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Overexpression of the Barley Nicotianamine Synthase Gene *HvNAS1* Increases Iron and Zinc Concentrations in Rice Grains

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Abstract In humans, iron (Fe) and zinc (Zn) deficiencies result in major worldwide health problems. Transgenic technologies to produce Fe- and Zn-biofortified rice varieties offer a promising potential solution. Nicotianamine, the precursor of phytosiderophores, chelates Fe^{2+} and Zn^{2+} and plays an important role in transporting these metals to both vegetative and reproductive organs within the plant. Our objective was to increase Fe and Zn contents in rice grains by overexpressing the barley nicotianamine synthase gene *HvNAS1*. *HvNAS1*-overexpressing transgenic rice showed increased *HvNAS1* expression and subsequent increases in endogenous nicotianamine and phytosiderophore content in shoots, roots, and seeds. Fe and Zn concentrations in polished T_1 seeds from transgenic plants increased more than three and twofold, respectively; Fe and Zn concentrations also increased in both polished and brown T_2 seeds. These results suggest that the overproduction of nicotianamine enhances the translocation of Fe and Zn into rice grains.

Keywords Nicotianamine · *HvNAS1* · Iron · Zinc · Transgenic rice · Biofortification

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Introduction

Iron (Fe) and zinc (Zn) are essential micronutrients in both plants and animals. Shortages in Fe and Zn are among the most prevalent human micronutrient deficiencies in the world, each affecting an estimated one third of the world's population (Sandstead 1991; Stoltzfus and Dreyfuss 1998) and resulting, separately, in 0.8 million deaths per year worldwide (WHO 2002). Fe and Zn deficiencies are the sixth and fifth highest health risk factors, respectively, in developing countries with high mortality rates (WHO 2002). Several approaches have been proposed to decrease the prevalence of micronutrient deficiencies. Biofortification (i.e., breeding micronutrient-fortified crops) is particularly advantageous for people living in rural areas because it does not require specific processing techniques after harvest or specialized infrastructure (Lucca et al. 2006). In addition, transgenic techniques offer the possibility of producing micronutrient-fortified rice varieties.

Transgenic Fe fortification has been attempted using ferritin, a protein that stores approximately 4,500 Fe atoms

in a complex. Goto et al. (1999) generated transgenic rice plants that expressed soybean ferritin in the endosperm using the endosperm-specific rice *GluB1* promoter; the transformants showed Fe accumulation in brown rice seeds. However, further enhancement of ferritin expression did not produce further increases in seed Fe content (Qu et al. 2005). In addition to increased Fe storage in seeds, enhanced Fe uptake from the soil and translocation within the plant are thought to be required to further improve Fe fortification in seeds.

Nicotianamine (NA), a chelator of metal cations such as Fe(II) and Zn(II), is biosynthesized from S-adenosyl methionine via NA synthase (NAS; Higuchi et al. 1994). All higher plants synthesize and utilize NA for the internal transport of Fe and other metals (Hell and Stephan 2003; Takahashi et al. 2003). Indeed, the NA-defective tomato mutant *chloronerva* (Rudolph et al. 1985) presents a phenotype indicative of Fe deficiency (Pich and Scholz 1996; Stephan et al. 1996). Takahashi et al. (2003) produced NA-deficient transgenic tobacco plants by constitutively expressing the barley NA aminotransferase (NAAT) gene, *HvNAAT-A*. The transformants showed young leaves with interveinal chlorosis, and as a result of disrupted internal metal transport, Fe and Zn concentrations in the leaves and flowers decreased. Conversely, overexpressing the barley NAS gene, *HvNAS1*, led to increased Fe and Zn concentrations in the leaves, flowers, and seeds of tobacco plants (Takahashi et al. 2003). Similarly, in *Arabidopsis AtNAS* quadruple mutant, Fe concentration in seeds was found to decrease (Klatte et al. 2009). These reports suggest that NA plays an essential role for Fe translocation to seeds.

In addition, Koike et al. (2004) identified the rice NA-Fe(II) transporter gene *OsYSL2*, which is preferentially expressed in leaf phloem cells and the vascular bundles of flowers and developing seeds, suggesting a role in internal Fe transport.

In graminaceous plants, NA is the precursor of mugineic acid family phytosiderophores (MAs), which are natural Fe(III) chelators used in Fe acquisition from the rhizosphere (Mihashi and Mori 1989; Takagi 1976). Under Fe-deficient conditions, graminaceous plants synthesize and secrete MAs into the rhizosphere where they form Fe(III)–MAs complexes that are absorbed into the root via YS1-like transporters (Curie et al. 2001). Rice produces 2′-deoxymugineic acid (DMA), which chelates Fe(III) and contributes to Fe uptake from the soil and internal transport (Kobayashi et al. 2008). Therefore, increased NA concentrations would also be expected to increase DMA concentrations in rice plants, with subsequent improvements in Fe uptake and transport.

We hypothesized that the overexpression of the *NAS* gene in rice would enhance the synthesis of NA and DMA, and thus increase Fe and Zn concentrations in seeds. Higuchi et al. (2001a) introduced the *HvNAS1* gene into rice under the control of the *35S* promoter. The resultant transgenic lines produced two to threefold greater concentrations of NA compared to non-transgenic lines. DMA production and secretion also increased under Fe-sufficient conditions. In this paper, we describe new transgenic rice lines that overexpress the *HvNAS1* gene under the control of the rice *Actin1* promoter. Using these lines and previously generated *35S-HvNAS1* transformants (Higuchi et al. 2001a), we analyzed *HvNAS1* gene expression, endogenous NA and DMA concentrations, and Fe and Zn contents in seeds. NA concentrations in the shoots, roots, and seeds of transgenic rice, and Fe and Zn concentrations in polished T_1 rice seeds, increased compared to non-transgenic plants. Fe and Zn concentrations also increased in polished and brown T_2 seeds in proportion to the expression of the *HvNAS1* transgene in T_1 plants.

Results

Fe and Zn concentrations in polished T_1 seeds from *OsActin1-HvNAS1* lines

To determine whether enhanced *NAS* expression increases Fe and Zn concentrations in rice seeds, *OsActin1-HvNAS1* (AN) transgenic lines were produced. After harvest from soil culture with slow-release fertilizer, polished and brown rice seeds from both non-transgenic rice (NT) and 12 AN lines were subjected to metal concentration analysis. Fe and Zn concentrations increased markedly in polished T_1 transgenic rice seeds compared to NT seeds (Fig. 1). In line AN1, Fe and Zn concentrations in polished seeds increased 4.5- and 2.5-fold, respectively, compared to the NT line. Fe and Zn concentrations also increased in brown rice seeds, although to a lesser degree (data not shown). Among 12 AN lines, we selected lines AN1, AN2, and AN3, which showed the highest Fe and Zn concentrations in polished rice seeds, for further analysis.

