Planta (2005) 222: 225–233
DOI 10.1007/s00425-005-1530-8

ORIGINAL ARTICLE

Le Qing Qu · Toshihiro Yoshihara · Akio Ooyama
Fumiyuki Goto · Fumio Takaiwa

Iron accumulation does not parallel the high expression level of ferritin in transgenic rice seeds

Received: 19 November 2004 / Accepted: 22 February 2005 / Published online: 9 April 2005
© Springer-Verlag 2005

Abstract To answer the question whether iron accumulation in transgenic rice seeds depends on the expression level of exogenous soybean ferritin, we generated two kinds of ferritin hyper-expressing rice lines by introducing soybean ferritin *SoyferH-1* gene under the control of the rice seed storage glutelin gene promoter, *GluB-1* and the rice seed storage globulin gene promoter, *Glb-1*, (*GluB-1*/*SoyferH-1* and *Glb-1*/*SoyferH-1*, DF lines), and by introducing the *SoyferH-1* gene under the control of *Glb-1* promoter alone (*Glb-1*/*SoyferH-1*, OF lines). Ferritin expression was restricted to the endosperm in both lines and protein levels determined by western blot analysis were up to 13-fold higher than in a construct previously reported FK22 (*GluB-1*/*SoyferH-1*, in genetically Kitaake bachground); however, the maximum iron concentrations in seeds of both of the new lines were only about 30% higher than FK22. The maximum iron concentration in the OF and DF lines was about threefold higher than in the non-transformant. The mean Fe concentration in leaves of ferritin over-expressing lines decreased to less than half of the non-transformant while that the plant biomasses and seed yields of the ferritin-transformed lines were not significantly different from those of the non-transformant, suggesting that accumulation of Fe in seeds of hyper-expression ferritin rice did not always depend on the expression level of exogenous ferritin but may have been limited by Fe uptake and transport. No obvious differences were observed for other divalent-metal concentrations (Ca, Cd, Cu, Mg, Mn and Zn) in the seeds among all experimental lines and non-transformant.

Keywords Gene over-expression · Iron fortification · Ferritin accumulation · Transgenic rice

Abbreviations bp: Base pair · CTAB: Cetyl trimethyl ammonium bromide · DF: Double transformation line with *GluB-1*/*SoyferH-1* and *Glb-1*/*SoyferH-1* · NT: Non-transformant · OF: Single transformation line with *Glb-1*/*SoyferH-1* · PCR: Polymerase chain reaction · SDS-PAGE: SDS-polyacrylamide gel electrophoresis

Introduction

Dietary iron deficiency, a major factor in the etiology of microcytic hypochromic anemia, is one of the most serious nutritional problems especially for woman and children in developing countries where iron supplements by tablet are not easily available (Gillespie and Haddad 2001). Development of high iron content staple crops either by a traditional breeding or cultivation strategy or by genetic engineering may be an alternative and sustainable approach for solving iron deficiency-related health problems. However, breeding of high iron content varieties from the common crop lines is time consuming and resource intensive, and may not be sufficient to yield agronomically acceptable high-iron content plants. In addition, high concentrations of free ionic iron could be extremely toxic to plants, injuring the plant by catalyzing the generation of cellular free-radicals. Therefore, it is essential not only to increase iron uptake

L. Q. Qu · A. Ooyama · F. Takaiwa (✉)
Laboratory of Genetic Engineering,
Department of Plant Biotechnology,
National Institute of Agrobiological Sciences,
Kannondai 2-1-2, Tsukuba,
Ibaraki 305-8602, Japan
E-mail: takaiwa@nias.affrc.go.jp
Fax: +81-29-8388397

T. Yoshihara (✉) · F. Goto
Plant Molecular Biology Group,
Laboratory of Environmental Science,
Central Research Institute Electric Power Industry,
1646 Abiko, Chiba 270-1194, Japan
E-mail: yoshiha@criepi.denken.or.jp

L. Q. Qu
Institute of Botany,
The Chinese Academy of Sciences,
Beijing, 100093, China

ability, but also to enhance the storage capacity of iron in a non-toxic and bio-available form. Ferritin, a conjugated protein with the molecular weight of about 540 kDa assembled from 24 subunits into a spherical shell (Laulhere et al. 1988), can store up to 4,500 iron atoms in its central cavity (Theil 1987, 2004; Harrison and Arosio 1996). Ferritin, with about 23% of its weight as iron and high stability and survivability in vitro digestion conditions, is thus a major iron storage protein in both plants and animals (Theil 1987, 2004; Harrison and Arosio 1996; Briat and Lobréaux 1997).

Rice is one of the most important staple foods for a large part of the world's population; therefore, varieties containing large amounts of bio-available iron would have the potential to ameliorate much of the global endemic dietary iron deficiency. To date, many efforts to accumulate iron in rice seeds have employed ferritin gene transformation strategies. Goto et al. (1999) expressed soybean ferritin gene (*soyferH-1*) in rice under the control of a rice endosperm-specific promoter, 1.3 kb *GluB-1*, and obtained stable transgenic rice that accumulated ferritin protein in the seed. Iron concentrations in transgenic rice seed were increased up to threefold over non-transformant seed. Lucca et al. (2001) obtained rice seed with double the iron content by introducing a ferritin gene from *Phaseolus vulgaris* under the control of a rice seed storage glutelin promoter, *Gt1*. Vasconcelos et al. (2003) introduced *soyferH-1* into *Indica* rice and examined the effect of *soyferH-1* expression on iron accumulation when grown in the field. Iron concentrations in T2 lines were at most 3.7-fold greater than in the non-transformant, even after commercial milling. Additionally, Drakakaki et al. (2000) reported a significant increase in iron concentration in vegetative organs but not in seeds by expressing a ferritin gene in rice and wheat under the control of a maize *Ubiquitin-1* promoter. High level of ferritin in rice tissues may contribute to improvement of the iron bioavailability since it absorbs iron and separates iron from phytate (Theil 2004). Ferritin-transformed rice seed with high bioavailability of iron was confirmed to be as effective as $FeSO_4$ in recovering from iron depletion by a rat feeding test (Murray-Kolb et al. 2002).

