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Development of iron-rich rice and ways to improve its bioavailability by genetic engineering

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Abbreviations

bp	basepair
CAMBIA	Center for the Application of Molecular Biology in International Agriculture
CaMV	Cauliflower Mosaic Virus
2,4-D	2,4-dichlorphenoxy acetic acid
(d)NTP	(deoxy) nucleoside triphosphate
Da	Dalton
DAP	days after pollination
DIG	digoxygenin
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
EDTA	ethylendiaminotetraacetic acid
FAO	Food and Agriculture Organisation of the United Nations
HPLC	high performance liquid chromatography
hph/ hpt	hygromycin phosphotransferase
hyg	hygromycin
IRRI	International Rice Research Institute
LB	Luria-Bertani (medium)
MS	Murashige-Skoog (medium)
NPK	nitrogen, phosphorous, potassium
OD	optical density
PAGE	Polyacrilamide gel electrophoresis
PBS-T	phosphate-buffered saline solution + Tween 20
PCR	polymerase chain reaction
PEG	polethylene glycol
PIG	particle inflow gun
prom	promoter
PMSF	Phenylmethylsulfonylfluoride
RNA	ribonucleic acid
rpm	rotation(s) per minute

RT	room temperature
RT-PCR	reverse transcriptase polymerase chain reaction
SDS	sodium dodecyl sulfate
SSC	salt/sodium citrate
TAE	Tris/acetic acid/EDTA buffer
Tris	tris(hydroxymethyl)aminomethane
UV	ultra violet
v	volume
WHO	World Health Organisation

Transgenes:

pfe	*Phaseolus vulgaris* ferritin
phyA	phytase
rgMT	rice genome metallothionein

Summary

The prevalence of iron deficiency is estimated to be about 30% of the world population or about 2 billion people. It is further estimated that about half of these people suffer from the more severe iron-deficiency anaemia, making iron by far the most widespread nutrient deficiency world-wide.

The most widely recognised strategies for reducing micronutrient malnutrition are supplementation with pharmaceutical preparations, food fortification, dietary diversification, and disease reduction. For various reasons, none has been very successful in reducing the prevalence of iron deficiency anaemia in developing countries.

Rice is characterised by a very low content of iron, and moreover, its absorption in humans is very poor. Since rice represents up to 80% of the daily calorie intake in Southeast Asia, rice rich in highly absorbable iron would be of great help.

To improve the iron storage capacity of the rice endosperm, the ferritin gene, isolated from *Phaseolus vulgaris*, has been introduced into *Japonica* rice variety TP 309. Moreover, two approaches to potentially increase iron absorption in humans have been taken. First, two fungal phytases from *A. niger* and *A. fumigatus* have been transferred into rice to reduce the level of the main inhibiting factor of iron absorption, phytic acid. Second, the amount of the absorption-enhancing amino acid cysteine has been increased with the overexpression of a cysteine-rich protein, the metallothionein-like protein from *Oryza sativa*.

Analysis of the hemizygous primary transgenic plants showed that the *Phaseolus* ferritin was expressed in the seeds increasing the iron content up to 2 fold. The Q27L mutant of the phytase enzyme from *A. fumigatus* and the metallothionein-like protein were similarly expressed in the transgenic grains increasing the cysteine content by 20% and giving a phytase activity theoretically sufficient to substantially degrade rice phytate during food preparation and digestion. The phytase from *A. niger* could not be detected in any transgenic rice plant.

Riassunto

E' stimato che la carenza di ferro concerne circa il 30% della popolazione, ossia 2 miliardi di persone. Inoltre, circa la meta' di queste persone soffre di anemia, la conseguenza piu' grave della carenza di ferro, per cui questa materia si rivela essere l' elemento maggiormente carente nell' alimentazione umana.

Le strategie maggiormente adottate per ridurre la carenza di micronutrienti sono la somministrazione di preparati farmaceutici, l' arricchimento degli alimenti, la diversificazione alimentare e la lotta contro le malattie delle malattie. Per diverse ragioni, nessuna delle stategie sopracitate ha ottenuto risultati soddisfacenti nei paesi in via di sviluppo.

Il ferro e' presente in quantita' molto scarsa nel riso e, inoltre, il suo assorbimento da parte del corpo umano e' molto limitato. Siccome il riso rappresenta piu' dell' 80% della disponibilita' calorica nel sud-est asiatico, una qualita' di riso ricca di ferro facilmente assorbibile sarebbe di grande aiuto per combattere l' anemia.

Per migliorare la capacita' di immagazzinamento di ferro nel chicco di riso, il gene della ferritina, isolato dal fagiolo, e' stato introdotto nella varieta' di riso Japonica TP309. Inoltre sono state utilizzate due strategie per aumentare potenzialmente l' assorbimento del ferro. Due fitasi fungali dell' aspergillo *niger* e *fumigatus* sono state introdotte nel riso per ridurre la concentrazione di acido fitico, la sostanza maggiormente responsabile della scarsa biodisponibilita' del ferro nei cereali. Inoltre il contenuto di cisteine, aminoacido che permette un assorbimento migliore del ferro, e' stato aumentato grazie alla sopraespressione di una proteina molto ricca in cisteine, la metallotioneina del riso.

L' analisi delle piante emizigote transgeniche rivelo' che la ferritina del fagiolo veniva espressa nei chicchi di riso, aumentandone piu' del doppio il contenuto di ferro. L' enzima della fitasi dell' aspergillo *fumigatus* e la metallotioneina erano pure espressi nei semi transgenici. Il contenuto di cisteine risultava maggiore del 20% e l' attivita' della fitasi era teoricamente sufficiente per degradare l' acido fitico durante la preparazione e la digestione del cibo. In nessuna pianta di riso e' stato possibile constatare la fitasi dell' aspergillo *niger*.

1 INTRODUCTION

1.1 Historical perspective

Among all of the micronutrients, iron has the longest and best-described history. Iron is the fourth most abundant terrestrial element, comprising approximately 4.7% of the earth's crust in the form of the minerals hematite, magnetite, and siderite. Primordial iron compounds were probably responsible for the catalytic generation of the atmospheric oxygen upon which most modern life forms depend. Iron is an essential nutrient for all living organisms with the exception of certain members of the bacterial genera *Lactobacillus* and *Bacillus*. In these organisms, the functions of iron are replaced by other transition metals, especially manganese and cobalt, which reside next to iron in the periodic table. In all other life forms, iron is an essential component of, or cofactor for, hundreds of proteins and enzymes.

Egyptians, Greeks, and Romans, although ignorant about the nutritional importance of iron, attributed therapeutic properties to iron. The ancient Greeks administered iron to their injured soldiers to improve muscle weakness, which probably derived from haemorrhagic anaemia. Alchemists and physicians of the 16th century prescribed iron for medicinal use. Iron salts were given to young women to treat what was described as chlorosis, an arcane term for anaemia due to iron or protein deficiency. Various physicians during this time also prescribed iron pills for anaemia and were unceremoniously ridiculed by their successors in the medical profession (Beard, *et al.*, 1996)

1.2 Iron deficiency

Despite its abundance in the earth's crust, iron deficiency is a serious health issue in many parts of the world.

About 2.15 billion people suffer from anaemia, 85% of which is attributed to iron deficiency. The overall prevalence of iron deficiency will be close to 34% of the 6.25 billion persons on our planet by the year 2000. 80% of the total population reside in the developing world, where the prevalence of iron deficiency and anaemia is almost fourfold that in the industrial world, which has an overall prevalence of 11% (Viteri, 1997).

Between 1970 and 1989 the amount of iron in diets decreased drastically in most of the developing countries, thus making iron deficiency a major health problem. The most dramatic fall in iron intake occurred in South and Southeast Asia, where it fell from 6.2 mg/ 1000 kcal to 5.8 mg/ 1000 kcal. In 1977 some 57% of the adult women (15-49 years old) were estimated to be anaemic and this increased to more than 70% in 1987 (Seymour, 1996).

1.3 Iron in the human body

Adult males normally have a total content of about 4.2 g of iron in their body. Iron is present in two pools, functional and storage iron. Functionally important forms of iron in the body are haemoglobin, myoglobin, cytochromes, iron-sulphur proteins, iron enzymes and lactoferrin. About two thirds of body iron is present in haemoglobin in red blood cells, where it is essential in the transport of oxygen. The storage iron compartment has no physiological function other than to serve as a reserve from which increased requirements can be met (e.g. pregnancy) and from which losses from the functional compartment can be replaced. Body iron stores exist as ferritin and hemosiderin in the liver, spleen, and bone marrow. In the iron-replete adult male, storage iron amounts to approximately one-quarter of body iron. In young children and menstruating women, where the physiological demands for iron are high, iron stores are often low or absent (Heinrich, 1975).

1.4 Iron absorption and bioavailability

The capacity of the body to excrete iron is extremely limited, therefore the absorptive process play the major role in the maintenance of iron homeostasis. In general, only a small proportion of dietary iron is absorbed, and the amount is quite variable both between and within individuals. Absorption is increased during deficiency and decreased when erytropoiesis is depressed.

Haem and non-haem iron in foods are absorbed by different pathways with different degrees of efficiency depending upon the chemical form, other dietary constituents and the level of iron stores in the individual.

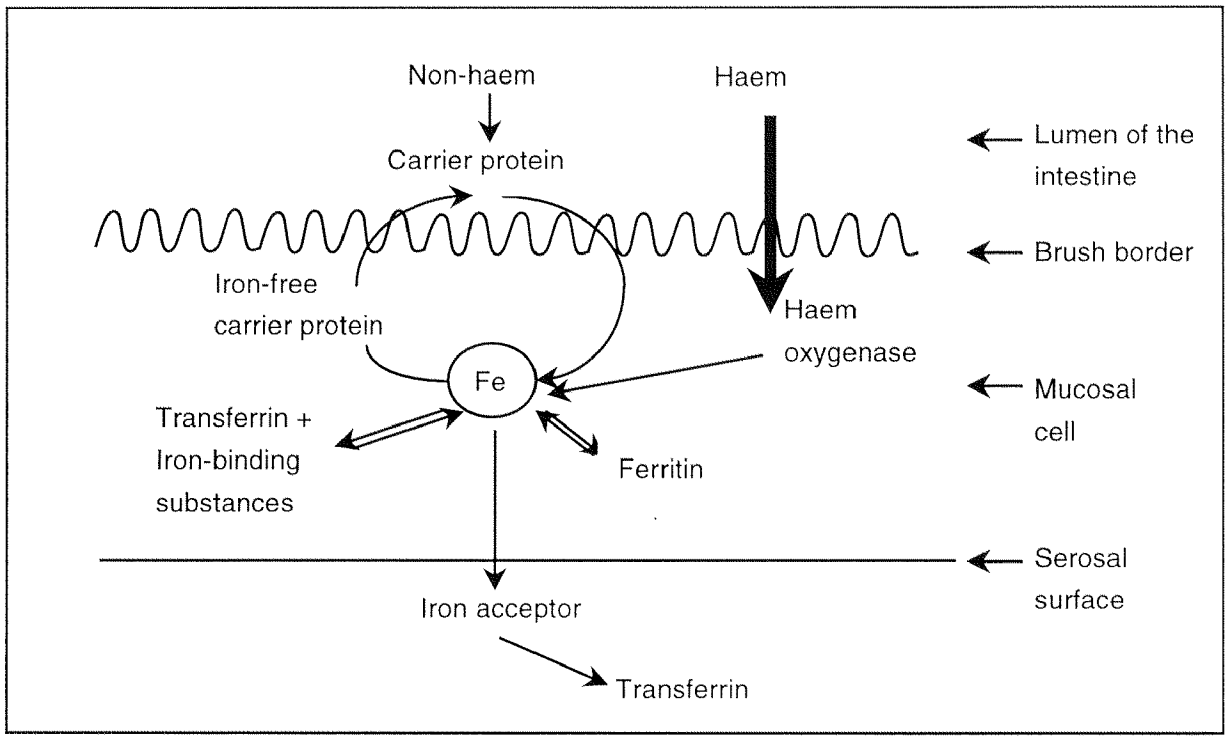


Figure 1: Different pathways of haem and non-haem iron absorption (Foundation, 1995).

Haem, which provides 10 to 15 percent of food iron consumed in industrialised countries, is contained in the haemoglobin and myoglobin present in food from animal sources. Haem iron is highly available (20 to 30 percent absorbed) and may

account for as much as one-fourth of the iron absorbed from diets, which have a high meat content. Moreover, haem iron absorption is relatively unaffected by other dietary or physiological variables (Foundation, 1995).

Non-haem iron, contained in cereals, pulses, fruits and vegetables, is the major source of absorbed dietary iron in developing countries. Absorption of non-haem iron is highly variable depending on the nature of the meal. In any given meal composed of various foods, a common pool of non-haem iron is formed in the intestinal lumen. Absorption from this pool is determined by the composite effect of several factors promoting or impairing iron availability.

The known inhibitors of iron absorption are phytic acid, polyphenolic compounds, calcium and, perhaps to a lesser extend, certain peptides from partially digested proteins. Phytic acid is widely present in cereal grains and legume seeds and is the major factor in the low bioavailability of iron from these foods. The phenolic compounds present in plant foods include phenolic acids, flavonoids and their polymerisation products and are particularly high in beverage such as tea, coffee, herb teas, cocoa and red wine. Calcium can inhibit iron absorption when fed as an inorganic calcium compound or when consumed in dairy products such as milk or cheese. During digestion, food proteins are transformed into peptides, which can bind iron in the intestinal lumen and influence its absorption. Peptides can both inhibit or enhance iron absorption depending on their nature. Studies in which different commercial protein sources were fed in a liquid formula meal have indicated that soy proteins, egg albumin and casein are important inhibitors.

