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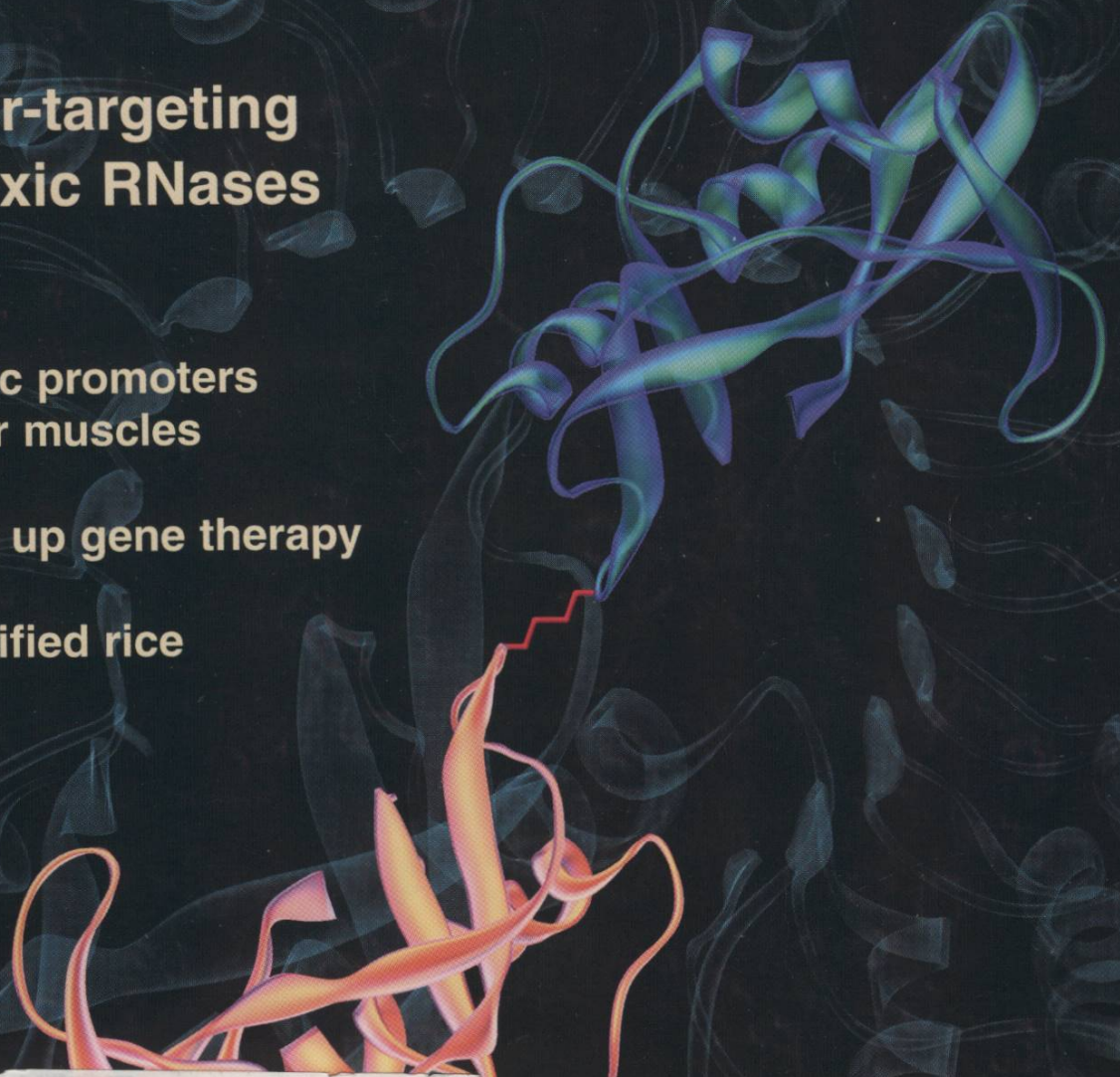

Cancer-targeting cytotoxic RNases

Synthetic promoters flex their muscles

Splicing up gene therapy

Iron-fortified rice

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Case No.: IPR2023-00207
U.S. Patent No. 11,224,241

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Structure of an engineered dimeric human ribonuclease (foreground) which has been altered to both facilitate targeting to cancer cells and to render it insensitive to its natural cytosolic inhibitor (shown in background). For more details, see Suzuki et al. p 265, and Wagner and Lipovsek, p 227. Illustration by Ken Eward (BioGrafx, Mount Vernon, Ohio).

