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Enhanced iron and zinc accumulation in transgenic rice with the *ferritin* gene

Marta Vasconcelos [a], Karabi Datta [a], Norman Oliva [a], Mohammad Khalekuzzaman [a], Lina Torrizo [a], Sellapan Krishnan [a,b], Margarida Oliveira [c], Fumyuki Goto [d], Swapan K. Datta [a,*]

[a] *Plant Breeding, Genetics and Biochemistry Division, International Rice Research Institute, DAPO 7777, Metro Manila 1277, Philippines*
[b] *Department of Botany, Goa University, Goa 403 206, India*
[c] *Instituto de Biologia Experimental e Tecnológica, (IBET/ITQB), Quinta do Marquês, 2784-505 Oeiras, Portugal*
[d] *Bio-Science Department, Central Research Institute Power Industry, Abiko, Abiko-shi, Chiba-ken 270-11, Japan*

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Abstract

In this report, we show that the expression of the soybean *ferritin* gene, driven by the endosperm-specific glutelin promoter, leads to higher iron and zinc levels in transgenic indica rice grains. Brown rice is rarely consumed, and polishing of the rice grain brings considerable loss of micronutrients by removing its outer layers. No data until now have shown that after commercial milling the micronutrient concentration remains higher than that of the control. In our experiment, expression of the soybean *ferritin* gene under the control of the glutelin promoter in rice has proven to be effective in enhancing grain nutritional levels, not only in brown grains but also in polished grains. Besides determining the iron levels in transgenic rice grains, we also checked for zinc concentration, and it was found to be higher in transgenic seeds than in the control. Moreover, we introduced this gene in an elite *indica* rice line that has highly desirable agronomic and field-performance traits. Prussian blue staining reaction clearly revealed the presence of iron in the endosperm cells of transgenic rice grains, and immunolocalization revealed the presence of the expression gene in the endosperm of the transgenic material.

Keywords: Iron accumulation; Milling; Genetic engineering; Nutrition; Elite *indica* rice

1. Introduction

Iron deficiency is the leading human nutritional disorder in the world. Compared with other micronutrients such as vitamin A and iodine, overall progress in reducing iron deficiency has been limited [1–6]. Plant foods contain almost all of the mineral and organic nutrients established as essential for human nutrition [7], but often these are not present in sufficient amounts. The cereal grains represent the single largest source of calories in the world [8] and, in countries where the staple food is rice, per capita consumption is so high that even a small increase in its nutritive value would be highly significant [9]. Until now, research has tended to focus on providing appropriate fortification of rice grains [8] or having oral supplementation programs [6]. Improving mineral nutrition through plant biotechnology may be a more sustainable strategy to combat deficiencies in human populations [10]. The ferritin protein takes up iron, stores it in a non-toxic form, and releases it when needed for metabolic functions, as iron stored in ferritin rice is bioavailable [11–14]. The removal of the outer layers of the rice seed by commercial milling dramatically reduces the level of iron in the grains because most of the iron is accumulated in the aleurone layer [15,16]. The rice glutelins compose up to 70–80% of the total seed protein [17,18] and represent the major storage protein of rice [19]. The *indica* rice line IR68144-3B-2-2-3 has high tolerance for rice tungro virus and excellent grain quality, yield, and

* Corresponding author. Tel.: +63-2-845-0563x657/430; fax: +63-2-845-0606.
E-mail address: sdatta@cgiar.org (S.K. Datta).

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has good tolerance for mineral-deficient soils, and a high iron level in the grain (ca. 15–17 μg/g in brown rice) [20]. We have transformed this elite *indica* line with the soybean *ferritin* gene driven by the glutelin promoter and have obtained transgenic rice plants with higher iron and zinc content in the grain even after the seeds have been polished.

2. Materials and methods

2.1. Seed milling

Four seed lots collected at random from three rice varieties, with high iron (IR68144), medium iron (Mamina), and low iron (IR64) and were subjected to four milling times (10, 30, 60, and 120 s) using a commercial bench-top miller (Kett Electrical Laboratory, Tokyo, Japan).