It has been reported that the activation of enzymes related to iron-uptake also increases the uptake of other divalent-metal cations (Grusak et al. 1990; Welch et al. 1993; Delhaize 1996; Vansuyt et al. 2000). Some divalent metal accumulation properties in leaves of ferritin-over-expressing tobacco have already been described (Vansuyt et al. 2000; Yoshihara et al. 2003). Vasconcelos et al. (2003) observed that zinc concentrations increased in ferritin-transformed rice along with the increased concentration of iron, but did not report the concentrations of other divalent metals. For seed quality and food safety reasons, it is necessary to quantify the other divalent metals in rice seeds prior to agronomic trials.

The US Recommended Dietary Allowance (1995) for iron is 18 mg/day. Given the iron content in ferritin transformed rice seed of 37 μg/g, the highest iron content in polished ferritin transformed rice seed (Vasconcelos et al. 2003), about 500 g of rice will be required for a daily diet. Therefore, higher iron content in ferritin-transformed rice seed is preferred. Goto et al. (1998) reported a positive correlation between ferritin levels and iron accumulation in ferritin-transformed tobacco, raising the possibility that higher iron concentrations *in planta* could be achieved by increasing the expression of ferritin in transgenic rice seeds. Further, use of strong promoters that could target to seed tissue not removed by mechanical de-hulling would allow high iron accumulation in ingested seed parts. Recently, we have isolated and characterized a promoter from the rice seed storage globulin gene *Glb-1* that gives expression more than tenfold higher than the 1.3 kb *GluB-1* promoter, and directs the GUS reporter gene to be expressed mainly in central parts of the endosperm rather than in the outer part of the endosperm where *GluB-1* is most active (Qu and Takaiwa 2004). In this study, we generated two kinds of ferritin-over-expressing rice lines (double ferritin, DF lines, *GluB-1*/*SoyferH-1* + *Glb-1*/*SoyferH-1* and one ferritin, OF lines, *Glb-1*/*SoyferH-1*) and compared ferritin expression levels and Fe accumulation levels in seeds and vegetative tissues with the ferritin over-expressing line FK 22 (Goto et al. 1999). In addition, accumulation levels of Ca, Cd, Cu, Mg, Mn and Zn in seeds were determined.

Materials and methods

Plant materials

Rice (*Oryza sativa* L.) cultivar Kitaake and ferritin-transformed rice line FK22 containing the *SoyferH-1* gene under the control of rice glutelin *GluB-1* promoter (GenBank accession No. AY427569) with *bar* as a selection marker (Goto et al. 1999), were used as plant materials.

Construction and transformation of the chimeric gene

Soybean ferritin cDNA, *SoyferH-1* encoding the ferritin H1 subunit (Goto et al. 1999) was linked to the rice 26 kDa globulin gene *Glb-1* promoter (GenBank accession No. AY427575). The fragment containing the promoter and the ferritin gene was inserted into a binary vector (pGPTV-35S-it HPT) containing hygromycin phosphotransferase (*HPT*) as a selection marker (Fig. 1). The binary vector was reconstructed from the pGPTV-it HPT binary vector (Becker et al. 1992) by exchanging the *Nos* promoter with the 0.8 kb CaMV 35S promoter as the promoter of *HPT* and by replacing *UidA* (GUS) with the *Glb-1*/*SoyferH-1* fragment. The binary vector was introduced into the non-transformant (Kitaake) and FK22 by *Agrobacterium tumefaciens* (strain EHA105) mediated transformation. The transformation was carried out as described previously (Goto et al. 1999) except for the hygromycin selection. The successful

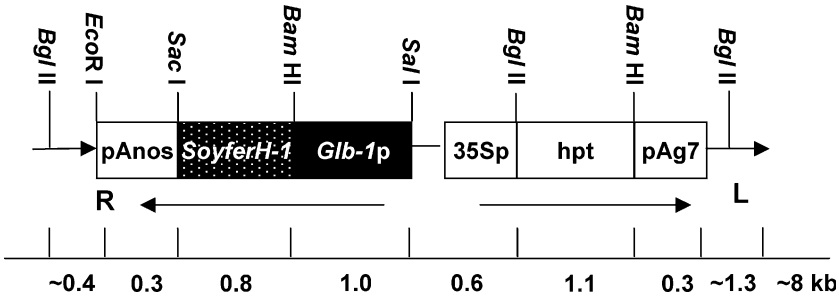

Fig. 1 The chimeric soybean ferritin gene construct. Soybean ferritin cDNA was fused to rice endosperm-specific *Glb-1* promoter. The chimeric genes were inserted into the pGPTV-35S-hpt binary vector between the restriction sites *Sal*I and *Sac*I

transformation was verified by PCR analysis using 5′-TCTACACGAAGCTCACCGTGCAC-3′ from *Glb-1* promoter and 5′-ATGGCTCTTGCTCCATCCAAAG-3′ from *SoyferH-1* as a set of primers.

Culture conditions for transgenic rice plants

Transformants were grown in a greenhouse at 30 ± 3 °C under natural illuminating conditions. One rice plant was grown in a pot containing about 3 l paddy field soil without fertilizing until seed harvest. All of the pots were submerged in a pool. Transformants were self-pollinated for five generations and the homozygous T3 to T6 plants were used for further studies.

Southern and northern blot analysis

Rice genomic DNA was extracted from leaves using the CTAB method. Ten micrograms of genomic DNA was digested with *Hin*dIII. DNA digests were fractioned on a 0.8% agarose gel, transferred onto a nylon membrane (Hybond N+) and hybridized with the specific probe to detect the soybean ferritin gene, *SoyferH-1*, prepared by PCR amplification using a primer set (5′-TGTGCC-TCAACGGTGCCTCTC-3′ and 5′-CTCTTAATCAA-GAAGTCTTTG-3′). Hybridization probes were chemically labeled with horseradish peroxidase. Signal detection was carried out using the ECL system (Amersham Biosciences). RNA was prepared from developing rice seeds 10–14 days after flowering. RNA extraction, probe labeling, hybridization and signal detection were carried as described previously (Qu et al. 2002).