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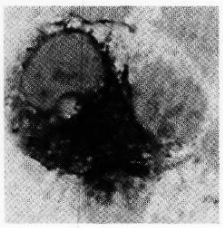

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Iron fortification of rice seed by the soybean ferritin gene

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To improve the iron content of rice, we have transferred the entire coding sequence of the soybean ferritin gene into *Oryza sativa* (L. cv. Kita-ake) by *Agrobacterium*-mediated transformation. The rice seed-storage protein glutelin promoter, *GluB-1*, was used to drive expression of the soybean gene specifically in developing, self-pollinated seeds (T1 seeds) of transgenic plants, as confirmed by reverse transcription PCR analysis. Stable accumulation of the ferritin subunit in the rice seed was demonstrated by western blot analysis, and its specific accumulation in the endosperm by immunologic tissue printing. The iron content of T1 seeds was as much as threefold greater than that of their untransformed counterparts.

Keywords: nutrition, iron content, genetic improvement, glutelin promoter, *Oryza sativa*

Iron deficiency is a serious nutritional problem that afflicts an estimated 30% of the world population[1], especially where vegetable-based diets are the primary food source[2]. Although supplements added to food or taken in tablet form are effective in preventing and controlling iron deficiency, such treatments are difficult to implement in developing countries because of the associated costs and the small number of primary health care programs[3]. Some crops (e.g., spinach and leguminous plants) are known for their high iron content[4]. However, these plants also contain oxalic acid and phytate-like substances that decrease their bioavailability[5]. The iron content of crops has been improved by increasing the iron concentration of the hydroponic culture media or soil. However, this method is costly and cannot be used to target iron accumulation to a desirable part of the plant. In addition, the excess iron or the chelator in the media suppresses plant growth and can also reduce commercial value and productivity[6].

Ferritin is an iron-storage protein found in animals, plants, and bacteria[7]. Comprising 24 homologous or heterologous subunits, it assembles to form a large complex (450 kDa) that stores up to 4500 iron atoms in its central cavity[7,8]. The ferritin gene has been isolated and sequenced in plants, including soybean[9], French bean[10], pea[11], and maize[12]. Although the amino acid sequence homology between plant and mammalian ferritin is low (39–49% within the transit and extension peptides[8]; 56–66% without the additional peptide[9]), its molecular conformation and iron-storage capacity is conserved.

Ferritin is thought to play two main roles in living cells, both through modulation of iron content[13]. One is to provide iron for the synthesis of iron proteins such as ferredoxin and cytochromes. The other is to prevent damage from free radicals produced by iron/dioxygen interactions. In animals, ferritin synthesis is regulated at the translational, not the transcriptional, level[13]. Translation of the ferritin mRNA increases in response to high levels of iron, thus producing more ferritin for storage, and conversely, translation is decreased by low levels of iron. In plants, however, iron-dependent regulation of phytoferritin synthesis occurs at the transcriptional level[14,15] and is also responsive to developmental cues[16,17].

Recent studies show that ferritin is used by both plants and animals as the storage form of iron[18] and that, orally administrated, it can provide a source of iron for treatment of rat anemia[19]. These findings suggest that increasing the ferritin content of cereals by genetic engineering may help to solve the problem of dietary iron deficiency. We have explored that possibility by introducing the soybean ferritin gene into rice plants (*Oryza sativa* L. cv. Kita-ake) by *Agrobacterium*-mediated transformation. The introduced ferritin gene was expressed under the control of a rice seed-storage protein glutelin promoter, *GluB-1* (-1302/+18)[20], to mediate the accumulation of iron specifically in the grain. Our results indicated that the soybean ferritin could be highly expressed and accumulated in the endosperm tissue of heterogeneous rice plants. Notably, the transgenic seeds stored up to three times more iron than the normal seeds.