Reducing agents such as ascorbic acid change the valency of iron from Fe (III) to Fe (II). They increase iron absorption as Fe (II) is more soluble than Fe (III) at pH values greater than three, like in duodenum. Ascorbic acid is the best-known and most potent enhancer of iron absorption both in its natural form in fruits and vegetables and when added as the free compound. The enhancing effect is dose related and at high concentrations, ascorbic acid can even overcome the inhibitory effect of phytic acid. Peptides, after muscle protein digestion, enhance the non-haem iron bioavailability, too. The mechanism is not fully known yet, but it appears to be related to the high level of cysteine-containing proteins in these tissues. Their

potential to chelate iron and, to reduce ferric iron to more soluble ferrous iron, could explain the enhancing effect of cysteine on iron absorption (Hurrell, 1997).

A simple monotonous diet containing cereals, roots, and/or tubers and negligible quantities of meat, fish or ascorbic acid-rich foods is a low bioavailability diet. This diet contains a preponderance of foods that inhibit iron absorption and is dominant in many developing countries, particularly among lower socio-economic groups (FAO, 1988).

1.5 Requirement for absorbed iron

Iron needs can be assessed in terms of the amount of iron that must be absorbed to replace body losses and that is needed to provide normal body iron accretion rates during growth and pregnancy.

Normally, dietary iron requirements depend on three factors: physiological requirements, bioavailability of dietary iron and body iron status. Requirements are first considered in terms of the needs for absorbed iron; then they are converted to estimates of dietary iron requirements, taking into account bioavailability, depending on the presence of enhancing and inhibiting factors in the food. In adult men, iron loss is due mainly to exfoliation of cells; about two-thirds of this loss occurs from the gastro-intestinal tract and most of the remainder from the skin. Iron losses in urine and sweat are negligible. When estimating the requirement of absorbed iron for infants, children and adolescents, iron required for expanding red cell mass and growing body tissues must be added to basal losses (FAO, 1988).

Table 1: Requirement for daily absorbed iron (FAO, 1988).

	Age (years)	Total requirement (mg/day)	Total requirement (µg/kg/day)
Children	0.25 - 1	0.96	120
	1 - 2	0.61	56
	2 - 6	0.70	44
	6 - 12	1.17	40
Boys	12 - 16	1.82	34
Girls	12 - 16	2.02	40
Adult men		1.14	18
Adult women:			
Menstruating		2.38	43
Post-menopausal		0.96	18
Lactating		1.31	24

1.6 Consequences of iron deficiency

The adverse effects of iron deficiency are usually defined by the anaemia that accompanies a marked reduction in body iron (FAO, 1988). Severe anaemia in pregnant women is estimated to be responsible for up to 40 per cent of the half a million deaths associated with childbirth each year (Seymour, 1996), sometimes by triggering heart failure by starving the hearth muscle of oxygen during labour.

In infants, lack of sufficient iron in the brain can seriously limit the intellectual potential by causing irreversible changes in mental and psychomotor development (Lozoff, *et al.*, 1991). Abnormalities in mental performance, including apathy, irritability, impaired attentiveness, and reduced learning capacity have also been observed in iron-deficient children.

In the case of less severe anaemia, a correlation between haemoglobin concentration and work capacity has been observed in both laboratory and field studies (Viteri, 1974; Basta, *et al.*, 1979). Iron deficiency causes debilitating

tiredness and poor concentration, and may even directly affect the development of the nervous system.

A number of immunological abnormalities such as reduced percentage of T-lymphocytes, defects in cell-mediated immune response, impaired lymphocyte transformation, fewer positive skin reactions to common antigens and a decrease in granulocyte myeloperoxidase with decreased microbial killing capacity have been identified in individuals with iron deficiency (Beisler, 1982; Vyas, 1984). However, the clinical significance of these abnormalities in terms of risk of infection is not yet clear.

Other severe symptoms of continuous iron deficiency are epithelial changes of nails, gums inflammation, atrophy of tongue papillae, gastritis, nasal mucosal atrophy, loss of hair and depigmentation.

1.7 Iron supplementation and fortification

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). Iron supplementation is useful for producing a rapid improvement in Fe status in anaemic individuals, but is expensive and usually has poor compliance because of the unpleasant side effects of medicinal iron (gastro-intestinal troubles). 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. Unfortunately the iron compounds of relatively high iron availability, such as ferrous sulphate, often provoke unacceptable colour and flavour changes, whereas those compounds, which are organoleptically inert, such as elemental iron, are usually poorly absorbed (Hurrell, 1992). While staple foods, such as wheat and maize flours, can be relatively easily fortified with iron, rice grains pose a much more difficult problem (Hunnell, 1985). For various reasons, none of the current intervention strategies has been very

successful in reducing the prevalence of iron deficiency anaemia in developing countries and iron deficiency is therefore a major public health problem.

An alternative more sustainable approach would be the enrichment of the food staples either by plant breeding or by genetic engineering (Bouis, 1996; Theil, *et al.*, 1997). Micronutrient-rich plants could reach a larger numbers of people than vitamin and mineral supplements, and they would be also far cheaper (Seymour, 1996).

1.8 Iron in rice, a major food staple

Rice provides the primary or secondary staple food for 50% of the world's population and, thus, may be the most important plant on earth. About 20 species of the genus *Oryza* are recognised, but nearly all cultivated rice is *Oryza sativa* L. and only a small amount of *Oryza glaberrina*, a perennial species, is grown in Africa. The so-called "wild-rice" (*Zizania aquatica*) grown in the Great Lakes region of the United States, is more closely related to oats than to rice (Juliano, 1993). Cultivated *O. sativa* is divided into three subspecies: Indica, Japonica and Javanica. Indica varieties, which are by far the economically most important ones, are widely grown in Southeast Asia as they are adapted to the tropics and represent approximately 80% of the world production of rice.

World-wide 530 million tons of rice were produced in 1994 (FAO, 1995), mostly in developing countries like China, India, Indonesia and Bangladesh (IRRI, 1993). Human consumption accounts for 85% of the total production, thus, making rice the major staple for more than 2 billion people in Southeast Asia (IRRI, 1993).

Rice in its milled form, as it is consumed by most people, is characterised by a very low content of iron (between 0.2 mg and 2.8 mg/100 g rice) and by its very low bioavailability. The milled rice kernel consists exclusively of the endosperm, which is completely filled with starch granules and protein bodies, low in iron and vitamins. The embryo and the aleuron layer, the only sources of minerals and vitamins in the grain, are removed during rice milling process. Milling is an important prerequisite

for long term storage in tropical and subtropical areas. During this process the hull and the bran are removed. The bran, the most important part in this respect, consists of the fruit and seed coat (pericarp and tegmen), as well as of the aleuron layer. The oil in the aleuron layer tends to turn rancid during storage even at moderate temperatures, which makes the grain inedible.

Since rice represents up to 80% of the daily calorie intake in Southeast Asia, an increase of the iron content in the rice endosperm tissue and an improvement of its bioavailability in order to optimise the daily iron intake and absorption in people predominantly living on rice would be highly desirably.

1.9 Ferritin, a tool to improve the iron content in the rice endosperm

A sustainable strategy to combat iron deficiency is amplification of the natural iron stores in existing commodities. Before determining the traits to be amplified, a survey was made of how nature provides storage of iron for developing organisms. The results shows that both plants (seeds, leaves and leguminous nodules) and animals (eggs, liver, red blood cells) use ferritin as the form of iron storage during development. Plant ferritin is composed of 24 subunits (Lobreaux, *et al.*, 1992) which together can store up to 4000 iron atoms in the central cavity of the ferritin complex (Korcz and Twardowski, 1992).

The concept to use ferritin as nutritional source of iron is not new. Earlier studies (Baynes and Bothwell, 1990; Derman, *et al.*, 1982; Hallberg, 1981; Layrisse, 1975) concluded that ferritin iron was relatively ineffective as a nutritional iron source, except when ingested with ascorbate (Derman, *et al.*, 1982). However, a recent revaluation of the results obtained so far concludes that increasing seed ferritin, the natural iron store, by biotechnology and breeding holds promise as sustainable solution to global dietary iron deficiency (Theil, *et al.*, 1997).

Seed ferritin is a suitable solution to enhance dietary iron for many reasons. First, ferritin is the natural iron source for normal development in plants and animals. Second, ferritin seed content appears to be under accessible genetic control. Third,

recent revaluation of dietary iron sources in rats made anaemic by dietary iron deficiency indicates that ferritin iron is bioavailable. Finally, there should be few if any cultural or culinary barriers to the use of seeds enriched in ferritin (Theil, *et al.*, 1997).

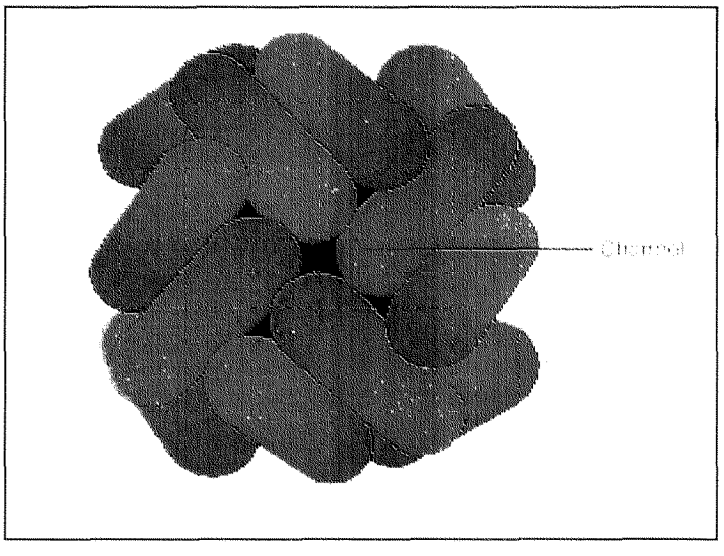


Figure 2: Structure of ferritin. Schematic representation of the 24-polypeptide assembled ferritin. Channels through the coat protein enable iron movement to the inside cavity (Briat and Lobreaux, 1997).

Recently, there have been reports demonstrating novel genetic transformation of iron-holding protein genes such as human lactoferrin and bacterial haemoglobin (VHb). Mitra and Zhang (Mitra and Zhang, 1994) transformed tobacco to express human lactoferrin, which had the effect of increased antibacterial activity for the host plant. Holmberg (Holmberg, *et al.*, 1997) introduced the gene for VHb into tobacco and observed enhanced growth of the transformants as well as changes in the chlorophyll content and the ratio of nicotine to anabasine. However, there was no mention of an increased capacity of the transformants to store iron.

High iron-content transgenic tobacco plants have been produced by transfer via *Agrobacterium tumefaciens* of soybean ferritin cDNA under control of a CaMV 35S promoter (Goto, *et al.*, 1998). The protein was expressed in the tissues of leaves

and stems and the iron content of transformant leaves was approximately 30% higher than in leaves from non-transformants. In this way, the potential of breeding high iron content crops by introduction of the ferritin gene has been demonstrated.

Plant ferritin cDNA have been cloned from many sources, including soybean hypocotyl (Ragland, *et al.*, 1990), soybean cell suspensions stimulated by iron starvation (Lescure, *et al.*, 1991), iron-induced maize roots (Lobreaux, *et al.*, 1992), young pea seed (Lobreaux, *et al.*, 1992), early expansion leaves of cowpea (Wicks and Entsch, 1993) and French bean seed (Spence, *et al.*, 1991). However, ferritin cDNAs could not be cloned from mature leaves of non-iron-induced plants, in which ferritin was barely detectable (Lobreaux and Briat, 1991). Recent studies have demonstrated that plant ferritin subunits encoded in the nucleus are synthesised in the cytoplasm with a transit peptide (TP), and subsequently transported into plastids such as chloroplats and amyloplasts (Van Der Mark, *et al.*, 1983; Sczekan and Joshi, 1978; Ragland, *et al.*, 1990; Waldo, *et al.*, 1995). Following transport, TP is cut off from ferritin subunit precursor and subunits are assembled into the mature protein (Ragland, *et al.*, 1990).

It is interesting to note the wide differences seen in the whole-plant partitioning of micronutrients between vegetative and reproductive tissues. Iron deposition in seeds represents 75% of total shoot Fe in pea, whereas only about 4% of shoot Fe is partitioned to grains in rice (Grusak, *et al.*, 1999). Clearly in rice, sufficient shoot iron is already in place to generate as much as 10-fold increase in seed Fe content.

1.10 Phytic acid, the major inhibiting factor for iron absorption

Phytic acid, myoinositol hexaphosphate, is a naturally occurring compound formed during the maturation of seeds and cereals grains. In the seeds phytin, a mixed potassium, magnesium, and calcium salt of phytic acid, is usually found in organelles called protein bodies, where it generally constitutes an inclusion, the globoid (Pernollet, 1978). In cereals these organelles are localised in the aleuron layer.

Seed germination is accompanied by an increased phytase activity, and phytic acid hydrolysis releases phosphate and free myoinositol for the use during plant development (Graf, 1986). Phytic acid is the storage form of phosphorous in plants and is required for efficient germination of seedlings.

During food processing and digestion, inositol hexaphosphate can be partially dephosphorylated to produce degradation products, such as penta-, tetra-, and triphosphate, by the action of endogenous phytases, which are found in most phytic-acid containing seeds from higher plants (Sandberg, *et al.*, 1987). The hydrolysis of phytic acid in the human gastrointestinal tract is carried out by the action of phytases from three sources: dietary plant phytases, phytases from bacterial flora in the gut, and intestinal mucosal phytases (Williams and Taylor, 1985). Mucosal phytases do not seem to play a significant role in phytic acid digestion in humans, whereas the dietary phytases seems to be an important factor for phytic acid hydrolysis (Sandberg and Andersson, 1988).

