

## Original Research

# Fighting Iron Deficiency Anemia with Iron-Rich Rice

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**Objective:** Iron deficiency is estimated to affect about 30% of the world population. Iron supplementation in the form of tablets and food fortification has not been successful in developing countries, and iron deficiency is still the most important deficiency related to malnutrition. Here we present experiments that aim to increase the iron content in rice endosperm and to improve its absorption in the human intestine by means of genetic engineering.

**Methods:** We first introduced a ferritin gene from *Phaseolus vulgaris* into rice grains, increasing their iron content up to twofold. To increase iron bioavailability, we introduced a thermo-tolerant phytase from *Aspergillus fumigatus* into the rice endosperm. In addition, as cysteine peptides are considered major enhancers of iron absorption, we over-expressed the endogenous cysteine-rich metallothionein-like protein.

**Results:** The content of cysteine residues increased about sevenfold and the phytase level in the grains about one hundred and thirtyfold, giving a phytase activity sufficient to completely degrade phytic acid in a simulated digestion experiment.

**Conclusions:** This rice, with higher iron content, rich in phytase and cysteine-peptide has a great potential to substantially improve iron nutrition in those populations where iron deficiency is so widely spread.

## INTRODUCTION

The prevalence of iron deficiency is estimated to be about 30% of the world population [1], making iron by far the most widespread nutrient deficiency world-wide. The functional effects of iron deficiency anemia result both from a reduction in the circulating hemoglobin and in the iron-containing enzymes and myoglobin. The major consequences are reduced psychomotor and mental development in infants [2], poor pregnancy outcome [3], decreased immune function [4], tiredness and poor work performance [5].

The amount of bioavailable iron is dependent both on the iron intake and absorption. Dietary iron in developing countries consists primarily of non-heme iron, whose poor absorption is considered a major factor in the etiology of iron deficiency anemia [6]. Grain and legume staples are high in phytic acid, which is a potent inhibitor of iron absorption [7,8]. In addition, the intake of foods that enhance non-heme iron absorption such as fruits, vegetables [9] or muscle tissue [10] is often limited.

The most widely recognized strategies for reducing micronutrient malnutrition are supplementation with pharmaceutical

preparations, food fortification, dietary diversification and disease reduction [11]. Iron supplementation is useful for producing a rapid improvement in Fe status in anemic individuals, but is expensive and usually has poor compliance because of the unpleasant side effects of medicinal iron. Food fortification has been considered the best long-term strategy for prevention, but there are technical problems related to the choice of a suitable iron compound. The iron compounds of relatively high iron availability, such as ferrous sulfate, often provoke unacceptable color and flavor changes, whereas those compounds which are organoleptically inert, such as elemental iron, are usually poorly absorbed [12]. While staple foods, such as wheat and corn flours, can be relatively easily fortified with iron, rice grains pose a much more difficult problem [13]. For various reasons, none of the current intervention strategies has been very successful in reducing the prevalence of iron deficiency anemia in developing countries. An alternative, more sustainable approach would be the enrichment of the food staples either by plant breeding or by genetic engineering [14,15]. Increasing seed ferritin, the natural iron store, had been suggested as a means to increase the iron content [15]. Ferritin is the iron storage protein found in animals, plants and bacteria,

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which can store up to 4500 iron atoms in a central cavity [16]. The concept of using ferritin as a nutritional source of iron is not new. Earlier studies [17–20] concluded that animal ferritin iron was relatively ineffective as a nutritional iron source, except when ingested with ascorbate [18]. However, a recent reevaluation of the results obtained so far concludes that iron from animal and plant ferritin can be utilized by anemic rats and man [21,22].

Increasing iron intake, however, will not be successful in eliminating iron deficiency anemia unless the diet is also low in iron absorption inhibitors or contains enhancers of iron absorption and utilization. The major inhibitor, phytic acid, can readily be degraded in cereal and legume foods by addition of exogenous phytases either during food processing [8] or during digestion [23], increasing iron absorption dramatically. In the same way, muscle tissue, through the action of the cysteine-containing peptides formed on digestion [24], improves iron absorption from cereal-based meals [25].