Western analyses

The expression pattern and strength of ferritin in mature seeds of transgenic rice were examined by in situ western analysis and western blot analysis as described previously (Goto et al. 1999; Qu et al. 2003). The image of western blot analysis was captured by a digital image analyzer, ChemiDoc XRS (Bio-Rad), and processed for analyzing ferritin expression levels according to the manufacturer's instructions. Ferritin expression was standardized using FK22 as a baseline.

Hydroponics of ferritin-transformed rice plants

Transgenic rice lines were cultured in green house in hydroponic solution using modified Kasugai's medium (Mori and Nishizawa 1987). The medium consisted of 0.35 mM $(NH_4)_2SO_4$, 0.27 mM K_2SO_4, 0.18 mM Na_2HPO_4 $12H_2O$, 0.36 mM $CaCl_2$ $2H_2O$, 0.46 mM $MgSO_4$ $7H_2O$, 18.4 μM H_3BO_3, 4.6 μM $MnSO_4$ $5H_2O$, 1.5 μM $ZnSO_4$ $7H_2O$, 1.5 μM $CuSO_4$ $5H_2O$ and 1 μM Na_2MoO_4 $2H_2O$. The Fe-EDTA concentrations of the medium were 45 μM (standard Fe concentration), 90 μM (2×Fe) and 225 μM (5×Fe, excessive Fe concentration), respectively. The hydroponic solution was changed completely every three days. The growing conditions were as described above.

Metal concentration analysis

Three plants from each line were used for metal concentration analyses. Rice seeds, leaves, and stalks were collected separately and dried for three days at 65°C. Ten seeds (about 200 mg) or 100 mg of pre-dried vegetative tissues from each plant were randomly selected, ground and wet-ashed with 2 ml HNO_3 and H_2O_2 overnight at 110°C. Ashing was repeated until the tissues whitened. These samples were then dissolved in 15 ml of 1 N HCl. Concentrations of Ca, Cd, Cu, Fe, Mg, Mn and Zn were measured using inductively coupled plasma atomic emission spectrometry (ICP; type P-4000, Hitachi, Japan) at wavelengths of 317.33 nm (Ca), 214.438 nm (Cd), 324.754 nm (Cu), 238.204 nm (Fe), 279.806 nm (Mg), 293.930 nm (Mn) and 213.856 nm (Zn). The means of three to five duplicates per line were compared using Student's *t*-test.

Results

Characterization of transgenic rice lines

Successful transformation of *SoyferH-1* under the control of the *Glb-1* promoter was determined by PCR analysis using genomic DNA extracted from leaves of T0 plants. Transformants contained a 900 bp fragment (150 bp for the *Glb-1* promoter and 750 bp for the coding region of *SoyferH-1*) (Fig. 2). Transformants derived by introducing *Glb-1*/*SoyferH-1* into FK22, a ferritin-transformed

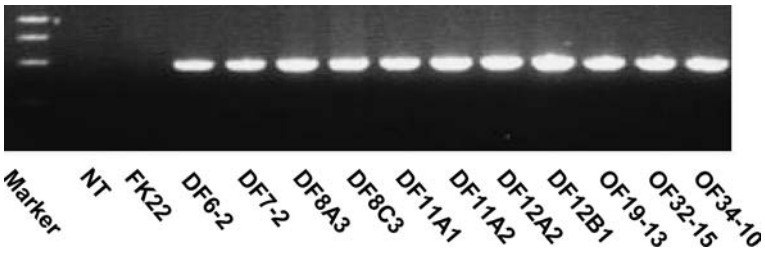

Fig. 2 PCR analysis of transgenic plants using specific primers in *Glb-1* promoter and *SoyferH-1* coding region. The expected 900 bp PCR products indicated the successful insertion of soybean ferritin genes in the transgenic plants. NT, Kitaake, Non-transformants; FK22, transformant with *SoyferH-1* gene directed by *GluB-1* promoter; DF, double tranformants with soybean ferritin gene *SoyferH-1* directed by *GluB-1* and *Glb-1* promoters, respectively; OF, transformants with *SoyferH-1* gene directed by *Glb-1* promoter

line containing *GluB-1*/*SoyferH-1*, in genetically Kitaake background (Goto et al. 1999), were denoted as double transformants (DF lines, *GluB-1*/*SoyferH-1* and *Glb-1*/*SoyferH-1*) and transformants derived by introducing *Glb-1*/*SoyferH-1* into cultivar Kitaake were denoted as single transformation lines (OF lines, *Glb-1*/*SoyferH-1*). Eight DF and three OF lines were obtained. Transgenic rice lines were self-pollinated through five generations and the resulting homozygous lines (T6) were used for further analyses. Southern hybridization with genomic DNA from T6 transgenic rice digested with *Hin*dIII (there is no *Hin*dIII site within *SoyferH-1*) showed one band in FK22 indicating that it contains a single copy of *SoyferH-1*. DF lines containing both constructs show up to three bands, indicating that these isolates contain *GluB-1*/*SoyferH-1* and at least one copy of *Glb-1*/*SoyferH-1* (Fig. 3a). Three to five bands were observed in the OF lines, reflecting the copy number of *SoyferH-1* (Fig. 3a). Northern hybridization using total RNA extracted from T6 seeds (10–14 days after flowering) gave a single band of 750 bp, corresponding to the expected size of *SoyferH-1* gene, indicating that the gene is expressed in seeds of each line (Fig. 3b), though the expression varied considerably.