2.2. Plasmid constructs and rice transformation

The plasmid pGPTV bar/Fer encoding for the soybean ferritin protein from *Glycine max* L. and controlled by the endosperm specific promoter *GluB-1* was combined for co-transformation with pGL2 [21] containing *hpt* as the selectable marker and used for biolistic-mediated transformation (Fig. 2). Transformation of IR68144 was done using modified MS medium and by the biolistic method following the modified procedure previously described [22–24].

2.3. DNA extraction, polymerase chain reaction (PCR) and Southern blot analysis

PCR analysis was conducted for initial screening of the regenerated rice plants. Genomic DNA was extracted from freshly collected freeze–dried or lyophilized rice leaves of putative transformants and non-transformed plants following the method previously described [25]. Genomic DNA for Southern analysis was extracted using the modified Dellaporta method [26] and used for detection of the *ferritin* gene. Five micrograms of DNA per sample were digested with *Sst* I-*Bam* HI restriction endonucleases (Gibco-BRL, Gaithersburg, MD) in a final volume of 50 μl. The digested DNA was separated by electrophoresis on 1% (w/v) agarose gels. After electrophoresis, DNA fragments were denatured and transferred to a Hybond-N^+ nylon membrane (Amersham, Arlington Heights, IL) according to the manufacturer's instructions. The ferritin coding sequence isolated from plasmid by digestion with *Sst* I-*Bam* HI, was labeled with (α-^{32}P) dCTP using the Rediprime labeling kit (Amersham, Arlinton Heights, IL) and used as a hybridization probe.

2.4. Western blot analysis

Protein was extracted from seeds of transgenic plants and the non-transgenic control by grinding the material in liquid nitrogen and adding 100 μl extraction buffer (0.05 M Tris–HCl, 10% glycerol, 10 mM PMSF, pH 7.0) for each 100 mg of ground tissue. The samples were then centrifuged twice for 10 min at $13\,000 \times g$. After each centrifugation, the clear supernatant fraction was transferred to a new eppendorf tube. The concentration of total soluble protein was estimated with BCA protein assay kit (PIERCE Company) and the SOFTMAX PRO software (Molecular Devices, CA) using bovine serum albumin (BSA) as standard, following the manufacturer's instructions. The resulting extracts were used for the western blot analysis.

For western blot analysis, 150 μg of total soluble protein was boiled in sample buffer [12.5 mM Tris, pH 6.8, 20% glycerol, 2% (w/v) SDS, 0.001% (w/v) bromophenol blue, 2% (v/v) of 0.3 M 2-ME] for 2 min. After electrophoresis on 12% (w/v) SDS-PAGE gel, the gel was blotted onto nitrocellulose membrane (Hybond-C extra, Amersham) using a semi-dry trans-blot SD transfer cell (BIO-RAD, Hercules, CA). Membranes were then blocked in 3% (w/v) TBST–BSA at room temperature for 2 h, probed with a ferritin antibody at room temperature overnight, and detected according to the protocol previously described [27].

2.5. Immunohistochemistry

Immunohistochemistry experiments were conducted for localization of ferritin protein in the transgenic and control materials. Seeds at different maturation stages were cut longitudinally and transversely and pressed against a nitrocellulose membrane. The membrane was blocked for 30 min in 3% BSA–TBST solution and transferred to primary antibody solution in BSA for 30 min. The manufacturer's protocol (Vector Labs) was followed, with the exception of using BSA in every step and having an alternative detection system. Detection was done using either an AP conjugate substrate kit with goat anti-rabbit IgG–AP conjugate or HRP color development reagent with goat anti-rabbit IgG–HRP conjugates (BIO-RAD).

2.6. Iron and zinc quantification

Mature dehusked brown and milled T_0 and T_1 seeds from all lines were analyzed for micronutrient concentration by the Inductively Coupled Argon Plasma (ICP) Spectrometer using a modified procedure for wet ashing digestion [28].