Transgene expression in *HvNAS1* overexpression lines

Three AN lines (AN1, AN2, and AN3) and the NT line were grown hydroponically under Fe-sufficient conditions. A previously generated transgenic line carrying *35S-HvNAS1* (35S; Higuchi et al. 2001a) was also grown under the same conditions. *HvNAS1* transgene expression in shoots was detected via Northern blot analysis (Fig. 2). *HvNAS1* expression was induced in the 35S line and three AN lines in Fe-sufficient shoots. The strongest transgene expression was observed in the 35S and AN1 lines.

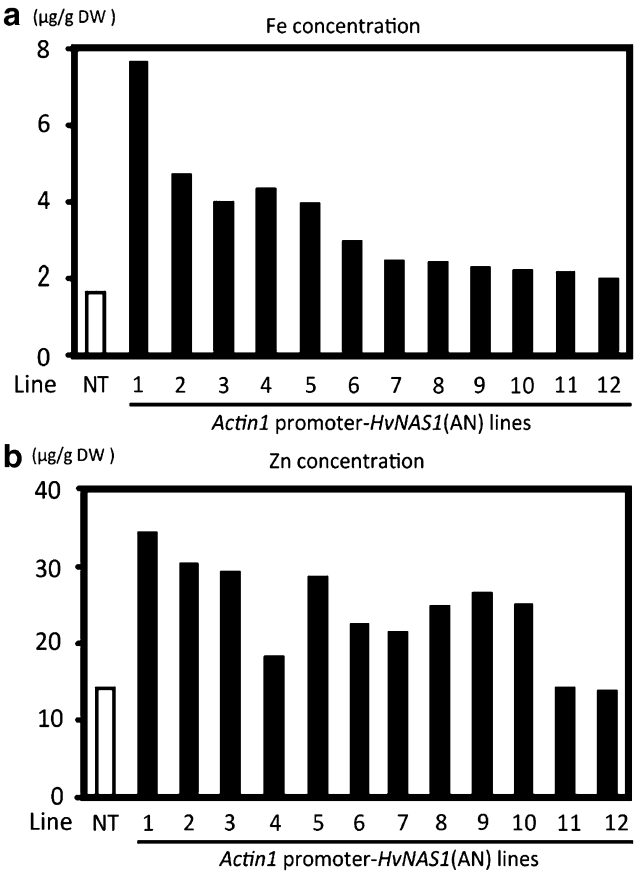

Fig. 1 Fe and Zn concentrations in polished T_1 seeds. Ten well-polished seeds from main tillers were subjected to metal concentration analysis. **a** Fe concentration in polished T_1 seeds. **b** Zn concentration in polished T_1 seeds. NT (*white bar*), non-transgenic line; numbers (*black bars*), *OsActin1-HvNAS1* (AN) lines.

Endogenous NA and DMA concentrations in T_1 plants

Endogenous NA and DMA concentrations in AN, 35S, and NT lines were measured via high-performance liquid chromatography (HPLC; Fig. 3). In the shoots and roots,

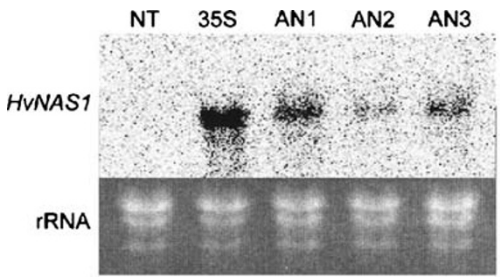

Fig. 2 Northern blot analysis of *HvNAS1* gene expression in transgenic lines. The *HvNAS1* ORF was used as a probe. Total RNA was extracted from the shoots of 44-day-old plants grown hydroponically under Fe-sufficient conditions, and 20 μg of total RNA was loaded into each lane. Ethidium bromide staining was used to ensure equal loading of rRNA. *NT* non-transgenic line; *35S*, *35S-HvNAS1* transgenic line; *AN1*, *AN2*, and *AN3*, *OsActin1-HvNAS1* transgenic lines.

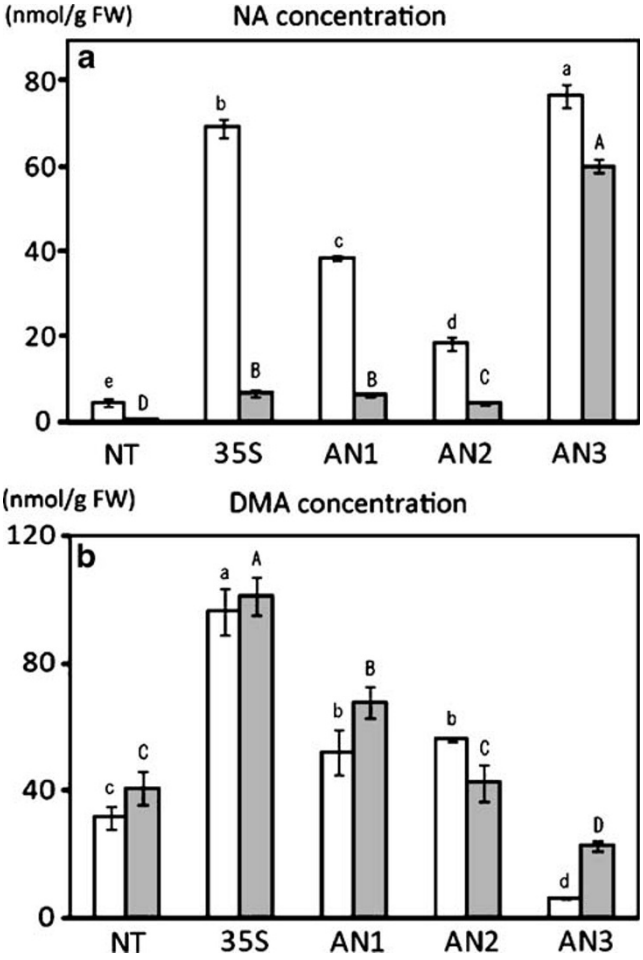

Fig. 3 Endogenous NA and DMA concentrations in T_1 plants determined via high-performance liquid chromatography (*HPLC*). **a** NA concentrations. **b** DMA concentrations. NA and DMA were extracted from 44-day-old plants grown hydroponically under Fe-sufficient conditions. The data represent the mean ± standard error of three measurements for each sample. *White bars*, shoot concentration; *gray bars*, root concentration; *NT* non-transgenic line; *35S*, *35S-HvNAS1* transgenic line; *AN1*, *AN2*, and *AN3*, *OsActin1-HvNAS1* transgenic lines. *Distinct letters above the bars* indicate significant differences ($p<0.05$).

endogenous NA concentrations were markedly higher in the 35S, AN1, AN2, and AN3 lines in T_1 plants compared to the NT line (Fig. 3a). The most marked increase (approximately 15-fold) was observed in shoots collected from lines 35S and AN3 (Fig. 3a). DMA concentrations were also higher in shoots and roots collected from the 35S, AN1, and AN2 lines compared to the NT line (Fig. 3b).