The first report of a nutritional aspect of phytic acid was the inhibitory effect of phytic acid on calcium absorption by Harrison and Mellanby (Harrison, 1934). Since then, the influence of phytic acid on mineral absorption has been extensively studied.

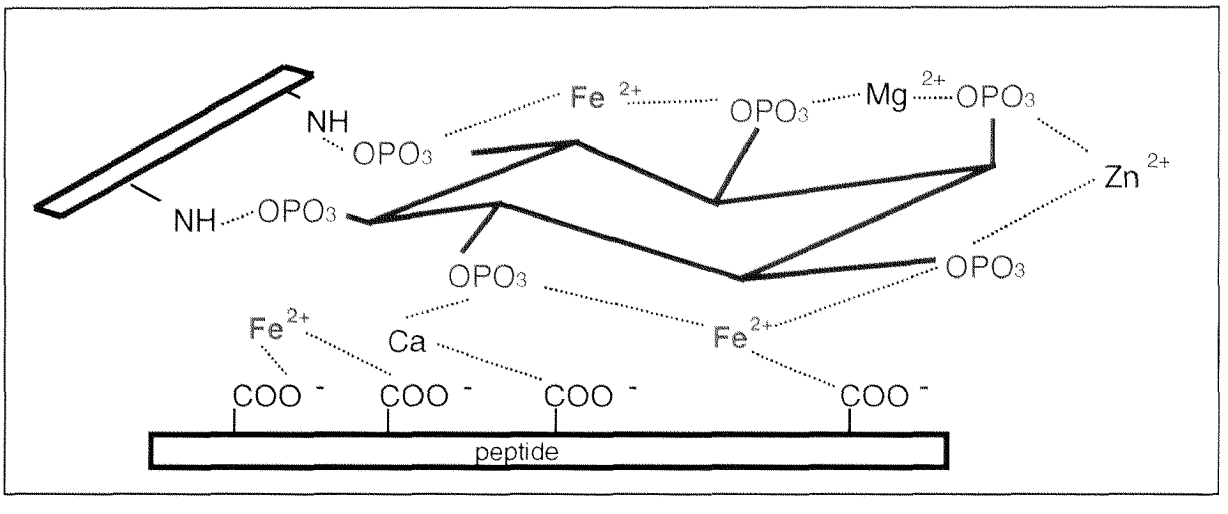


Figure 3: Complex formation during the digestion. Phytic acid binds minerals and peptides, strongly decreasing the iron bioavailability.

A half century ago, McCance (McCance, 1943) reported that phytic acid inhibited iron absorption. Later, numerous experiments were carried out to study the effect of phytic acid on iron absorption. Many human studies indicated that phytic acid had a very strong inhibitory effect on iron absorption (Brune, *et al.*, 1992; Hallberg, *et al.*, 1989; Tuntawiroon, *et al.*, 1990), which was due only to phytic acid and other inositol phosphates in bran and not to fiber and other constituents (Brune, *et al.*, 1992). Studies also revealed that the removal of phytic acid in bran by endogenous phytase significantly increased iron absorption, and the inhibition could be restored to a marked extent by restitution of the phytic acid content (Hallberg, *et al.*, 1987). Hurrell and co-workers (Hurrell, *et al.*, 1992) found that the reduction of phytic acid from soybean protein isolates resulted in increased iron absorption. Furthermore, phytic acid reduction in soybean and soy foods by fermentation has been shown to result in the prevention of iron-deficiency anaemia in children (Qin, 1989). The inhibition of non-haem iron absorption by phytate is dose-dependent and already with small amounts a strong inhibition is exerted (Hallberg, *et al.*, 1989).

In humans, iron absorption after *Aspergillus niger* phytase addition to the meal indicated that no inhibitors were present at the site of iron absorption, suggesting that effective and complete phytate degradation occurred in the stomach (Sandberg, *et al.*, 1996). This assumption was supported by previous results from in vitro studies of addition of *A. niger* phytase at pH and temperature conditions similar to that of the stomach, which showed a complete phytate degradation (Tuerk, 1992).

The expression of phytase in transgenic tobacco plants proved that the available phytase clone from *Aspergillus niger* is suitable for expression in higher plants, although it comes from a fungal origin. Phytase-expressing tobacco plants showing high content of phytase in the seeds (1% of soluble protein) were visually indistinguishable from non transgenic plants, and the constitutive expression of the enzyme did not affect the morphology nor the growth-rate. Similarly, germination was not affected by the presence of the enzyme. Analysis for phytase activity of seeds stored at 4°C, as well as at room temperature showed that no significant

decrease in the activity occurred over a period of a year, demonstrating that active phytase can be produced and stably stored in plant seeds (Verwoerd, *et al.*, 1995).

Most of the phytate and endogenous phytase in rice grains are deposited in the aleuron layer, the outermost cell layer of the endosperm, which is lost during milling and polishing of the rice grains. Therefore after these processes, almost no phytase is present in the milled rice for the hydrolysis of the remaining phytate. Endosperm (milled rice) contains only 0.02-0.07% of phytic acid, but even these relatively small quantities of residual phytate do strongly inhibit iron absorption. Phytic acid concentration has to be reduced to <0.01% before a meaningful increase in iron absorption can be observed (Hurrell, *et al.*, 1992).

Two cDNA encoding two different fungal phytases (the Q27L mutant from *Aspergillus fumigatus* and the *A. niger* phytase) were available for rice transformation. Phytase from *A. niger* shows two pH optima, one at 2.5-3.0 and one at pH 5.5, whereas *A. fumigatus* phyA enzyme catalyses phytic acid hydrolysis over the broadest pH range (2.5 to 8.0) of all known phytases. The Q27L mutant is a pointmutation of the *A. fumigatus* phytase, which results in a much higher specific activity. At pH 6.0 the wild-type *A. fumigatus* phytase has an activity of 30 U/mg (free µmol P/ min/ mg protein), whereas the pointmutant reveals an activity of 200 U. *A. fumigatus* phytase displays a high resistance to heat inactivation, similar to enzymes from hyperthermophilic organisms, as this enzyme has the ability to refold properly after denaturation. Investigation of the resistance of both phytases to heat inactivation revealed that even after 20 min of exposure to 100°C, *A. fumigatus* enzyme still retained 90% and *A. niger* 40% of their initial activity (Wyss, unpubl.). The glutelin promoter, responsible for the expression of the protein in the rice endosperm, ensures that even after milling of the rice, the fungal phytase will be present in the grains to catalyse the hydrolysis of phytate into inorganic phosphate and myoinositol.

1.11 A cysteine-rich polypeptide to potentially increase the iron bioavailability

Many studies confirmed the enhancing effect of muscle tissue on the iron absorption since Layrisse (Layrisse, *et al.*, 1968) first showed that the addition of veal to a meal doubled non-haem iron absorption.

In rat, studies using ligated duodenal segments *in vivo* found that histidine, ornithine, lysine and cysteine enhanced iron uptake, whereas methionine, glutamic acid, glutamine, glycine and norleucine have no effect (Van Campen and Gross, 1969; Van Campen, 1973). Studies in humans have shown that cysteine and peptides containing reduced cysteine, such as glutathione, enhance iron absorption from extrinsically and intrinsically labelled foods (Layrisse, *et al.*, 1984; Martinez Torres, *et al.*, 1981; Taylor, *et al.*, 1986). Thus, a common thread between rat and human studies is that cysteine enhances Fe absorption.

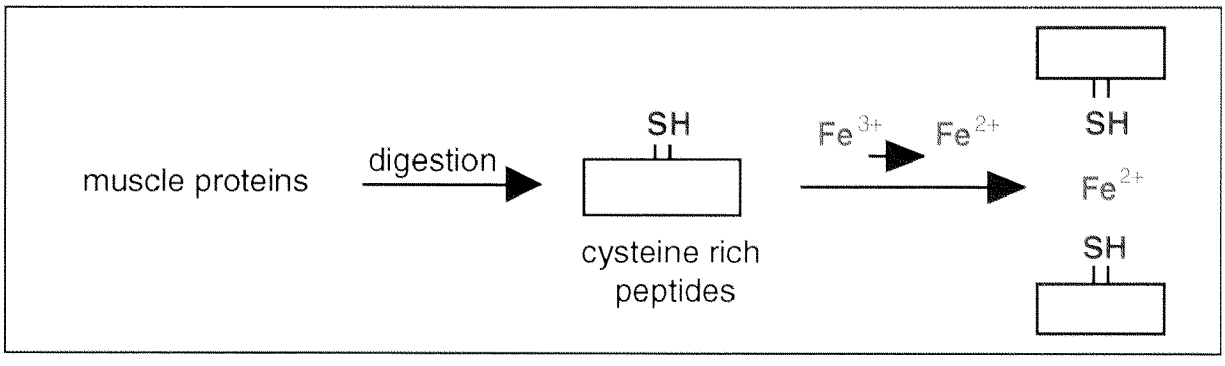


Figure 4: Enhancing effect of cysteine on the iron bioavailability. Cysteine rich peptides, after muscle protein digestion, reduce and bind iron in a soluble form, protecting it from other inhibitory factors present in the meal.

Cysteine is the only free amino acid to have an enhancing effect on iron absorption in man (Martinez-Torres and Layrisse, 1997) and at equivalent quantities of cysteine, free cysteine, glutathione or beef similarly increased iron absorption from a maize meal (Layrisse, *et al.*, 1984). The potential to chelate iron and, to reduce ferric iron (FeIII) to the more soluble ferrous iron (FeII) could explain the enhancing effect of cysteine-containing peptides on the iron absorption.

One of the possible approaches to increase iron availability is therefore the overexpression of a cysteine-rich protein in rice endosperm.

A group of cysteine-rich, low molecular weight polypeptides, are the metallothionein proteins (MTs) (for review: (Robinson, 1993). MTs are small, metal-binding proteins that are present in animals, fungi, cyanobacteria, and plants. They are encoded by genes inducible by high levels of metals (Palmiter, 1987) and are believed to play a role in the metabolism and detoxification of trace metals (Kagi, 1987).

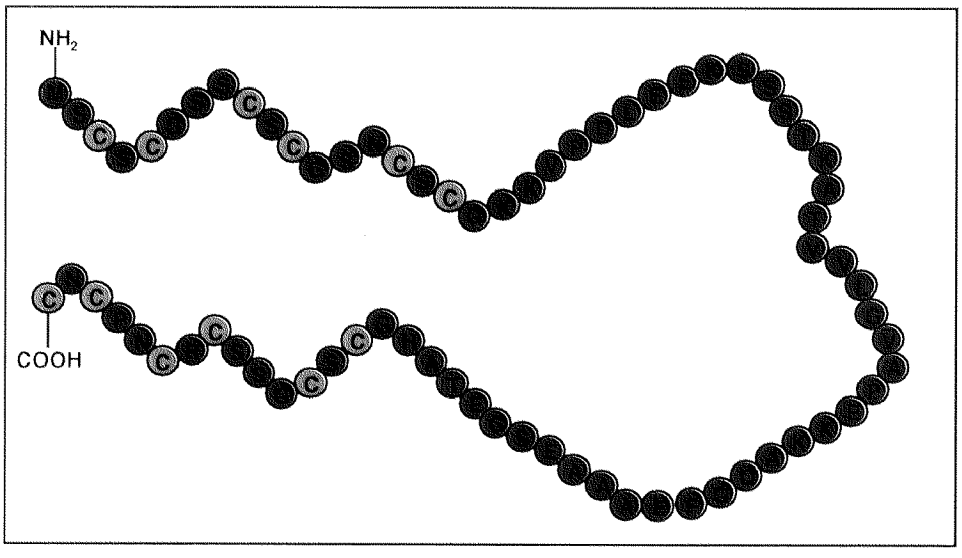


Figure 5: Putative structure of the metallothionein-like protein from *Oryza sativa*. Cysteine residues are shown in red.

Genes encoding MT-like proteins have been identified in wheat (Kawashima, *et al.*, 1992), maize (De Framond, 1991), barley (Okumura, 1991), rice (Hsieh, *et al.*, 1995), Arabidopsis (Zhou and Goldsbrough, 1994), pea (Evans, 1990) and soybean (Kawashima, *et al.*, 1991).

As the metallothionein-like protein (Hsieh, *et al.*, 1995) is already present in the rice grains, no side effects for the plant and for the grain quality are expected as consequences of its overexpression. Northern blot analysis of rice suspension cells after different metal ion treatment showed that there was no increased MT-like gene expression after 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 is not expected.

The availability of sulphur, which is needed for the biosynthesis of metallothioneins, is guaranteed, as sulphur deficiency is not widespread in rice farming (Patrick *et al.*, 1985).

1.12 Importance of the project

Iron deficiency is a global problem afflicting an estimated 30% of the world's total population. The negative effects of iron-deficiency anaemia on work efficiency have a large economic impact. The World Bank estimates that pregnancy-related problems account for 18 per cent of healthy years of life lost in women aged between 15 and 44, more than any other single cause (Seymour, 1996). That loss cuts deep into a country's resources, because in the developing world women provide most of the unpaid labour and one third of the wage labour. The cost benefit of alleviating iron deficiency in the developing countries has been analysed by Levin, who estimated that with each 10% increase in haemoglobin concentration, a 15% improvement in worker productivity of anaemic labourers can be expected (Levin, 1986). As the affected persons are usually the family money earner, the economic impact of iron deficiency on the family and society may be far greater than is immediately apparent (Levin, 1986).

Defect in learning capacity and cognitive performance associated with iron deficiency anaemia during the first years of life (Pollitt, 1993) also have social and economic effects of enormous proportions (Beard, *et al.*, 1996).