2.7. Histochemical localization of iron in the rice endosperm

Pearl's Prussian blue technique was employed for the localization of iron [29]. Thin microtome sections of transgenic and non-transgenic rice grains were stained with potassium ferrocyanide in acidic condition to form an insoluble blue color after reaction with iron. Observation and documentation were done with an Axiophot-II microscope under bright-field mode.

3. Results

3.1. Effect of milling time in iron content of the rice grain

Before transformation, the effect of four different milling times on iron content was determined by ICP analysis. We used three genotypes known to have high iron (IR68144), medium iron (Mamina), and low iron (IR64) content, besides a few other cultivars popularly grown in Asian countries. Without milling, iron levels in the seeds ranged from about 10 μg for IR64 to 17 μg for IR68144 per gram of seeds. Cultivar Mamina had an iron concentration of about 15 μg/g of grains (Fig. 1). However, all varieties showed a sharp decrease in iron content with very short milling time. At commercial milling time (30 s), the three varieties exhibited more than a 50% decrease in iron content. IR 68144 had about 7 μg of iron per gram of seed, while the more commercial high-yielding variety IR64 showed a very low amount of iron in the milled grain (about 2 μg/g of seeds).

3.2. Integration of the transgene in rice

A total of 375 putative primary transformant hygromycin-resistant plants (T_0 plants) were obtained after bombardment and calli selection. PCR analysis indicated the integration of the *ferritin* gene (Fig. 3). Sixteen plants were selected for further analysis. The plants produced self-pollinated seeds (T_1 and T_2 seeds). Four independent T_1 lines carrying the *ferritin* gene were obtained. Twenty plants from each line were grown for the T_2 generation and analyzed for gene segregation pattern. All plants obtained showed normal phenotype, morphology and fertility (Fig. 4).

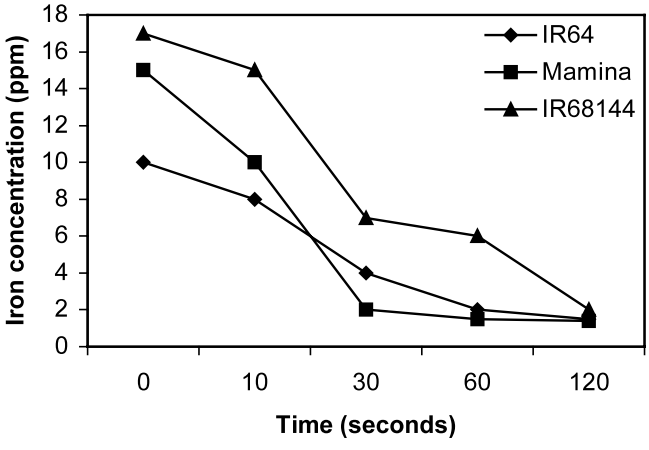

Fig. 1. Effect of different milling times on the iron content of rice seed. Milling times of 10, 30, 60, and 120 s were counted. One gram of seed powder was analyzed for iron content by ICP.

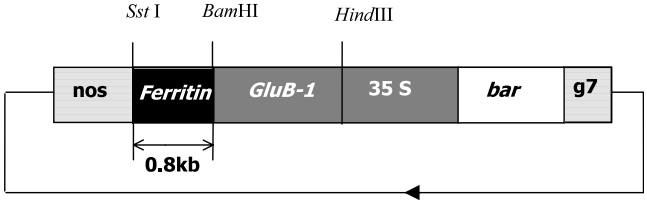

Fig. 2. Soybean *ferritin* chimeric gene in the pGPTV-bar/Fer vector, showing the expected 0.8 kb fragment detected by Southern blot [2].

Southern blot analysis was carried out with genomic DNA from self-pollinated progenies of the positive transformants, non-transformed plants, and T_1 positive and control plants. Genomic DNA was digested with *Sst* I-*Bam* HI, which excised the intact *ferritin* sequence. A band of 0.8 kb confirmed the integration of the intact *ferritin* cDNA into the rice genome (Fig. 5).

The segregation pattern was analyzed in more detail with the Southern blot analysis of 20 T_1 plants from each progeny.

