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Genetic engineering approaches to improve the bioavailability and the level of iron in rice grains

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Abstract Iron deficiency is the most widespread micronutrient deficiency world-wide. A major cause is the poor absorption of iron from cereal and legume-based diets high in phytic acid. We have explored three approaches for increasing the amount of iron absorbed from rice-based meals. We first introduced a ferritin gene from *Phaseolus vulgaris* into rice grains, increasing their iron content up to two-fold. To increase iron bioavailability, we introduced a thermotolerant phytase from *Aspergillus fumigatus* into the rice endosperm. In addition, as cysteine peptides are considered a major enhancer of iron absorption, we overexpressed the endogenous cysteine-rich metallothionein-like protein. The content of cysteine residues increased about seven-fold and the phytase level in the grains about 130-fold, giving a phytase activity sufficient to completely degrade phytic acid in a simulated digestion experiment. High phytase rice, with an increased iron content and rich in cysteine-peptide, has the potential to greatly improve iron nutrition in rice-eating populations.

Keywords Bioavailability · Genetic engineering · Iron · Rice

Introduction

The prevalence of iron deficiency is estimated to be about 30% of the world population (WHO 1992), making iron by far the most-widespread nutrient deficiency world-wide. The major consequences are poor pregnancy outcome including increased mortality of mother and children, reduced psychomotor and mental development in infants, decreased immune function, tiredness and poor work performance (Cook et al. 1994).

The amount of bioavailable iron is dependent both on iron intake and on its absorption. Dietary iron in developing countries consists primarily of non-haem iron, whose poor absorption is considered as a major factor in the aetiology of iron-deficiency anaemia (Taylor et al. 1995). Grain and legume staples are rich in phytic acid, which is a potent inhibitor of iron absorption (Hurrell et al. 1992). In addition, the intake of foods that enhance non-haem iron absorption, such as fruits, vegetables or muscle tissue, is often limited.

The most-widely recognised strategies for reducing micronutrient malnutrition are supplementation with pharmaceutical preparations, food fortification, dietary diversification and disease reduction (Maberly et al. 1994). For various reasons, none of these have been very successful in reducing the prevalence of iron-deficiency anaemia in developing countries. An alternative more sustainable approach would be the enrichment of food staples either by plant breeding or by genetic engineering (Bouis 1996; Theil et al. 1997). Increasing seed ferritin, the natural iron store, had been suggested as a means to increase the iron content (Theil et al. 1997). Ferritin is the iron-storage protein found in animals, plants and bacteria, which can store up to 4500 iron atoms in a central cavity (Theil 1987). Recent studies have shown that iron from animal and plant ferritin can be utilised by anaemic rats and man (Beard et al. 1996; Skikne et al. 1997).

However, increasing iron intake will not be successful in eliminating iron-deficiency anaemia unless the diet is also low in iron-absorption inhibitors or contains enhancers of iron absorption and utilisation. The major inhibitor, phytic acid, can be readily degraded in cereal and legume foods by the addition of exogenous phytases either during food processing (Hurrell et al. 1992) or during digestion (Sandberg et al. 1996), increasing iron absorption dramatically. In the same way, muscle tissue, through the action of the cysteine-containing peptides

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formed on digestion, improves iron absorption from cereal-based meals (Cook et al. 1997).

We have, therefore, 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* (Spence et al. 1991). To improve its bioavailability we have introduced a thermotolerant phytase from *Aspergillus fumigatus* (Pasamontes et al. 1997; Tomschy, unpublished) and overexpressed the endogenous cysteine-rich metallothionein-like protein (Hsieh et al. 1995). 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

Plasmid construction

For *Agrobacterium*-mediated rice transformation, two binary vectors were constructed. After *Bgl*II/*Hind*III digestion of plasmid pKS1 (Okita et al. 1989), the 1.8-kb glutelin promoter Gt1 was introduced into pCAMBIA 1390 (CAMBIA, Canberra, Australia) in the opposite direction to the *hpt*II gene. Using PCR, two *Sma*I sites were introduced at the ATG and downstream from the stop codon present in the full-length ferritin clone (*pfe*) isolated from *Ph. vulgaris* (Spence et al. 1991) and in the metallothionein-like clone (*rgMT*) isolated from the genomic library of *Oryza sativa* (Hsieh et al. 1995). The *pfe* and *rgMT* genes were introduced downstream from the glutelin promoter, resulting in the clones pAGt1Fe and pAGt1Me (see Fig. 1).