Anything that increases the survival of pregnant women and the nutrition quality in infants can have a massive impact on a country's productivity.

If improved iron nutrition can reduce misery and mortality among millions of people in South and Southeast Asia, we should consider this project as a humanitarian effort under the sponsorship of industrialised and developed countries.

1.13 Aim of the work

For various reasons, none of the strategies for reducing micronutrient malnutrition has been very successful in decreasing the prevalence of iron deficiency anaemia in developing countries.

Increasing seed ferritin, the natural iron storage protein found in animals, plants and bacteria, had been suggested as a means to increase the iron content of cereals and, therefore, to improve the iron nutrition in cereal-eating population (Theil, *et al.*, 1997).

Increasing iron intake, however, 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. The major inhibitor phytic acid can readily be degraded in cereal and legume foods by activation of the native phytases or addition of exogenous phytases either during food processing (Sharma and Kapoor, 1996) or during digestion (Sandberg, *et al.*, 1996), increasing iron absorption dramatically (Hurrell, *et al.*, 1992). In the same way, muscle tissue, through the action of the cysteine-containing peptides formed on digestion (Layrisse, *et al.*, 1984), and ascorbic acid will enhance iron absorption from cereal based meals (Cook, *et al.*, 1997).

The aim of this work was to increase the iron bioavailability from rice. As first step, the iron storage capacity of the rice endosperm was to be improved. For this purpose, the ferritin protein of *Phaseolus vulgaris* was to be expressed into the rice grains. Moreover, two approaches to potentially increase iron absorption in humans were taken into consideration. First, to reduce the level of phytic acid, the main inhibiting factor of iron absorption, a fungal phytase had to be introduced into the rice endosperm. Second, to increase the amount of the absorption-enhancing amino acid cysteine, a cysteine-rich protein, the metallothionein-like protein from *Oryza sativa*, had to be overexpressed in the grains.

The sequences encoding the genes of interest were to be combined with an endosperm specific promoter, the glutelin promoter (Gt1) (Okita, *et al.*, 1989), so

that the novel characters will be exclusively in the rice grains. Even after rice milling, the three proteins will be present in the edible part to improve the iron content and to potentially increase the iron bioavailability from a rice-based meal.

Transgenic rice plants should be obtained in order to analyse whether the introduced genes have a potential for improving iron nutrition in rice eating populations.

2 MATERIALS and METHODS

Table of buffers

F1 extraction buffer	50 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 1 mM PMSF
F2 extraction buffer	50 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 1 mM PMSF, 0.1% o-phenantroline
Lysis buffer	25 mM Tris-HCl, pH 7.5, 25 mM Na_2EDTA, 0.5 mg/ml lysozyme, 0.1 mg/ml RNase, 10 % v/v glycerol and bromophenol blue
MAE (10x)	0.2M MOPS, 50 mM Na-acetat, 10 mM EDTA
Neutralisation buffer	0.5 M Tris-HCl, pH 7.2, 1.5 M NaCl, 1 mM Na EDTA
PBS-T (1x)	20 mM NaH_2PO_4, 80 mM Na_2HPO_4, 100 mM NaCl, 0.1% Tween 80
RNA denaturation buffer	1x MAE, 1.85% formaldehyd, 50% formamide
RNA sample buffer	1 x MAE buffer saturated with bromphenolblue/ glycerol 1:1
TAE	40 mM Tris-acetate, 1 mM Na_2EDTA, pH 8.0

SSC (1x)	15 mM sodium-citrate, 150 mM NaCl, pH 7.6

Table of media

LB (liquid)	1% bacto tryptone (Difco), 0.5% bacto yeast extract (Difco),1% NaCl
LB (solid)	LB liquid supplemented with 1.5 % agar (Difco, Detroit)
MS salts	(Murashige, 1962) 0.025 mg/l $CoCl_2$, 0.025 mg/l $CuSO_4$, 36.7 mg/l FeNaEDTA, 6.2 mg/l H_3BO_3, 0.83 mg/l KI, 16.9 mg/l $MnSO_4$, 0.25 mg/l Na_2MoO_4, 8.6 mg/l $ZnSO_4$, 332.02 mg/l $CaCl_2$, 170 mg/l KH_2PO_4, 1900 mg/l KNO_3, 180.54 mg/l $MgSO_4$ and 1650 mg/l NH_4NO_3 (Duchefa, The Netherlands)
MS vitamins	(Murashige, 1962) 2 mg/l glycine, 100 mg/l myo-inositol, 0.5 mg/l nicotinic acid, 0.5 mg/l pyridoxine HCl and 0.1 mg/l thiamine HCl (Duchefa, The Netherlands)
MS1 medium	MS salts and vitamins, 3% sucrose, 2 mg/l 2,4-D, 50 mg/l cefotaxime (Duchefa, The Netherlands), and 0.35% agarose (Type I, low EEO, Sigma, St, Louis, MO)
MS2 medium	MS vitamins, 10 % sucrose, 2 mg/l 2,4-D and 0.4 % agarose, pH 5.8
MS rooting medium	MS salts, 1.5% sucrose and solified with 0.3% gelrite (Duchefa)

R2 salts	(Ohira, 1973) 1012.5 mg/l KNO_3, 62.5 mg/l $MgSO_4$, 82.5 mg/l $(NH_4)_2SO_4$, 68.75 mg/l NaH_2PO_4, 0.55 mg/l $ZnSO_4$, 0.5 mg/l $MnSO_4$, 0.05 mg/l $CuSO_4$, 37.5 mg/l $CaCl_2$, 0.715 mg/l H_3BO_3, 0.0325 mg/l Na_2MoO_4, 3.1 mg/l $FeSO_4$, 4.175 mg/l Na_2EDTA
R2I	R2 salts, MS vitamins, 100 mg/l inositol, 2 mg/l 2,4-D, 6 % sucrose, 0.5 % agarose, 30 mg/l hygromycin and 50 mg/l cefotaxime, pH 5.8
R2r culture medium	R2 salts, 1 mg/l 2,4-D, 3 % sucrose, 1 mg/l thiamine, pH 5.8
Rzs regeneration medium	R2 salts, MS vitamins, 2% sucrose, 3% sorbitol, 1 mg/l zeatin (Duchefa, The Netherlands), 0.5 mg/l IAA (Sigma), 30 mg/l hygB and solified with 0.65% agarose (Sigma)

Media for protoplast culture and transformation

Oc-medium	4.4 g/l MS salts and vitamins, 6% sucrose, 2.5 mM 2(N-morpholino)ethanesulfonic acid (MES), 2 mg/l 2,4-D, pH 5.6
Enzyme solution	3% (w/v) Cellulase "Onozuka" RS (Yakult Inc., Japan), 1.0% (w/v) Macerozyme, 1mM $CaCl_2$ and 8% (w/v) mannitol, pH 5.8

KMC	116 mM KCl, 85 mM $CaCl_2$, 79.5 mM $MgCl_2$ and 0.5% MES, pH 5.8
Sucrose solution	0.5M sucrose, 0.1% MES, pH 5.8
MMM	15 mM $CaCl_2$, 0.1% MES, 0.5 M mannitol, pH 5.8
PEG solution	40% (w/v) PEG 4000 (Merck), 400 mM mannitol, 100 mM $Ca(NO_3)_2$ and 0.1% MES, pH 7.0
K3 solution	250 mM KNO_3, 30 mM NH_4NO_3, 10 mM NaH_2PO_4, 60 mM $CaCl_2$, 10 mM $MgSO_4$, 10 mM $(NH_4)_2SO_4$

Media for *Agrobacterium* culture and *Agrobacterium*-mediated rice transformation

AB buffer (20x)	6% K_2HPO_4, 2% NaH_2PO_4
AB salts (20x)	2% NH_4Cl, 0.6 % $MgSO_4$, 0.3% KCl, 0.02% $CaCl_2$, 50 mg/l $FeSO_4$
AB medium (1x)	50 ml/l AB buffer, 50 ml/l AB salts, 5 g/l glucose
CHU(N6) medium	Micro and Macro elements including vitamins (Duchefa, The Netherlands)
Cocultivation medium	CHU(N6) medium, 500 mg/l proline, 1 g/l enzymatic caseinhydrolysate, 3% sucrose, 1% glucose, 2 mg/l 2,4-D, 300 µM acetosyringone, 0.6% agarose, pH 5.2

Induction media	CHU(N6) medium, 500 mg/l proline, 1 g/l enzymatic caseinhydrolysate, 7% sucrose, 3.6% glucose, 300 µM acetosyringone, pH 5.2
MA1 medium	CHU(N6) medium, 500 mg/l proline, 1 g/l enzymatic caseinhydrolysate, 2 mg/l 2,4-D, 30% sucrose and solidified with 0.4% agarose
Proliferation medium	CHU(N6) medium, 500 mg/l proline, 1 g/l enzymatic caseinhydrolysate, 6% sucrose, 2 mg/l 2,4-D, 30 mg/l hygromycin, 250 mg/l cefotaxime, 0.6% agarose, pH 5.6)
Selection medium	CHU(N6) medium, 500 mg/l proline, 1 g/l enzymatic caseinhydrolysate, 3% sucrose, 2 mg/l 2,4-D, 30 mg/l hygromycin, 250 mg/l cefotaxime, 0.6% agarose, pH 5.6
YEB (liquid)	1 g/l bacto yeast extract, 5 g/l bacto beef extract, 1 g/l bacto peptone, 5 g/l sucrose, 240 mg/l $MgSO_4$, pH 7.2
YEB (solid)	liquid YEB solified with 1.5 % agar

2.1 Microbiological methods

2.1.1 Escherichia coli

Escherichia coli strains XL-1 blue (Stratagen, La jolla, Ca) were used for production of recombinant plasmid DNA molecules. *E. coli* were grown at 37°C either on solid

or in liquid LB broth. Liquid cultures were grown on a shaker at 250 rpm in test tubes for pre-culture or in Erlenmeyer flasks for plasmid isolation.

E. coli transformed with pUC19- (Stratagene, La Jolla) or pCAMBIA-derivatives (Cambia, Canberra, Australia) were grown in media supplemented with 100 µg/ml ampicillin (ampicillin sodium salt, Fluka, Switzerland).

Preparation of competent cells of *E. coli*

E. coli cells competent for uptake of foreign plasmid DNA were produced using a calcium chloride procedure (Sambrook, *et al.*, 1989).

Transformation of competent *E coli*

1 ng of plasmid DNA or ca. 100 ng ligation products was added to 100 µl competent *E. coli* cells immediately after thawing of the frozen bacteria on ice. The suspension was carefully mixed and incubated on ice for 40 min. A heat shock of 42°C for 90 s was applied, followed by an incubation on ice for 5 min. 500 µl of LB broth were added and the bacterial suspension was incubated at 37°C and 100 rpm for 60 min. Aliquots of the suspension were spread on solid LB media supplemented with the selective agent. The bacteria were incubated at 37°C overnight.

Storage of bacteria

For short-term storage, bacteria were grown on solid LB supplemented with ampicillin and stored at 4°C.

For long-term storage, single colonies of bacteria were picked and inoculated into LB supplemented with ampicillin as the selective agent. After overnight growth, 625 µl of the bacteria suspension were added to 375 µl 40% glycerol to bring the final

concentration to 15%. The bacteria were quick-frozen in liquid nitrogen and stored at –80°C.

2.1.2 Agrobacterium tumefaciens

Agrobacterium tumefaciens strain LBA 4404 (Hoekema, 1983) containing a disarmed Ti-plasmid was used for *Agrobacterium*-mediated gene transfer to *Oryza sativa*.

Preparation of competent cells

Agrobacteria were grown in 50 ml YEB medium at 28°C under shaking 250 rpm. When its OD600 reached 0.5, the culture was put on ice for 30 min. The bacteria were collected, suspended in 10 ml 0.5 M NaCl, centrifuged and resuspended in 1 ml of pre-cooled 20 mM $CaCl_2$. 50 µl aliquots were transferred into 1.5 ml Eppendorf tubes, frozen in liquid nitrogen and stored at –80°C.

Transformation of competent *Agrobacterium tumefaciens*

0.5 – 1 µg plasmid was added into thawed competent Agrobacteria and gently mixed. The mixture was incubated on ice for 30 min, frozen in liquid nitrogen for 1 min, and thawed in 37°C water bad for 5 min. 1 ml YEB medium was added and cultured at 28°C for 2 – 4 hours on a shaker with 250 rpm. Aliquots were applied onto agar medium containing 100 mg/l rifampicin (Duchefa, Haarlem, NL) and 50 mg/l kanamycin (kanamycin sulphate, Duchefa, Haarlem, NL) for the selection of the transformed bacteria and incubated at 28°C.

2.2 Isolation of plasmid DNA

Single colony plasmid DNA isolation from *E. coli*

A rapid and simple method was used to isolate very small amounts of plasmid DNA from a single bacterial colony in order to determine the success of plasmid ligations (Rusconi, pers. comm.). Overnight-grown colonies were picked with plastic inoculation loops, streaked out on a masterplate and incubated in 12 µl lysis buffer. The bacteria were lysed for 5 min and 2 µl phenol/chloroform (1:1, v/v) were added. The mix was vortexed for 30 s and centrifuged at 12,000 rpm in a minicentrifuge (Hettich, Tuttlingen, FRG) for 3 min. The supernatant was examined by gel electrophoresis and compared with the original plasmid. The masterplate was incubated over-night at 37°C.

Small-scale plasmid DNA isolation

Small amounts of DNA suitable for restriction analysis and molecular cloning were isolated by a small-scale preparation, using the Quiagen "Mini"-DNA-isolation kit (Quiagen, Hilden, FRG), following the manufacturers protocol.