Fe and Zn contents in T_2 seeds

To determine whether Fe and Zn accumulated in T_2 seeds, we cultivated *HvNAS1*-overexpressing lines in two separate experimental designs. In the first experiment, three AN

lines (AN1, AN2, and AN3; T_1 generation), the 35S line, and NT plants were grown in small pots fertilized with nutrient solution alone. In polished T_2 seeds collected from the AN1 and 35S lines, Fe and Zn concentrations increased significantly compared to NT plants (Fig. 4a, b). Fe concentrations in polished rice seeds collected from the 35S and AN1 lines increased 2.3- and 1.6-fold, respectively (Fig. 4a). Zn concentrations in polished rice seeds collected from the 35S, AN1, and AN3 lines increased 1.5-, 1.2-, and 1.3-fold, respectively, compared to NT plants (Fig. 4b). Fe and Zn concentrations also increased in brown rice seeds collected from the 35S, AN1, and AN3 lines (Fig. 4c, d). Fe concentrations increased only slightly in rice bran collected from 35S and AN3 plants (Fig. 4e), and Zn concentrations

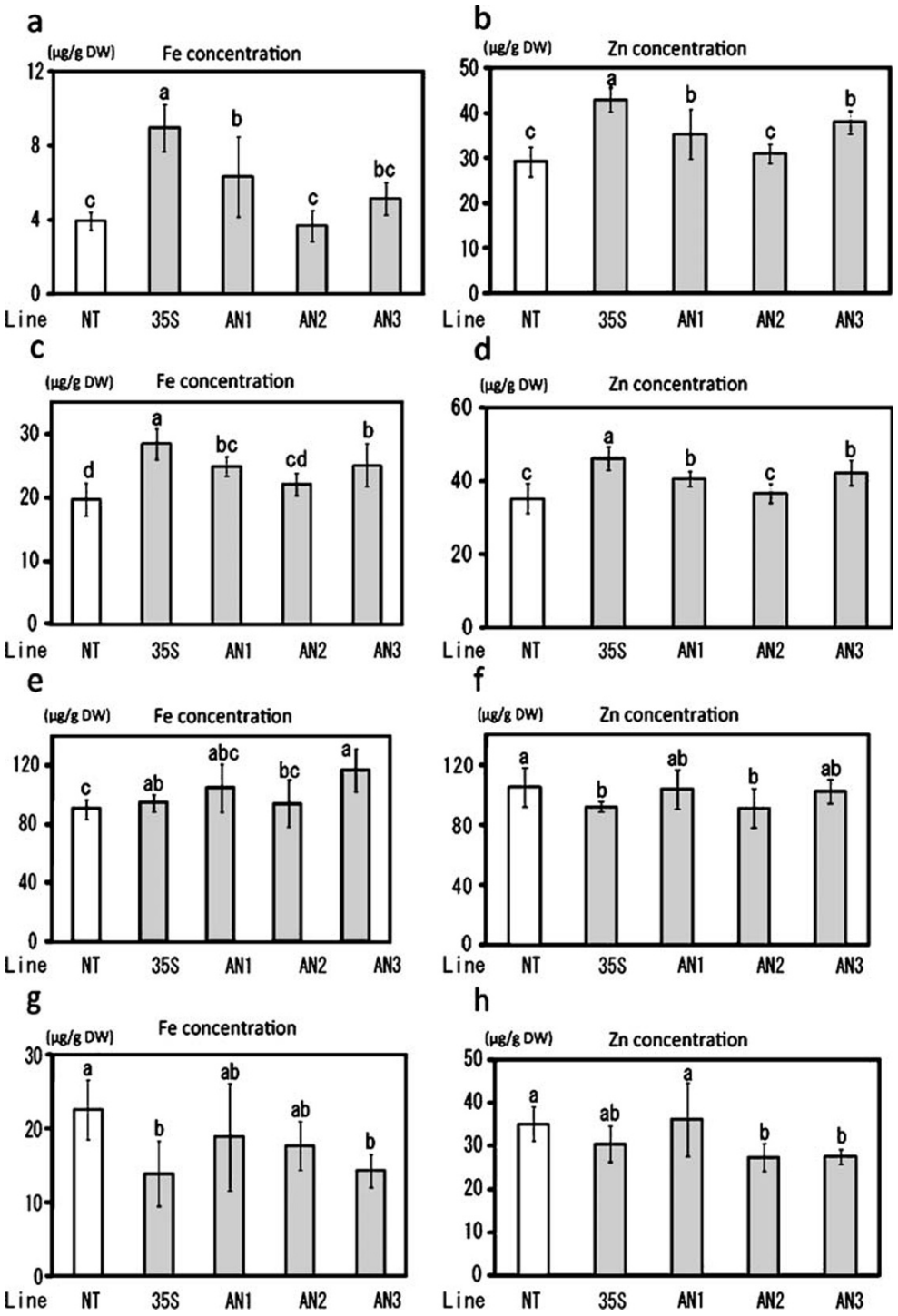

Fig. 4 Fe and Zn concentrations in T_2 seeds obtained from plants cultured in small pots fertilized with nutrient solution. T_2 seeds from the main tillers of NT, *35S-HvNAS1* (35S), and *OsActin1-HvNAS1* (AN1, AN2, and AN3) lines were harvested and subjected to metal concentration analysis. **a** Fe concentration in polished rice seeds. **b** Zn concentration in polished rice seeds. **c** Fe concentration in brown rice seeds. **d** Zn concentration in brown rice seeds. **e** Fe concentration in bran. **f** Zn concentration in bran. **g** Fe concentration in hulls. **h** Zn concentration in hulls. The data represent the mean ± standard error of six independent pots. *Distinct letters above the bars* indicate significant differences ($p<0.05$).

did not increase (Fig. 4f). In rice hulls, Fe and Zn concentrations decreased in the 35S and AN lines (Fig. 4g, h). Fe and Zn contents per seed were calculated using the metal concentration data from T_2 seeds (Fig. 5a, b). Fe content per seed for polished, brown, and full grains increased in the 35S, AN1, and AN3 lines, whereas Fe content decreased in hulls per seed in the 35S, AN1, and AN3 lines. Zn contents showed a similar distribution among the 35S and AN3 lines.