3.3. Inheritance of the transgenes

Twenty T_1 progenies from four selected T_0 plants were analyzed for the mode of inheritance and segregation of the transgene. Southern blot analysis of 20 T_1 plants derived from each transgenic line showed the Mendelian segregation of 3:1 as expected for single-

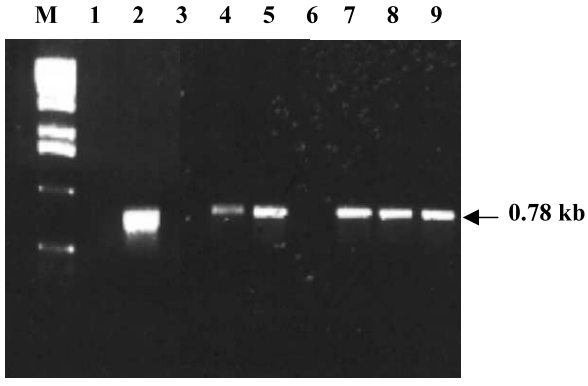

Fig. 3. PCR analysis showing amplification of 0.78 kb size band of *ferritin* gene. Lane 1, non-transformed control plant; lane 2, plasmid control; lane 3, blank; lane 4, 5, 7, 8 and 9, five individual transformants; lane 6, negative regenerated plant. M, 1 kb DNA molecular marker.

Fig. 4. IR68144 control (right) and transgenic (left) plants showing similar phenotype at late tillering stage.

locus integration. However, one of the lines showed a different inheritance pattern of 1:3 (data not shown).

3.4. Immunoblot analysis

Expression of the *ferritin* gene was assessed by immunoblotting of T_1 progenies (Fig. 6). The 28 kDa soybean ferritin protein was detected in all Southern-positive T_1 lines, but not in the non-transformed control. Moreover, when immunoblotting was performed with protein extract from individual immature seeds, all seeds of one line showed ferritin expression, suggesting a homozygous condition of the transgene.

3.5. Immunological tissue printing

Immunological tissue printing was carried out to determine the distribution of the ferritin protein in the endosperm of the rice grain. A positive reaction was observed in the transgenic material. In fact, clear differences could be observed between the control seeds and the transformed seeds. Dark brown coloration was observed in the endosperm of the transformed seeds, whereas no color appeared in the control material (Fig. 7).

3.6. Histochemical localization

Prussian blue staining reaction clearly revealed the presence of iron in the endosperm cells of transgenic rice grains as indicated by the blue color staining (Fig. 8B). In non-transgenic rice grains, iron was localized only in the aleurone cells and embryo (Fig. 8A), whereas the endosperm did not show any detectable color reaction. Moreover, the transgenic rice grains showed a higher accumulation of iron in all tissues than the non-transgenic grains.

3.7. Iron and zinc content in brown and milled transgenic rice plants

Iron and zinc levels in the transformed T_1 seeds obtained from T_0 plants and control seeds were deter-