Large-scale plasmid isolation

To obtain plasmid DNA amounts in the mg range for gene transfer experiments, large volumes (400 ml) of bacteria suspensions were lysed by alkaline treatment, and the nucleic acids purified by column chromatography (Quiagen, Hilden, FRG). For the lysis and purification the plasmid Maxi Protocol of Quiagen was followed.

2.3 Enzymatic manipulation of DNA

DNA was cleaved by restriction endonucleases (Boehring, Mannheim, FRG, New England Biolabs, Beverly, MA, GibcoBRL, Gaitersburg, MD) according to the instruction of the manufacturer.

DNA fragments were ligated overnight at 20°C with T4 DNA ligase (New England Biolabs) in the appropriate buffer provided by the manufacturer. Control treatments included incubation of vector alone without and with ligase to monitor vector linearization and vector religation, respectively.

5' phosphate residues of linearised vector DNA molecules used for molecular cloning were removed to prevent religation of the molecule without an insert DNA fragment. Calf intestine phosphatase (CIP, Boehringer) was used for this purpose. Before CIP incubation restriction enzymes were deactivated. DNA molecules were incubated at 37°C for 1 h. CIP was inactivated by heat treatment at 75°C for 10 min (Ausubel, 1994).

2.4 DNA fragment isolation

DNA molecules were separated by horizontal gel electrophoresis in TAE buffer. For analytical purposes and for DNA fragment isolation, agarose (Sigma chemicals, St. Louis, USA) and low melting agarose (Sigma chemicals, St. Louis, USA) were used. Ethidium bromide was added to the gel to a final concentration of 0.1 μg/ml.

Linear DNA fragments used for further enzymatic treatment were isolated from agarose gels after electrophoresis. Agarose slabs containing the desired DNA fragment were excised with a scalpel blade under UV light, keeping the exposure to UV irradiation at a minimum. Excess agarose was removed. DNA fragments were isolated by melting the agarose in a high salt buffer and subsequently binding the DNA to glass particles (Geneclean, Bio 101 Inc.). DNA fragments shorter than 8000

bp were isolated after centrifugation of the agarose slabs at 12000 rpm for 10 min with the agarose spin columns (Gen Elute ^{TM} ,Supelco, Bellenfonte, USA).

Small DNA fragments were separated by 5% nondenaturing polyacrylamide gel electrophoresis (Sambrook, *et al.*, 1989).

2.5 Plasmid development

2.5.1 Constructs for protoplast transformation

For the transient transformation of protoplasts with the fungal phytases, three constructs were used, all containing the cauliflower mosaic virus (CaMV) 35S promoter. This promoter gives constitutive expression throughout all rice tissues (Terada and Shimamoto, 1990; Battraw and Hall, 1990).

Plasmid C4GL$_{m2}$BgNdBGILP (J. Fütterer, ETH Zürich, Switzerland) contains the uidA gene under control of the CaMV35S promoter. For a better expression, a part of the intron and untranslated leader sequence from rice tungro bacilliform virus (RTBV) was fused to the promoter. The signal peptide from the β-glucanase (Leah, *et al.*, 1991) was also present in this construct, allowing the secretion of the fungal protein. Using PCR, a *Hind* III site was introduced at the ATG and a *Pst* I site was introduced downstream of the stop codon present in the full length phytase clones isolated from *Aspergillus niger* (Mullaney, *et al.*, 1991, Van Hartingsveldt, *et al.*, 1993) and from *Aspergillus fumigatus* (Pasamontes, *et al.*, 1997; Tomschy, *et al.*, unpubl.).

After *Hind* III / *Pst* I digestion of C4GL$_{m2}$BgNdBGILP, the Uid A gene was removed and replaced with the phyA genes from *A. niger* and *A. fumigatus*, resulting in plasmid pPhyNC and pPhyFC, respectively.

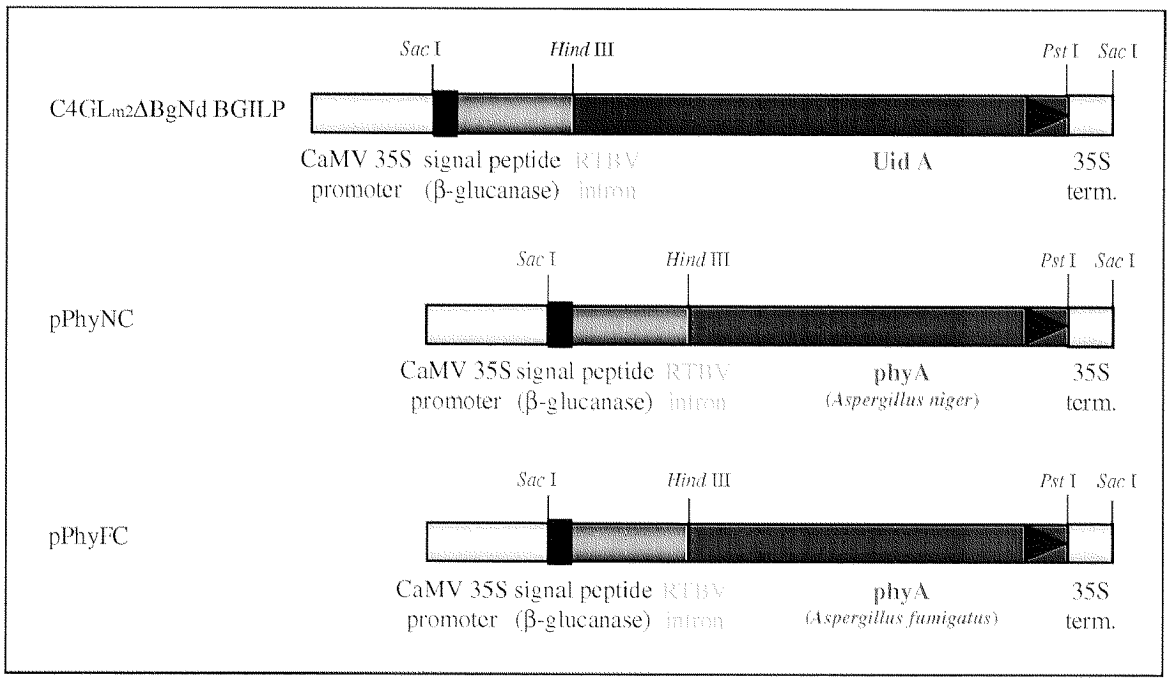


Figure 6: Plasmids used for transient transformation of protoplasts.

2.5.2 Constructs for biolistic transformation

All the constructs made use of a rice glutelin promoter (Gt1, (Okita, *et al.*, 1989) that guarantees a high level of specific expression in the rice endosperm (Kim, *et al.*, 1993; Zheng, 1993). The origin of the Gt1 promoter was plasmid pKS1 (a generous gift of T. Okita, Washington State University, WA) containing the Gt1 promoter inserted into vector pBSK (Stratagene, La Jolla, CA). pKS1 was digested with *Bgl* II and *Eco* RI and the isolated Glutelin promoter was inserted into vector V34. Vector V34 carried a pUC19 plasmid backbone, lacking the lacz-region and the polylinker cloning site. Instead it included a self-designed polylinker sequence and two different Scaffold Attachment Regions (SAR); one from Petunia and the other one from soybean, which are supposed to increase gene expression in transformed plant cells (Allen, *et al.*, 1993). SAR DNA elements play an important role in the spatial organisation of DNA and in the regulation of transcription resulting in a high-level of the transgene expression (Allen, *et al.*, 1993; Allen, *et al.*, 1996).

As the selection system is based on hygromycin B, a hygromycin phosphotransferase expression cassette (35S-prom/*hph*IV/35S-term) was introduced into all plasmids used for rice transformation. The *hph*-cassette was excised from pCIB900 (Wunn, *et al.*, 1996) with *Sal* I and *Sac* I and introduced into plasmid V34 containing the glutelin promoter, and resulting in vector V34-Gt1.

Using PCR, two Sac I sites were introduced at the ATG and downstream of the stop codon present in the full length ferritin clone (pfe) isolated from French bean, *Phaseolus vulgaris* L. cv. Tendergreen (Spence, *et al.*, 1991) and in the metallothionein-like clone (rgMT) isolated from *Oryza sativa* genomic library (Hsieh, *et al.*, 1995). After *Sac* I digestion, the pfe and the rgMT genes were introduced into the vector containing the glutelin promoter and the hygromycin phosphotransferase expression cassette resulting in vector pGt1Fe and pGt1Me respectively.

The chimeric phytase genes obtained after *Sac* I digestion of plasmids pPhyNC and pPhyFC were separately introduced into vector V34-Gt1, resulting in pGt1PN and pGt1PF respectively.

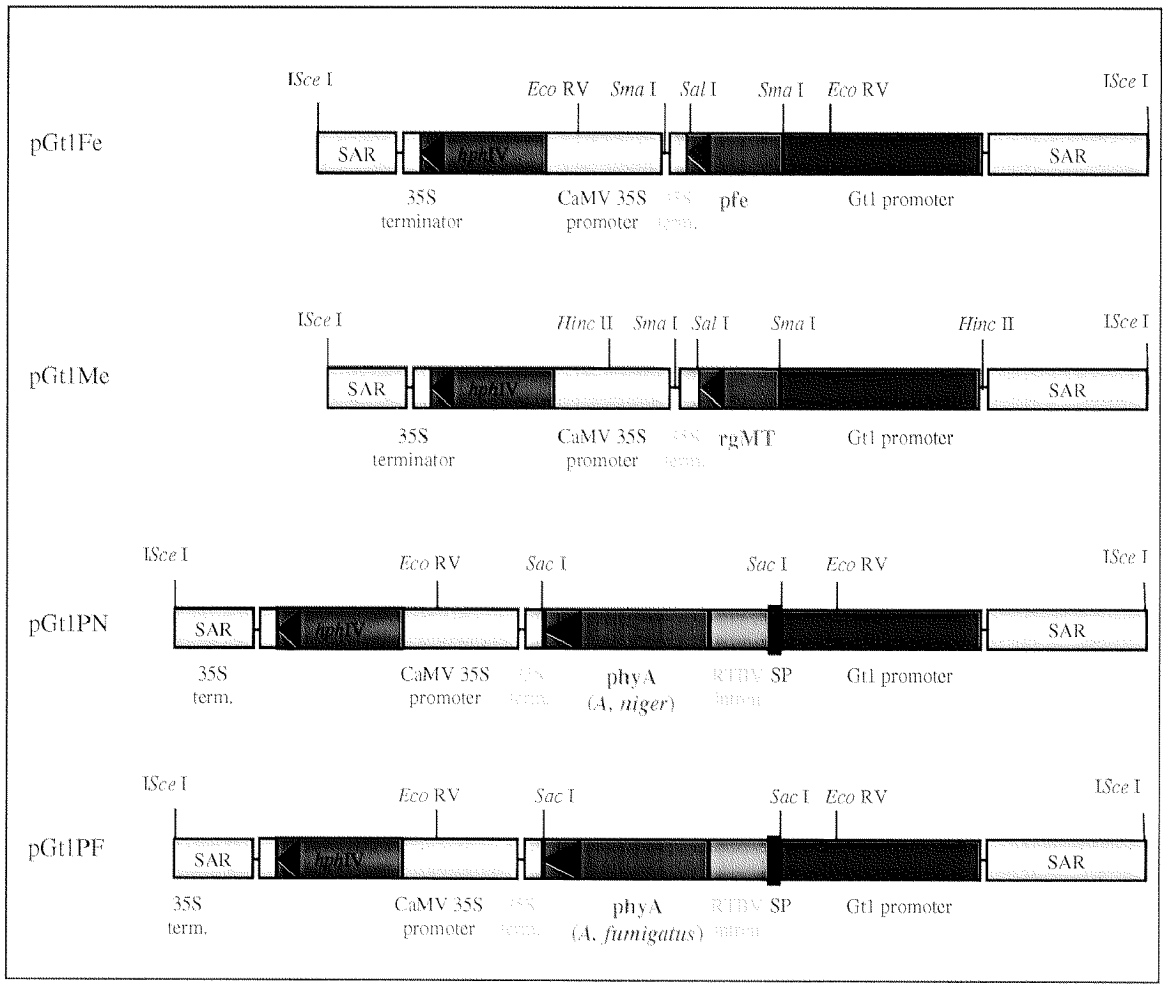


Figure 7: Gt1 promoter constructs for biolistic transformation of calli derived from immature embryos and rice cell suspensions.

2.5.3 Constructs for *Agrobacterium*-mediated transformation

For stable, *Agrobacterium* mediated transformation of rice mature embryos two plasmids were constructed, containing the pfe and the rgMT genes.

After *Bgl* II/ *Hind* III digestion of plasmid pKS1, the isolated Glutelin promoter was introduced into pCAMBIA 1390 in the opposite direction of the hygromycin phosphotransferase expression cassette (35S-prom/*hph*II/35S-term).

Plasmid pGt1Fe and pGt1Me were digested with *Sma* I. After gel electrophoresis, the fragments were eluted and introduced downstream of the glutelin promoter by

sticky end ligation with the 8.60 kb *Sma* I fragment from pCAMBIA 1390 vector. This created the plasmid pAGt1Fe and pAGt1Me.