NA and DMA contents in T_2 seeds

Next, endogenous NA and DMA concentrations in T_2 brown rice seeds of 35S, AN1, AN3, and NT lines were measured using liquid chromatography/electrospray ionization time-of-flight mass spectrometry (LC/ESI-TOF-MS). NA concentrations in T_2 seeds of 35S, AN1, and AN3 lines increased 10.6-, 5.1-, and 4.7-fold, respectively, compared to the NT line (Fig. 6a). DMA concentrations in T_2 seeds of 35S, AN1, and AN3 lines also increased 4.8-, 2.8-, and 1.7-fold, respectively, compared to the NT line (Fig. 6a). The NA concentration in seeds was correlated with Fe or Zn concentration in polished rice seeds in each line (Fig. 6b, c). A correlation between DMA concentration in seeds and Fe or Zn concentration in polished seeds in each line was also observed (Fig. 6d, e).

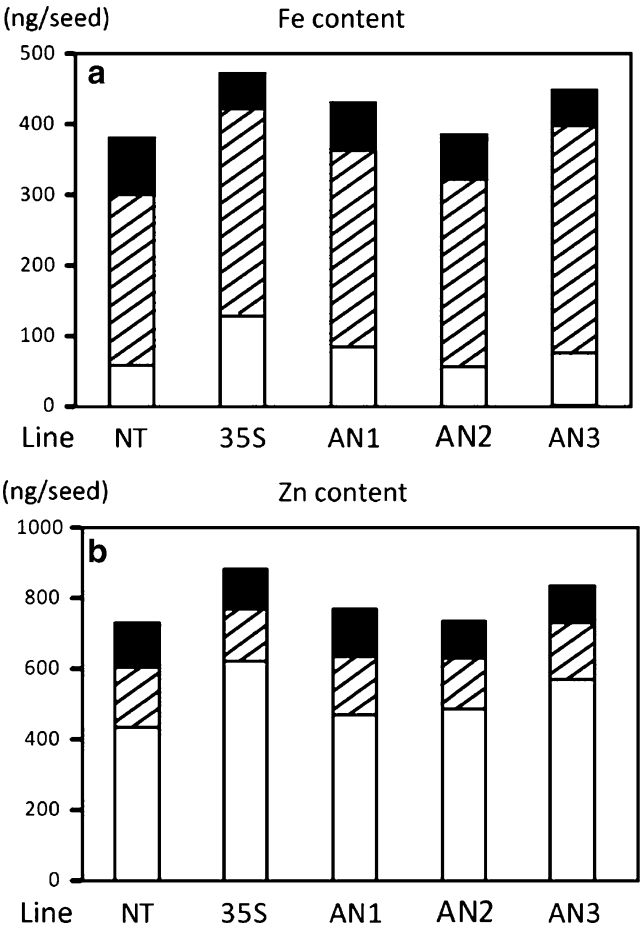

Fig. 5 Fe and Zn contents per seed in T_2 seeds obtained from plants cultured in small pots fertilized with nutrient solution. Fe and Zn contents per seed were calculated from the metal concentration data shown in Fig. 4. **a** Fe content per seed. **b** Zn content per seed. *White bar*, polished seed parts (endosperm); *hatched bar*, bran and embryo; *black bar*, hulls.

Fe and Zn concentration and localization in T_2 seeds of soil culture

In the second experiment, we cultivated six T_1 sublines from AN1, AN2, and AN3 in soil culture with slow-release fertilizer to obtain T_2 seeds and then measured Fe and Zn contents in seed and leaves (Fig. 7). In polished T_2 seeds collected from sublines AN1-1, AN3-2, and AN3-6, Fe concentrations increased up to 3.4-, 2.5-, and 2.8-fold, respectively, compared to NT plants (Fig. 7a). AN1-2, AN1-5, AN2-6, AN3-1, and AN3-4 also produced polished T_2 seeds with up to 1.5-fold increases in Fe concentration compared to NT plants. In the remaining sublines, Fe concentrations in polished seeds showed only slight increases, or no increase, compared to NT plants (Fig. 7a). Zn concentrations in T_2 polished rice seeds collected from the AN1-1, AN1-4, AN 3-2, and AN 3-6 sublines increased up to 2.0-, 1.8-, 1.9-, and 2.3-fold, respectively, compared to NT plants (Fig. 7b). The remaining sublines, except for AN2-3, AN2-4, and AN2-5, also produced Zn-enriched polished seeds, with increases of approximately 1.3–1.7 times that observed in NT plants (Fig. 7b). Fe and Zn content per seed in T_2 polished seeds also increased in AN lines compared to the NT line (Fig. 7c, d). Fe and Zn concentrations in leaves of AN lines after seed harvest were not significantly increased compared to NT (Fig. 7e, f).

Next, T_2 seeds were subjected to Prussian blue staining to observe the Fe localization in seeds (Fig. 8). Histochemical analysis showed that Fe was localized mostly in bran and embryo in NT seeds (Fig. 8a). On the other hand, more Fe was localized in endosperm of AN1-1 and AN3-2 line seeds compared to that of NT (Fig. 8b, c).

Discussion

In rice, NA is thought to function as an Fe(II) chelator in phloem and to participate in long-distance Fe transport within the plant (Inoue et al. 2003; Koike et al. 2004). We cultivated transgenic rice lines carrying *OsActin1*promoter-*HvNAS1* (AN lines) and *35S* promoter-*HvNAS1* (35S line), which overexpress the *HvNAS1* transgene (Fig. 2). In the AN lines, Fe and Zn concentrations increased in polished