Results

Expression of the soybean ferritin in rice seeds. Thirty independent transgenic rice lines (T0 plants) were obtained by *Agrobacterium*-mediated transformation, and the insertion of the ferritin cDNA in their chromosomes was confirmed by PCR analysis of leaves from T0 plants (data not shown). All lines produced self-pollinated seeds (T1 and T2 seeds), and heterozygous lines were determined by screening with bialaphos and/or PCR, except in experiments to determine iron content of seeds. Southern blot analysis of total DNA isolated from T2 leaves of several lines (Fk1, 4, 7, 11, 16, 22, and 25) showed that independent transformants contained one to several copies of the introduced gene per haploid genome (Fig. 1A and B).

To examine the expression of the ferritin gene, total RNA isolated from T2 immature seeds of three lines (Fk1, 11, and 22) was analyzed by reverse transcriptase PCR (RT-PCR) analysis using primers specific for soybean ferritin. Figure 1C shows that a 566 bp band corresponding to a portion of the soybean ferritin cDNA could be amplified from cDNA prepared from seeds, but not leaves, of transformed plants. This suggested that the expression of the ferritin gene was tissue-specific. The specificity of the RT-PCR reaction for the soybean ferritin gene was confirmed by the absence of an amplification product from cDNA preprared from seeds of nontransformed plants (N4).

Total protein homogenates isolated from T1 seeds (Fk1, 4, 7, 11, 16, 22, and 25) were analyzed by western blotting. A polyclonal anti-

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body directed against the soybean ferritin gene specifically immunodecorated a 28 kDa band, confirming that the soybean ferritin protein accumulates in seeds (Fig. 2A and B). The 28 kDa band specific to soybean ferritin was detected in protein homogenates from soybean, but not from untransformed rice and tobacco. Though ferritin levels varied between different individual lines, the protein could be detected in almost all the transformants assayed (Fig. 2B), and the amount was estimated to be between 0.01–0.3% of the total seed protein (data not shown). The 28-kDa band is very similar in size to that observed in the native soybean cotyledon and transgenic tobacco leaf containing the ferritin gene (Fig. 2A). The absence of proteolytic products indicated that the soybean ferritin was stable in the heterogeneous rice seed. In addition, another specific hybridized band about 32 kDa in molecular size was observed in *Escherichia coli* and native soybean, but was barely detectable in transgenic rice and tobacco plants (Fig. 2A). This band may correspond to a precursor