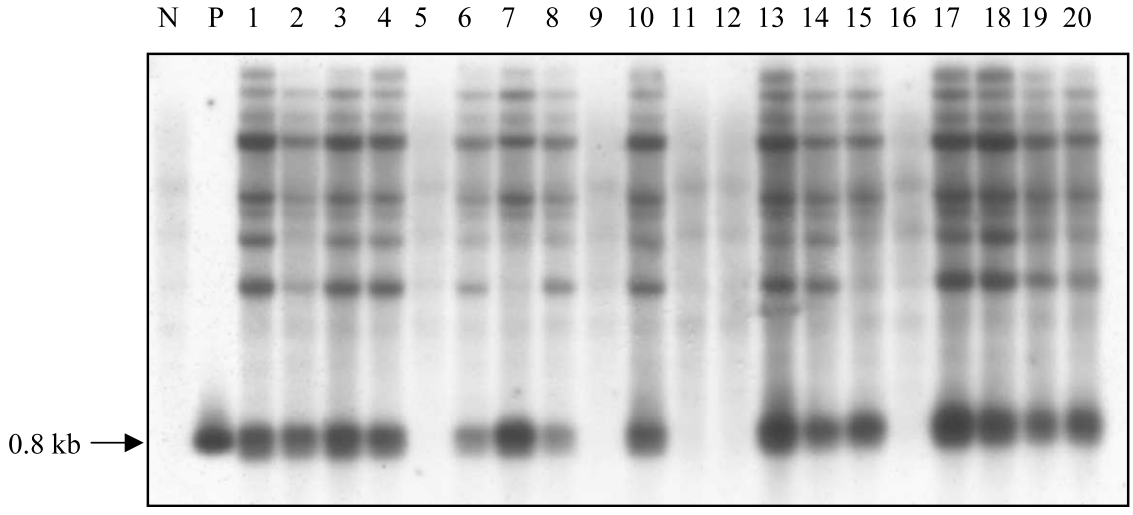

Fig. 5. Southern blot analysis showing 0.8 kb band expected for *ferritin* in transgenic T_1 plants, absent in non-transformed control and non-transgenic segregants. Lanes, N (negative control); P (positive control); and 1–20 (T_1 progeny from Fr22). The bands of higher molecular weight seen in non-transformed control and non-transgenic segregants correspond to endogenous *ferritin* gene of rice leaves.

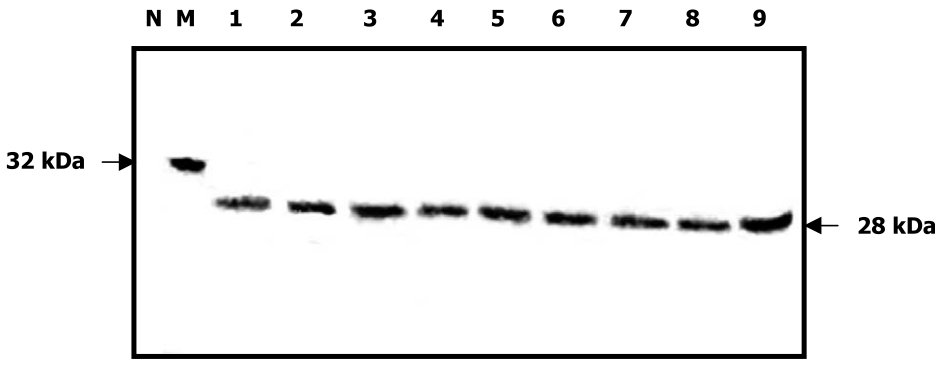

Fig. 6. Immunoblot analysis of ferritin in individual T_1 seeds of IR68144 transgenic material (lanes 1–9). Total soluble protein was extracted from nine random immature seeds, resolved by SDS-PAGE, and electroblotted to a nitrocellulose membrane. Fifty microgram of total protein was loaded in each well. Lane N, control non-transformed seed; lane 1–9, transformed seeds; lane M, Protein marker. Arrow indicates the size in kDa.

mined by ICP. For brown rice, results showed significant differences between the control and transgenic material ($P < 0.05$) (Table 1). Iron levels were 17.0 ± 0.1 μg/g total dry weight (DW) in control seeds and varied from 16.2 to 34.7 μg/g DW in transgenic seeds. All transgenic plants showed higher levels of iron in the rice grain and, in one plant, Fr18, the iron concentration was more than double its content in control seeds. When comparing the pooled mean values between different lines and the control, three of the four lines obtained were significantly different from the control at $P < 0.05$ (Table 2). All transgenic plants contained higher zinc levels in the seed than the control material. The values for zinc concentration varied from 34.9 ± 0.1 to 55.5 ± 2.7 in transgenic seeds and were 33.6 ± 0.2 μg/g in the control material. Pooled mean values between different lines and the control showed that the transformed lines also had significantly higher zinc levels than the control plants (Table 2).

