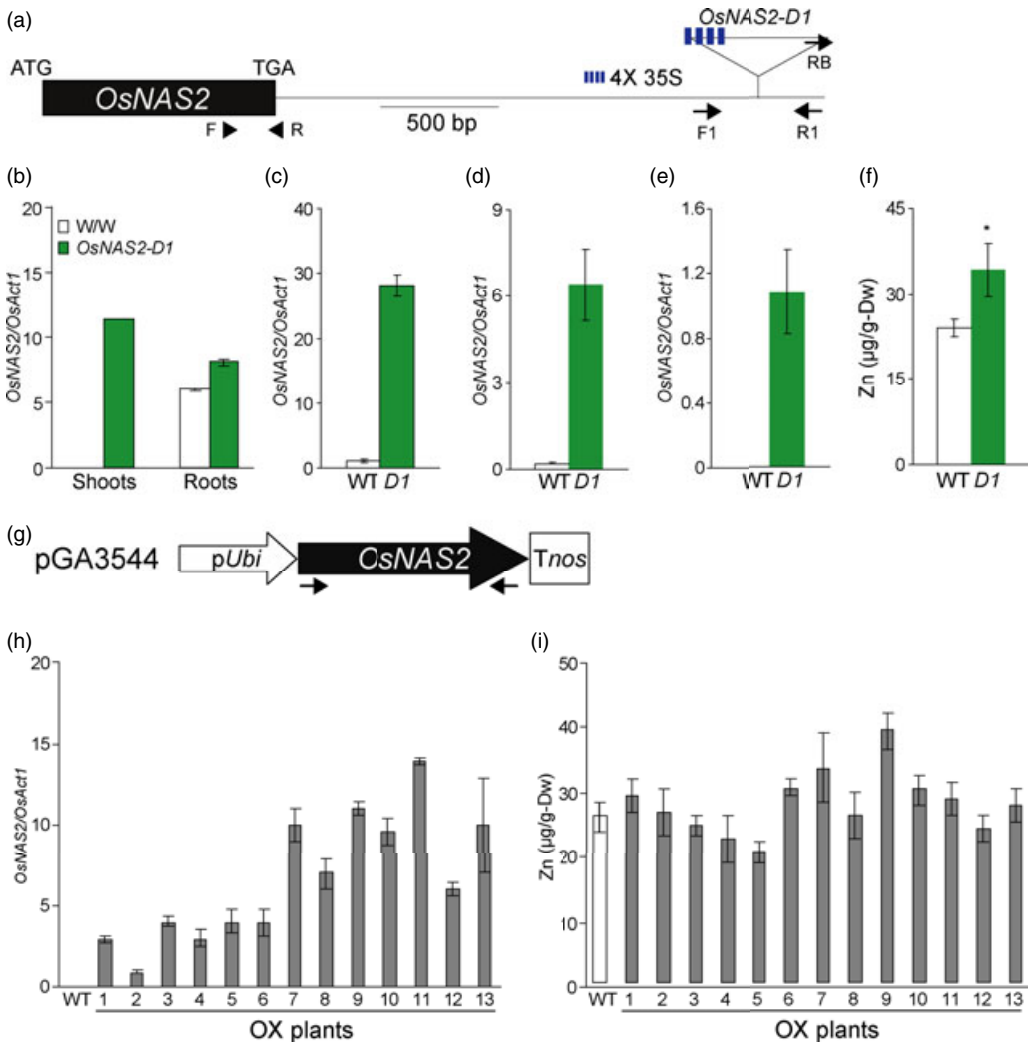

Figure 1 Characterization of *OsNAS2* transgenic plants. (a) Schematic diagram of *OsNAS2-D1* showing position of inserted T-DNA. Black bar indicates coding region. Gene-specific (F1 and R1) and T-DNA-specific (RB) primers used in genotyping are shown with arrows. Primers (F and R) for qRT-PCR are indicated by arrow heads. 4 × 35S, four copies of *35S* enhancer elements from cauliflower mosaic virus. Real-time PCR analyses of WT and *OsNAS2-D1* (indicated as *D1*) plants, using RNA from shoots and roots (b), flag leaves (c), flowers (d) and immature seeds (e). Gene expression was described as relative transcript levels between *OsNAS2* and *OsAct1*. W/W, segregating WT siblings of *OsNAS2-D1* line. (f) Zn concentrations in flag leaves from WT and *OsNAS2-D1*. (g) Schematic representation of pGA3544 expressing *OsNAS2* under control of maize *ubiquitin* promoter (p*Ubi*) and *nopaline synthase* terminator (T*nos*). *OsNAS2*-specific primers (OxF and OxR) were used to measure transcripts in transgenic plants. (h) qRT-PCR analysis of *OsNAS2* in WT and *OsNAS2*-overexpressing (OX) transgenic plants, using RNA from flag leaves. (i), Zn concentrations in flag leaves from WT and *OsNAS2*-overexpressing transgenic plants. Values are means ± SD.

OsNAS2-D1, OX-9 and OX10 under different metal supplies. When placed on a standard MS medium with sufficient Zn, growth of *OsNAS2-D1*, OX-9 and OX-10 plants was indistinguishable from WT siblings (Figure 2a and Figure S1a). However, *OsNAS2-D1*, OX-9 and OX-10 plants contained more Zn in their shoots (83%, 84% and 101%, respectively) and roots (38%, 55% and 61%, respectively) (Figure 2c). In contrast, when Zn was limiting, plants over-expressing *OsNAS2* grew better than the corresponding WT (Figure 2b). Plants of *OsNAS2-D1*, OX-9 and OX-10 under Zn deficiency were 9%, 16% and 10% taller, respectively, than the WT (Figure S1a). Furthermore, at reduced Zn supply, Zn concentrations in *OsNAS2-D1,* OX-9 and OX-10 were 71%, 78% and 40% higher in shoots, and 68%, 81% and 59% higher in roots, respectively (Figure 2d). Thus, greater *OsNAS2* expression resulted in enhanced tolerance to a Zn deficiency.