The pGt1PF plasmid (see Fig. 1) used for biolistic rice transformation was constructed on the basis of pGluChi (Bliffeld et al. 1999), which contained two scaffold-attachment regions (SARs). The expression cassette was replaced by the chimeric phytase gene encoding the barley β-glucanase signal peptide (Leah et al. 1991) and the Q27L mutant (Tomschy, unpublished) of the mature *A. fumigatus* phytase gene (Pasamontes et al. 1997), which shows a higher specific activity than the native one. The chimeric gene was placed under the control of the 1.8-kb Gt1 promoter. The *hpt*IV gene from Escherichia coli, placed under the control of the CaMV35 S promoter and the CaMV polyadenylation sequence, was isolated from plasmid pCIB900 (Wünn et al. 1996) and employed for the selection of the transgenic tissue.

Plant material and transformation

Japonica rice variety Taipei 309 was used as target plant. Embryogenic calli, derived from mature zygotic embryos, were inoculated with *Agrobacterium tumefaciens* strain LBA 4404 (Hoekema et al. 1984). Callus and bacterial induction, transformation, selection and regeneration of the transgenic tissues were performed as previously reported (Ye et al. 2000).

Rice-suspension cell aggregates, derived from immature zygotic embryos, were used for biolistic transformation. Protocols for callus induction and initiation of the cell suspension culture were identical to those described by Zhang et al. (1996). Transformation, selection of the transformants and regeneration were all performed as reported (Burkhardt et al. 1997).

Analysis of transgenic rice plants

RNA analysis

Total RNA was isolated from immature T1 seeds about 10–14 days after pollination by the standard method with guanidine thiocyanate (McGookin 1984), using the Tiazol Extraction kit (GibcoBRL).

After agarose electrophoresis, the RNA was blotted onto a 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 *rgMT* DNA was used as a probe. Hybridisation, washing and detection were performed as described by Burkhardt et al. (1997).

Western blot

Mature transgenic T1 seeds were dried at 50°C, de-husked and ground to a fine powder. Proteins were extracted from seeds transformed with the *pfe*-gene by homogenisation of 0.2 g of rice powder in 2 ml of 50 mM Tris-HCl, pH 7.5, containing 1 mM of PMSF. Protein extraction from seeds harbouring the fungal phytase gene was performed as described (Verwoerd et al. 1995). Thirty micrograms of total protein extract were separated by SDS-PAGE and transferred electrophoretically onto a nitrocellulose 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 de-husked 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 and for zinc by thermal ionization mass spectrometry after microwave digestion (Davidsson et al. 1996; Engelmann et al. 1998). Proteins were extracted from seeds transformed with the *rgMT* gene by homogenisation of 0.2 g of rice powder in 2 ml of 10 mM Tris-HCl, pH 8.0. Cysteine residues were oxidised to cysteic acid and quantified by HPLC analysis with a HP-Amino Quant II analyser provided with a fluorescence detection system.

Phytase activity was determined in samples containing 0.2 g of rice powder. The sample was diluted in 2 ml of 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 min 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 (Van Veldhoven et al. 1987). Acid- and thermo-tolerance were tested by incubating the rice samples for 2 h at pH 2.5 and 37°C or 20 min 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 1-h incubation at pH 6.5 and 37°C by extraction of inositol phosphates from the rice seeds with 0.5 M HCl and subsequent 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 (Sandberg and Ahderinne 1986).