Accumulation and distribution of ferritin in transgenic rice seeds

The expression pattern of soybean ferritin directed by two rice endosperm-specific promoters, *GluB-1* and/or *Glb-1*, was determined by in situ western hybridization. In FK22, soybean H1 ferritin expression was limited to the endosperm with higher concentrations in the outer cells of the endosperm (Fig. 4). In OF lines, *SoyferH-1* directed by the *Glb-1* promoter was expressed in inner starchy endosperm tissue. In DF lines, ferritin was expressed almost equally throughout the endosperm. Non-transformant rice seed (NT) used as a control remained unstained. in situ western hybridization patterns observed in transgenic lines were consistent with previously reported tissue-specific expression of the promoters (Qu and Takaiwa 2004). Western blot analysis using total protein extracted from mature seed was performed to investigate the expression levels of ferritin in the DF and OF lines (Fig. 5). Polyclonal antibody directed against soybean H1 ferritin bound to a 28 kDa band from transgenic plants, while no band was observed in proteins from the non-transformant. Ferritin levels in DF and OF lines were 3.1- to 11.5-fold (5.8-fold on average) and 8.4- to 13.4-fold (11.4-fold on average) higher than FK22, respectively. Levels of ferritin accumulation corresponded to *SoyferH-1* mRNA levels determined by northern hybridization analysis. It is noteworthy that soybean H1 ferritin was not expressed in any tissues other than the endosperm in any transgenic line.

Iron and other divalent metal concentrations in ferritin-transformed lines

Iron concentrations in seeds and leaves of T6 plants were measured for three kinds of ferritin over-expressing lines (DF, OF and FK22) and non-transformant (NT). The iron concentrations (dry weight) in seeds of the DF and OF lines ranged from 12.4 ± 1.8 μg/g to 20.1 ± 4.9 μg/g (15.1 ± 2.8 μg/g on average) and from 15.4 ± 1.3 μg/g to 16.0 ± 1.2 μg/g

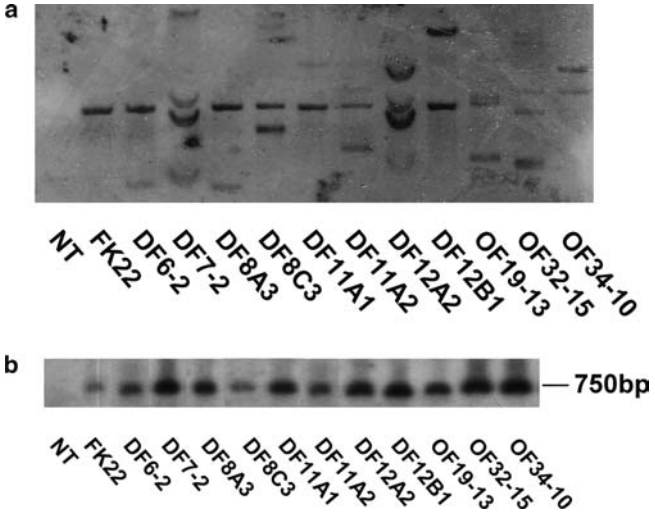

Fig. 3 Southern and northern hybridization of rice lines. **a** 6 μg genomic DNA was digested by *Hin*dIII, fractioned by 0.8% agarose gel, transferred onto a nitrocellose membrane and hybridized with a fragment encoding ferritin subunit. **b** 10 μg total RNA was fractioned by 1.2% agarose-formaldehyde gel, transferred onto a nitrocellose membrane and hybridized with a fragment encoding mature ferritin subunits. NT, FK22, DF and OF as described in Fig. 2

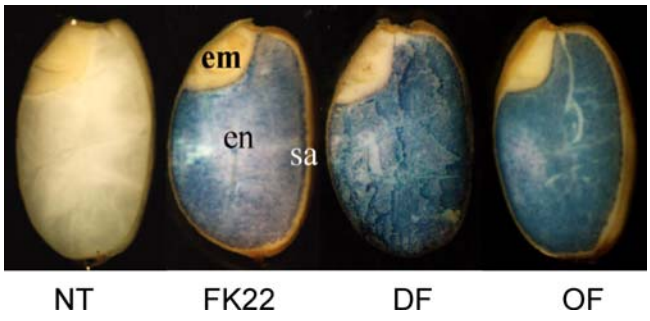

Fig. 4 In situ western hybridization of ferritin transgenic rice seeds. NT, FK22, DF and OF as described in Fig. 2. *em* embryo, *en* endosperm, *sa* subaleurone

(15.7 ± 0.3 μg/g on average), respectively (Fig. 6a). Mean iron concentrations in the seeds of FK22 and NT were 15.1 ± 1.7 and 11.2 ± 1.8 μg/g, respectively. Compared to the NT line, iron concentrations in the T6 ferritin over-expressing lines increased up to about 80% with an average of about 40%. Statistical analysis revealed that the averaged seed iron concentrations of all ferritin-transformed lines (DF, OF and FK22) were significantly higher than that of NT ($P < 0.05$); however, there was little difference among most of the ferritin-transformed lines and FK22; although, some lines showed about 30% higher iron than FK22. Iron concentrations in leaves of DF and OF lines after seed matured ranged from 0.6 ± 0.2 μg/g to 10.3 ± 0.7 μg/g (2.3 ± 3.5 μg/g on average) and from 3.0 ± 2.9 μg/g to 4.2 ± 3.7 μg/g (2.7 ± 2.2 μg/g on average) (Fig. 6b). The iron concentrations in leaves of FK22 and NT at the same physiological stage of development were 1.2 ± 1.4 and 27.2 ± 2.6 μg/g, respectively. Pooled data showed that the leaf iron concentrations of each ferritin over-expressing line (DF, OF and FK22) were significantly lower than NT ($P < 0.05$); however, there was almost no difference among the ferritin-transformed lines (DF, OF and FK22). Concentrations of Ca, Cd, Cu, Mg, Mn and Zn in the seeds of each line were not significantly different among all lines, although concentrations of Mg and Zn were slightly higher in ferritin over-expressing lines (Table 1). Furthermore, metal concentrations in leaf and stalks were essentially the same in every line (data not shown).