To investigate whether greater *OsNAS2* expression affects Zn homeostasis, we examined representative genes involved in Zn transport and acquisition. Transcript levels of *OsZIP4* (Ishimaru *et al.*, 2005) and *OsZIP5* (Lee *et al.*, 2010) were markedly increased in transgenic roots (Figure 2e,f). These genes encode a membrane protein that transports Zn (Ishimaru *et al.*, 2005; Lee *et al.*, 2010). The expression of *nicotianamine aminotransferase* (*OsNAAT1*) (Suzuki *et al.*, 2008) and *2′-deoxymugineic acid synthase* (*OsDMAS1*) (Bashir *et al.*, 2006) was likewise higher in transgenic plants (Figure 2g,h). These two genes act downstream of *OsNAS* and are involved in the production of phytosiderophores (PS). Similar results were obtained from the shoots (Figure S1b–e).

Increased expression of *OsNAS2* enhances the Zn content in mature seeds

Transcript levels of *OsNAAT1* and *OsDMA1* were also higher in transgenic seeds (Figure 3a,b). This resulted in 19.8- and 3.5-fold higher levels of NA and DMA, respectively, relative to the WT (Figure 3c,d). Whole mature seeds from engineered plants contained 2.3- to 3.2-fold more Zn (Figure 3e). Polishing decreased the Zn concentration in mature grains about 16%, but the grains from engineered plants still contained 2.4- to 2.9-fold more Zn than those from the WT (Figure 3f).

Increased expression of *OsNAS2* results in a new Zn pool complexed with NA and DMA in the endosperm of mature seeds

To elucidate the chemical speciation of Zn, we polished seeds and extracted their remaining endosperm in aqueous buffer prior to analysis by size exclusion chromatography hyphenated with inductively coupled plasma mass spectrometry (SEC-ICP-MS; Persson *et al.*, 2009). In the WT, all Zn in the endosperm eluted as a single fraction at 770 s (Figure 4a). The ion intensity of Zn in this fraction was similar to that observed for *OsNAS2-D1* seeds. However, a major additional Zn pool, eluting at 840 s, was present in seeds of the activation-tagged lines (Figure 4a). Thus, activation of *OsNAS2* resulted in the formation of a new pool of low-molecular-weight Zn species *in planta*, resulting in 16.4 ± 1.2 times higher extractable amount of Zn in *OsNAS2-D1* compared with the WT. To verify the identity of the ligand(s) responsible for the novel pool, the Zn-binding fraction eluting at 840 s was collected, lyophilized and re-injected onto a hydrophilic interaction liquid chromatography (HILIC) column coupled to an electro spray ionization-time of flight-mass spectrometer (ESI-TOF-MS). The newly obtained mass spectrum confirmed the main compounds to be NA (LC-ESI-TOF-MS tR = 8.96; m/z calculated: 304.1509, m/z found: 304.1566; $C_{12}H_{22}N_3O_6$) and DMA (tR = 8.58; m/z calculated: 305.1349, m/z found: 305.1510; $C_{12}H_{21}N_2O_7$) (Figure 4b,c).

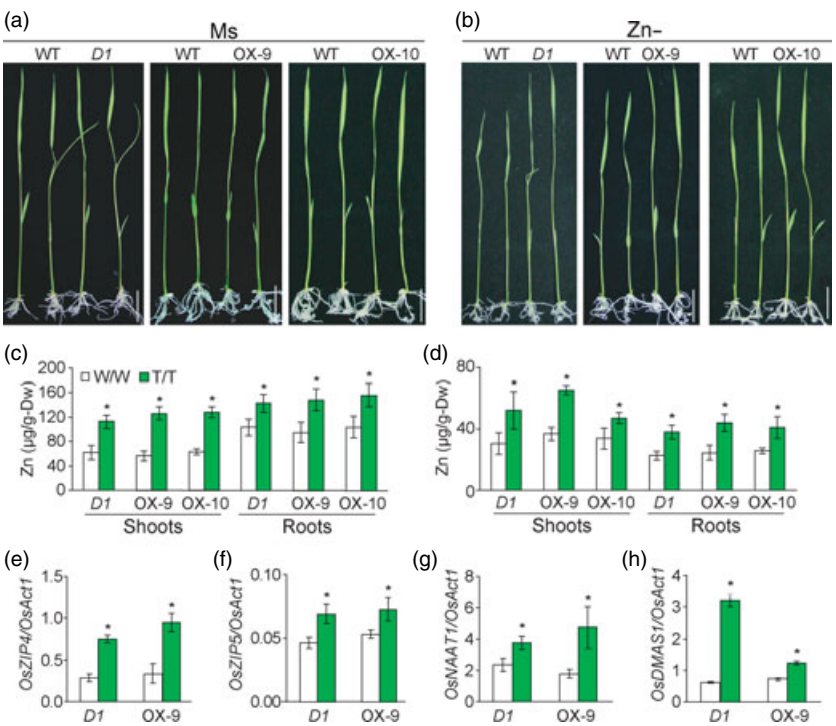

Figure 2 Enhanced tolerance to Zn deficiency. Phenotypes of *OsNAS2-D1*, OX-9, OX-10 and WT plants grown for 8 days on control MS (a) or Zn-limited (b) media. Scale bars = 2.5 cm. Zn concentrations in shoots and roots (n = 4 each) from *OsNAS2-D1*, OX-9, OX-10 and WT grown on MS (c) or Zn-free (d) media. Expression levels of *OsZIP4* (e), *OsZIP5* (f), *OsNAAT1* (g) and *OsDMAS1* (h) in roots of *OsNAS2-D1* and OX-9 plants grown on normal MS media. T/T, homozygous transgenic plants; W/W, segregating WT siblings. Values are means ± SD. Significant differences from WT were determined by Student's t-tests; $^*P < 0.05$.