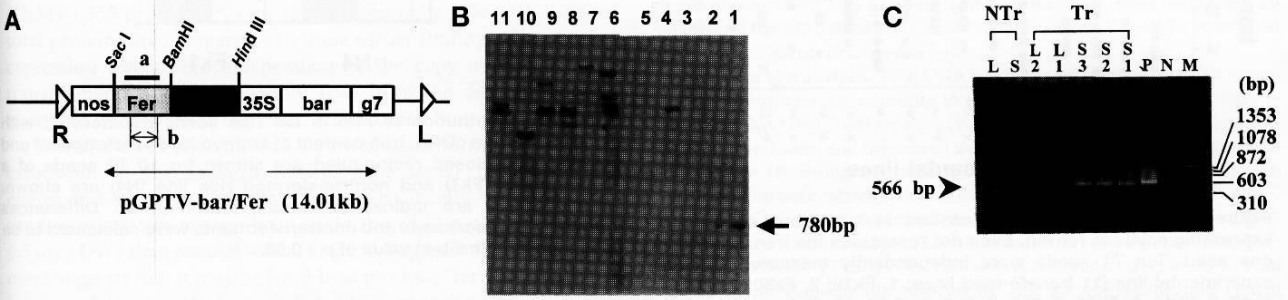

Figure 1. (A) Construction of *GluB-1* promoter—soybean ferritin chimeric gene in the pGPTV-35S-bar binary vector. (a) Deduced hybridization position of digoxigenin-labeled ferritin cDNA probe. (b) Annealing sites of RT-PCR primers, which amplified a fragment of 566 bp, for detecting the introduced soybean ferritin gene expression. (B) Southern blot hybridization of DNA prepared from individual T2 and a nontransformed control plants. Total DNA (10 μg) was digested with *Hind*III and hybridized with digoxigenin-labeled soybean ferritin cDNA probe. Lane 1 and 2, two and one copy (per a haploid genome) markers; lane 3, nontransformant (N4); lane 4, Fk1; lane 5, Fk1n (the ferritin cDNA was not detected by PCR method); lane 6, Fk4; lane 7, Fk7; lane 8, Fk11; lane 9, Fk16; lane 10, Fk22; and lane 11, Fk25. (C) RT-PCR analysis of total RNA from transgenic rice expressing the soybean ferritin gene. NTr, nontransformant (N4); Tr, transformant (1, Fk1; 2, Fk11; and 3, Fk22); M, ϕX174/*Hae*III; N, no template; P, pGPTV-bar/Fer; S, seed; L, leaf.

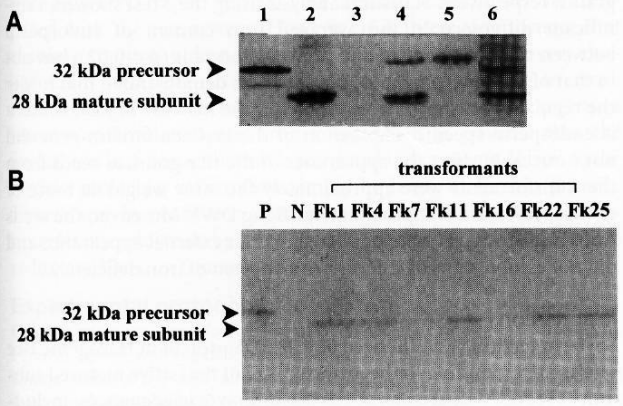

Figure 2. Western blot analysis of soybean ferritin protein in the transgenic rice seeds. (A) Total protein extracts from *E. coli*, rice (T1-Fk1), and tobacco (lanes 1, 2, and 4) transformed with the soybean ferritin cDNA, and from nontransformed rice, tobacco, and soybean (lanes 3, 5, and 6) were separated by SDS-PAGE and immunoblotted with the soybean ferritin rabbit polyclonal antibody. Twenty micrograms were loaded in each lane, except for the *E. coli* extract, which used 28 μg. (B) Variability in soybean ferritin expression in different transgenic rice lines. 3.75 μg of total protein from one matured seed of each transformant (T1-Fk1, 4, 7, 11, 16, 22, and 25) and nontransformant (N; N4) was analyzed by western blot analysis with soybean ferritin antibody. Soybean ferritin subunit produced by *E. coli* (4.75 μg) was used as the positive control (P).

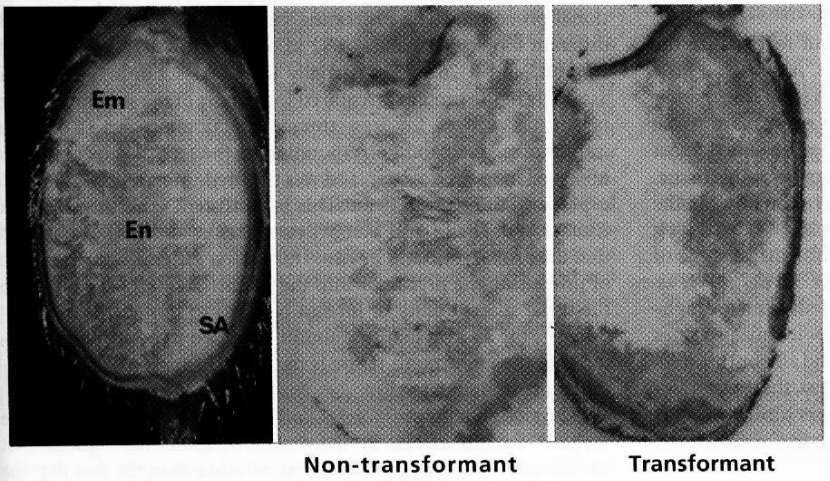

Figure 3. Immunological tissue printing of a seed from transgenic rice expressing soybean ferritin cDNA. Em, embryo; En, endosperm; SA, subaleurone layer. Each of three grains of three T1 transgenic lines (Fk1, 11, and 22) were analyzed by tissue printing using the soybean ferritin polyclonal antibody. Representative staining from a typical line (Fk1) is shown, with tissues that accumulate soybean ferritin staining dark brown.