Variation of iron accumulation in ferritin over-expressors over four generations

To test the stability of iron accumulation, iron concentrations in seeds of newly generated ferritin over-expressing lines were measured through four generations from T3 to T6 (Fig. 7). Iron concentrations in seeds of DF varied from 7.9 μg/g to 27.0 μg/g with a mean of 15.5 ± 4.6 μg/g and from 8.9 to 22.4 μg/g with a mean of 16.7 ± 3.8 μg/g in OF from generation T3 to T6. In contrast, FK22 lines varied from 10.0 μg/g to 15.0 μg/g with a mean of 12.8 ± 2.2 μg/g and non-transformants varied from 7.3 to 17.0 μg/g with a mean of 11.3 ± 4.1 μg/g. Iron concentrations in each of the ferritin over-expressing lines increased up to about threefold with an average of 1.3-fold above NT and up to about twofold with an average of 33% above FK22 (in the case of the OF19–13 line, T3 generation). Though iron concentrations of ferritin-transformed lines were always higher than NT in all generations, they varied from generation to generation within an individual line, and the relative order for iron concentrations in these experimental lines was also unfixed except for in the NT line. For example, the iron content of line DF12A2 was the highest in generation T5 with 27.0 μg/g, while it was the lowest in the T4 generation with 10.0 μg/g.

Effect of medium iron concentration on iron accumulation in the ferritin-transformed rice seeds

To determine if the iron accumulation in the ferritin-transformed rice seeds is affected by the medium iron concentration, the ferritin-transformed rice plants were grown in hydroponic solution with iron concentrations of 45 μM (standard Fe concentration), 90 μM (2×Fe) and 225 μM (5×Fe, excessive Fe concentration), respectively. The seed iron contents of the ferritin-transformed lines showed no significant differences among the three treatments (Fig. 8), indicating that the iron accumulation in ferritin-transformed rice seeds were not affected by iron concentrations in the hydroponic solution.

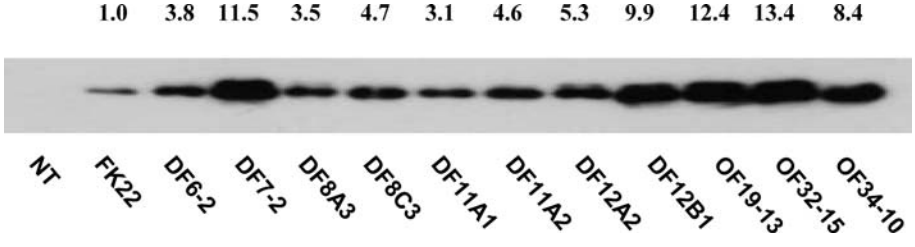

Fig. 5 Western blot analysis of ferritin in transgenic rice seeds. Total proteins extracted from each transgenic and nontransgenic seeds were fractioned by SDS-PAGE and immunoblotted with the soybean ferritin rabbit polyclonal antibodies. The predicted 28 kDa protein band is ferritin. The *numerals above each lane* indicate the multiples of H1 ferritin levels compared with FK22. NT, FK22, DF and OF as described in Fig. 2

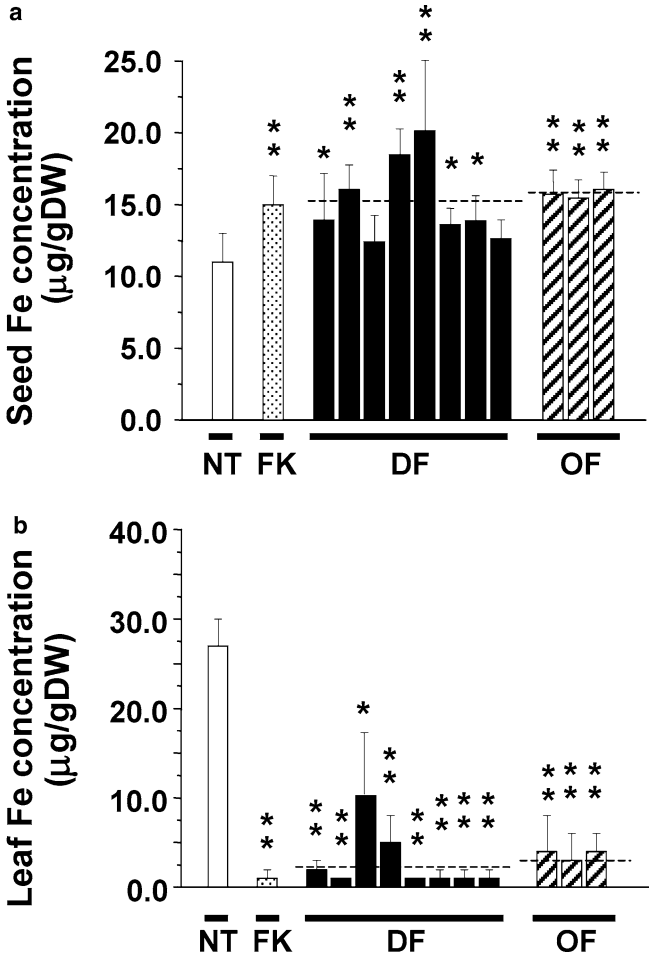

Fig. 6 Iron concentrations in seeds and leaves of rice plants. **a** Iron concentrations of ferritin-transformed rice seeds were increased by 80 and 30% compared to non-transformed control and FK22. **b** Iron concentrations of leaves of all ferritin-transformed plants were much less than half of the control. *Asterisk* and *double asterisks* show significance at 0.05 and 0.01 levels, respectively, comparing to the non-transformed control. *Dotted lines* indicate an average value of experimental lines

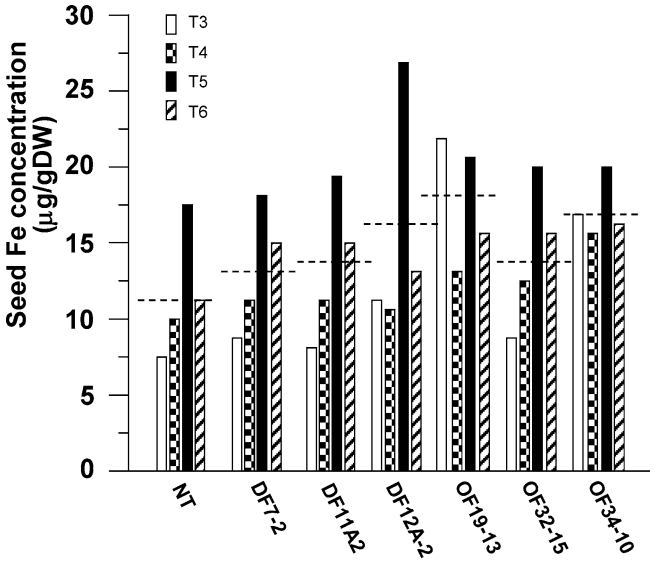

Fig. 7 Seed iron concentrations from T3 to T6 generations. Though the seed iron concentrations of ferritin-transformed rice lines varied significantly throughout four generations within individual lines, they were always higher than that of the nontransformant. *Dotted lines* indicate an average value of each experimental line