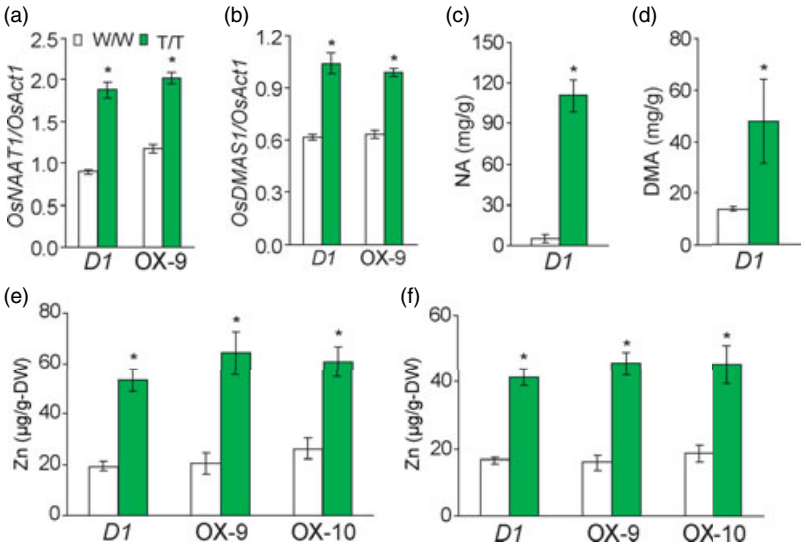

Figure 3 Characterization of mature seeds. Transcript levels of *OsNAAT1* (a) and *OsDMAS1* (b) in *OsNAS2-D1* and OX-9 plants (T/T) and segregating WT siblings (W/W). Nicotianamine (c) and DMA (d) concentrations in *OsNAS2-D1* and WT seeds. (e) Zn concentrations in mature seeds obtained from *OsNAS2-D1*, OX-9, OX-10 and WT siblings. (f) Concentrations of Zn in milled seeds. Values are means ± SD from four replicates per line. Significant differences from WT were determined by Student's *t*-tests; $*P < 0.05$.

Mice fed with *OsNAS2-D1* seeds recover more quickly from a Zn deficiency

The bioavailability of Zn in *OsNAS2-D1* seeds was tested in a feeding experiment employing mice. To induce zinc deficiency, 4-week-old mice were fed either a control diet (CD; 30 mg Zn per kg) or a Zn-depleted diet (ZD; 0.5 mg Zn per kg) for 2 weeks. Because reduction in zinc level in serum is a major symptom of Zn deficiency (King *et al.*, 2005), we measured these parameters as an indicator of the Zn status. During the 2-week period, CD mice continuously gained weight, whereas ZD mice failed to grow (Figure 5a,b). After 2 weeks, the weight of ZD mice was only 76% of that of control mice (Figure 5a), and their Zn levels in plasma and muscles were decreased to 68% and 88%, respectively, of the control values (Figure 6a,b). Thus, Zn deficiency was clearly induced in mice receiving the Zn-depleted diet.

The ZD mice were then divided into three new groups for feeding with the control diet, *OsNAS2-D1* seeds, or WT seeds. During recovery, body weights returned to normal within 6 day when *OsNAS2-D1* seeds were supplied, whereas it took 13 day with WT rice (Figure 5c). Plasma Zn levels from the first and second groups increased rapidly and were restored within 15 days to the status of the control mice, which had never been exposed to a deficient diet (Figure 6a). By contrast, the plasma Zn level in the third group receiving WT seeds increased slowly and did not recover to normal (Figure 6a). In muscle tissue, Zn levels also rose more rapidly in mice receiving *OsNAS2-D1* seeds compared with those fed WT grains (Figure 6b). These results show that *OsNAS2-D1* seeds had markedly higher amounts of bio-available Zn.

Enhanced expression of *OsNAS2* in rice plants leads to improved tolerance towards heavy metals but does not increase cadmium and lead levels

Nicotianamine forms coordination complexes with several cations in plant cells. Therefore, we investigated whether increased *OsNAS2* expression also influences plant development in the presence of excess heavy metals. *OsNAS2-D1*, OX-9 and OX-10 plants showed improved tolerance to a high level of Zn (5 mM). Whereas WT-seedling growth was reduced under that condition, engineered plants grew better and showed less chlorosis (Figure 7a and Figure S2d). The *OsNAS2-D*, OX-9 and OX-10 plants also accumulated higher levels of Zn in their shoots (37%, 47% and 25%, respectively) and roots (46%, 45% and 31%, respectively) compared with the WT (Figure 7d).

Transgenic plants also tolerated higher concentrations of Cu, Ni and Co (Figure S2a–d) and accumulated more of these elements (Figure S2e–g). Under excess Cd and Pb, however, WT and transgenic plants did differ in neither their growth nor their concentrations of heavy metals in shoots, roots and grains (Figure 7b,c,e,f and Figure S2d,h,i). In mature seed, the content of Fe, Cu and Ni associated with the NA/DMA pool was increased 4.9-, 9.7- and 2.4-fold, respectively, relative to the WT (Figure S3). However, this pool did not contain more Cd (data not shown).

Discussion

Biofortification offers a cost-effective and sustainable approach to alleviation of micronutrient malnutrition, a major health burden for more than half of the world's population (Mayer *et al.*, 2008). We here show that the content of bioavailable Zn in the endosperm of rice grains can be drastically enhanced by genetic engineering of *NAS*. Enhanced expression of *OsNAS2* resulted in elevated NA levels, greater total content of Zn and better plant tolerance to Zn deficiency. Transcripts of genes involved in Zn uptake and PS biosynthesis were increased in transgenic plants grown with sufficient Zn. This suggests that the higher amount of NA in engineered plants led to a greater exudation of PS from the roots and stimulated Zn uptake, translocation and seed-loading.