Results

Introduction of ferritin (*pfe*), metallothionein-like (*rgMT*) and phytase (*phyA*) genes into rice

The genes encoding the ferritin protein from *P. vulgaris* and the rice metallothionein-like protein were separately

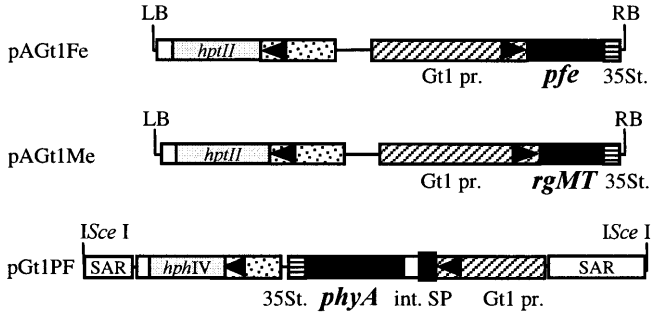

Fig. 1 Schematic representation of the plamids employed. The ferritin (*pfe*), the metallothionein-like (*rgMT*) and the phytase (*phyA*) genes were placed under the control of the glutelin promoter (*Gt1 pr.*), whereas the selective antibiotic resistance genes (hygromycin phosphotransferase, *hpt*II and *hpt*IV) were driven by a constitutive promoter (cauliflower mosaic virus 35 S promoter). The pAGt1Fe and pAGt1Me plasmids were used for *Agrobacterium*-mediated, and pGt1PF for biolistic, transformation of rice

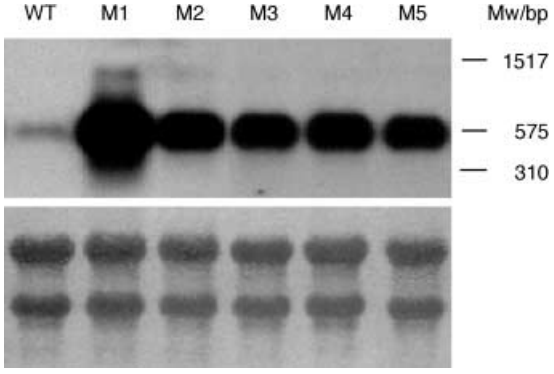

Fig. 2 Northern-blot analysis of seeds of plants transformed with pAGt1Me. Total RNA (12 µg) from five transformants (*M1–M5*) and non-transgenic rice (*WT*) were electrophoresed, transferred onto a nitrocellulose membrane, and hybridised with a fragment of the metallothionein-like protein cDNA. The membrane after the transfer of RNA, and stained with 0.04% methylene blue, is shown in the lower panel

cloned into pCAMBIA 1390 under the control of the endosperm-specific promoter Gt1. The resulting plasmids pAGt1Fe and pAGt1Me (Fig. 1) were used for *Agrobacterium*-mediated transformation of pre-cultured mature embryos. Forty hygromycin-resistant clones were obtained after transformation with either pAGt1Fe or pAGt1Me, 20 of which were regenerable. All clones analysed were transgenic and most of the plants revealed a single insertion (data not shown). Twelve independent plants carrying the ferritin or the metallothionein gene respectively were further analysed.

A chimeric phytase gene encoding a cDNA fragment of the mature *A. fumigatus* phytase driven by the Gt1 promoter and the barley β-glucanase signal peptide for the secretion of the fungal protein into the apoplast was constructed and linked to the *hpt*IV sequence. The resulting plasmid pGt1PF (Fig. 1) was used for the biolistic transformation of suspension cells. Ten hygromycin-resistant calli were regenerated, four of which developed transgenic fertile plants carrying three to multiple-copy insertions. All plants described showed a normal phenotype and fertility.