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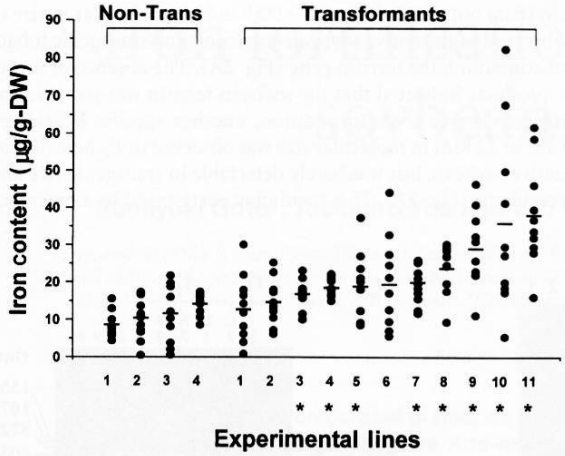

Figure 4. Comparison of iron content in transgenic rice seeds expressing soybean ferritin. Each dot represents the iron content of one seed. Ten T1 seeds were independently measured in each experimental line (11 transformed lines: 1, Fk19; 2, Fk30; 3, Fk4; 4, Fk17; 5, Fk13; 6, Fk25; 7, Fk2; 8, Fk7; 9, Fk22; 10, Fk11; and 11, Fk1; and four nontransformed rice lines: 1, N1; 2, N3; 3, N2; 4, N4). Bars indicate an average value of each experimental line. Asterisks indicate significant differences between the pooled mean values of all nontransformants and that of each independent transgenic lines at $p<0.05$ (*t*-test).

Table 1. Iron content in some tissues of a transgenic rice line expressing soybean ferritin cDNA.

	Seed	Leaf	Stem	Root
		(μg-Fe/g-DW)		
Transformants				
(Fk 1)	38.1±4.5[a]	104.8±7.9[b]	162.8±8.1[b]	962.1±65.6[b]
(Fk 11)	35.9±7.7[a]	98.8±25.8[b]	175.5±17.2[b]	966.8±105.7[b]
Nontransformants				
(N4)	14.3±3.0	119.3±8.9	170.0±5.3	956.0±37.4

Numerical status shows means of iron concentration in transformants (Fk1 and Fk11) and nontransformants (N4) from three (nonseed tissues) or ten (seed) independent replications. [a]Significant or [b]nonsignificant differences between the mean values were calculated by *t*-test ($p<0.05$).

form of the protein (see Discussion). Bands of greater than 32 kDa in transformed and nontransformed tobacco (Fig. 2A, lanes 4 and 5) may be due to the nonspecific reaction.

Immunological tissue printing was carried out to visualize the distribution of soybean ferritin in T1 seed (Fk1). Although a little background staining could be seen in the nontransgenic seed, darker brown staining was visible in the transgenic rice endosperm tissue (Fig. 3). The subaleurone layer of the endosperm stained most intensely, indicating a high accumulation of the ferritin in this tissue.

Tissue-specific enrichment of the iron content. Iron levels in the 11 mature whole T1 seeds varied from 13.3 ± 2.8 to 38.1 ± 4.5 μg/g dry weight (DW) (Fig. 4). Given that the iron contents in seeds of nontransformants ranged from 8.6 ± 3.3 to 14.3 ± 3.0 μg/g DW, we estimated a threefold increase in iron content of seeds from the highest-expressing transgenic rice lines. When the pooled mean values between all transformants (22.5 ± 1.3 μg/g DW) and all nontransformants (11.2 ± 0.9 μg/g DW) were compared by *t*-test, there was a significant difference ($p = 1.53\ E^{-11}$). Iron content was similar in nonseed tissues (i.e., leaf, stem and root) from T1 transformants and nontransformants (Table 1).