Engineered plants also showed greater tolerance to elevated Zn, Cu, Ni and Co, and they accumulated more of these metals. Plants with elevated *OsNAS2* expression may thus complex metal ions more effectively, thereby reducing the activities of the free ions and their accompanying phytotoxic effects. It is an advantage that our engineered plants did not have elevated levels of Cd and Pb relative to the WT, indicating that NA does not form stable coordination complexes with these two metals *in planta*. Logarithms of NA stability constants for Cu, Ni, Co, Zn and Fe are 18.8, 16.1, 14.8, 14.7 and 12.1, respectively

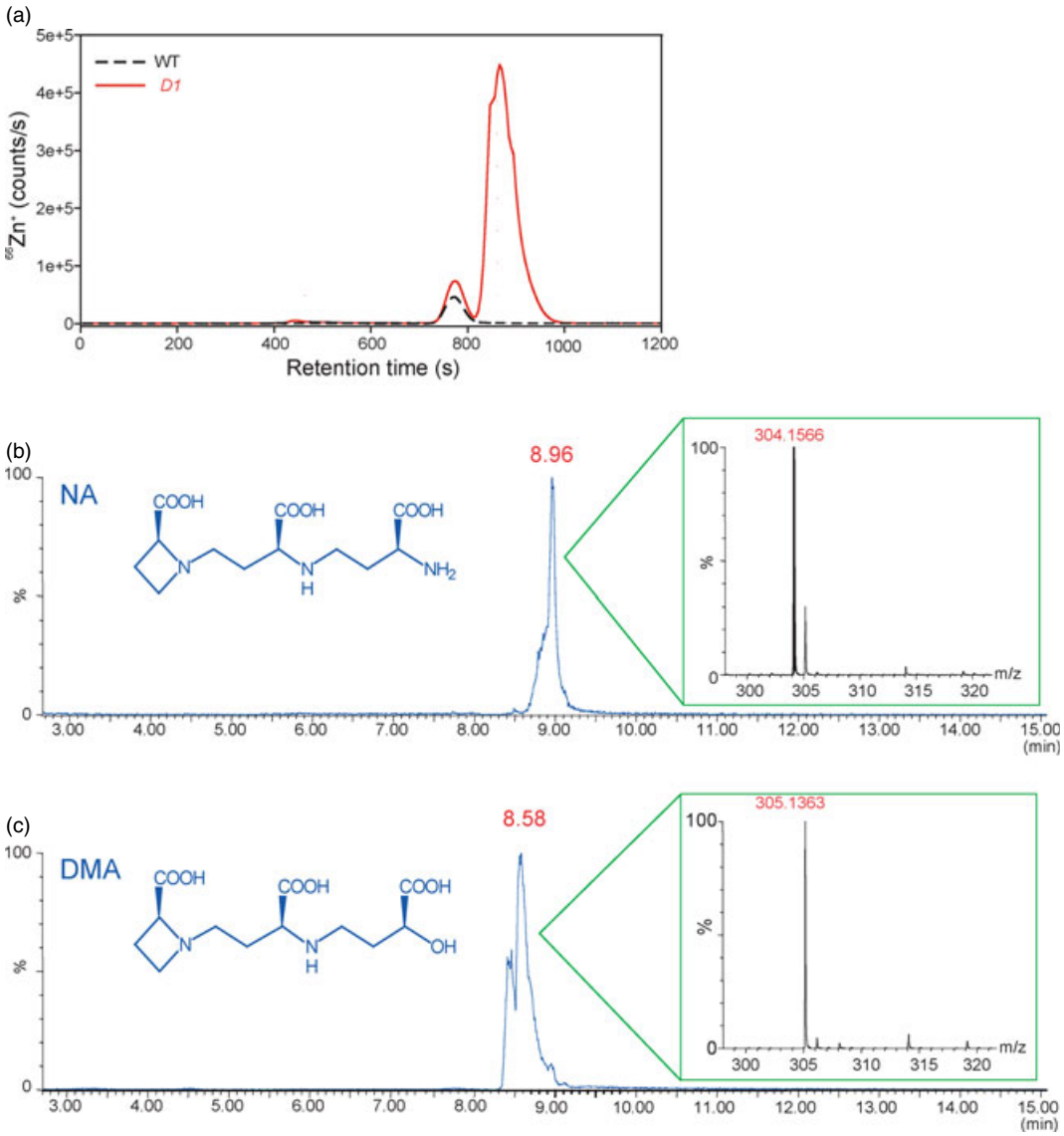

Figure 4 Chemical speciation of Zn in endosperm. (a) SEC-ICP-MS chromatograms of *OsNAS2-D1* (red) and WT (black). Zn was monitored as $^{66}Zn^{+}$, and chromatograms were averaged from triplicate samples. (b, c) HILIC-ESI-TOF-MS ligand analysis of Zn fractions collected from SEC column. Peak eluting at 8.96 min (b) was identified as nicotianamine; peak at 8.58 min (c) was identified as DMA. ESI-TOF-MS signals in positive mode from Zn fractions collected on SEC column. Signals strongly suggest the presence of both NA (m/z calculated: 304.1509) and DMA (m/z calculated: 305.1349).

(Benes *et al.*, 1983). We are not aware of any data for the stability constant of NA-Cd complexes, but their *in-vivo* affinity seems to be considerably lower than that of other characterized complexes. This has also been demonstrated in wheat and barley seedlings, where plant-produced PS does not lead to elevated Cd uptake (Shenker *et al.*, 2001).

Grains from cereal plants over-expressing *ZIP*, *AtHMA4* and *ferritin* contain more Zn (Gómez-Galera *et al.*, 2010). Foliar application of Zn-containing fertilizers may also increase grain Zn concentrations (Cakmak, 2009). However, these earlier studies did not investigate the binding forms of the elevated Zn content. Although the total amount of Zn is an important for the nutritional quality, information about changes in the molecular speciation of Zn is crucial because this determines how well the extra Zn is absorbed in the human gut (Zhu *et al.*, 2007). Within food plants, the availability of dietary Zn is reduced considerably by phytic acid and, possibly, by other constituents (Hunt, 2003). Here, we document that activation of *OsNAS2* generates a new pool of low-molecular-weight Zn complexes dominated by NA and DMA ligands. This is the first evidence that bio-fortified Zn may be directly bound to NA and DMA in the endosperm of rice grains. Furthermore, seeds from the activation-tagged line contained 4.9-fold more Fe bound to NA/DMA relative to the WT. The corresponding values for Cu and Ni were increased 9.7- and 2.4-fold. Importantly, the Cd level in the NA/DMA pool of the activation-tagged plants was not significantly higher than in the WT. This shows that Zn biofortification can be achieved without a simultaneous increase in Cd levels, thus avoiding potential toxicity problems in human nutrition.