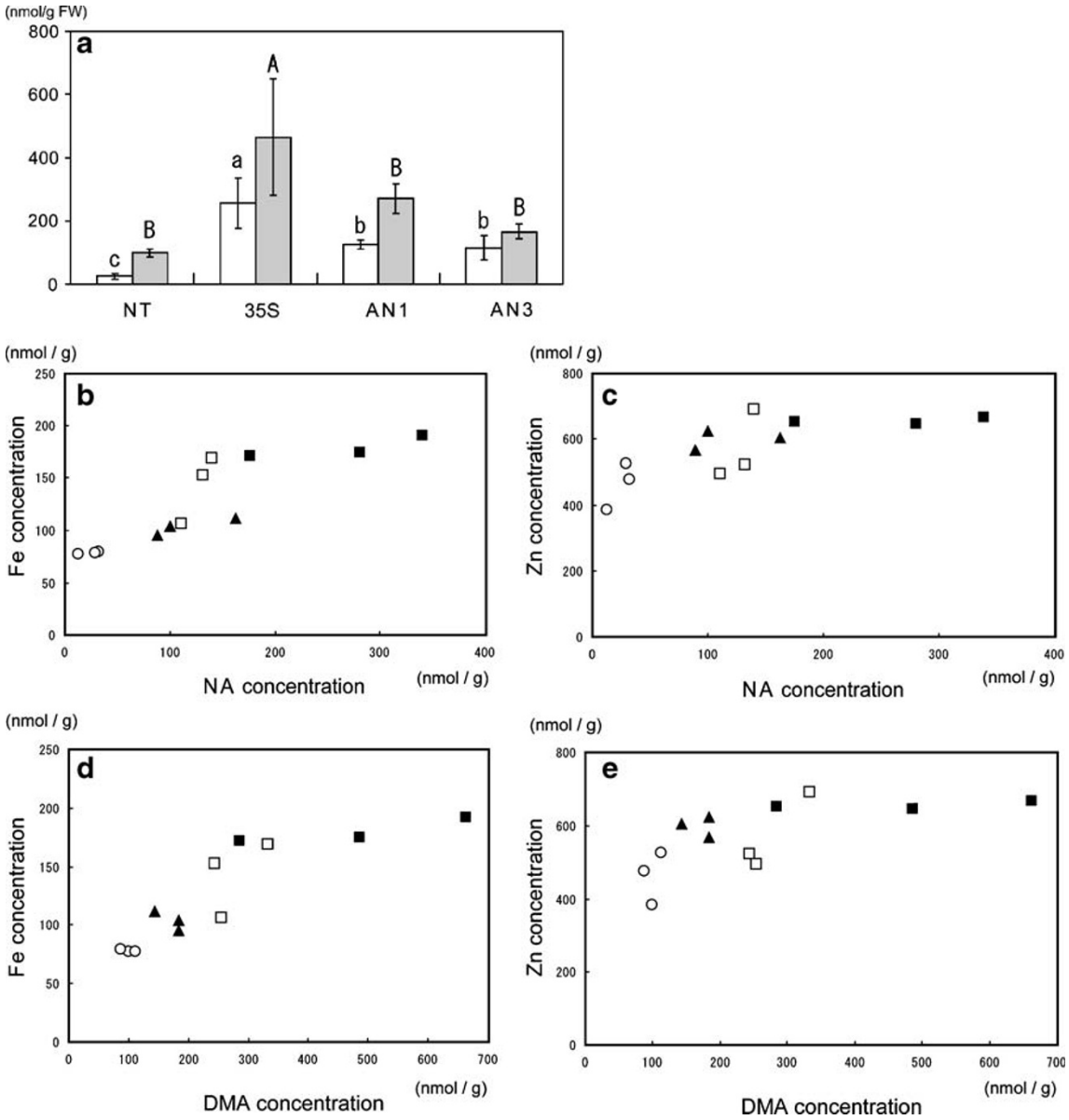

Fig. 6 **a** NA and DMA concentration in T_2 seeds obtained from plants cultured in small pots fertilized with nutrient solution. *White bars*, NA; *gray bars*, DMA. *Distinct letters above the bars* indicate significant differences ($p<0.05$). **b**–**e** Scatter diagram between NA or DMA concentration in T_2 seeds and Fe or Zn concentration in polished T_2 seeds for each plant cultured in small pots fertilized with nutrient solution. **b** NA and Fe concentrations. **c** NA and Zn concentrations. **d** DMA and Fe concentrations. **e** DMA and Zn concentrations. *White circle*, NT lines; *black square*, 35S lines; *white square*, AN1 lines; *black triangle*, AN3 lines.

T_1 and T_2 seeds cultivated via two distinct methods (Figs. 1, 4, and 7). We observed a positive correlation between the expression of *HvNAS1* in the shoots and Fe and Zn concentrations in polished and brown rice seeds (Figs. 2 and 4). In addition, a positive correlation was observed between Fe or Zn concentration and NA or DMA concentration in seeds (Fig. 6). These results suggest that enhanced NA production increases the translocation of Fe and Zn into seeds. Bashir et al. (2006) suggested that DMA-Fe is transported from the roots to shoots in Fe-sufficient rice plants. In Zn-deficient rice plants, Suzuki et al. (2008b) showed that DMA promotes increased Zn translocation. We observed increased DMA concentrations in the 35S and AN1 lines (Fig. 3b), which may also have contributed to increased Fe and Zn concentrations in these plants.

NA concentration in T_2 seeds of the 35S line and AN lines also increased compared to the NT line (Fig. 6). Usuda et al. (2009) produced transgenic rice that expressed *HvNAS1* under the control of an endosperm-specific

promoter, *OsGluB-1*. NA concentration in brown seeds of this transgenic line increased threefold compared to the NT line, and Fe concentration in polished seeds also increased 1.5-fold (data not shown). Higher NA productivity in the endosperm of this transgenic rice might have improved Fe accumulation in seeds. Thus, enhanced NA synthesis in the 35S and AN lines may have improved not only Fe translocation to polished seeds but also Fe accumulation in polished seeds.

In contrast to polished and brown rice seeds, Fe and Zn contents decreased in hulls per seed (Fig. 4). The vascular bundle inside a seed is divided and runs in two directions: one that reaches the ovary, which develops into the bran and endosperm, and another that reaches the hull in a rachilla (Takane and Hoshikawa 1993). NA-Fe(II) and NA-Zn(II) may be more easily transported into the bran and endosperm than into the hull.

Cheng et al. (2007) produced a rice mutant with increased endogenous NA concentrations via knockout of the *OsNAAT1* gene, which is the sole Fe deficiency-induced *NAAT* gene in rice (Inoue et al. 2008). The *OsNAAT1* knockout mutant accumulated greater amounts of Fe, but not Zn, in polished rice seeds when cultivated under waterlogged conditions. Because the mutant expressed a wide range of Fe deficiency-responsive genes, the *OsNAAT1* knockout mutant appears to induce physiological Fe deficiency. In contrast, we found that the overexpression of the *NAS* gene alone is sufficient to increase Fe and Zn concentrations in seeds.

Higuchi et al. (2001a, b) produced rice lines expressing a genomic fragment of *HvNAS1*, resulting in increased NA content in the roots and leaves under Fe-sufficient conditions. However, Fe and Zn concentrations did not increase in seeds collected from *HvNAS1* genome insertion rice plants when grown under Fe-deficient conditions in calcareous paddy fields or under Fe-sufficient conditions in andosol paddy fields (Masuda et al. 2008; Suzuki et al. 2008a). This difference in Fe accumulation in rice seeds is thought to be related to the expression of the *NAS* transgene and subsequent NA content. Under Fe-sufficient conditions, NA concentrations in *35S-HvNAS1* lines were higher compared to *HvNAS1* genome insertion lines (Higuchi et al. 2001a). Moreover, in *35S-HvNAS1* lines, the *NAS* transgene is presumably expressed throughout the rice plant, which would promote widespread Fe circulation and bioavailability.