Rice provides the primary or secondary staple food for 50% of the world's population; it is, thus, one of the most important plants on earth. Worldwide 530 million tons of rice were produced in 1994 [26], mostly in developing countries like China, India, Indonesia and Bangladesh [27]. Rice seeds, which are usually milled to remove the oil-rich aleurone layer that turns rancid upon storage, are characterized by a very low content of iron (between 0.2 mg and 2.8 mg/100 g rice) and by its very low bioavailability. We have explored three different approaches to increase the amount of iron absorbed from rice. We have attempted to increase the iron content with the introduction of the ferritin gene from *Phaseolus vulgaris* [28]. To improve its bioavailability we have introduced a thermo-tolerant phytase from *Aspergillus fumigatus* [29] (Tomschy, unpubl.) and over-expressed the endogenous cysteine-rich metallothionein-like protein [30]. All genes were regulated by an endosperm-specific promoter to ensure and restrict expression to the endosperm, the tissue constituting the milled rice grains.

## MATERIALS AND METHODS

### Plant Material and Transformation

*Japonica* rice variety Taipei 309 was used as the target plant. Embryogenic calli, derived from mature zygotic embryos, were inoculated with *Agrobacterium tumefaciens* strain LBA 4404 [31] containing the ferritin [28] or the metallothionein-like [30] gene. Callus and bacteria induction, transformation and selection of transgenic tissues were performed as reported [32]. Regeneration of plants from the selected calli was carried out as described [33]. Rice suspension cells, derived from immature zygotic embryos, were used for biolistic transformation with the fungal phytase [29]. Protocols for callus induction and initiation of cell suspension culture were identical to those previously described [34,35]. Transformation,

selection of the transformants and regeneration were performed as reported [33].

### Analysis of Transgenic Rice Plants

**RNA Analysis.** Total RNA was isolated from immature T1 seeds about 10 to 14 days after pollination by the standard method with guanidine thiocyanate [36], by using the Tiazol Extraction kit (GibcoBRL). After agarose electrophoresis, the RNA was blotted onto nylon membrane (Hybond-N, Amersham, Zürich, Switzerland) and UV cross-linked. A PCR-amplified, DIG-labelled (Boehringer, Rotkreuz, Switzerland) fragment of the coding region of the metallothionein DNA was used as probe. Hybridization, washing and detection were performed as described [33].

**Western Blot.** Mature transgenic T1 seeds were dried at 50°C, dehusked and ground to a fine powder. Proteins were extracted from seeds transformed with the pfe-gene by homogenization of 0.2 g rice powder in 2 mL 50 mM Tris-HCl, pH 7.5 containing 1 mM PMSF. Protein extraction from seeds harboring the fungal phytase gene was performed as described [37]. Thirty µg of total protein extract were separated by SDS-PAGE and transferred electrophoretically onto a nitro-cellulose membrane (Schleicher and Schuell, Dassel, Germany). The primary antibodies used were raised in rabbit against the pea ferritin (kindly provided by Prof. Briat, Montpellier, France) and against the phytase from *A. fumigatus* (kindly provided by Dr. Lehmann, Hoffmann-La Roche, Basel). Detection of the protein was performed with an ECL chemiluminescence western blotting kit (Amersham), according to the instructions of the manufacturer.

**Biochemical Analysis.** Dried and dehusked T1 rice seeds were ground to a fine powder with an oscillating mill (Retsch MM2, Schieritz and Hauenstein AG, Arlesheim, Switzerland) equipped with agate cups and balls. Aliquots were analysed for iron by atomic absorption spectrophotometry after microwave digestion [38]. Proteins were extracted from seeds transformed with the rgMT gene by homogenization of 0.2 g rice powder in 2 mL 10 mM Tris-HCl, pH 8.0. Cysteine was oxidized to cysteic acid and quantified by HPLC analysis with a HP-Amino Quant II analyser provided with a fluorescence detection.