We used a mouse system to assess the Zn bioavailability of Zn in grains from the activation-tagged plants. Hallmarks of a

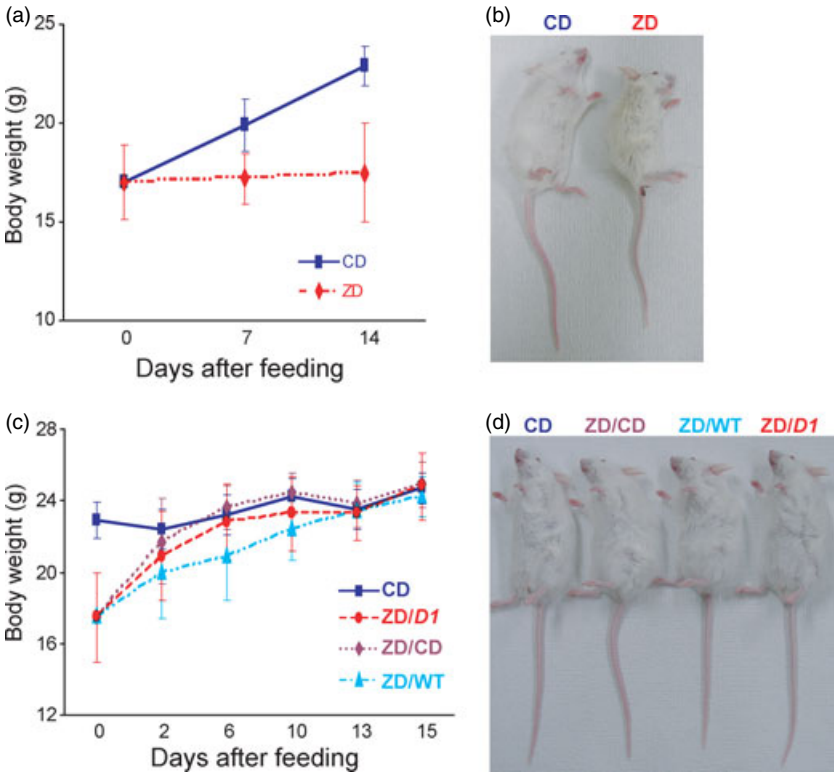

Figure 5 Body weight during Zn-bioavailability test. (a) Mice fed with Zn-adequate (control diet; CD) or Zn-deficient (ZD) diet for 2 weeks. (b) Representative photograph of mice after 2 weeks of Zn-starvation. (c) Changes in body weight of mice fed with control diet (ZD/CD), *OsNAS2-D1*seeds (ZD/D) or WT seeds (ZD/WT). (d) Representative photograph of mice after 15-day recovery period.

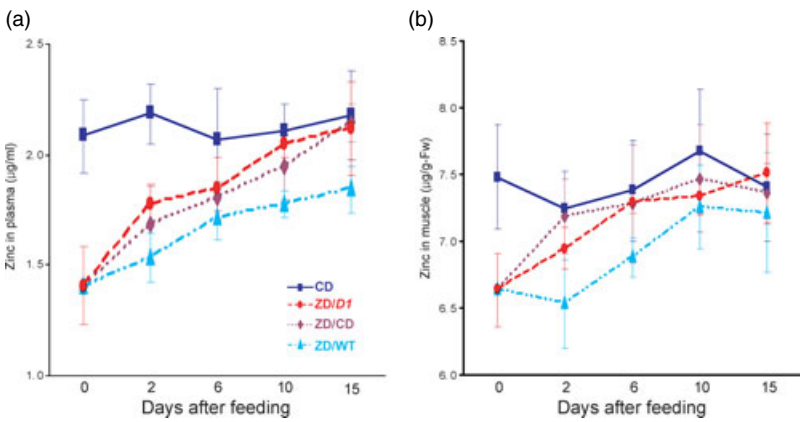

Figure 6 Feeding experiments for Zn bioavailability. Recovery of Zn levels in plasma (a) and muscle (b). Mice were fed for 2 weeks with control diet (CD; 30.0 mg Zn per kg) or Zn-depleted diet (ZD; 0.5 mg Zn per kg). Afterwards, ZD mice were divided into new groups treated with control diet (ZD/CD), WT seeds (ZD/WT) or *OsNAS2-D1* seeds (ZD/*D1*).

chronic Zn deficiency are severe growth retardation and reduced serum Zn levels (King *et al.*, 2005). Substantial amounts of Zn are taken up by the liver and, subsequently, re-distributed to the bones, muscles and appropriate cellular sites (Humphrey *et al.*, 1997). Here, during a period for recovery from that deficiency, Zn levels in the plasma and muscles of mice fed with seeds from the activation-tagged plants increased rapidly during recovery from Zn deficiency and reached normal values after 2 weeks, whereas mice fed with WT seeds did not improve significantly. This shows that the new Zn pool complexed with NA/DMA in *OsNAS2-D1* seeds has higher bioavailability and can alleviate Zn deficiency more efficiently than the native Zn pool.

We have here demonstrated that activation of a single *NAS* gene can drastically improve the amount of bio-available Zn in the endosperm of rice grains. By activation-tagging, only native NA genes are enhanced at their specific locations in the root pericycle or in phloem companion cells (Inoue *et al.*, 2003). This targeted approach seems to overcome some of the bottlenecks in Zn loading into the grain, while a more systemic increase of NA biosynthesis may create unbalances in Zn handling within the plant. This knowledge is important for the design of future strategies for bio-fortification strategies by plant biotechnology and plant breeding.