Discussion

Iron deficiency anemia is one of the most prevalent nutritional problems in the world today, affecting about 30% of the world population. Developing an iron-enriched staple plant food either through traditional plant breeding methods or by molecular biology would be a powerful tool for the health problems caused by iron deficiency. Ferritin was evaluated and found to be effective as a dietary treatment for iron deficiency in rats (Beard et al. 1996). Murry-Kolb et al. (2002) found that ferritin over-expressing rice seeds were as effective as $FeSO_4$ in recovering from iron deficiency in rats, suggesting that ferritin over-expression might contribute to the alleviation of human health problems caused by iron deficiency.

It has been suggested that increasing iron storage capacity in cells may act as an effective signal to

Table 1 Major divalent metal concentrations other than Fe in ferritin-overexpressing rice seeds

Line/	Ca	Cd	Cu	Mg	Mn	Zn
NT	119 ± 5^a	0.2 ± 0.2^a	13 ± 0^a	625 ± 41^b	69 ± 4^a	37 ± 3^b
FK22	93 ± 8^b	0.1 ± 0.1^a	14 ± 1^a	680 ± 32^b	68 ± 11^a	39 ± 2^b
DF	119 ± 18^a	0.0 ± 0.1^a	13 ± 1^a	769 ± 59^a	60 ± 7^a	41 ± 3^b
OF	119 ± 12^a	0.1 ± 0.0^a	14 ± 1^a	780 ± 25^a	64 ± 1^a	46 ± 2^a

Each value is showing in a unit of μg/g DW. Averages of three duplicate per line were statistically analyzed using *t*-test
[a, b]Show a significant difference against each metal concentration of NT ($P < 0.05$)

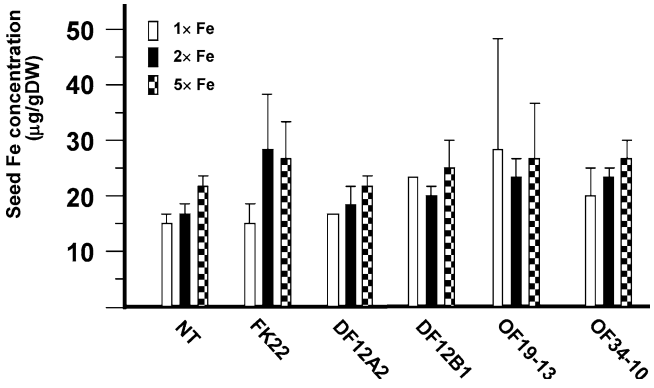

Fig. 8 Seed iron contents of ferritin-transformed rice plants grown in hydroponic media with different Fe concentrations. The seed iron contents of the ferritin-transformed rice did not show significant difference among the three treatments with medium iron concentrations of 45 μM (1×Fe, standard Fe concentration), 90 μM (2×Fe) and 225 μM (5×Fe, excessive Fe concentration), respectively

stimulate iron uptake into the cells, and to sequester the excess iron which subsequently flows into the cells (Van Wuytsuwinkel et al. 1999; Yoshihara et al. 2003). Based on this hypothesis, we successfully developed two new kinds of ferritin hyper-expression transgenic rice lines using the strong endosperm specific promoter, *Glb-1*. The average expression levels of *SoyferH-1* in seeds of DF and OF lines were 5.8-fold and 11.4-fold above FK22 as reported previously (Fig. 5). Those levels corresponded to the level of the ferritin transcripts determined by northern hybridization analysis. It is notable that mRNA and ferritin levels are stable through generations T3 to T6 (data not shown). It should be noted that ferritin expression in DF lines was significantly lower than in the OF lines at the RNA and protein levels (Figs. 3b, 5). These results were far from the expected additive effects. The tissue specificity of these two constructs, mainly in sub-aleurone layer and central parts of endosperm, respectively, partially overlapped; so, it was possible that a kind of interference between products of these two constructions may reduce the expression level. The lower expression level of ferritin in DF lines might be also caused by homologous gene co-suppression, which is a common effect of adding multiple copies of the same gene (Flavell 1994; Meyer and Saedler 1996).

Ferritin expression levels in the seeds of DF and OF lines were much higher than in FK22. However, the iron contents in the seeds of DF and OF lines were not significantly different from FK22 though some lines showed about a 30% increase comparing to FK22 (Fig. 6a). Little correlation between ferritin expression levels and the iron concentrations in seed could be observed from the present results, although our previous report showed that there was a correlation in leaves of ferritin-transformed tobacco (Goto et al. 1998). The maximum iron concentration (i.e. about 27 μg/g, about threefold higher than non-transformant) was also similar to concentrations reported previously (Goto et al. 1999; Lucca et al. 2001; Vasconcelos et al. 2003). In addition, although the iron concentration of the ferritin over-expressing line was always higher than NT, iron concentrations varied from generation to generation even within individual lines, and the relative order for the iron concentration in these experimental lines was also unfixed except for the NT line (Fig. 7). Though environmental conditions like soil, light, temperature and microbial activity have been reported to influence iron uptake and storage (Kokot and Phuong 1999; Lueders and Friedrich 2000; Vansuyt et al. 2000), genetic regulatory factors, such as DNA methylation and co-suppression may also play a role (Finnegan et al. 2000; Meins 2000). The problem of variable expression across generations would need to be better understood before iron-enhanced varieties could be employed in the field.