T_2 seeds derived from four transgenic independent T_1 lines were used to analyze the micronutrient levels of the non-transgenic control and transgenic IR68144. The iron and zinc levels of the grains before and after polishing (commercial milling) of the seeds were determined. In line Fr19, the iron values in milled rice ranged from 8 to 19 μg/g and in line Fr18 varied from 9 to 37 μg/g (Fig. 9). Control material had only 10 μg/g of iron after polishing. Although a decrease in the concentration was observed in some lines, in many cases it still remained higher than in the non-milled control material.

Zinc content in both milled and brown rice was higher in transgenic seeds than in the non-transgenic control, as already observed in T_1 seeds.

4. Discussion

In this report, we showed that the expression of the soybean *ferritin* gene, driven by the endosperm-specific glutelin promoter, led to higher iron and zinc levels in transgenic rice grains. Although similar observations have been made previously [5,10,14,30], no data have been shown after milling of the rice grains. Brown rice is rarely consumed [15], and polishing of the rice grain brings considerable loss of micronutrients by removing its outer layers. In our experiment, expression of the soybean *ferritin* gene under the control of the glutelin promoter in rice has proven to be effective in enhancing grain nutritional levels, not only in brown grains but also in polished grains. Moreover, we introduced this gene in an elite *indica* rice line that has highly desirable agronomic and field-performance traits.

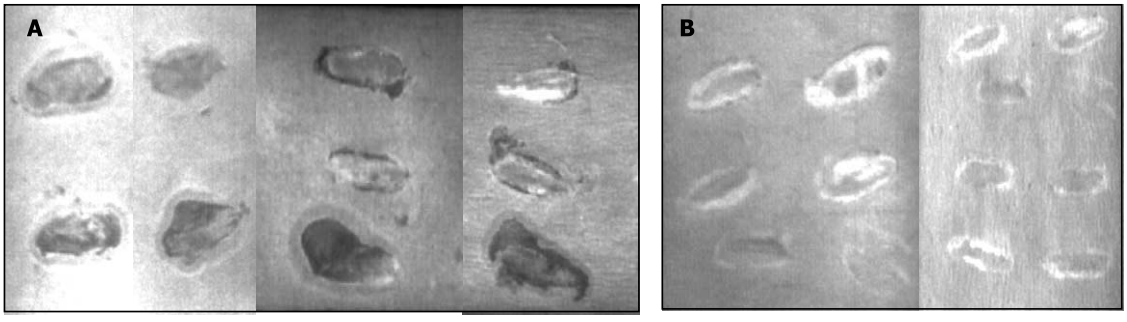

Fig. 7. Immunolocalization of the ferritin protein by pressing longitudinally cut seeds in nitrocellulose membrane and detection with specific antibody. (Panel A) Transgenic seeds showing the characteristic dark brown staining that reveals the ferritin protein localization both in the aleurone layer and in the endosperm. No coloration was found in seeds that were not transformed with the *Glycine max* L. *ferritin* gene (B).

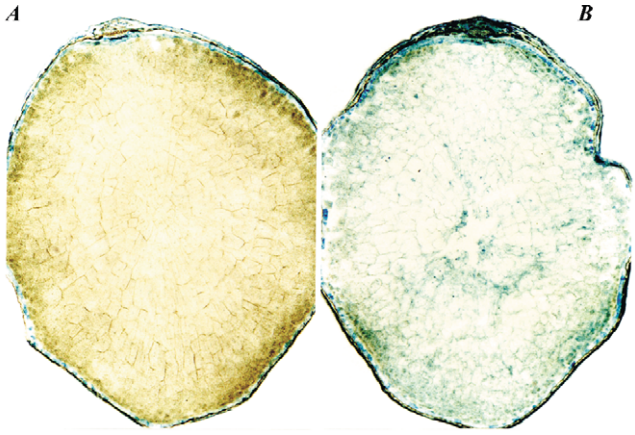

Fig. 8. Iron detection in transverse sections of non-transgenic control (A) and transgenic (B) rice grains. The accumulation of iron in the control material is restricted to the aleurone layer while in transgenic seeds iron is present in the entire grain, including the endosperm.