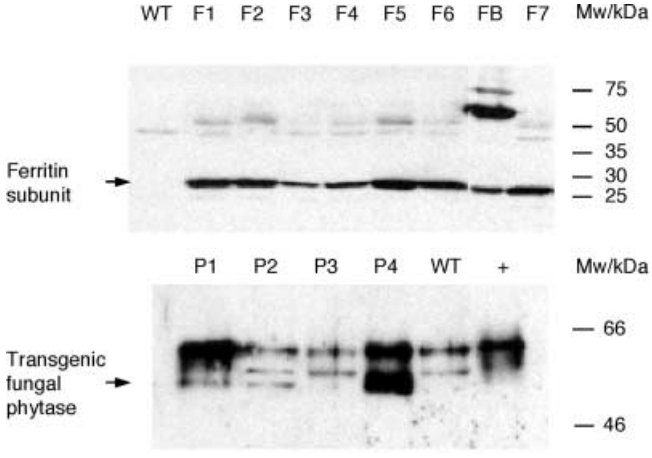

Fig. 3 Western blot analysis of 30 µg of protein extract from transgenic seeds containing the *pfe*-gene (*F1–F7*) and the *phy*-gene (*P1–P4*) and an untransformed control (wild-type, *WT*). French bean protein extract (FB) and the purified fungal phytase (+) were used as positive controls

Gene expression

Northern-blot analysis of T1 seeds from plants transformed with pAGt1Me demonstrated that *rgMT* was clearly overexpressed in all the lines obtained (Fig. 2). 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. 3). The 26.5-kDa ferritin subunit was detected in all transformants and in *P. vulgaris*, but not in the non-transformed rice. An additional band at 55 kDa, which was also detected in the control extract from bean, probably represents ferritin dimers.

Using phytase antiserum, three different immunoreactive proteins with apparent molecular weights of 65, 58 and 55 kDa were detected. The purified *A. fumigatus* phytase showed the same electrophoretic mobility as the larger 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 (Hayakawa et al. 1989). Lines P1, P2 and P4 showed an additional band at 55 kDa not present in line P3 or in the untransformed seeds. The variation in molecular weight of 10 kDa compared to the positive control was expected because of the different glycosylation pattern of fungal phytase in plants (Verwoerd et al. 1995). 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 due to a different processing of the transgenic protein or an overexpression of the endogenous phytase.

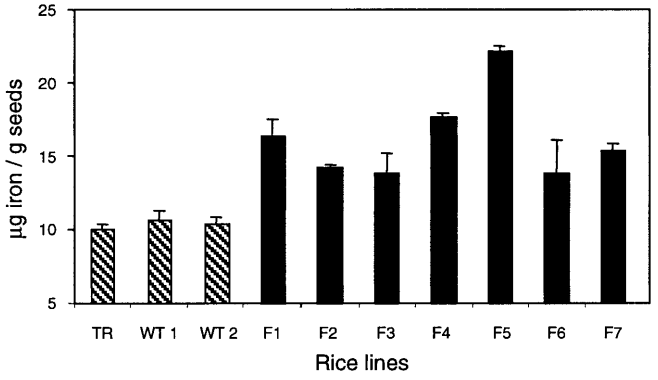

Fig. 4 Iron content of de-husked seeds from seven transgenic rice lines (*F1–F7*) were measured by Graphite Furnace Atomic Absorption Spectrometry. Two untransformed plants (*WT1, WT2*) and a plant transformed with the *rgMT*-gene (*TR*) were used as controls

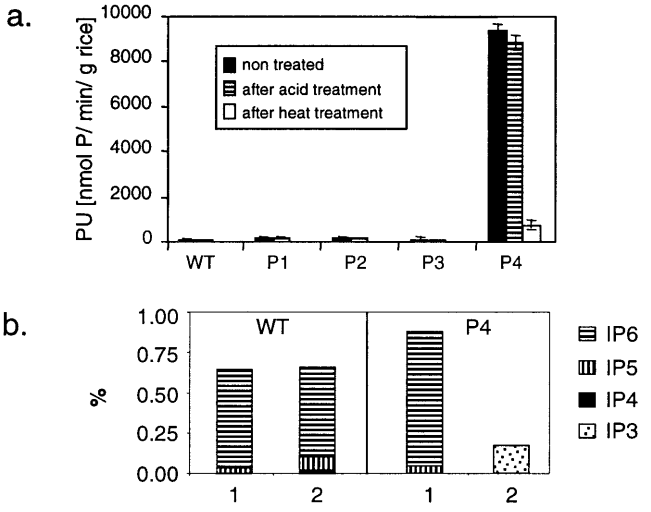

Fig. 5a, b Analysis of seeds from plants transformed with the fungal phytase (*P1, P2, P3, P4*) and an untransformed control (*WT*). **a** The phytase activity was measured at pH 6.5 and 37°C before and after acid or heat treatment. **b** The phytate content from seeds of a transgenic plant (*P4*) and an untransformed control was determined by HPLC analysis before (*1*) and after (*2*) simulated small-intestine conditions

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 transgenes.