It is interesting to note that the iron concentrations in leaves of all ferritin-transformed lines were much less than half of the non-transformant after seed matured, most of them were even less than one-tenth of the non-transformant (Fig. 6b) while the iron contents in leaves before heading, stalks and roots of the transgenic plants were not significantly different from those of the non-transformed control (data not shown). It is noteworthy that the biomasses and seed yields of the ferritin-transformed lines were not significantly different from those of the non-transformed control (data not shown). The leaf is a sink for nitrogen and mineral nutrients during the early stage of plant development. However, the leaf becomes a mineral nutrient source once leaf senescence begins and the iron is redistributed from leaves to seeds at reproductive stage of plant development (Mauk and Nooden 1992; Himelblau and Amasino 2001). Himelblau and Amasino (2001) reported that the iron was mobilized from senescing leaves to developing seeds and the iron content in senescent leaves was reduced to less than half of that in pre-senescent leaves in *Arabidopsis*. Plant endogenous ferritin is almost undetectable except when plants are under stress or during iron loading (Proudhon et al. 1989). Free and ferritin-bound irons in plants exist in equilibrium, so over-expression of ferritin may disturb this equilibrium, resulting in the sequestration of essential free iron. It has been suggested that iron atoms captured by ferritin in seed amyloplasts may decrease free iron in the cytoplasm (Briat and Lobreaux 1997; Gueriont and Yi 1994). The seed then sends a signal of iron starvation and accelerates the iron flow from leaves to seeds, resulting in the reduction of iron in the leaves. This hypothesis was also supported by the observation that the leaves of ferritin-transformed rice plants, but not the non-transformed control, gradually exhibited chlorosis, a symptom of iron deficiency (data not shown), during seed maturation. In spite of chlorosis, the ferritin-transformed rice showed little morphological difference from the non-transformed control, and the ferritin-transformed rice plants did not show premature senility. Drakakaki et al. (2000) expressed soybean ferritin gene under the control of the maize *ubiquitin-1* promoter and reported that the iron content in leaves of ferritin-transformed rice was about twofolds higher than that of the non-transformed control irrespective of similar iron contents in the seeds among them. This might be due to the expression property of the *ubiquitin-1* promoter since that the soybean ferritin gene directed by *ubiquitin-1* promoter was also expressed in leaves which fixed the iron in the leaves and inhibited the iron in leaves from flowing to seeds. Although the total iron balance between seeds and leaves (and other parts of the plant) is still unknown, iron translocation from leaves to seeds may be very important for iron accumulation in seeds. In the hydroponic experiments with iron concentrations of 45 μM (standard Fe concentration), 90 μM (2×Fe) and 225 μM (5×Fe) in the medium, it was shown that there were no significant differences in iron levels of seed treated by different concentrations of iron within individual ferritin-transformed line (Fig. 8). It should be noted that the ferritin-transformed plants gradually showed chlorosis after flowering even grown in the medium with excessive Fe concentration of 225 μM

(data not shown), indicating that iron accumulation in ferritin-transformed rice seeds was not limited by medium iron concentration, but might be limited by iron uptake. The iron content of transgenic seeds may also be limited by the transport of iron from vegetative tissues to seeds. Further investigation on activation of Fe uptake/transportation-related enzymes in leaves, stalks and roots and iron translocation in rice plant will be required.

Rice is usually consumed after polishing the outer layers and embryo. Vasconcelos et al. (2003) reported that iron concentrations in ferritin-transformed rice seeds with *SoyferH-1* under control of the *GluB-1* promoter increased even after polishing. Since the ferritin gene directed by the *GluB-1* promoter is mainly expressed in the sub-aleurone layer, polishing will cause considerable loss of fixed iron. Therefore, the two newly developed constructs, DF and OF, are useful for expression mainly in the central part of endosperm or whole endosperm (Fig. 4). The expression patterns of ferritin in seeds directed by the *GluB-1* and *Glb-1* promoters agreed well with previous studies using GUS as the reporter gene (Qu and Takaiwa 2004).

It has been reported that the enzymes related to iron uptake (e.g. Fe^{3+}-chelate reductase and Fe^{2+}-transporters) enhance not only iron uptake, but also the uptake of other divalent-metal cations, especially Cd and Mn (Grusak et al. 1990; Welch et al. 1993; Delhaize 1996; Cohen et al. 1998; Vasconcelos et al. 2003). The accumulation properties of some divalent metals in leaves of ferritin-over-expressing tobacco have been investigated, and an obvious accumulation of an undesirable metal was sometimes observed (Vansuyt et al. 2000; Yoshihara et al. 2003). However, the major divalent metal concentrations in ferritin-transformed rice seeds were not significantly different from the non-transformant, although the concentrations of Mg and Zn tended to be higher in ferritin over-expressing lines. These findings indicate that ferritin over-expression in gramineous plants does not increase the concentration harmful metals *in planta*.

Although high storage ability of iron in rice seeds could be achieved by ferritin over-expression under the control of a strong endosperm-specific promoter *Glb-1*, the increase of iron accumulation did not parallel the high expression of *SoyferH-1*. The limiting factor for the iron accumulation may be iron uptake and/or transport. In addition, even though the demand for iron uptake is increased by ferritin over-expression, the accumulation of undesirable metals may be restricted under normal culture conditions.

Acknowledgements We thank Ms. K. Tsunokwa and S. Sumino (CRIEPI) and Ms. M. Utsuno, Y. Suzuki and X. Wang (NIAS) for their outstanding technical assistance. This research was supported in part by a research grant from the Ministry of Agriculture, Forestry and Fishery of Japan to F. Takaiwa, Core Research for Evolutional Science and Technology (CREST) and by a Grant-in-aid for Scientific Research from the Japan Society for the Promotion of Science (JSPS) to T. Yoshihara.