Zinc deficiency has been associated with complications in pregnancy and delivery, as well as with growth retardation, congenital abnormalities, and retarded neurobehavioral and immunological development in the fetus [31]. We do not yet know whether the *ferritin* gene has any influence on zinc accumulation in rice grains. If this is true, a possible explanation might be that the absorption of either zinc or iron enhances the absorption of the other by affecting their transport to the seed. It has been suggested that iron and zinc in the rice grain are correlated, because when the level of one is high, the other appears to follow the same pattern. It has also been shown phytosiderophores mobilize not only iron but also zinc [32]. This suggests a possible common regulatory mechanism for both these nutrients. Our results further suggest that there seems to be a regulatory mechanism for plants with higher iron and also to have higher zinc content. We obtained as much as 71 μg/g iron and 55.5 μg zinc per gram of unpolished transgenic seed. This accounts for a 4.4-fold increase in iron compared with that of the control and a 2–3-fold extra iron content of transgenic rice grains would already be of significant difference as reported earlier [2,5]. Moreover, when analyzing the iron content in milled transgenic and control seeds, it can be observed that the concentration remained very high in several lines. The iron content in polished seeds of many transgenic lines was the same or higher than the iron content of the control non-polished seeds. The plant with the highest iron content exhibited as much as 37 μg/g in polished seeds. A good food source of iron contains a substantial amount of iron in relation to its calorie content, and contributes at least 10% of the US Recommended Dietary Allowance (US RDA) for iron in a selected serving size. The US RDA for iron is 18 mg/day. If we take into consideration a daily serving of about 300 g of rice per day, then we would obtain around 33% of the RDA by ingesting *ferritin* rice.

However, bioavailability needs to be studied, since there is no advantage in having high iron/zinc rice if the micronutrients are not available for absorption. Since ferritin is used as a natural source of iron in the early development of animals and plants, ferritin-iron bioavailability should not be a problem. In fact, iron in transgenic rice with ferritin has already been tested in iron-deficient rats, and rice diets were as effective as the $FeSO_4$ diet in replenishing hematocrit, Hb concentration, and liver iron concentration [14]. The body absorbs iron more efficiently when iron stores are low and during growth spurts or pregnancy [39].

Hodges et al. [33] suggested that vitamin A deficiency significantly contributed to the prevalence of anaemia, in both humans and in vitamin A deficient rats. More recently, several authors have corroborated this theory and indicated that iron in the presence of vitamin A is more bioavailable [34–38]. Vitamin A appears to be involved in the pathogenesis of anaemia through diverse

Table 1
Iron and zinc concentrations in brown seeds of transgenic T_0 lines (Fr) and control of IR68144

	Micronutrient concentration (μg/g DW)	
T_0 plant	Iron	Zinc
Fr 18	34.7±11.8[a]	46.8±0.2[a]
Fr 19	27.9±1.0[a]	55.5±2.7[a]
Fr 25	25.4±0.7[a]	51.3±1.6[a]
Fr 68	22.8±0.7[a]	45.1±0.3[a]
Fr 77	21.1±0.3[a]	38.9±0.3[a]
Fr 76	20.6±0.7[a]	36.1±1.1[a]
Fr 15	20.5±0.1[a]	42.9±0.9[a]
Fr 20	19.7±3.2[a]	40.8±0.6[a]
Fr 78	18.6±0.1[a]	34.9±0.1[a]
Fr 22	18.0±0.6[a]	37.4±0.3[a]
Fr 28	16.2±0.2[b]	36.7±0.5[b]
Control	15.7±0.1	33.6±0.2

Results were obtained from two independent replications of 0.6 g seeds. [a], Significant or [b], non-significant differences between the mean values were calculated by RCBD and Duncan's multiple range test at $P \leq 0.05$; 0.6 g seeds were used for each sample analyzed.

Table 2
Average iron and zinc concentrations in transgenic (lines 1–4) and control materials of IR68144

	Micronutrient concentration (μg/g DW)	
Sample	Iron	Zinc
Line 1	17.1±1.1[b]	36.2±0.2[b]
Line 2	27.9±1.0[a]	55.5±2.7[a]
Line 3	21.3±2.3[a]	41.5±5.4[a]
Line 4	34.1±11.9[a]	46.8±0.2[a]
Control	15.7±0.1	33.6±0.2

[a], Significant or [b], non-significant differences between the mean values were calculated by RCBD and Duncan's multiple range test ($P < 0.05$).