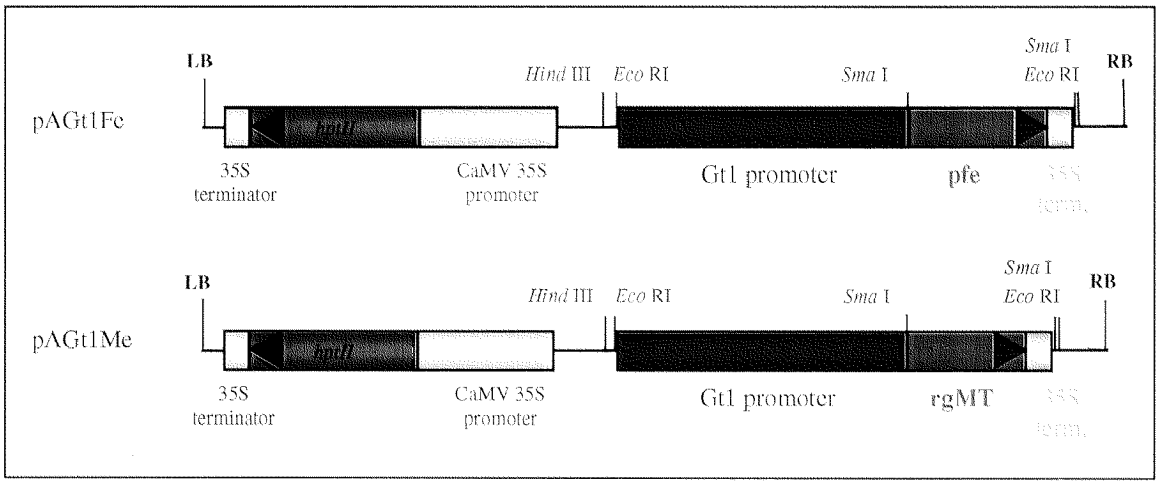


Figure 8: Gt1 promoter plasmids for *Agrobacterium*-mediated transformation of calli derived from mature embryos.

2.6 Tissue culture

2.6.1 Plant material

Rice plants (*Oryza sativa*) japonica (Taipei 309) cultivar were grown in a greenhouse at 28°C (day) and 21°C (night) temperatures. The photoperiod was 12 h light supplemented with 400 W fluorescent lamps (MT 400 DL/BH, 400 W mercury lamp, Iwasaki, Tokyo, JP) if a minimum of 100,000 lx sunlight measured outside the greenhouse was not reached during the day. The humidity was set to 80% and 60% during the day and night, respectively. Three rice plants were grown together in one hydroponics pot (12/18, Migros, Zürich, Switzerland) filled with 0.25 l Perlite and 2.5 l sterilised, sieved soil. The soil was supplemented with 1 g/l initial fertiliser (Plantamaag 4 D, NPKMg 20-10-15-3.6, Maag AG, Dielsdorf, Switzerland), 1 g/l long time fertiliser (Nutricote, NPK 16-10-10, Maag AG, Switzerland), and 0.25 g/l

NaFeEDTA. The rice plantlets were transferred into these pots when they had reached an age of three weeks. Initially they were germinated and grown under sterile conditions on solid half streng MS medium without hormones in a culture chamber (WIESS, Reiskirchen, FRG) at 30°C. After one week the seedling were transferred to the greenhouse and planted into open pots filled with sterilised soil without fertilisers. After another two weeks plantlets were transferred to the hydroponics pots as described above.

2.6.2 Protoplasts

50 ml rice suspension Oc-cells were harvested after 5 days of culture in Oc-medium sedimented and transferred to a 140 mm plastic petri dish. 50 ml of enzyme solution were added to the cells and the mixture was incubated in the dark for 16 - 20 at 27°C under shaking at 30 rpm.

A 100 µm and a 50 µm sieves (Saulas, St. Louis, Paris, France) were autoclaved in a plastic box and were prerinsed with KMC immediately before use. The incubated protoplast-enzyme suspension was passed through 100 and 50 µm sieves and collected by centrifugation (800 rpm, 10 min). Protoplasts were resuspended in 10 ml sucrose solution, overlaid with 1 ml KMC and after centrifugation at 1000 rpm for 10 min, the protoplasts floating at the interphase were collected with a wide tip pipette. After washing with 10 ml KMC, the cells were collected and resuspended in 1 – 2 ml MMM at a density of 2.0 x 10^6 intact protoplasts per ml.

As cells tend to aggregate, protoplasts were use as soon as possible for transformation.

2.6.3 Isolation and culture of mature zygotic embryos

Mature seeds were dehusked and sterilised by rinsing in 70% ethanol for 2 min and subsequently incubating in 6% $Ca(ClO)_2$, 0.1% (v/v) Tween 80 for 20 min followed

by washing with sterilised water for three times. After incubation at 30°C for 5 days on MA1 medium, the embryos were dissected from the endosperm without hurting the scutellum, the emerged coleoptiles were removed and the embryo was placed scutellum-up on fresh medium. Subcultures were carried out at weekly intervals for a total of 2-3 weeks. This tissue was used for *Agrobacterium*-mediated transformation.

2.6.4 Isolation and culture of immature zygotic embryos

Immature caryopses were collected between 10 – 12 DAP. The caryopses were surfaced-sterilised as described above. Embryos were squeezed out of the caryopsis after cutting off the ventral end with a scalpel and placed scutellum side up on MS1 medium. The embryos were cultured at 30°C for 4-6 days in the dark. Callus produced from the scutellum was selected and subcultured. This tissue was used for microprojectile bombardment or for preparation of rice suspension cells.

2.6.5 Embryogenic cell suspension culture

Immature zygotic embryos of rice cultivar TP 309 were precultured for 2 weeks on MS medium as described before. Five to ten of the large embryo-derived calli were transferred to 25 ml liquid R2r culture medium. Cultures were maintained at 30°C on a rotary shaker at 90 rpm. Subcultures were carried out at weekly intervals to obtain meristematically active, embryogenic cell clusters. These embryogenic cell suspensions were used for transformation by particle bombardment.

2.7 Transformation

2.7.1 Protoplast transformation

For transformation 10 µg plasmid DNA were added to 0.3 ml aliquots of protoplast suspension (6.0 x 10^5 intact protoplasts) in a 13 ml centrifuge tube and left for 2 min at RT. An equal volume of PEG solution was added drop-wise and gently mixed with the protoplast suspension. The mixture was then left at room temperature for 5 min with sporadic agitation. To stop the transformation, 6 ml K3 solution were added step-wise over a time period of 10 min. Protoplasts were pelleted by centrifugation, resuspended in 2 ml K3 medium and incubated overnight for expression of the transgene at 25°C in the dark.

2.7.2 Biolistic gene transfer

DNA-coated gold particles (gold powder spherical, 1.5-3 µm diameter, Aldrich, Buchs, CH) were used for transformation. Prior to coating particles were washed with water and sterilised in 50% glycerol at a concentration of 50 µg/ml. 50 µl particle suspension and 5 µg of linearised plasmid DNA were mixed and vortexed at full power. After addition of 50 µl 2.5 $CaCl_2$ and 20 µl 0.1 M spermidine, the particles were strongly mixed for 3 min. 300 µl cold ethanol 100% were added while vortexing the tube. After incubation of the particles for 1 h at –20°C, the suspension was centrifuged for 3 min, the supernatant was removed and replaced by 40 µl H_2O.

Prior to microprojectile bombardment the rice tissue was plasmolysed on solid MS2 medium for 1 h. Immature, precultured, and plasmolysed embryos and suspension cells were stably transformed by microprojectile bombardment with a particle inflow gun (PIG, (Finer, *et al.*, 1992). Before bombardment, as much as possible of the

remaining liquid was removed from the cell clusters by pipetting and by drying the plates for 5 minutes in the air flow. The tissue was placed into the vacuum chamber of the PIG at a distance of 14 cm from the filter. A 500 µm nylon mesh was positioned 9 cm from the filter. 8 µl coated particle suspension corresponding to 400 µg gold particle and 0.8 µg DNA were loaded on the filter. The chamber was evacuated to 100 mbar and the particles were accelerated by a helium jet generated by a 6 bar pressure for 50 ms. The vacuum was released and the tissue was incubated for another 24 h on the plasmolysis medium. Subsequently, the embryos and the suspension cells were transferred to liquid R2r supplemented with 50 mg/l cefotaxime, and cultured for 1 week at 30°C in the dark under shaking.

Selection of the bombarded tissue

To select the transformed cells, bombarded tissues were exposed to hygromycin B (Duchefa, Haarlem, NL) as a selective agent one week after transformation. This was done in liquid R2r medium supplemented with 30 mg/l HygB and 50 mg/l cefotaxime. The liquid cultures were placed in an incubation shaker (Infors, Bottmingen, CH) at 90 rpm and 30°C in the dark. The tissue was subcultured in weekly intervals for 3-4 weeks until the first resistant clones were visible. The resistant calli were incubated 2 weeks on solid R2I until embryogenic structures were obtained.

Regeneration of transgenic tissue.

Resistant calli were transferred to Rzs regeneration medium. The regenerating tissue was subcultured in intervals of 3 weeks until the green shoots developed. Shoots that had reached a length of 2-3 cm were transferred to MS rooting medium. After cultivation for 2 weeks at 30°C in a Weiss incubator, plantlets were transferred directly to the greenhouse and planted in soil.

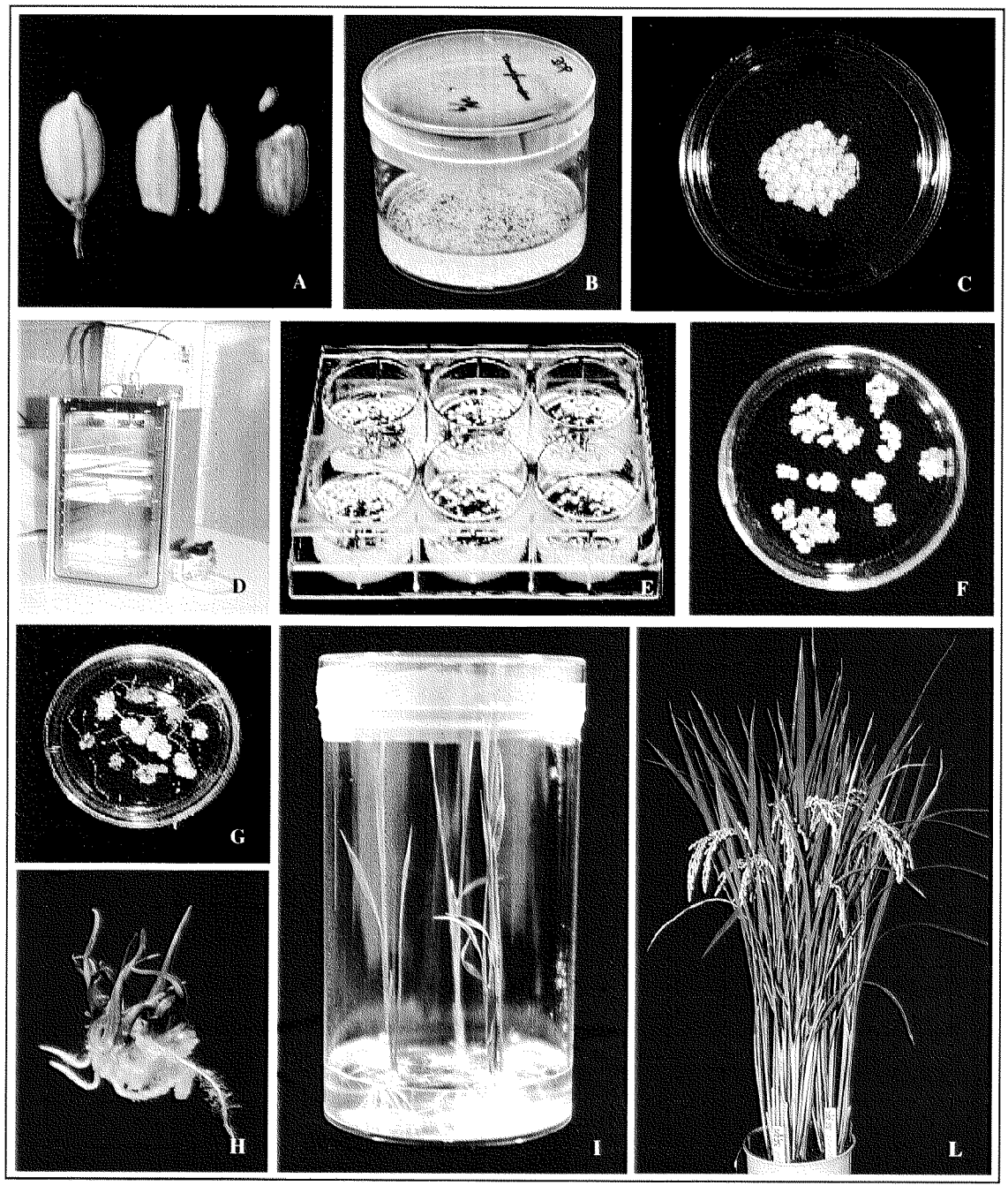


Figure 9: Plant regeneration after biolistic transformation of rice suspension cells. A: Immature rice grain from *Japonica* rice variety Taipei 309, dissected; arrow marks the embryo. B: Rice suspension cells obtained from scutellum tissue. C: Suspension cells on high osmotic medium before transformation. D: PIG. E: Liquid selection of the transformed cells. F: Embryogenic hygromycin-resistant calli. G, H: Shoot induction. I: Root induction. L: Fertile plant.

2.7.3 *Agrobacterium*-mediated transformation

Agrobacteria containing the pAGt1Fe or pAGt1Me plasmids were grown in liquid AB medium supplemented with selection antibiotica (100 mg/ml rifampicin and 50 mg/ml kanamycin). When its OD600 reached 0.8, the cultures were centrifuged for 10 min at 3000 rpm, the supernatant removed, and the bacteria were incubated for 90 min at 28°C in 25 ml induction media. The rice tissue was plasmolysed for 30 min in induction medium without acetosyringone before transformation.