Regenerated plants expressing the *Phaseolus* ferritin protein showed an improved iron accumulation in the seeds (Fig. 4). The iron content in mature T1 seeds varied between 11.53±0.16 and 22.07±0.70 µg/g per seed. As the iron level in seeds of negative controls ranged from 9.99±0.37 to 10.65±0.60 µg/g per seed, we observed a two-fold increase in the iron content of seeds from the transgenic plant with the highest iron level.

The three transgenic plants expressing the fungal protein showed an increased phytase activity in the grains (Fig. 5a). Compared to the non-transformed grains, two plants produced seeds with double phytase activity, whereas in the third plant (line P4, Fig. 5a) the phytase activity of the grains increased about 130-fold, from 72 to 9415 phytase units/g of rice. After simulated stomach conditions, transgenic grains retained the same enzymatic activity as before acidic treatment. In addition, the phytase activity correlated well with the amount of the 55-kDa protein detected by Western-blot analysis (Fig. 3).

Seeds from line P4 were also analysed for their inositol phosphate content (Fig. 5b). No phytic acid decrease could be observed in these transgenic seeds, because 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 still be detected, whereas no inositol hexa- and penta-phosphate were present in the digested rice.

Thermostability analysis of the purified fungal protein cooked together with rice flour revealed that rice components partially affected the thermotolerance of the engineered phytase. In fact, the protein retained 50% of its initial activity (data not shown), making it a promising enzyme candidate, which would withstand rice cooking.

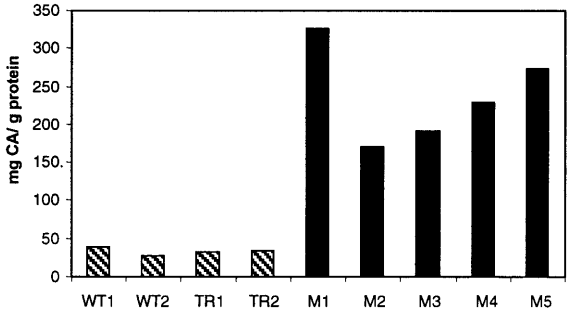

Fig. 6 The cysteic acid (*CA*) content of de-husked seeds from lines overexpressing the *rgMT*-gene (*M1–M5*) was determined by HPLC analysis after oxidation of cysteine residues. Untransformed plants (*WT1, WT2*) and two plants transformed with the *pfe*-gene (*TR1, TR2*) were used as controls

Unfortunately, these preliminary results were not confirmed with the transgenic rice seeds as only 8% of the phytase activity was retained after boiling the seeds for 20 min in water (Fig. 5a).

The cysteic-acid content in proteins of seeds overexpressing the *rgMT* gene increased significantly (Fig. 6). The cysteine residues content varied from 27.4 to 38.9 mg/g of protein in the control seeds and from 170.0 to 323.8 mg/g of protein in the transgenic seeds. Both the iron and zinc contents of *rgMT*-transgenic grains were measured and did not differ significantly from those in control seeds (data not shown), indicating that the overexpression of the endogenous metallothionein-like protein did not lead to an increased metal content of the transgenic grains.

Discussion

We have generated transgenic rice plants, which produce seeds of higher iron content and with a potentially improved iron 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 normally, indicating that the transgenic protein did not negatively affect the phosphorous content of the seeds and, thereby, germination. Recently it has been reported that transgenic lettuce plants constitutively expressing the iron-binding protein ferritin grew larger and faster compared with the controls (Goto et al. 2000). These characteristics could not be observed in our transgenic rice plants, probably because the ferritin gene was driven by the glutelin promoter, restricting its expression to the rice endosperm.