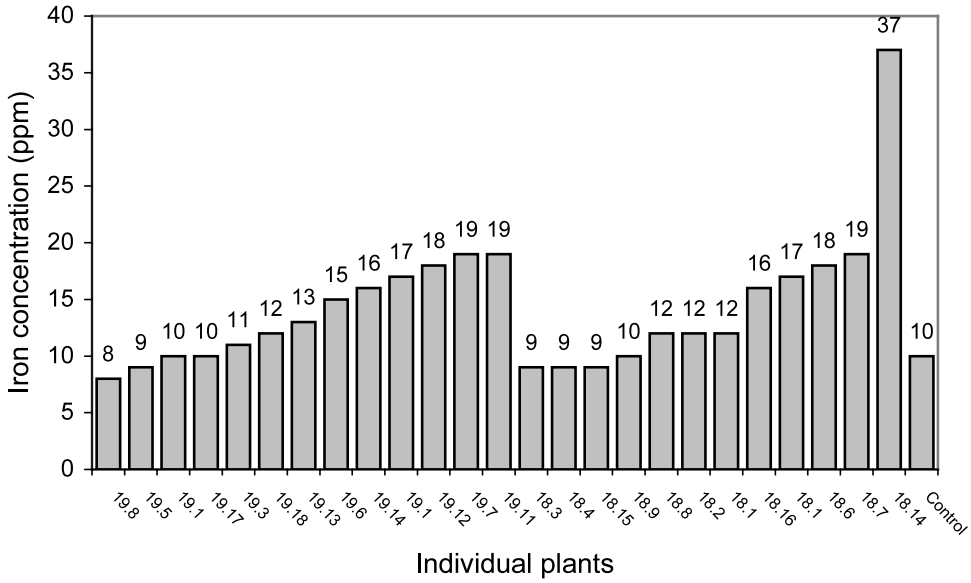

Fig. 9. Iron concentration in T_2 seeds of lines Fr19 and Fr18, obtained by transformation of IR68144, and of control seeds, after commercial milling. Values represent an average of two independent extractions of 0.6 g of seed.

biological mechanisms such as reduction of infection and mobilization of iron from tissues [36]. Work is already in progress to introduce the genes for the β-carotene pathway in the same *indica* rice variety (IR 68144) (Datta et al., submitted) and the combination of ferritin and β-carotene would result in increased iron bioavailability.

The levels of iron and zinc in rice grains may not always be exactly the same for the same variety, but variations are moderate [20]. Soil properties and weather, for example, can affect mineral content in the grain for any particular line of a crop [20].

One can speculate on the difficulties in obtaining the exact amount of iron and zinc in the milled grain to completely fulfil the recommended daily allowances. The homeostatic processes that control iron influx and movement through the plant appear to be tightly matched to minimize iron toxicity at all points within the system [7]. Moreover, the plant's ability to translocate and absorb iron is genetically controlled [40]. These two factors in conjunction might prevent an unlimited translocation of iron to the seed and, therefore, reduce the theoretical amount of iron that could be accumulated.

A nutritional approach whereby available dietary iron is held at an adequate level is the ultimate solution to prevent iron deficiency [41]. Enhancing the iron content of rice is a good strategy as an alternative to iron fortification, which is commonly not available to the most needy population [42]. Our *indica* rice variety, which already has very good agronomic and nutritional traits, and expresses the *ferritin* gene from *Glycine max* L., has a nutritionally added value. The homozygous lines of our transgenic plants that express ferritin would be suitable for consumption after assessing their agronomic performance in the field. After having combined the traits of iron enhancement and β-carotene production, we will have a variety with higher iron availability and that is richer in nutritional terms.

These data suggest that biotechnological approaches to manipulate *ferritin* expression in the rice seed may contribute to a sustainable solution to global problems of iron deficiency.

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