In addition to seed Fe and Zn concentration, Fe and Zn content per seed in T_2 seeds of AN lines also increased compared to those of the NT line (Fig. 5). There was no significant correlation between yield and Fe or Zn concentration in polished seeds in each T_2 plant of NT, AN1, and AN3 lines (data not shown). Therefore, it can be postulated that increased Fe and Zn concentration in seeds of transgenic lines was not due to decreased grain size or yield. Fe and Zn concentration in leaves of AN lines were not significantly increased compared to NT (Fig. 7e, f). Overexpression of *HvNAS1* might not lead to Fe and Zn overaccumulation in leaves. We observed no symptom of Fe toxicity in transgenic rice lines.

Actin promoter is reported to induce generally stronger expression than *35S* promoter when introduced into graminaceous plants such as rice (McElroy et al. 1990). Therefore, we produced not only 35S lines but also AN lines in order to obtain new lines with higher Fe and Zn concentrations. However, 35S line accumulated more NA, DMA than AN lines (Fig. 3). Fe and Zn concentration in seeds were also higher in 35S line than AN lines (Fig. 4). Nevertheless, AN lines also showed higher Fe and Zn concentrations in seeds than NT line.

In lines AN1, AN2, and AN3, Fe accumulation was relatively low in T_2 polished rice seeds compared to T_1 seeds (Figs. 1, 4, and 7). However, marked increases in Fe and Zn concentrations, similar to T_1 seeds, were achieved in some T_2 sublines (Fig. 7). These results may reflect differences in the genotype of each subline. Fe concentrations in the 35S line were consistently high because of its stable homozygous genotype. Further selection of AN sublines with high Fe and Zn content in T_2 seeds such as AN1-1 and AN3-6 would produce stable increases in micronutrient content.

Our results clarified the effect of overexpression of *NAS* to increase Fe and Zn concentrations in rice seeds. In previous studies, we generated a variety of transgenic rice lines bearing one or more of the genes involved in MA biosynthesis (Suzuki et al. 2008a). Among these, a transgenic rice line carrying a barley genomic fragment containing the *IDS3* gene showed increased Fe and Zn contents when grown in calcareous or andosol paddy fields (Masuda et al. 2008; Suzuki et al. 2008a). Therefore, we assume that introduction of barley *IDS3* genome fragments into rice also has high potential to increase Fe concentration in rice seeds. Vasconcelos et al. (2003) produced transgenic rice plants with introduced *Ferritin* genes into high Fe variety IR68144, which resulted in increase of both Fe and Zn concentration in polished rice up to 3.7 or 1.4 times. Wirth et al. (2009) also reported that constitutive overexpression of *AtNAS1* in combination with endosperm-specific expression of *Ferritin* is an effective method to increase Fe concentration in polished rice seeds. Because the *IDS3* genome fragment might enhance Fe and Zn uptake from the soil, concomitant *NAS* overexpression might also increase Fe and Zn transport to the seeds, while endosperm-specific expression of *Ferritin* might further enhance Fe storage ability in seeds. Thus, we postulate that combination of these three genes may allow further improvement in Fe and Zn accumulation in seeds.

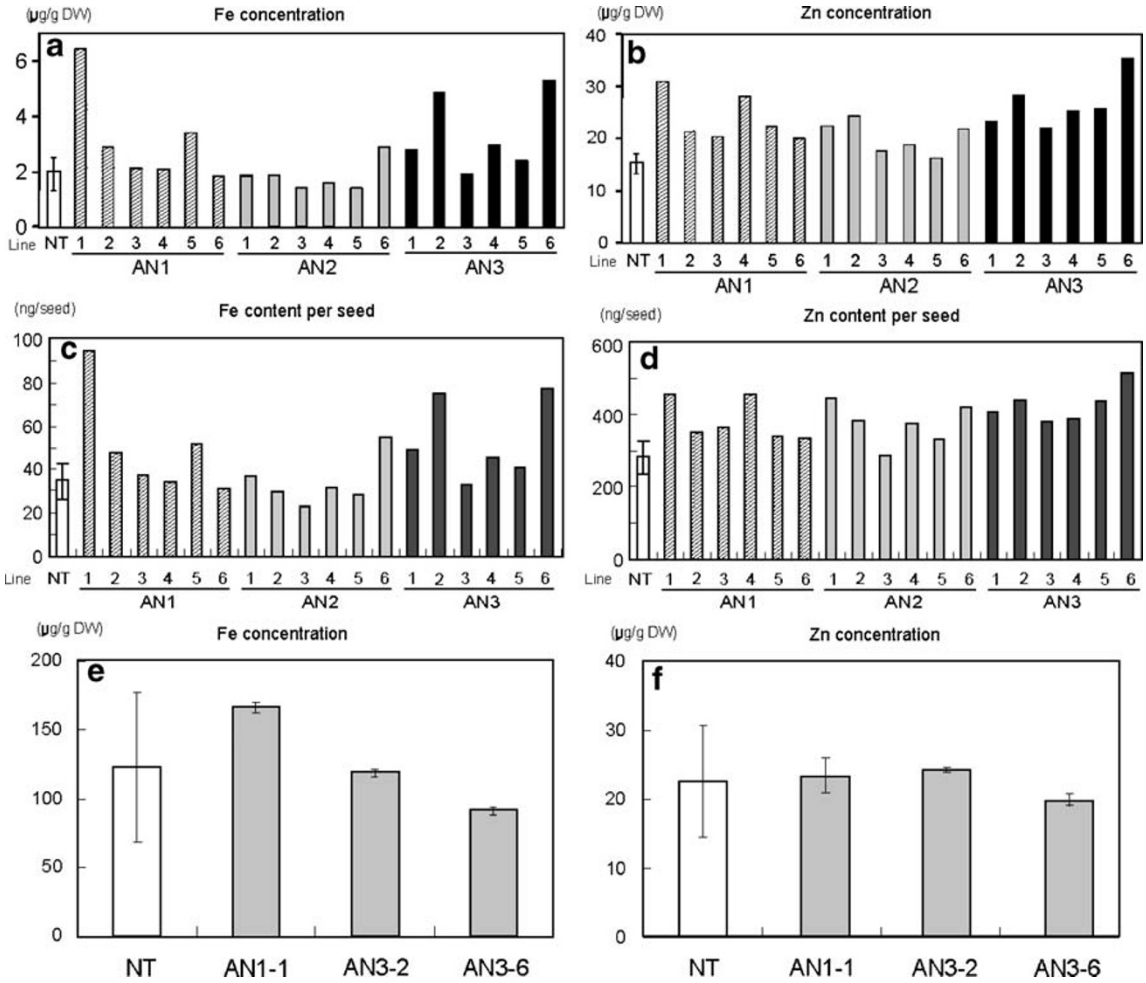

Fig. 7 Fe and Zn concentrations in polished T_2 seeds and leaves obtained from soil culture with slow-release fertilizer. Ten well-polished seeds obtained from the main tillers of six plants in each of six T_1 sublines were analyzed. **a** Fe concentration. **b** Zn concentration. **c** Fe contents per seed. **d** Zn contents per seed. The data shown for the NT line represent the mean ± standard error for six plants cultivated in independent pots (**a**–**d**). **e** Fe concentration in leaves. **f** Zn concentration in leaves. After T_2 seeds were harvested, leaf sheaths and leaf blades were also harvested and Fe and Zn concentration were measured. The data shown for each line represent the mean ± standard error for three replication of sample digestion (**e**, **f**).