Experimental procedures

Plant growth

Surface-sterilized rice seeds were germinated and grown 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 micronutrients. For

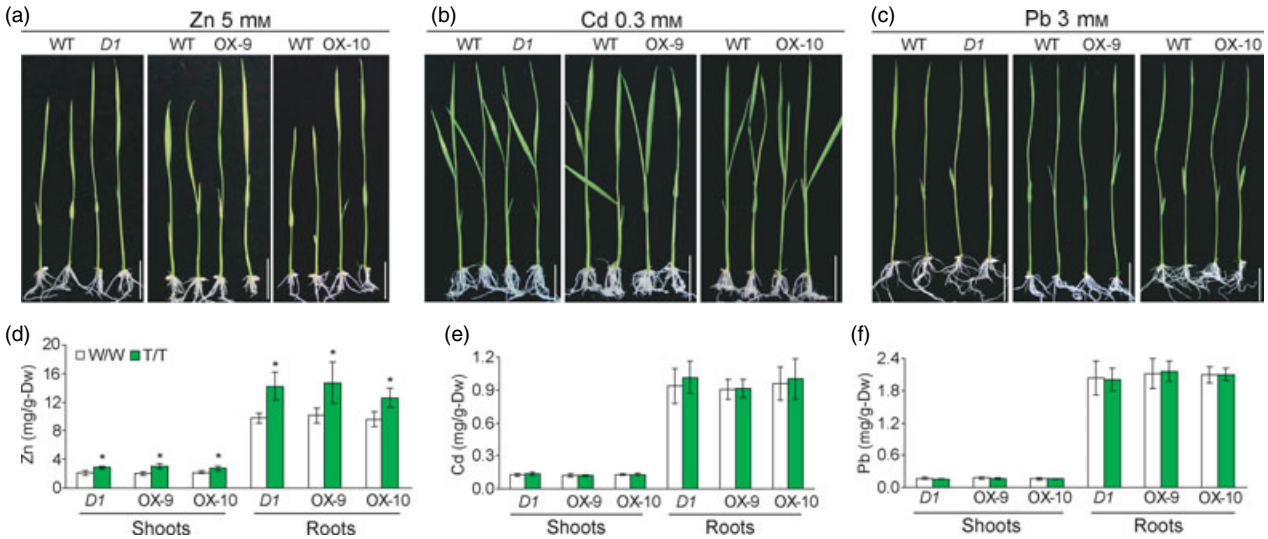

Figure 7 Effect of elevated levels of heavy metals in the growth medium. WT sibling, *OsNAS2-D1*, OX-9 and OX-10 plants were grown for 10 days on solid agar containing half-strength MS medium supplemented with 5 mM $ZnCl_2$ (a), 0.3 mM $CdCl_2$ (b) or 3 mM lead tartrate (c). Bars = 2.5 cm. Concentrations of Zn (d), Cd (e) and Pb (f) from shoots and roots of WT, *OsNAS2-D1*, OX-9 and OX-10 (n = 4 each). Significant differences from WT were determined by Student's t-tests; $^*P < 0.05$.

deficiency tests, seeds were instead germinated and grown for 8 day on an MS medium lacking $ZnSO_4$ (Zn-deficient). For the excess-metal treatments, seeds were germinated and grown for 10 day on solid media containing half-strength MS salts and supplemented with 5 mM $ZnCl_2$, 0.3 mM $CuCl_2$, 0.5 mM $NiCl_2$, 0.5 mM $CoCl_2$, 0.3 mM $CdCl_2$ or 3 mM lead tartrate.

RNA preparation and mRNA quantification

Shoots and roots were collected and frozen in liquid nitrogen. Total RNA was isolated with RNAiso Plus (Takara Bio, Shiga, Japan) and treated with RNase-free DNase I (Takara Bio) to prevent genomic DNA contamination. First-strand cDNA was synthesized from 2 μg of total RNA in a 25-μL reaction mixture with M-MLV reverse transcriptase (Promega, Madison, WI, USA). qRT-PCR was performed on a Rotor-Gene 6000 real-time rotary analyzer (Corbett Life Science, Sydney, Australia), using a SYBR premix ExTaq kit (Takara Bio). The levels of *OsActin1* mRNA served to normalize the expression ratio for each gene. Changes in expression were calculated via the $\Delta\Delta_{Ct}$ method (Han *et al.*, 2006). Primers for PCR are listed in Table S1.

Isolation of the *OsNAS2* mutant and generation of overexpressing transgenic plants

A putative *OsNAS2* mutant line was isolated from our flanking sequence-tag database as confirmed by inverse PCR analyses (Jeong *et al.*, 2006). For genotyping, we used two gene-specific primers (F1 and R1) and one T-DNA-specific primer (RB). Transcript levels of *OsNAS2* were determined by qRT-PCR, using cDNA prepared from 8-day-old seedling leaves, roots, flag leaves, panicles (~10 cm) and immature seeds collected at 5 days after pollination. To make our *OsNAS2*-overexpression construct, we amplified the full-length cDNA sequence with primer pair OxF and OxR (Table S1). The PCR products were inserted into pGA1611, generating pGA3544. *Oryza sativa* cv. Kitaake was used for producing transgenic plants by *Agrobacterium*-mediated cocultivation (Lee *et al.*, 1999; Kim *et al.*, 2009). Among thirteen transgenic lines, we selected two representative lines (#9 and #10), which expressed the *OsNAS2* gene at high levels and contained high amounts of Zn. Homozygous lines were isolated by growing the T2 seeds on MS media with hygromycin (80 mg/mL).

Metal analyses

Metal contents were measured as described (Lee *et al.*, 2009a). Samples of roots, shoots and seeds were dried for 2 day at 70 °C before they were weighed. Afterwards, they were digested in 1 mL of 11 N HNO_3 for 3 day in an oven at 180 °C. Following dilution, metal concentrations were determined by atomic absorption spectrometry (AAS; SpectrAA-800; Varian, Palo Alto, CA) and inductively coupled plasma mass spectroscopy (ICP-MS, ELAN6100; Perkin Elmer, Shelton, CT, USA).

Determination of NA and DMA levels

Mature seeds were finely crushed with a Multi-Beads Shocker (Yasui Kikai, Osaka, Japan). Samples were mixed with 400 μL of 80% ethanol and centrifuged at 12 000 ***g*** for 5 min, and the supernatants were collected for measurements. This step was repeated three times. Concentrations of NA and DMA were analysed by LC/ESI-TOF-MS, as previously described (Kakei *et al.*, 2009).