Phytase activity was determined in samples containing 0.2 g rice powder. The sample was diluted in 2 mL 0.2 M imidazol-HCl buffer at pH 6.5 containing 1% phytic acid (Sigma, Buchs, Switzerland) and incubated on a shaker at 37°C. Samples were taken at 0, 15 and 30 minutes, and the reaction was stopped by the addition of an equal volume of 15% trichloroacetic acid. Free inorganic phosphate was measured at 610 nm with a procedure based on the complex formation of malachite green with phosphomolybdate [39]. Acid and thermo-tolerance were tested by incubating the rice samples two hours at pH 2.5 and 37°C or 20 minutes at 100°C respectively before testing for phytase activity. Thermostability of the purified phytase was

determined by adding 1% of the fungal protein to ground rice prior to cooking and testing for activity.

The inositol phosphate content was determined before and after one hour's incubation at pH 6.5 and 37°C by extraction of inositol phosphates from the rice seeds with 0.5 M HCl and subsequently separation from the crude extract by ion-exchange chromatography. The quantification was performed by ion-pair C18 reverse phase HPLC analysis using formic acid/methanol and tetrabutylammonium hydroxide in the mobile phase [40,41].

## RESULTS

### Introduction of Ferritin (*pfe*), Metallothioneinlike (*rgMT*) and Phytase (*phyA*) Genes into Rice

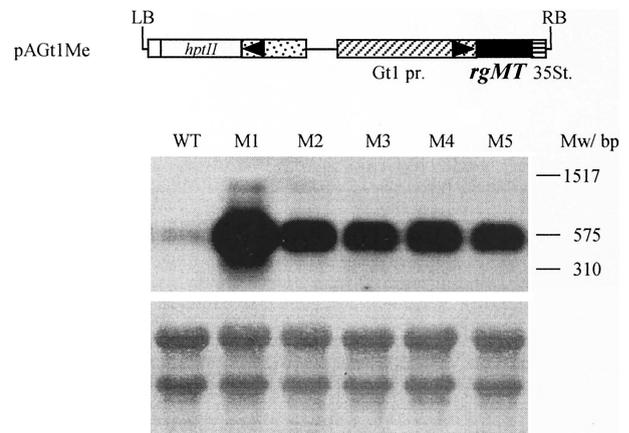
Constructs pAGt1Fe containing the gene for the ferritin protein from *Phaseolus vulgaris* [28] and pAGt1Me with the rice metallothionein-like protein [30] were cloned following standard procedures and used for *Agrobacterium*-mediated transformation of mature rice embryos [42]. Forty hygromycin-resistant clones were obtained after transformation with either pAGt1Fe or pAGt1Me, twenty of which were regenerable. Twelve independent plants carrying the ferritin or the metallothionein gene respectively were further analysed. pGt1PF containing the Q27L mutant (Tomschy, unpubl.) of the mature phytase gene from *A. fumigatus* [29] was used for biolistic transformation of suspension cells. Ten hygromycin-resistant calli could be regenerated, four of which developed transgenic fertile plants.

### Gene Expression

Northern blot analysis of T1 seeds from plants transformed with pAGt1Me demonstrated that *rgMT* was clearly over-expressed in all lines obtained (Fig. 1). Non-transgenic rice showed a weak signal indicating the background expression of the endogenous gene.