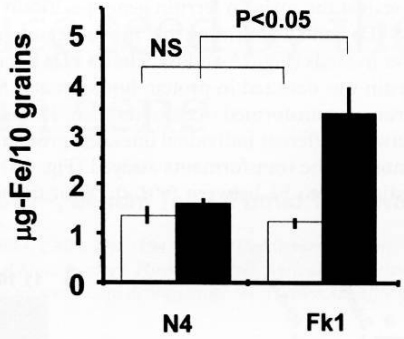

Figure 5. Distribution of iron in the rice seeds transformed with soybean ferritin cDNA. Iron content of embryo (open rectangular) and endosperm (closed rectangular) are shown for 10 T2 seeds of a transformed (Fk1) and nontransformed rice line (N4) are shown. Mean values are indicated ± standard error (n=3). Differences between transformants and nontransformants were calculated to be significant, with *t*-test value of $p = 0.02$.

To determine whether the transformation affected the iron content of seed tissue, we separately determined iron content in the embryo and endosperm of T2 seeds. The average iron content in the endosperm of a transformant (Fk1) was 3.4 ± 0.5 μg/10 grains, and that of a nontransformant (N4) was 1.6 ± 0.1 μg/10 grains (Fig. 5). In contrast, the iron content of the embryo of the transformant and nontransformant were 1.2 ± 0.1 μg/10 grains and 1.3 ± 0.2 μg/10 grains, respectively. Statistical analysis using the *t*-test showed a significant difference in the averaged iron content of endosperm between the transformant and nontransformant ($p = 0.02$), but not in that of embryo ($p = 0.64$). These results demonstrated that under the regulation of the glutelin promoter, the increase in iron content is endosperm specific. Expression of the soybean ferritin gene did not noticeably affect the appearance of the rice grain, as seeds from the transformants were approximately the same weight as those of nontransformants (approximately 18 mg DW). Moreover, the seeds from transformants were unaltered in their external appearance and did not exhibit signs of chlorosis, a symptom of iron deficiency.

Discussion

Soybean ferritin is synthesized as a 28 kDa protein in transgenic rice seeds, corresponding to the expected size of the native matured subunit[21]. From comparisons of ferritin amino acid sequences, including soybean ferritin, Ragland et al.[9] proposed that the plant ferritin subunits could be generated from a single precursor by posttranslational processing. The processing yields three types of subunits of different molecular masses: a 32 kDa precursor, a 28 kDa matured subunit, and a 26.5 kDa partially degraded subunit. The proper assembly of the protein is required for import of the subunit into the plastid, as well as for iron storage function. In fact, a 32 kDa immunoreactive band corresponding to the ferritin precursor was observed in native soybean and was the predominant form in *E. coli* expressing the ferritin gene. This is consistent with the results of Kimata and Theil[22] and Van Wuytswinkel et al.[23,24], who observed that the prokaryotic and in vitro translation systems were incapable of processing the precursor into the mature subunit. In contrast to the usual susceptibility of foreign proteins to proteolytic degradation[25–27], the 28 kDa mature subunit appeared to be stable, suggesting that the endosperm tissue is particularly appropriate for accumulation of foreign ferritin.

The rice glutelin promoter, *GluB-1*, which is used to express the soybean ferritin cDNA in the transformation, has two distinct advantages in this experimental system other than the fact that the

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gene originated from rice. One is that it conferred endosperm-specific expression of the ferritin gene, which led to specific accumulation of the protein within that tissue. This accumulation was consistent with the β-glucuronidase staining pattern directed by the *GluB-1* promoter[28,29]. The other advantage was the ability of the promoter to induce a high level of expression. Takaiwa et al.[27] indicated that glycinins expressed under the control of *GluB-1* promoter accumulated at the level of 0.02–4% of total proteins in dry seed of tobacco. This level was more than 40-fold higher than that achieved with a constitutive expression system using the cauliflower mosaic virus (CaMV) 35S promoter[30]. Our results (approximately 0.01–0.3% of total protein) are comparable to these earlier findings. The varying expression levels were independent of the copy numbers in the transformants and may possibly arise from the different sites of transgene integration in the rice chromosome[31].