Methods

Plant materials

Japonica rice (*Oryza sativa* L.), cultivar Tsukinohikari, was used as the NT (non-transgenic) control and for transformation. The transgenic rice line bearing *35S-HvNAS1* (35S) was originally produced by Higuchi et al. (2001a) and designated S11. This S11 line was cultured through several generations to select a homozygous line, which was then used in all analyses.

Construction of *OsActin1-HvNAS1* and rice transformation

The *OsActin1-HvNAS1*-nopaline synthase gene 3′-UTR (*tNOS*) cassette was constructed as follows. The *OsActin1* promoter was amplified using rice genomic DNA and the primers 5′-AAG CTT CGA GGT CAT TCA TAT GCT TG-3′ and 5′-GTC GAC CTT CTA CCT ACA AAA AAG CT-3′ and then subcloned using the *Hin*dIII and *Sal*I sites in the pUC18 vector (TaKaRa Bio, Shiga, Japan). This promoter sequence was excised from the vector using *Hin*dIII and *Xba*I and then ligated into pBluescript SK(+) (Stratagene, La Jolla, CA, USA). The *HvNAS1* open reading frame (ORF) -*tNOS* sequence was amplified via polymerase chain reaction using the *35S-HvNAS1-tNOS* cassette (Higuchi et al. 2001a) as a template and the primers 5′-GCA CTA GTA GCC ATG GAT GCC CAG AAC AGG AGG-3′ and 5′-GCA CTA GTA TTC CCG ATC TAG TAA CAT AGA TG-3′, both of which contained a *Spe*I site at the 5′-end. After sequence verification, the *HvNAS1-tNOS* fragment was digested with *Spe*I, whereas the *OsActin1* promoter in the SK(+) vector was

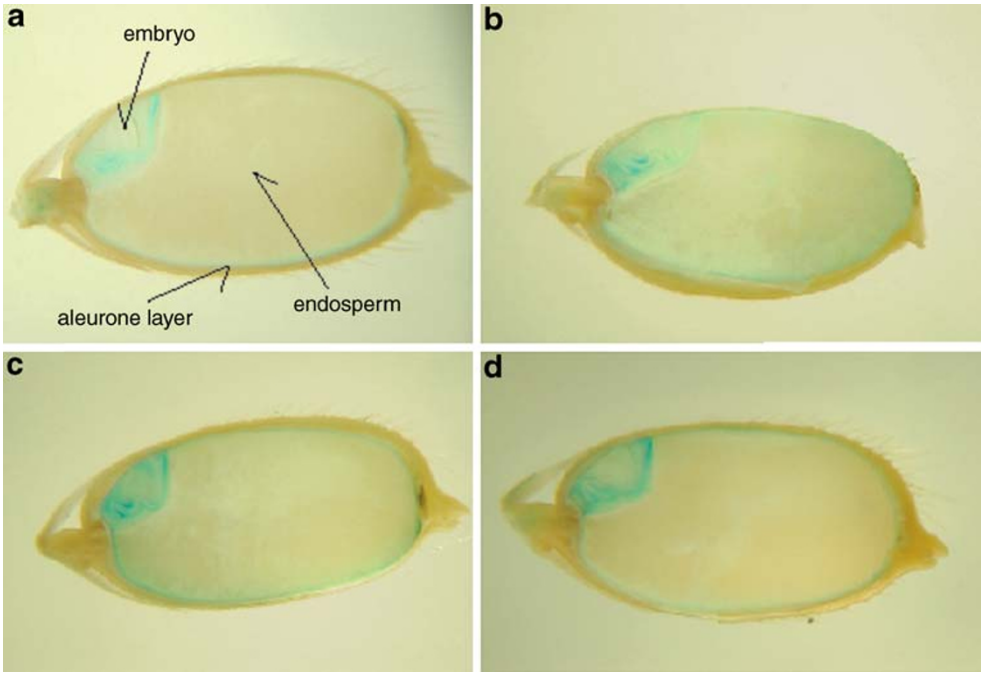

Fig. 8 Fe localization of T_2 seeds obtained from soil culture. Brown seeds were cut and stained by prussian blue staining. **a** NT. **b** AN1-1. **c** AN3-2. **d** AN3-6.

digested with *Xba*I. These two fragments were then ligated together to produce the *OsActin1-HvNAS1-tNOS* cassette. To create transgenic lines, this cassette was excised using *Not*I and *Kpn*I and then cloned into the same sites in the pBIGRZ1 vector (Akiyama et al. 1997). *Agrobacterium tumefaciens* (C58) was used to carry this construct into *O. sativa* L. cv. Tsukinohikari using the method developed by Hiei et al. (1994) and modified by Akiyama et al. (1997). Twenty *OsActin1-HvNAS1-tNOS* transgenic (AN) lines were obtained as T_0 transgenic plants.

Plant growth conditions

Twenty T_0 AN lines carrying *OsActin1-HvNAS1* and control NT plants were cultivated in 3.5-CL pots (1,000-ml volume; Kaneya, Aichi, Japan) containing artificial soil composed of a 2:1 mixture of bonsol-ichigou (Sumitomo Kagaku, Tokyo, Japan) and vermiculite (Green Tec, Tochigi, Japan). The soil was evenly fertilized with 3.5 g of a slow-release fertilizer, LongTotal-70 (Chisso-Asahi, Tokyo, Japan; N/P/K, 13:11:13), and 3.5 g of Long Total-140 (Chisso-Asahi; N/P/K, 13:11:13) per plant. Plants were grown in a greenhouse under natural light conditions, with 14 h of light at 30 °C and 10 h of dark at 25 °C. Plants were transplanted on 18 December 2006 and seeds harvested on 4 June 2007. Twelve lines were subjected to metal concentration analysis.