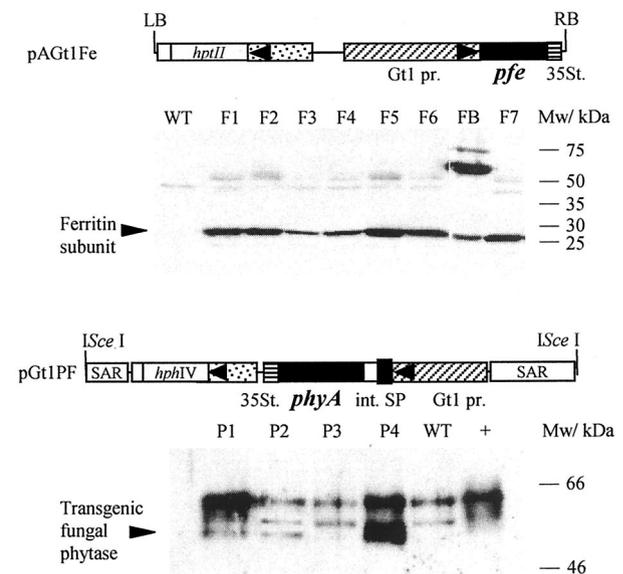
Expression of ferritin and phytase was assessed by immunoblotting (Fig. 2). The 26.5 kDa ferritin subunit was detected in all transformants and in *Phaseolus vulgaris*, but not in the non-transformed rice. An additional band at 55 kDa, which is also detected in the control extract from bean, probably represents ferritin dimers.

Using phytase antiserum, three different immunoreactive proteins with an apparent molecular weight of 65, 58 and 55 kDa were detected. The purified *A. fumigatus* phytase showed the same electrophoretic mobility as the bigger endogenous rice phytase (65 kDa). All plants showed a second band having a lower molecular weight (58 kDa), indicating the presence of a further endogenous phytase [43]. Lines P1, P2 and P4 showed an additional band at 55 kDa not present in line P3 and in the untransformed seeds. The variation in molecular weight of 10 kDa compared to the positive control was expected because of



**Fig. 1.** Northern blot analysis of seeds of plants transformed with pAGt1Me, plasmid containing the rice metallothionein-like gene (*rgMT*) under the control of the glutelin promoter (*Gt1 pr.*). Total RNA (12 mg) from five transformants (M1–M5) and non transgenic rice (WT) were electrophoresed, transferred onto nitrocellulose membrane, and hybridised with a fragment of the metallothionein-like protein cDNA. The membrane after the transfer of RNA, stained with 0.04% methylene blue, is shown in the lower panel. [42 (Reproduced with permission.)]

the different glycosylation pattern of the fungal phytase in plants [37]. Two transformed plants (P1 and P4) showed not only the presence of the additional phytase, but also an increased amount of the 65 kDa phytase. This increment could be



**Fig. 2.** Western blot analysis of 30 mg protein extract from transgenic seeds containing the *pfe*-gene (F1–F7) and the *phy*-gene (P1–P4) and a untransformed control (wild-type, WT). French bean protein extract (FB) and the purified fungal phytase (+) were used as positive controls. Both the ferritin (*pfe*[upper panel]) and the phytase (*phyA* [lower panel]) genes are placed under the control of the glutelin promoter (*Gt1 pr.*). [42 (Reproduced with permission.)]

due to a different processing of the transgenic protein or an over-expression of the endogenous phytase.

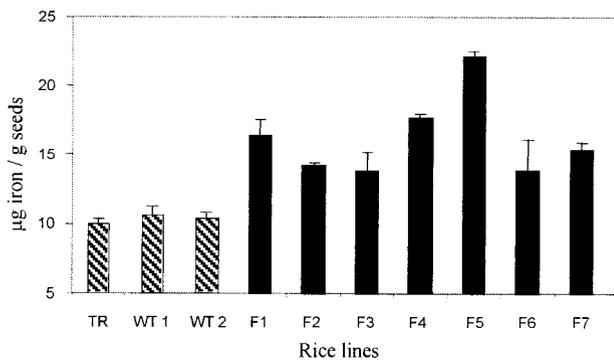
**Effect of Transgenes on Seed Composition**

Rice plants expressing the transgenic proteins were tested for iron, cysteine and for phytase activity to investigate the effect of the transgene.