The iron content of the rice seed was significantly increased by expression of the soybean ferritin gene under control of the endosperm-specific promoter, *GluB-1*. Specifically, the seeds from the transgenic rice accumulated up to threefold more iron (38.1 ± 4.5 μg/g DW) than normal seeds (11.2 ± 0.9 μg/g DW). This achievement suggests that it may be feasible to produce "ferritin rice" as an iron supplement in the human diet. The iron content in a meal-size portion of "ferritin rice" (approximately 5.7 mg-Fe/150 g-DW) would be sufficient to provide 30–50% of the daily adult iron requirement (approximately 13–15 mg Fe [ref. 4]). We recently demonstrated that expression of the soybean ferritin gene in tobacco under the control of the CaMV 35S-regulated promoter resulted in a maximum iron content in transformed leaves that was about 1.3 times that of nontransformed leaves (109.3 μg/g DW compared with 84.0 μg/g DW, respectively)[32]. This increase seems to be low compared with the rice system. However, it is interesting to note that absolute amounts of iron increase in these two systems are very similar (24.3 μg/g DW in tobacco versus 26.9 μg/g DW in rice). Although a positive correlation can be observed between exogenous ferritin accumulation and iron content[32], it is possible that the amount of iron accumulation is restricted by transport of iron to the ferritin molecule, rather than simply by levels of ferritin protein. Thus, it may be possible to store larger amounts of iron in the exogenous ferritin molecule by cointegrating the ferritin gene and the iron reductase-like iron transporter gene[33].

Experimental protocol

Construction and transformation of chimeric gene and plant cultivation. Ferritin cDNA was isolated from soybean cotyledons (Glycine max L. cv. Kitano-shiki) a few days after germination. polyCAT mRNA was isolated from 1 g fresh weight (FW) of the cotyledons and the ferritin cDNA (0.84 kb) was amplified by RT-PCR. The primers for the reverse transcription were random primers (p[dN]6), and those for the PCR corresponded to the ferritin cDNA sequence reported by Lescure et al.[15] Forward primer: 5′-ATGGCTCTTGCTCCATCCAAAGTT; Reverse primer: 5′-TTGATCAAAGTGCCAAACACCGTG. The ferritin cDNA fragment was then cloned into the pCR II vector (Invitrogen, San Diego, CA) and the sequence was confirmed. Alignment of the fragment sequence with the known soybean ferritin sequence[15] showed 95% identity in the conserved regions (data not shown). The divergence may have resulted from the use of different soybean cultivars. Next, the ferritin cDNA was inserted into a modified binary vector pGPTV-35S-bar, replacing the *uidA* gene. This modified vector was reconstructed from the pGPTV-bar binary vector[34] by exchange of the *Nos* with the CaMV *35S* as the promoter of the bar gene. The ferritin cDNA was introduced between the restriction enzyme site of *Bam*HI and *Sac*I under control of the rice seed-storage protein glutelin promoter, *GluB-1* (-1302/+18)[20]. It was flanked at the 3′ end by the *Nos* polyadenylation signal (Fig. 1A). The plasmid pGPTV-35S-bar, containing the soybean ferritin gene, was transferred into *Agrobacterium tumefaciens* strain C58 or EH105 by electroporation. This *Agrobacterium* was used to infect 3-week-old calli derived from a mature rice seed (*Oryza sativa* L. cv. Kita-ake) for three days. Next the calli were successively cultured in N6 selection medium[35] (supplemented with 3% sucrose, 0.4% gelrite, 100 mg/l myoinositol, 300 mg/l casamino acids, 2878 mg/l proline, 2 mg/l 2,4-D, 500 mg/l Claforan and 5 mg/l bialaphos) for 6 weeks, and MS regeneration medium[36] (supplemented with 3% sucrose, 3% sorbitol, 0.8% agar, 2 g/l casamino acids, 0.2 mg/l NAA, 2 mg/l kinetin, 250 mg/l Claforan, and 5 mg/l bialaphos) for 5 weeks at 28 ± 1°C, under continuous light condition. Each line was designated Fk (Ferritin/Kita-ake) followed by an ordered number from 1 through 30, respectively. Total DNA was isolated from leaves of regenerated plantlets and tested for the integration of the soybean ferritin gene by the PCR method (using the same primer set as described for gene construction). These transformants (T0 plants) were subsequently acclimated in the MS medium without hormones and antibiotics for 3 weeks, transplanted to soil, and grown in a greenhouse (30 ± 3°C, 12 hours light/12 hours dark) until harvesting. T0 plants and the self-pollinated heterozygous progenies (T1 and T2) were used for the experiments described below.