The induced bacteria were distributed into Petri dishes and incubated with the plasmolysed rice tissue in induction medium for 20 min at 25°C. Calli were blotted dry on sterile Kimwipes and then placed without rinsing on cocultivation medium during three days at 25°C. Bacteria were removed by several washes in cefotaxime solution (250 mg/l cefotaxime in sterile water) and the rice tissue was transferred onto selection medium until the first resistant clones were visible. The resistant calli were incubated for 2 weeks on proliferation medium until embryogenic structures were obtained. Somatic embryogenic calli were transferred to regeneration medium as described for the regeneration of rice tissue after biolistic transformation.

Growth of T1 transgenic plants

To exclude from further cultivation those plants that had lost the transgene due to Mendelian segregation, seedlings were grown on selective medium. Seeds were dehusked, briefly rinsed with 70 % ethanol and sterilised by incubation in 6% $CaClO_2$/0.1% (v/v) Tween 80 for 50 min. After washing with sterilised water, seeds were germinated on MS medium for 4 days at 30°C in the light. This was followed by 10 day incubation on MS media containing 25 mg/l hygromycin. The rice plantlets were screened for growth and root development. Vigorously developing, hygromycin-resistant plants were transferred to the greenhouse and planted in soil.

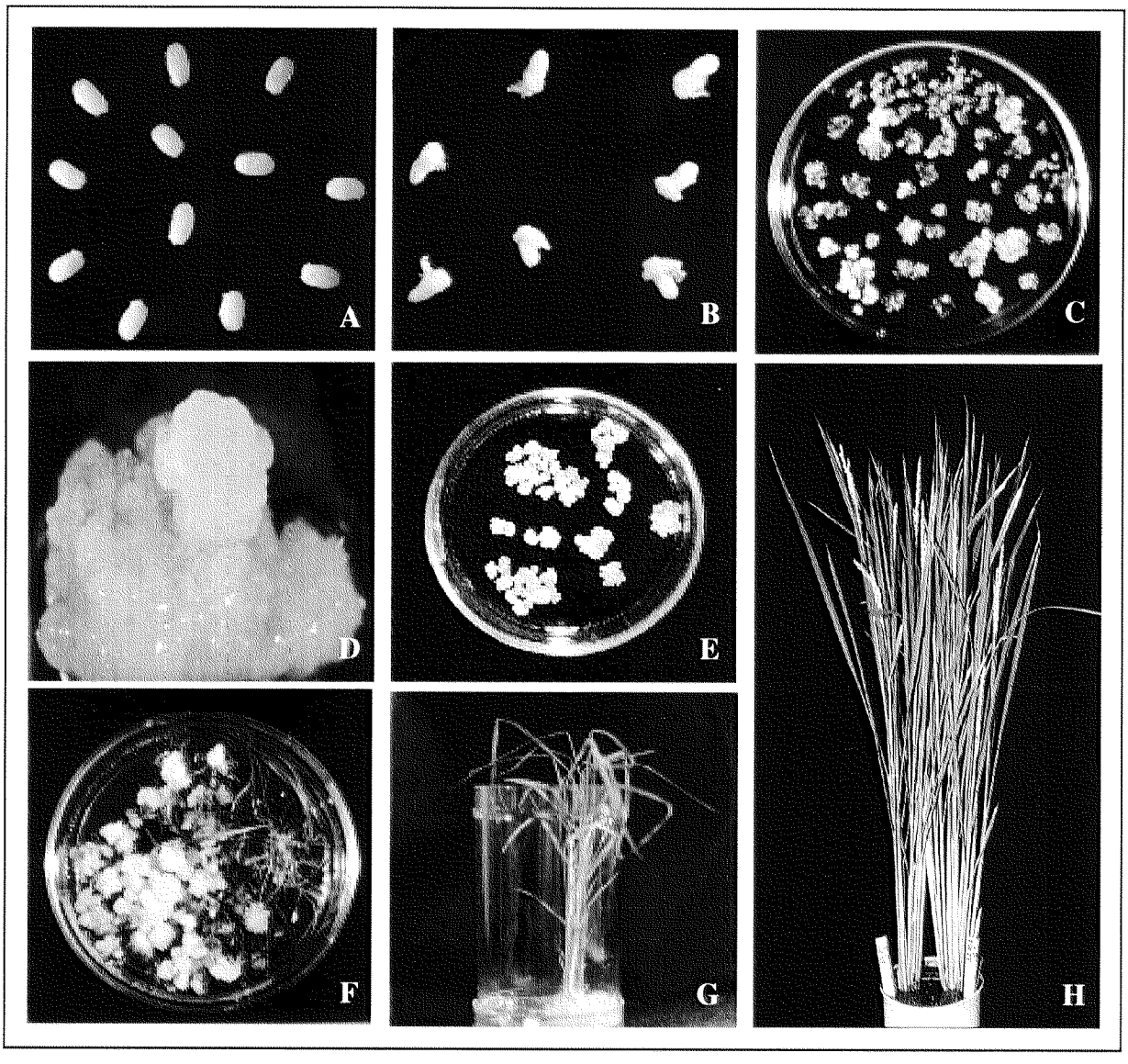


Figure 10: Plant regeneration after *Agrobacterium*-mediated transformation. A: Mature embryos. B: Developing calli from scutellum cells. C, D: Selection of the transformed cells. E: Embryogenic hygromycin resistant calli. F: Shoot induction. G: Root induction. H: Fertile plant.

2.8 Analysis of putative transgenic rice plants

2.8.1 DNA detection by Southern blot analysis

10-20 cm of young rice leaves (six to eight weeks old) were shock-frozen and ground with a mortar and pestle to a fine powder in the presence of liquid nitrogen. The powdered material was thoroughly suspended in 800 µl of extraction buffer and the DNA was extracted following the Quiagen Protocol. The extracted DNA was resuspended in 80 µl purified water.

Specific plant DNA sequences were detected by preparation of Southern blots and hybridisation (Southern, 1975) to DIG-labelled DNA probes at 42°C in the presence of formamide.

For this purpose 10 mg plant DNA were cleaved by treatment with restriction endonucleases and loaded on a 0.8% agarose gel. After electrophoretic separation, the gel was incubated for 10 min at RT in 0.25 M HCl and subsequently denaturated in 0.5 M NaOH, 1.5 M NaCl for 30 min. Finally the gel was washed in neutralisation buffer and blotted using 20 x SSC on a positively charged nylon membrane (Hybond N, Amersham). A PCR-amplified, DIG-labelled (Boehringer) fragment of the coding region was used as a probe for hybridisation. Following hybridisation, the membrane was washed twice in 2 x SSC, 0.1% SDS for 5 min at RT, once in 0.2 x SSC, 0.2% SDS for 15 min at 67°C and finally in 0.1 x SSC, 0.1% SDS for 15 min at 67°C. Washing was followed by an immunoreaction of DIG with an anti-DIG-alkaline phosphatase (AP)-conjugate and chemiluminescent detection reaction with a 1,2 dioxyethane enzyme substrate (CDP-Star, Topix, Bedford, MA). The DIG detection was performed as described in (Neuhaus-Url and Neuhaus, 1993). Hybridisation signals were visualised by exposing the filters to a Kodak X-Biomax film (Kodak, Rochester, USA) for 10 to 60 min at RT.

2.8.2 RT-PCR

RNA isolation from transgenic seeds

RNA for cDNA synthesis and subsequent PCR analysis was isolated from immature seeds about 10-14 days after pollination by the standard method with guanidine thiocyanate (McGookin, 1984).
Immature rice seeds were shock-frozen and ground with mortar and pestle to a fine powder in the presence of liquid nitrogen. 100 mg of the powder were thoroughly suspended in 1 ml Trizol® reagent (GibcoBRL) and the RNA was extracted according to the instruction of the manufacturer.

To remove any trace of DNA the solution was treated with RNase-free DNase (Boehringer) for 30 min. After extraction of the enzyme, the RNA was stored at –80°C.

Reverse transcription of RNA

Isolated RNA from immature seeds was used as a template to generate cDNA.

1 µg RNA, 50 ng 3' gene specific primer, 10 mM DTT, and 0.5 mM of each deoxynucleotide were incubated in a reaction mixture of 20 µl total volume at 42°C for 50 min. The reaction was stopped by heating 70°C for 15 min. After incubation of the reaction mix with *E. coli* RNase H (GibcoBRL) for 20 min, 2 µl of the cDNA obtained were used for PCR.

DNA amplification

cDNA sequences were amplified by PCR (Mullis, 1987) in a thermal cycler (Progene, Techne, Cambridge, England).

2 µl of a reverse transcriptase reaction mixture, 50 ng 5' and 3' primers, 0.5 mM of each deoxynucleotide, 3 mM $MgCl_2$ and 0.4 U Taq polymerase (Boehringer) were incubated in a reaction mixture of 50 µl total volume. DNA molecules were denatured at 94°C for 2 min followed by 35 amplification cycles of primer annealing (for temperatures see table 2), extension at 72°C for 1 min and denaturation at 94°C for 1 min/. The terminal delay was set for 7 min at 72°C.

Primers for amplification of the DNA obtained after the reverse transcriptase reaction were designed to recognise cDNA regions not present in the wild type rice. In this way a differentiation of the endogenous rice gene expression with the transgene expression was easily possible.

Table 2: Primers used for RT-PCR

Primer	Sequence	Fragment	Annealing temp.	Gene
RT-M1 5'	GGGTACCGA GCTCGCTTG	300	50°C	rgMT
RT-M2 5'	TTAAGGCCGT CGAGAATTCGA			
RT-P1 5'	GGGATCCACAATGGC TAGAAAAGATGTTGCC	1500	60°C	phyA (*A. fumigatus*)
RT-P2 5'	GACTGGTGATTT CAGCGGGCATGCC			

2.8.3 Northern blot analysis

Total RNA was extracted as described above. 12 µg of total plant RNA were lyophylised and dissolved in 20 µl denaturation buffer. Samples were denatured for 10 min at 60°C and placed on ice. After shortly spinning the denatured samples, 5 µl of sample buffer were added. Samples were electrophoresed under denaturing condition (1.2% agarose gel containing 2% formaldehyde) and were transferred overnight with 20xSSC to Hybond N membrane (Amersham). To visualise the RNA after the transfer, the membrane was stained in 0.5 M Na-acetate (pH 5.2-5.6) containing 0.04% methylene blue under constant shaking for 5- 10 minutes at RT. The membrane was destained in distilled water until the ribosomal RNA bands were visible. Filters were hybridised with a digoxigenin-labelled DNA probe and were washed and detected as described for Southern blot analysis.

2.8.4 Protein and immunological methods

Seed protein isolation

For the isolation of proteins from rice plants, containing the ferritin gene, mature dehusked seeds were used. 8 seeds were ground for 20 s at full speed with a oscillating mill (Retsch MM2, Schieritz and Hauenstein AG, Arlesheim, Switzerland). 150 µg of the resulting powder was suspended in 2 ml F1 extraction buffer and incubated in ice for 1 h. After centrifugation at 11.000 rpm for 20 min at 4°C, the supernatant was heated for 15 min at 65°C. The protein mixture was centrifuged as described above, the supernatant was removed, lyophilised, and dissolved in 100 µl water.

For the extraction of the mature ferritin protein, 150 µg rice powder were incubated for 1 hour in ice with 2 ml F2 extraction buffer. After centrifugation at 11.000 rpm for 20 min at 4°C, the supernatant was lyophilised and redissolved in 80 µl water.

Protein extraction from seeds harboring the fungal phytase gene was performed as described (Verwoerd, *et al.*, 1995). Seed tissue was homogenised in 25 mM sodium acetate buffer, pH 5.5 and left on ice for 20 min. The homogenate was centrifuged for 5 min, the supernatant collected and lyophilised to concentrate the extracted proteins.

Protein gel electrophoresis and staining

Polyacrylamide gel electrophoresis (PAGE) of proteins was performed with the Mini Protean II electrophoresis cell (Bio-Rad, Hercules, California). 8% denaturing polyacrylamide gels were prepared with 29:1 acrylamide/bis-acrylamide mixture according to Molecular Cloning protocol (Sambrook, *et al.*, 1989).

For the detection of the ferritin subunit and of the fungal phytase, 30 µg of total protein extract were denatured in SDS gel-loading buffer (Sambrook, *et al.*, 1989) at 95°C for 3 min and analysed by SDS-Page. Proteins were loaded onto the gel and separated by electrophoresis at RT and 100 V for 2h.

For the detection of the mature ferritin protein, 15 µg of total protein extract were mixed with loading buffer (50 mM Tris-HCl, pH 6.8, 10% glycerol, 0.1% bromphenol-blue). After centrifugation at 12.000 rpm for 10 min at RT, the sample were loaded on native gradient (4-20%) gel (Ready Gels, Biorad, Hercules, California) and the electrophoresis was performed at 150 Volt for 5 h. The ferritin proteins were visualised on the native gel by a silver staining procedure. To prevent protein diffusion, the gel was fixed after electrophoresis in 10 % trichloroacetic acid for 30 min and washed three times for 10 min in 5% acetic acid and 10 % ethanol. The proteins were oxidised 5 min with 3.4 mM $K_2Cr_2O_7$, 3.2 mM NHO_3 and then washed 2 times with water. After decanting water, the gel was stained with 12 mM $AgNO_3$ solution and then shortly washed with water. The stain was developed

soaking the gel in reducing solution (0.28M Na_2CO_3, 0.037% formaldehyde). As soon as proper staining was obtained, the reaction was stopped with 5% acetic acid.