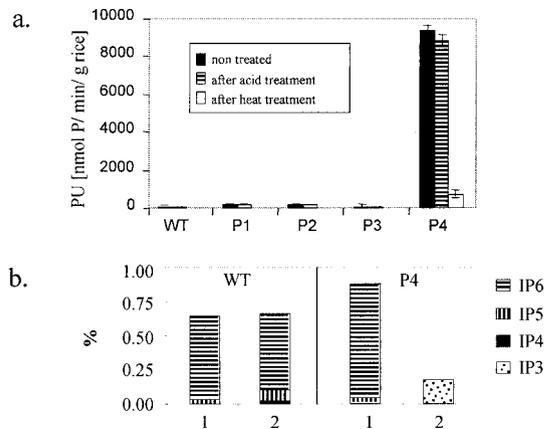
Regenerated plants expressing the *Phaseolus* ferritin protein showed an improved iron accumulation in the seeds (Fig. 3). Iron content in mature T1 seeds varied between  $11.53 \pm 0.16$  to  $22.07 \pm 0.70 \mu\text{g/g}$  seeds. As the iron levels in seeds of negative controls ranged from  $9.99 \pm 0.37$  to  $10.65 \pm 0.60 \mu\text{g/g}$  seeds, we estimated a twofold increase in iron content of seeds from the transgenic rice with the highest iron level.

The three transgenic plants expressing the fungal protein showed an increased phytase activity in the grains. Compared to the non-transformed grains, two plants produced seeds with double phytase activity, whereas the third plant (line P4, Fig. 4) increased the phytase content of its grains about one hundred and thirtyfold from 72 to 9415 phytase units/g rice. Phytase activity correlated well with the amount of the 55 kDa protein detected by phytase antibodies (Fig. 2). After simulated stomach conditions, transgenic grains retained the same phytase activity as before acidic treatment.

Seeds from line P4 were also analysed for their inositol phosphate content. No phytic acid decrease could be observed in the transgenic seeds, as the fungal protein was engineered for secretion into the apoplast, preventing its activity during the maturation of the seeds. However, after simulated small intestine conditions, only 0.2% inositol triphosphate could be detected, whereas no inositol hexa- and pentaphosphate were present in the digested rice. Thermo-stability analysis of the purified fungal protein cooked together with rice flour revealed that rice components affected the thermo-tolerance of the phytase. Nevertheless, the protein still retained 50% of its



**Fig. 3.** Iron content of dehusked seeds from 7 transgenic rice lines (F1–F7) were measured by Graphite Furnace Atomic Absorption Spectrometry. Two untransformed plants (WT1, WT2) and a plant transformed with the *rgMT*-gene (TR) were used as controls. [42 (Reproduced with permission.)]



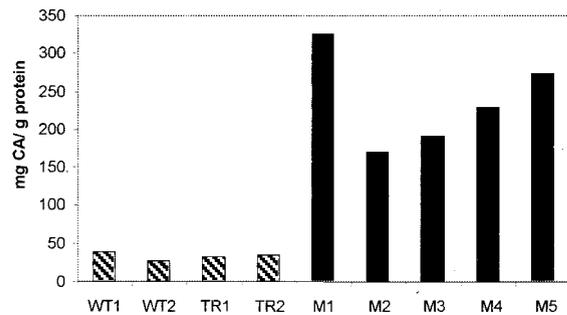
**Fig. 4.** Analysis of seeds from plants transformed with the fungal phytase (P1, P2, P3, P4) and an untransformed control (WT). (a) The phytase activity was measured at pH 6.5 and 37°C before and after acid or heat treatment. (b) The phytate content from seeds of a transgenic plant (P4) and an untransformed control was determined by HPLC analysis before (1) and after (2) simulated small intestine conditions. [42 (Reproduced with permission.)]

initial activity (data not shown), making it a promising candidate for an activity in rice even after cooking. However, these preliminary results were not confirmed after cooking the transgenic rice seeds for 20 minutes in boiling water and only 8% of the initial phytase activity was retained.

The cysteic acid content in proteins of seeds over-expressing the *rgMT* gene increased significantly (Fig. 5). The cyst(e)ine content varied from 27.4 to 38.9 mg/g protein in the control seeds and from 170.0 to 323.8 mg/g protein in the transgenic seeds.