References

Beard JL, Burton JW, Theil EC (1996) Purified ferritin and soybean meal can be sources of iron for treating iron deficiency in rats. J Nutr 126:154–160

Becker D, Kemper E, Schell J, Masterson R (1992) New plant binary vectors with selectable markers located proximal to the left T-DNA border. Plant Mol Biol 20:1195–1197

Briat JF, Lobréaux S (1997) Iron transport and storage in plants. Trends Plant Sci 2:187–193

Cohen KC, Fox TC, Garvin DF, Kochian LV (1998) The role of iron-deficiency stress in stimulating heavy-metal transport in plants. Plant Physiol 116:1063–1672

Delhaize E (1996) A metal-accumulator mutant of *Arabidopsis thaliana*. Plant Physiol 111:549–855

Drakakaki G, Christou P, Stoger E (2000) Constitutive expression of soybean ferritin cDNA in transgenic wheat and rice results in increased iron levels in vegetative tissues but not in seeds. Transgenic Res 9:445–452

Finnegan EJ, Peacock WJ, Dennis ES (2000) DNA methylation, a key regulator of plant development and other processes. Curr Opin Genet Dev 10:217–223

Flavell RB (1994) Inactivation of gene expression in plants as a consequence of specific sequence duplication. Proc Natl Acad Sci USA 91:3490–3496

Gillespie S, Haddad L (2001) Malnutrition in Asia and the Pacific. In: Nutrition and development series, attacking the double burden of malnutrition in Asia and the Pacific. Asian Development Bank, International Food Policy Research Institute, pp 5–14

Goto F, Yoshihara T, Saiki H (1998) Iron accumulation in tobacco plants expressing soybean ferritin gene. Transgenic Res 7:173–180

Goto F, Yoshihara T, Shigemoto N, Toki S, Takaiwa F (1999) Iron fortification of rice seed by the soybean ferritin gene. Nat Biotech 17:282–286

Grusak MA, Welch RM, Kochian LV (1990) Physiological characterization of a single-gene mutant of *Pisum sativum* L. exhibiting excess iron accumulation. I. Root iron reduction and iron uptake. Plant Physiol 93:976–981

Guerinot ML, Yi Y (1994) Iron: nutritious, noxious, and not readily available. Plant Physiol 104:815–820

Harrison P, Arosio P (1996) The ferritin: molecular properties iron storage function and cellular regulation. Biochim Biophys Acta 1275:161–203

Himelblau E, Amasino RM (2001) Nutrients mobilized from leaves of *Ababidopsis thaliana* during leaf senescence. J Plant Physiol 158:1317–1323

Kokot S, Phuong TD (1999) Elemental content of Vietnamese rice. Part2. Multivariate data analysis. Analyst 124:561–569

Laulhere JP, Lescure AM, Briat JF (1988) Purification and characterization of ferritins from maize, pea, and soybean seeds. J Biol Chem 263:10298–10294

Lucca P, Hurrell R, Potrykus I (2001) Genetic engineering approaches to improve the bioavailability and the level of iron in rice grains. Theor Appl Genet 102:392–397

Lueders T, Friedrich M (2000) Archaeal population dynamics during sequential reduction processes in rice field soil. Appl Environ Microbiol 66:2732–2742

Mauk CS, Nooden LD (1992) Regulation of mineral redistribution in pod-bearing soybean explants. J Exp Bot 43:1429–1440

Meins FJ (2000) RNA degradation and models for post-transcriptional gene silencing. Plant Mol Biol 43:261–273

Meyer P, Saedler H (1996) Homology-dependent gene silencing in plants. Annu Rev Plant Mol Biol 47:23–48

Mori S, Nishizawa N (1987) Methionine as a dominant precursor of phytosiderophores in Graminaceae plants. Plant Cell Physiol 28:1081–1092

Murray-Kolb LE, Theil EC, Takaiwa F, Goto F, Yoshihara T, Beard JL (2002) Transgenic rice is a source of iron for iron-depleted rats. J Nutr 132:957–960

Proudhon D, Briat JF, Lescure AM (1989) Iron induction of ferritin synthesis in soybean. Plant Physiol 90:586–590

Qu LQ, Takaiwa F (2004) Evaluation of tissue specificity and expression strength of rice seed component gene promoters in transgenic rice. Plant Biotech J 2:113–125

Qu LQ, Wei XL, Satoh H, Kumamaru T, Ogawa M, Takaiwa F (2002). Inheritance of alleles for glutelin α-2 subunit genes in rice and identification of their corresponding cDNA clone. Theor Appl Genet 105:1099–1108

Qu LQ, Tada Y, Takaiwa F (2003) In situ western hybridization: a new highly sensitive technique to detect protein distribution in seeds. Plant Cell Rep 22:282–285

Theil EC (1987) Ferritin: structure, gene regulation and cellular function in animals, plants and microorganisms. Annu Rev Biochem 56:289–315

Theil EC (2004) Iron, ferritin, and nutrition. Annu Rev Nutr 24:327–343

US Department of Agriculture, US Department of Health and Human Services Nutrition and your health (1995) Dietary guidelines for Americans, 4th edn. US Government Printing Office, Home and Garden Bulletin no. 232, Washington

Van Wuytsuwinkel O, Vansuyt G, Grignon N, Fourcroy P, Briat JF (1999) Iron homeostasis alteration in transgenic tobacco overexpressing ferritin. Plant J 17:93–97

Vansuyt G, Mench M, Briat JF (2000) Soil-dependent variability of leaf iron accumulation in transgenic tobacco overexpressing ferritin. Plant Physiol Biochem 38:499–506

Vasconcelos M, Datta K, Oliva N, Khalekuzzaman M, Torrizo L, Krishnan S, Oliveira M, Goto F, Datta SK (2003) Enhanced iron and zinc accumulation in transgenic rice with the ferritin gene. Plant Sci 164:371–378

Welch RM, Norvell WA, Schaefer SC, Shaff JE, Kochian LV (1993) Induction of iron (III) and copper (II) reduction in pea (*Pisum sativum* L.) roots by Fe and Cu status: does the root cell plasmalemma Fe (III)-chelate reductase perform a general role in regulating cation uptake? Planta 190:555–561

Yoshihara T, Masuda T, Jiang T, Goto T, Mori S, Nishizawa NK (2003) Analysis of some divalent metal concentrations in tobacco expressing the exogenous soybean ferritin gene. J Plant Nutr 26:2253–2265