SEC-ICP-MS and HILIC-ESI-TOF-MS analyses

Seeds were polished, and the remaining endosperm was freeze-dried and homogenized. These tissues (100 mg) were ground with a pestle and mortar, and 2 mL of 50 mM TRIS (pH 7.5) and 500 mg of acid-washed quartz sand were added to extract the water-soluble fraction. All extractions were performed on ice under a flow of N_2 gas to reduce enzymatic activity and to prevent oxidation. After 1 h, the extracts were centrifuged for 20 min at 5 °C and 11 180 ***g*** before the supernatant was decanted. The SEC-ICP-MS analysis was based on the procedures described in Persson *et al.* (2009). The latest-eluting Zn

peak (retention time 840 s) in the chromatogram was collected five times from the outlet of the Superdex Peptide column. The solution was then lyophilized overnight until all the water was removed. Each sample was re-suspended in 0.5 mL of a 90% MeOH solution and was injected on a HILIC (hydrophilic interaction liquid chromatography) column (Atlantis; 2.1 × 100 mm, 3 μm diam.), coupled to an ESI-TOF-MS (Micromass LCT; Waters, Saint-Quentin, France). The flow rate was set to 0.4 mL/min, and the mobile phases were A, 90% MeOH/5 mM ammonium acetate/0.2% formic acid (pH 2.9); and B, 5 mM ammonium acetate/0.2% formic acid. The following gradient was used: 0–3.5 min, 100% A; 3.5–5.0 min, 0%–10% B; 5–10 min, 10%–60% B; 10–20 min, 100% A. This analysis was performed in the positive mode. MassLynx version 4.0 software (Micromass) was used for data acquisition and processing.

Bioavailability tests

For assessing Zn bioavailability, 4-week-old male Balb/c mice (ORIENT BIO Inc., Seongnam, Korea) were maintained in pathogen-free facilities at Pohang University Science and Technology (POSTECH). All live-animal experiments were approved by the POSTECH Ethics Committee. Separate groups of mice were fed with a Zn-depleted (ZD) or control (CD) diet, containing 0.5 or 30.0 mg Zn per kg, respectively. After 2 weeks, Zn levels were measured in the plasma and muscles of animals from each group. Afterwards, mice that had initially been treated with the ZD diet were divided into three new groups (n = 20 each). The first was now fed with the control diet (ZD/CD); the second, with *OsNAS2-D1* seeds (ZD/*D1*); and the third, with wild-type seeds (ZD/WT). At 2, 6, 10, 13 and 15 days after feeding began, all mice were weighed. Plasma and muscle samples were collected daily for measuring Zn. Zinc levels in plasma and liver were analysed using atomic absorption spectrometry (AAS; SpectrAA-800; Varian, Palo Alto, CA) after digestion in 11 N HNO_3.

Acknowledgements

We thank In-Soon Park and Kyungsook An for plant transformation, Jongdae Kyung for technical assistance with the AAS measurements, Mary Lou Guerinot for discussion and critical comments 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 grants from the Crop Functional Genomic Center, the 21st Century Frontier Program (Grant CG1111); from the Biogreen 21 Program, Rural Development Administration (20070401-034-001-007-03-00); from the Korea Research Foundation Grant funded by the Korean Government (MOEHRD, Basic Research Promotion Fund, KRF-2007-341-C00028); from Kyung Hee University (20100791); from the EU-FP6 projects META-PHOR (FOOD-CT-2006-03622); and from PHIME (FOOD-CT-2006-016253).

References

Bashir, K., Inoue, H., Nagasaka, S., Takahashi, M., Nakanishi, H., Mori, S. and Nishizawa, N.K. (2006) Cloning and characterization of deoxymugineic acid synthase genes from graminaceous plants. *J Biol Chem.* **281**, 32395–32402.

Benes, I., Schreiber, K., Ripperger, H. and Kircheiss, A. (1983) Metal complex formation by nicotianamine, a possible phytosiderophore. *Cell. Mol. Life Sci.* **39**, 261–262.

Cakmak, I. (2008) Enrichment of cereal grains with zinc, agronomic or genetic biofortification? *Plant Soil* **302**, 1–17.

Cakmak, I. (2009) Agronomic approaches in biofortification of food crops with micronutrients. UC Davis, The Proceedings of the International Plant Nutrition Colloquium XVI. Retrieved from, http://escholarship.org/uc/item/3d46b1th.

Douchkov, D., Gryczka, C., Stephan, U.W., Hell, R. and Baumlein, H. (2005) Ectopic expression of nicotianamine synthase genes results in improved iron accumulation and increased nickel tolerance in transgenic tobacco. *Plant Cell Environ.* **28**, 365–374.

Fitzgerald, M.A., McCouch, S.R. and Hall, R.D. (2009) Not just a grain of rice, the quest for quality. *Trends Plant Sci.* **14**, 133–139.

Gómez-Galera, S., Rojas, E., Sudhakar, D., Zhu, C., Pelacho, A.M., Capell, T. and Christou, P. (2010) Critical evaluation of strategies for mineral fortification of staple food crops. *Transgen Res.* **19**, 165–180.

Han, M.J., Jung, K.H., Yi, G., Lee, D.Y. and An, G. (2006) Rice immature pollen 1 (RIP1) is a regulator of late pollen development. *Plant Cell Physiol.* **47**, 1457–1472.

Hoppler, M., Schönbächler, A., Meile, L., Hurrell, R.F. and Walczyk, T. (2008) Ferritin-iron is released during boiling and *in vitro* gastric digestion. *J. Nutr.* **138**, 878–884.

Humphrey, P.A., Ashraf, M. and Lee, C.M. (1997) Growth of trypanosomes *in vivo*, host body weight gains, and food consumption in zinc-deficient mice. *J. Natl Med. Assoc.* **89**, 48–56.

Hunt, J.R. (2003) Bioavailability of iron, zinc, and other trace minerals from vegetarian diets. *Amer J Clin Nutr.* **78**, 633S–639S.

Inoue, H., Higuchi, K., Takahashi, M., Nakanishi, H., Mori, S. and Nishizawa, N.K. (2003) Three rice nicotianamine synthase genes, OsNAS1, OsNAS2 and OsNAS3, are expressed in cells involved in long-distance transport of iron and differentially regulated by iron. *Plant J.* **36**, 366–381.

Ishimaru, Y., Suzuki, M., Kobayashi, T., Takahashi, M., Nakanishi, H., Mori, S. and Nishizawa, N.K. (2005) OsZIP4, a novel zinc-regulated zinc transporter in rice. *J. Exp. Bot.* **56**, 3207–3214.