**DISCUSSION**

We have generated transgenic rice plants that produce seeds of higher iron content and with a potentially improved iron



**Fig. 5.** The cysteic acid (CA) content of dehusked seeds from lines overexpressing the *rgMT*-gene (M1–M5) was determined by HPLC analysis after oxidation of cysteine residues. Untransformed plants (WT1, WT2) and two plant transformed with the *pfe*-gene (TR1, TR2) were used as controls. [42 (Reproduced with permission.)]

bioavailability. All transformed plants were visually indistinguishable from the non-transgenic plants, indicating that the newly expressed proteins do not affect morphology, growth or fertility. Rice grains expressing the fungal phytase germinated without any problems indicating that the transgenic protein did not negatively affect the phosphorus content of the seeds and, thereby, germination. HPLC analysis of seeds strongly expressing the phytase from *Aspergillus fumigatus* revealed that the level of phytic acid did not decrease in the mature transgenic rice seeds. It would therefore seem that the fungal protein was probably not in contact with the phytate in the seeds and that phytate hydrolysis did not occur during seed maturation. In support of this hypothesis, it has been shown that the glucanase signal peptide used in the transgenic construct secretes the protein to the extracellular fluid (Fuetterer, personal communication).

The increased iron content in our transgenic plants is assumed to be the result of the expression of *Phaseolus* ferritin. As the amount of iron in the seed is only a small part (ca. 4%) of the total iron present in the stem part of the rice plant [44], it seems that transfer of iron and accumulation in the seed might be the limiting step. Recently Goto [45] has reported the expression of soybean ferritin in rice seeds increasing the iron content of up to threefold. The twofold to threefold extra iron consumed by eating the transgenic rice grains would appear to be of nutritional significance. In fact, the iron intake from a daily consumption of about 300 g rice by an adult [27] would be increased from around 3 mg, for normal rice, to about 6 mg for our transgenic rice with the highest iron content. This daily 3 mg increase in iron intake represents 20% of the recommended intake for an adult woman of child-bearing age [46], one of the population groups most at risk of iron deficiency.

Of some concern is the possible poor bioavailability of ferritin iron in man. It has been reported that part of the iron contained within the ferritin molecule is not released into the gastrointestinal tract during digestion and thus is not available for absorption. Human studies with intrinsically radio-labeled animal ferritin indicated that the iron contained within ferritin molecules added to a meal is only about half as well absorbed as vegetable iron [47,48] and as ferrous sulfate [22]. Beard *et al.* [21], however, suggested that intrinsically labeled animal ferritin might not be typical of the ferritin found in normal animal tissues. They recommended a reevaluation of ferritin bioavailability and showed that iron content in horse spleen ferritin was as bioavailable as ferrous sulfate in anemic rats. The anemic rat has been shown to predict well the relative bioavailability in man of different fortification compounds [49], although it is not a good model to predict the magnitude of enhancers and inhibitors of iron absorption [50]. Therefore, it remains to be shown to what extent the iron contained in plant ferritin can be utilized in man.

The insertion of the phytase gene into rice has however a

great potential to improve iron nutrition in rice-eating populations. The phytase activity in the transgenic rice highly expressing the fungal protein is extremely high (9415 units/g) compared to other cereal grains and legume seeds, which we have analyzed using the same methodology (Egli, Davidsson, Hurrell, unpublished). We demonstrate that the fungal protein retained 92% of its activity after incubating the transgenic ground rice under stomach conditions, indicating the acid tolerance of the protein. After simulated small intestine conditions, the phytic acid content strongly decreased in seeds, and only inositol triphosphate could be detected in the digested rice. As only inositol hexa- and pentaphosphate are responsible for iron chelation and prevention of its absorption, no inhibition can be expected after rice digestion.