The two-fold increase of iron content in our transgenic plants resulted from the expression of *Phaseolus* ferritin. In fact, the positive correlation between ferritin expression and iron content in leaves of transgenic tobacco plants proved that the extra iron present in the transgenic plants was stored in the ferritin derived from the introduced gene (Goto et al. 1998). Recently Goto et al. (1999) reported the expression of soybean ferritin in rice seeds. In this study, transgenic seeds had a lower, a similar and a higher iron content than non-transformed controls, with a maximal three-fold iron increase in one transformant. A 2–3-fold extra iron content of the transgenic rice grains would appear to be of nutritional significance. In fact, the iron intake from a daily consumption of about 300 g of rice by an adult (IRRI 1993) would be increased from around 3 mg, for wild-type rice, to about 6 mg for our transgenic rice with the highest iron content. However, only increasing the iron intake will not be successful in eliminating iron-deficiency anaemia unless the diet is also low in iron-absorption inhibitors or contains enhancers of iron absorption. Thus, while providing a useful increase in iron intake, there is still room for further improvement in iron bioavailability.

The insertion of the phytase gene into rice has a great potential to improve iron nutrition in rice-eating populations, as even trace amount of phytic acid strongly inhibit iron absorption. The phytase activity in the transgenic rice grains highly expressing the fungal protein is extremely high (9415 units/g) compared to other cereal grains and legume seeds, which we have analysed using the same methodology (Egli, Davidsson and 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 penta-phosphate are responsible for iron chelation and prevention of its absorption, no inhibition can be expected after rice digestion. These results indicated the suitability of the transgenic protein for an enzymatic activity in the gastro-intestinal tract.

Unlike cereal and legume phytases, the enzyme from *A. fumigatus* is reported to be thermotolerant and to have a broad pH optimum (Pasamontes et al. 1997). According to the literature, after boiling the fungal protein for 20 min, only 10% of the phytase activity was lost (Pasamontes et al. 1997). However, the thermotolerance of our transgenic rice was surprisingly low. As this residual activity will be insufficient to completely degrade phytic acid during the digestion process, further attempts to heat-stabilise the phytase need to be found.

Cysteine (Layrisse et al. 1984) and cysteine-containing peptides from meat (Taylor et al. 1986) enhance the absorption of non-haem iron in man. When 210 mg of cysteine, or an equivalent amount of cysteine, were added to a maize meal, iron absorption approximately doubled. By overexpressing metallothionein in rice, we increased the cysteine-residues content of the soluble seed protein about 7-fold. Cysteine is thought to increase the absorption of non-haem iron by binding the iron through its thiol group (Taylor et al. 1986); therefore only cysteine, and not cystine, has an enhancing effect on iron absorption. Since each metallothionein molecule is reported to contain 12 cysteines out of 74 amino acids (Hsieh et al. 1995), the increased cysteine-residues content can be attributed to a higher cysteine amount in the transgenic seeds. By overexpressing metallothionein in rice, we increased the cysteine content of the seed protein in the endosperm to a level which could further enhance iron bioavailability.

Accumulation of mammalian and of some plant metallothioneins in response to an elevated metal ion concentration indicates a role of these proteins in sequestering an excess amount of a certain metal (Robinson et al. 1993). Northern-blot analysis of rice suspension cells after different metal ion treatment showed that there was no increased MT-like gene expression in response to the addition of aluminium, cadmium, lead and zinc to the culture medium (Hsieh et al. 1995). Therefore, an accumulation of toxic metals in the rice endosperm due to the overexpression of the protein was not expected and could not be observed.

The different transgenic lines have been crossed to combine the newly introduced quality improvements, and these plants are now growing under greenhouse conditions. As an adequate vitamin A nutrition has a beneficial effect on iron utilisation, the traits of the recently developed β-carotene rice (Ye et al. 2000) have also been crossed. A low vitamin A status is known to impair iron metabolism and the provision of vitamin A or β-carotene to vitamin A-deficient individuals will improve haemoglobin biosynthesis (Suharno et al. 1993). This rice, with an increased iron content, rich in phytase, cysteine-peptide and β-carotene, has great potential to substantially improve iron nutrition in rice-eating populations.

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