Southern blot analysis. Total DNA was prepared from leaves of T2 progenies of transformants, according to the method of Shure et al.[37] *Hin*dIII-digested DNA (10 μg) was separated by agarose gel electrophoresis, blotted to a nylon membrane, and hybridized with a ferritin cDNA probe labeled with digoxigenin (Boehringer Manheim, Mannheim, Germany). Hybridization and detection were carried out according to Engler-Blum et al.[38]

RNA isolation from the transformants and characterization. Total RNA was extracted from immature T2 seeds collected about 14–21 days after pollination and from T1 leaves (harvested with the seeds) using a commercial kit based on guanidinium thiocyanate and a column filtration system (RNAqueous kits; Ambion, Austin, TX). After purification by lithium chloride precipitation and DNase digestion, expression of the introduced soybean ferritin gene was analyzed by the RT-PCR method, using the total RNAs and a set of primers as follows: Forward primer: 5′-CAGTGTTGGGGATGCTCTGAA; Reverse primer: 5′-GGTCATTGTTGCGATCTGCCA. These primers were designed to amplify a 566 bp portion of the soybean ferritin cDNA (Fig. 1A).

Protein extraction and immunologic detection. One T1 immature seed (about 14–21 days after pollination) from each of the transformants was separately homogenized with mortar and pestle in extraction buffer (80 mM Tris-HCl, pH 7.0, 2% SDS, 2% 2-mercaptoethanol, and 20% glycerol). After 3 min boiling, the resulting extracts were centrifuged at 12,000 rpm for 20 min, and the supernatant fractions were collected. Proteins in these extracts were measured according to the method of Bradford[39], separated by SDS-PAGE, and transferred to PVDF membrane (Immobilon-P^{SQ}; Millipore, Tokyo) using a buffer system consisting of 25 mM Tris-H_3BO_3, pH 9.5, and 20% methanol. The production of the soybean ferritin was confirmed by western blot analysis based on the ordinary method[40], except for using TBS buffer (Tris-HCl, pH 7.5, 0.15 M NaCl). For the western blotting procedure, 1/2000 diluted polyclonal antiserum diluted in TBS buffer containing 0.05% Tween 20 and 5% nonfat dried milk. The antiserum was raised in a male rabbit against the soybean ferritin subunit[32], which was produced in *E. coli* harboring the ferritin gene in expression vector pQE30 (Qiagen, Hilden, Germany). Protein extracts from nontransformed soybean cotyledons and transgenic *E. coli* and tobacco[32] harboring the ferritin gene were processed in parallel as positive controls.

In situ localization of the ferritin in transgenic rice was observed by the immunologic tissue printing method of Kamachi et al.[41] An immature seed (about 14–18 days after pollination, T1) from transgenic rice was cut in half at the vertical section and pressed against a nitrocellulose membrane. Then the membrane was treated with the soybean ferritin polyclonal antiserum used for the western blotting, diluted for 1/2000 with TBS buffer. For detection, a biotinylated goat antirabbit IgG solution and ABC reagent containing avidin DH and biotinylated alkaline phosphatase H or horseradish peroxidase (Vectastain ABC Elite or ABC-AP kit; Vector Laboratories, Burlingame, CA) was used according to the protocol recommended by the manufacturer. Substrate kit II (Vectastain ABC-AP kit; Vector Laboratories) or Immunostain HRP-1000 (Konica, Tokyo) were used for visualizing the signal.

Iron content determination. Iron content was determined from 11 independent transformed lines and four nontransformed lines. Ten dry mature T1 seeds from each transformant and nontransformant were individually weighed and wet-ashed with HNO_3 and H_2O_2 solution at 110°C for 2 days. In addition, 10 T2 seeds each from a transformant and a nontransformant were separated into the endosperm, and embryo tissues and wet-ashed as described above. The dried precipitate was consequently dissolved in 2 ml of 1N HCl solution. The iron content was then measured by absorbance of Fe-*O*-phenanthroline at 510 nm, and calculated against the standard of $FeSO_4$ (ref. 41).

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Acknowledgments

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