Unlike cereal and legume phytases, the enzyme from *Aspergillus fumigatus* is reported to be thermo-tolerant and to have a broad pH optimum [29]. According to the literature, after heating the fungal protein 20 minutes at 100°C, only 10% of the phytase activity was lost [29]. Preliminary results obtained with the purified fungal protein cooked with rice flour demonstrate that, although the thermo-tolerance was somehow affected by the presence of rice components, 50% of the activity was still retained (data not shown). Despite these encouraging results, the thermo-tolerance of our transgenic rice was surprisingly low. Since the residual phytase activity present after cooking the transgenic seeds would be insufficient to degrade significantly the phytic acid in the rice endosperm, a further approach to decrease the phytic acid content in a rice meal was considered.

The amount of inositol penta- and hexaphosphate has to be degraded to an extremely low level in order to eliminate any inhibitory effect on iron bioavailability [51]. Such a reduction of the phytic acid present in the rice grains would alter the normal phosphorus storage form of the seeds and, thereby, their germination efficiency. However, if the phytic acid were specifically degraded in the inner part of the endosperm, without affecting the outer aleuron layer, which is particularly rich in phytic acid, sufficient phosphorus would be available for the germination of the seedlings. The rice milling process, performed before consuming rice, would discard the residual phytic acid present in the outer part of the rice seeds, and, therefore, no negative effects on iron bioavailability in humans are expected. This approach requires that the phytase introduced is active during the formation of the seeds and present in the protein storage vacuoles where the phytic acid is stored. Furthermore, the expression of the transgenic protein should be restricted to the inner part of the rice endosperm, the tissue eaten after rice milling. The globulin promoter is reported to be responsible for the specific expression of the transgenic protein in the inner part of the rice endosperm, as confirmed after  $\beta$ -glucuronidase staining of transgenic rice seeds transformed with the GUS gene driven by the globulin (−980 bp) promoter [52]. Therefore, the globulin promoter (−980 bp) with its signal peptide (+75 bp) was fused to the *A. fumigatus* phytase

gene, which has a high activity at pH 6–7, the pH of the protein storage vacuole during the maturation of the seeds [53]. In contrast, cereal phytase has a pH optimum between 4 and 5, the pH of the protein storage vacuole during the germination of the seeds [53], the period when free phosphorus is required. Fifty transgenic rice plants were regenerated after *Agrobacterium*-mediated transformation. The transgenic protein was expressed in all plants analyzed. Biochemical analyses are now in progress to determine whether the introduced phytase was indeed able to reduce the phytic acid in the rice grains during their development.

Cysteine [24] and cysteine-containing peptides from meat [10] enhance the absorption of non-heme iron in man. When 210 mg cysteine, or the equivalent amount of cysteine, were added to a corn meal, iron absorption approximately doubled. By over-expressing metallothionein in rice, we increased the cyst(e)ine content of the soluble seed protein of about sevenfold. Cysteine is thought to increase the absorption of non-heme iron by binding the iron through its thiol group [10]; therefore, only cysteine and not cystine has an enhancing effect on iron absorption. Since metallothionein is reported to contain 12 cysteine/72 amino acid [30], the increased cyst(e)ine content can be attributed to a higher cysteine amount in the transgenic rice seeds. By over-expressing metallothionein in rice, we increased the cysteine content of the seed protein in the endosperm to a level which could further enhance iron bioavailability.

Bioavailability tests with animals are considered to be of little use to predict iron bioavailability in man [54], and *in vitro* models, such as dialysability or uptake by Caco-2 cells also have their limitations [55]. Human studies, in which the plant foods are labeled intrinsically are necessary and will be made as soon as sufficient material is available.

## CONCLUSIONS

Transgenic rice grains have been produced that could potentially increase both iron intake and iron bioavailability. Further work remains to be done to evaluate the bioavailability in humans of iron from *Phaseolus* ferritin, to confirm that the fungal phytase is indeed able to reduce the phytic acid in the rice grains during their maturation and to measure the influence of the rice metallothionein-like protein on iron absorption.

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