NT, T_1 AN, and 35S seeds were germinated on Murashige and Skoog (MS) medium. After 3 weeks, six seedlings from each line were transferred into nutrient solution [2 mM $Ca(NO_3)_2$, 0.5 mM $MgSO_4$, 0.7 mM K_2SO_4, 0.1 mM KCl, 0.1 mM KH_2PO_4, 0.1 mM Fe(III)-EDTA, 10 mM H_3BO_3, 0.5 μM $MnSO_4$, 0.5 μM $ZnSO_4$, 0.2 μM $CuSO_4$, and 0.01 μM $(NH_4)_6Mo_7O_{25}$] and grown in a greenhouse under the conditions described above. The pH of the culture solution was adjusted daily to between 5.5 and 5.8 with 1 N HCl. Twenty-three days after transplanting, leaves and roots were harvested for Northern blot analysis and the determination of NA and DMA concentrations.

For the metal concentration analysis, T_1 transgenic and NT lines were grown using two different methods. In the first method, three lines of T_1 AN seeds (AN1, AN2, and AN3), 35S line seeds, and NT line seeds were germinated on MS medium for 3 weeks, and then seedlings were transplanted into small ceramic pots (250-ml volume) filled with bonsol-ichigou, but no additional fertilizer. Six pots containing three seedlings each were prepared for each line, and each pot was submerged in nutrient solution. The plants were settled on 6 August 2007 and harvested on 28 November 2007. T_2 seeds were used in the metal concentration analysis.

In the second method, NT seeds and T_1 seeds from lines AN1, AN2, and AN3 were germinated on MS medium on 17 August 2007. On 3 September 2007, six T_1 seedlings from each line were settled in 3.5-CL pots containing artificial soil (2:1 mixture of bonsol-ichigou and vermiculite). The soil was fertilized evenly with 3.5 g of LongTotal-70 and 3.5 g of Long Total-140 per plant. Plants were grown in a greenhouse under natural light conditions, with 14 h of light at 30 °C and 10 h of dark at 25 °C. The plants were harvested on 5 January 2008 and the seeds used in the metal concentration analysis.

Northern blotting

The ORF sequence for *HvNAS1* (AB010086) was used to create an *HvNAS1* probe (Suzuki et al. 2006). This fragment was labeled with [a-^{32}P]-dATP using the random labeling method; labeled DNA was purified using a ProbeQuant G-50 micro-column (Pharmacia, Uppsala, Sweden). Total RNA in shoots was extracted using the SDS-phenol method. Total RNA (20 µg per lane) was separated on 1.4% (*w*/*v*) agarose gels. Blotting, hybridization and radioactive detection, and quantification were performed as previously described (Ogo et al. 2006).

Determination of NA and DMA concentrations

For determination of endogenous NA and DMA concentrations in the T_1 plant body, approximately 5 g of frozen shoots and roots from each transgenic and NT line was ground and homogenized with a mortar and pestle and then suspended in 100 ml of deionized hot water (80 °C) for 20 min. After suspension, the samples were centrifuged at 8,000×*g* for 15 min and the supernatants collected. These extraction steps were the same for both NA and DMA measurement. For HPLC analysis of NA, the supernatants were subjected to ion-exchange purification, evaporated, and filtered as previously described (Wada et al. 2006). For HPLC analysis of DMA, the supernatants were subjected to ion-exchange purification, evaporated, and filtered under different conditions as previously described (Suzuki et al. 2006). All steps for DMA measurement were performed in the dark. One sample was prepared from each line and analyzed in triplicate to determine the concentrations of NA and DMA.

For determination of NA and DMA concentrations in T_2 seeds, ten brown seeds from each of three lines of 35S, AN1, and AN3, whose Fe concentrations in T_2 polished seeds were higher than in the other three lines, were selected randomly. These seeds were crushed in a Multi-Beads Shocker (Yasui Kikai, Osaka, Japan). Samples were mixed with 400 µl of 80% ethanol, centrifuged at 12,000×*g* for 5 min, and the supernatants were collected for measurement. This step was repeated three times. One hundred microliters of supernatant was filtered in a 10 K Nanosep centrifugal device (Pall, Ann Arbor, MI, USA). One sample was prepared from each line and the concentrations of NA and DMA were analyzed by LC/ESI-TOF-MS (JEOL, Tokyo, Japan) as previously described (Kakei et al. 2007; Wada et al. 2007).

Metal concentration analysis

Brown seeds were collected randomly from the ear of the main tiller (the tiller in the center or the largest among all tillers in one plant). Ten T_1 seeds from each T_0 transgenic line or ten T_2 seeds from each T_1 transgenic lines were dried overnight at 80 °C in a heat-dry machine. After determining the dry weight of each sample, the seeds were digested in 1 ml of 13 M HNO_3 and 1 ml of 8.8 M H_2O_2 (Wako, Osaka, Japan) at 200 °C for 20 min with MARS Xpress (CEM, Matthews, NC, USA). After digestion, the samples were diluted to a volume of 5 ml and analyzed via inductively coupled plasma atomic emission spectrometry (SPS1200VR; Seiko, Tokyo, Japan).

To polish seeds, 30 brown seeds from the ear of the main tiller were placed into a 2-ml tube and shaken vigorously for 150 s at 2,500 rpm for at least four cycles using a Multi-Beads Shocker (Yasui Kikai). Ten well-polished seeds from each plant were selected. After removing the embryos, polished seeds were dried overnight, weighed, digested, and then analyzed as described above.

Rice bran (approximately 30–50 mg) was obtained as a by-product of the polishing process. Rice bran was dried overnight, weighed, and then digested at 220 °C for 20 min in 1 ml of 13 M HNO_3 and 1 ml of 8.8 M H_2O_2. Metal analysis was performed as described above. Rice hulls (approximately 80–160 mg) were also dried overnight, weighed, digested, and subjected to metal analysis as described above. Leaf sheaths and leaf blades were harvested, dried, and crashed by homogenizer (Fujiwara Seisakusyo, Tokyo, Japan). About 200 mg of crashed leaves was then digested and subjected to metal analysis as described above.

Fe localization by prussian blue staining

T_2 brown seeds were soaked in water overnight and were cut by a razor. Then, the samples were soaked in 2% HCl (Wako) and 2% potassium hexacyanoferrate(II) trihydrate (Wako) for 1.5 h. Stained samples were washed with distilled water and observed by stereoscopic microscope (AX10 canHRP, ZEISS).

Statistics

Analysis of variance with the Student–Newman–Keuls test was used to compare data. The level of significance was set at $p<0.05$. The statistical software package JMP7 (SAS Institute, Cary, NC, USA) was used in all analyses.

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