Jeon, J.S., Lee, S., Jung, K.H., Jun, S.H., Jeong, D.H., Lee, J., Kim, C., Jang, S., Yang, K., Nam, J., An, K., Han, M.J., Sung, R.J., Choi, H.S., Yu, J.H., Choi, J.H., Cho, S.Y., Cha, S.S., Kim, S.I. and An, G. (2000) T-DNA insertional mutagenesis for functional genomics in rice. *Plant J.* **22**, 561–570.

Jeong, D.H., An, S., Kang, H.G., Moon, S., Han, J.J., Park, S., Lee, H.S., An, K. and An, G. (2002) T-DNA insertional mutagenesis for activation tagging in rice. *Plant Physiol.* **130**, 1636–1644.

Jeong, D.H., An, S., Park, S., Kang, H.G., Park, G.G., Kim, S.R., Sim, J., Kim, Y.O., Kim, M.K., Kim, S.R., Kim, J., Shin, M., Jung, M. and An, G. (2006) Generation of a flanking sequence-tag database for activation-tagging lines in japonica rice. *Plant J.* **45**, 123–132.

Kakei, Y., Yamaguchi, I., Kobayashi, T., Takahashi, M., Nakanishi, H., Yamakawa, T. and Nishizawa, N.K. (2009) A highly sensitive, quick and simple quantification method for nicotianamine and 2′-deoxymugineic acid from minimum samples using LC/ESI-TOF-MS achieves functional analysis of these components in plants. *Plant Cell Physiol.* **50**, 1988–1993.

Kim, S.R., Lee, D.Y., Yang, J.I., Moon, S. and An, G. (2009) Cloning vectors for rice. *J. Plant Biol.* **52**, 73–78.

King, L.E., Frentzel, J.W., Mann, J.J. and Fraker, P.J. (2005) Chronic zinc deficiency in mice disrupted T cell lymphopoiesis and erythropoiesis while B cell lymphopoiesis and myelopoiesis were maintained. *J. Amer. Coll. Nutr.* **24**, 494–502.

Lee, S., Jeon, J.S., Jung, K.H. and An, G. (1999) Binary vector for efficient transformation of rice. *J. Plant Biol.* **42**, 310–316.

Lee, S., Chiecko, J.C., Kim, S.A., Walker, E.L., Lee, Y., Guerinot, M.L. and An, G. (2009a) Disruption of *OsYSL15* leads to iron inefficiency in rice plants. *Plant Physiol.* **150**, 786–800.

Lee, S., Jeon, U.S., Lee, S.J., Kim, Y.K., Persson, D.P., Husted, S., Schjørring, J.K., Kakei, Y., Masuda, H., Nishizawa, N.K. and An, G. (2009b) Iron fortification of rice through activation of the nicotianamine synthase gene. *Proc. Natl Acad. Sci. USA*, **106**, 22014–22019.

Lee, S., Jeong, H.J., Kim, S.A., Lee, J., Guerinot, M.L. and An, G. (2010) OsZIP5 is a plasma membrane zinc transporter in rice. *Plant Mol. Biol.* **73**, 507–517.

Masuda, H., Usuda, K., Kobayashi, T., Ishimaru, Y., Kakei, Y., Takahashi, M., Higuchi, K., Nakanishi, H., Mori, S. and Nishizawa, N.K. (2009) Overexpression of the barley nicotianamine synthase gene HvNAS1 increases iron and zinc concentrations in rice grains. *Rice*, **2**, 155–166.

Mayer, J.E., Pfeiffer, W.H. and Beyer, P. (2008) Biofortified crops to alleviate micronutrient malnutrition. *Curr. Opin. Plant Biol.* **11**, 166–170.

Palmgren, M.G., Clemens, S., Williams, L.E., Krämer, U., Borg, S., Schjørring, J.K. and Sanders, D. (2008) Zinc biofortification of cereals, problems and solutions. *Trends Plant Sci.* **13**, 464–473.

Persson, D.P., Hansen, T.H., Laursen, K.H., Schjoerring, J.K. and Husted, S. (2009) Simultaneous iron, zinc, sulphur and phosphorus analysis of barley grain tissues using SEC-ICP-MS and IP-ICP-MS. *Metallomics*, **1**, 418–426.

Raboy, V. (2003) Myo-inositol-1,2,3,4,5,6-hexakisphosphate. *Phytochemistry*, **64**, 1033–1043.

Shenker, M., Fan, T.W. and Crowley, D.E. (2001) Phytosiderophores influence on cadmium mobilization and uptake by wheat and barley plants. *J. Environ. Qual.* **30**, 2091–2098.

Suzuki, M., Tsukamoto, T., Inoue, H., Watanabe, S., Matsuhashi, S., Takahashi, M., Nakanishi, H., Mori, S. and Nishizawa, N.K. (2008) Deoxymugineic acid increases Zn translocation in Zn-deficient rice plants. *Plant Mol. Biol.* **66**, 609–617.

Wirth, J., Poletti, S., Aeschlimann, B., Yakandawala, N., Drosse, B., Osorio, S., Tohge, T., Fernie, A.R., Günther, D., Gruissem, W. and Sautter, C. (2009) Rice endosperm iron biofortification by targeted and synergistic action of nicotianamine synthase and ferritin. *Plant Biotechnol. J.* **7**, 631–644.

Zheng, L., Cheng, Z., Ai, C., Jiang, X., Bei, X., Zheng, Y., Glahn, R.P., Welch, R.M., Miller, D.D., Lei, X.G. and Shou, H. (2010) Nicotianamine, a Novel Enhancer of Rice Iron Bioavailability to Humans. *PLoS ONE*, **5**, e10190.

Zhu, C., Naqvi, S., Gomez-Galera, S., Pelacho, A.M., Capell, T. and Christou, P. (2007) Transgenic strategies for the nutritional enhancement of plants. *Trends Plant Sci.* **12**, 548–555.

Supporting information

Additional Supporting information may be found in the online version of this article:

Figure S1 Quantification of phenotypes under Zn deficiency.
Figure S2 Phenotypes of plants under elevated levels of heavy-metal ions.
Figure S3 SEC-ICP-MS chromatogram of *OsNAS2-D1* and WT.
Table S1 Primers used in this study.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.