Western blot analysis and immunodetection

Proteins separated by SDS-PAGE were blotted onto a nitro cellulose membrane (Schleicher and Schüll, Dassel, Freiburg) at 50 mA for 1 h using a Mini Transblot® Electrophoretic transfer Cell equipment (Bio-Rad, Hercules, California). The membrane was washed in PBS-T, blocked in 5% blocking solution (5% (w/v) dried milk in PBS-T) for 2 hour and incubated with 20 µl antiserum in 20 ml blocking solution at 4°C over-night. The primary antibodies used for the detection of the ferritin subunit were raised in rabbits against the pea ferritin (kindly provided by Prof. Briat, INRA, Montpellier, France). For the detection of the transgenic fungal protein, antibodies raised against the *A. fumigatus* or *A. niger* phytase were used (kindly provided by Dr. Lehmann, Hoffmann-La Roche, Basel and Dr Ullah, New Mexiko). After washing the membrane with PBS-T, antigen-antibody complexes were detected with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulins, using the enhanced chemiluminescence procedure (ECL Western blotting, Amersham, UK). Signals were detected with ECL Chemiliminescence Western Blotting Kit (Amersham, Little Chalfont, Buckinghamshire, UK) and visualised by exposing the filters to a Kodak X-Biomax film (Kodak, Rochester, USA) for 5 to 20 min at RT.

2.9 Biochemical analysis

2.9.1 Iron concentration

For the analysis of the iron content, mature transgenic seeds were dried at 50°C and dehusked. The 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 to prevent iron contamination. About 0.5 g of the resulting powder were weighted accurately in preweighted, high pressure digestion vessels (MLS 1200, MLS GmbH, Leutkirch, Switzerland). After addition of 5 ml HNO_3 and 2 ml H_2O_2, the rice samples were left 20 min at RT and then mineralised by microwave digestion (MLS 1200, MLS GmbH, Leutkirch, Switzerland) following the conditions described in table 3.

Table 3: Conditions used for mineralisation of the rice samples.

Step	Time [min]	Power [W]
1	2	250
2	2	0
3	5	250
4	5	0
5	5	400
6	2	0
7	5	600
8	10	0

Iron present in the resulting solution was measured by graphite furnance atomic absorption spectroscopy. The operating parameters are summarised in table 4. A 20 µl portion of sample solution was injected into the graphite tube with an autosampler. Argon was used as purge gas. All absorption measurements were made in triplicates.

Table 4: Operating parameters used for graphite furnance atomic absorption spectroscopy.

Instruments:	
Atomic absorption spectrophotometer (AAS):	SpectrAA-400, Varian, (Mulgrave, Australia)
Graphite furnance + autosampler:	GTA-96 graphite tube atomizer, Varian

Atomic absorption spectrophotometer conditions:	
Lamp current:	5 mA
Wavenlenght:	248.3 nm
Slit width:	0.2 nm
Instrument mode:	absorbance
Measurement mode:	peak area
Background correction:	deuterium lamp
Replicates:	3

Graphite furnance operation:	
Atomization tube:	coating partition tube
Purge gas:	Argon, 3 L/min
Sample volume:	20 µl
Calibration: 5 points calibration curve	

Program:

Step	temp [°C]:	ramp [s]:	hold [s]:	remarks:
1	85	15	-	drying
2	110	30	-	drying
3	150	10	-	drying
4	800	5	10	ashing
5	2400	0.8	1.5	atomization
6	2600	2	-	cleaning

2.9.2 Phytase activity

Phytase assay

Phytase thermostability assay

1 g of ground milled rice japonica (Taipei 309) cultivar were suspended in 10 ml water. After addition of 10 mg of the purified phytases (*A. niger*, Fluka, A *fumigatus* Q27L, Hoffmann-La Roche) the rice suspension was cooked for 20 min at 100°C.
After addition of 20 ml substrate solution (15 mg phytic acid (24.6 mg phytic acid dodecasodium salt, Sigma / ml 0.3M glycin-HCl, pH 2.5 or 0.3 sodium-acetate, pH 4.5) to the rice mixture, samples were incubated at 37°C (Incubator KB 240, WTB Binder, E. Merck, Dietikon) on the electric stirrer (600 rpm, Variomag multipoint HP15, H + P Labortechnik, Oberschleissheim) for 30 min. 0.5 ml samples were taken every 10 min and the reactions were stopped with an equal volume of 15% trichloroacetic acid (p. a., Merck).

The endogenous phytase activity was measures without addition of the fungal phytase.

The liberated phosphate was determined colorimetrically as described below.

Transient expression in rice protoplasts

2 ml of protoplasts suspension were tested for phytase activity 24 h after transformation. Protoplasts were suspended in 8 ml substrate buffer (6.25 mg phytic acid in 0.2 M 0.4 glycine-HCl for pH 2.5, 0.2 M sodium acetate between pH 4 and 5 and 0.2 M imidazole-HCl for pH 6.0) and incubated at 37°C on the electric stirrer. Samples were taken as described above and the phosphate present was determined colorimetrically.

Transgenic rice seeds

For the analysis of the phytase activity from fertile rice plants, containing the fungal phytase gene, mature dehusked seeds were used. Rice seeds were ground for 20 s at full speed with a micro-dismembrator and 0.5 g of the resulting powder were suspended in 5 ml suspension buffer (pH 2.5 and pH 6.0). After addition of 5 ml substrate solution (10 mg/ml phytic acid in suspension buffer), the samples were incubated at different temperatures on the electric stirrer (600 rpm). 0.5 ml samples were taken as described above.

Colorimetric determination of the liberated phosphate

A procedure based on the complex formation of malachite green with phosphomolybdate under acidic conditions was used to measure the liberated inorganic orthophosphate (Van Veldhoven and Mannaerts, 1987)
150 µl of the reaction samples were mixed with 150 µl 0.5M H_2SO_4 in PS-microplates and after serial dilutions, 30 µl Ammonium molybdate-solution (1.75% Ammonium molybdate tetrahydrate) were added. The microplates were gently shaken and left at room temperature for 10 minutes. 30 µl Malachite green (0.035% Malachite green in 0.35 Polyvinylalcohol) were added to each well, and after shaking, the reactions were left for 45 min at RT. The absorbance was read at 610 nm with a microplate reader (Microtech MRX 1.2, Microtec Products AG).

2.9.3 HPLC Method for the determination of the inositol-phosphates

The method included extraction of inositol phosphates from the rice seeds with HCl, separation of the inositol phosphates from the crude extract by ion-exchange chromatography, and ion-pair C18 reverse phase HPLC analysis using

formic/methanol and tetrabutylammonium hydroxide in the mobile phase (Sandberg, *et al.*, 1989; Sandberg and Ahderinne, 1986).

0.30 ground rice seeds, were extracted under vigorous mechanical agitation (250 rpm/min) with 20 ml 0.5M HCl for 3 h at RT. The extract was centrifuged 10 min at 3500 rpm and the supernatant was evaporated in a waterbad at 40°C under a stream of air. After dissolving the residue in 10 ml 25 mM HCl, the inositol phosphates were separated from the filtrate by a ion-exchange procedure. Plastic columns with glass filter were filled with anion exchange resin (AG 1-X8, 200-400 mesh, chloride form, Bio Rad Laboratories). The inositol phosphates solution was loaded onto the column, washed with 10 ml 25 mM HCl and eluted 5 times with 4 ml 2M HCl. The filtrate was evaporated to dryness as described above and diluted in 2 ml of water.

The mobile phase consisted of 50 mM formic acid: methanol: tetrabutylammonium hydroxide solution 40% (48:60:1.74). To achieve separation between the inositol tri- to hexaphosphates, the pH was adjusted at 4.3 with 50% H_2SO_4.

A phytate standard solution was made by dissolving 17 mg phytic acid dodecasodium salt (Sigma P-8810) in 100 ml water. For the determination of the concentration of inositol penta-, tetra- and triphosphates correction factors were used (Sandberg and Ahderinne, 1986).

2.9.4 Cysteine content

Amino acids were separated by ion-exchange chromatography and spectrophotometrically detected after a post-column reaction with ninhydrin (Alpha Plus Amino Acid Analyser, Pharmacia Biosystem).

40 mg powdered rice seeds were suspended in 2 ml 6 N HCl under argon atmosphere to prevent oxidation of the sulphur group. After hydrolysis for 24 h at 110°C the hypochloric acid was evaporated (Rotavap Büchi, Switzerland, water temperature < 50°C). The dried residue was dissolved in 6 ml sodium citrate loading

buffer (pH 2.20, O.16 M, Pharmacia Biotech Ltd., Biochrom, Cambridge, England) and filtered through a 0.45 µm microfilter (Acrodisk 13, Skan, Basel, CH).

50 µl of the filtered solution were loaded onto the column (200 x 4.6 mm, Pharmacia Biosystems). For the derivatisation of the separated amino acid, ninhydrin-acetate reagens (10 g ninhydrin in 300 ml acetat buffer, 5 M, pH 4.95 and 700 ml Ethylenglycol) was added to the eluate. The flow of the buffer was set to 28 ml/h and the flow of the ninhydrin-acetate buffer at 22 ml/h.

The quantification was performed with external standard (AA-S-18, Sigma, St.-Louis, USA). 5 nmol of each amino acid and 2.5 nmol of cystin were used. The evaluation of the chromatogram was done with the chromatography-software program Chromatography Data System, Model 2600, Rev. 5.1, P.E. Nelson, Cupertino, USA.

3 RESULTS

3.1 Phytase thermostability assay

It is known from the literature that after 20 min exposure to 100°C, *Aspergillus fumigatus* phytase still retained 90% of its initial activity (Pasamontes, *et al.*, 1997). Investigation of the thermoresistance of the fungal phytase was performed after diluting the purified enzyme in sodium acetate buffer at pH 5.0.

In order to assess the thermostability of the fungal proteins in the rice grains to withstand the cooking process, the purified phytases from *A. niger* and *A. fumigatus* Q27L were separately mixed with ground rice. After cooking the protein/rice mixture for 20 min in water, the phytase activity was measured at pH 2.5 and 4.5. This was a more reliable assay for the prediction of the thermostability of the proteins in the rice endosperm, as all factors that could affect the stability of the transgenic proteins in rice were present in the broth.

The analysis revealed that the thermostability of the fungal protein was in fact affected by the presence of the rice components. Nevertheless, the two fungal proteins still retained part of their initial activity. *A. niger* phytase showed 15%, *A. fumigatus* phytase 42% of their activity, when the activity was measured at pH 2.5. When the activity was determined at pH 4.5, *A. niger* phytase retained 34%, *A. fumigatus* 50% of the initial activity. It is unclear how the different pH conditions for the measurements of the phytase activity affected the thermostability of the protein, as the proteins were both cooked in water.

These results indicated that the *A. fumigatus* protein was probably the best candidate for an activity after processing and cooking of rice.

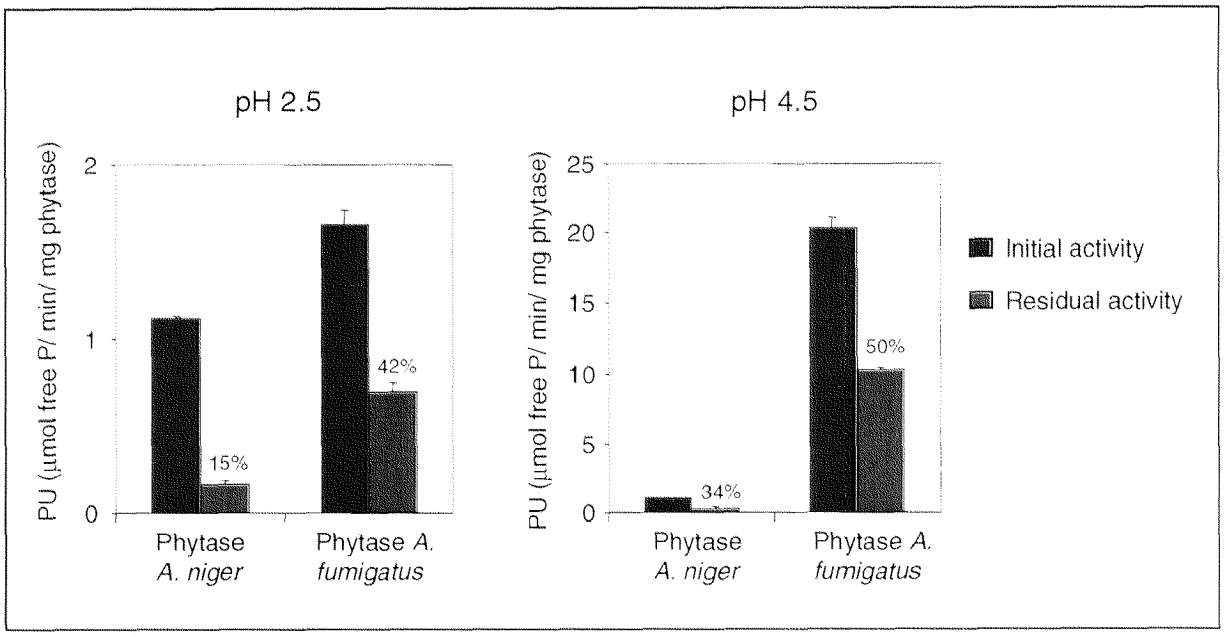


Figure 11: Thermostability assay of the purified phytases from *A. niger* and *A. fumigatus*. Both phytases were heated for 20 min at 100°C together with ground rice. The activity was measured at pH 2.5 and 4.5 at 37°C.

3.2 Transient gene expression in rice protoplasts

The suitability of the phytase plasmids for expression of the fungal proteins in rice was tested by measuring the activity of transformed protoplasts compared to the activity of protoplasts transformed with a Gus contruct as negative control. Moreover, the potential activity of the fungal phytases during the digestion was analysed exposing the transformed rice protoplasts at 37°C to different pH conditions. The investigation of the activity of the fungal phytases revealed that the transformed rice protoplasts had a higher phytase activity as the control, indicating that the fungal phytase plasmids were suitable for expression in rice.

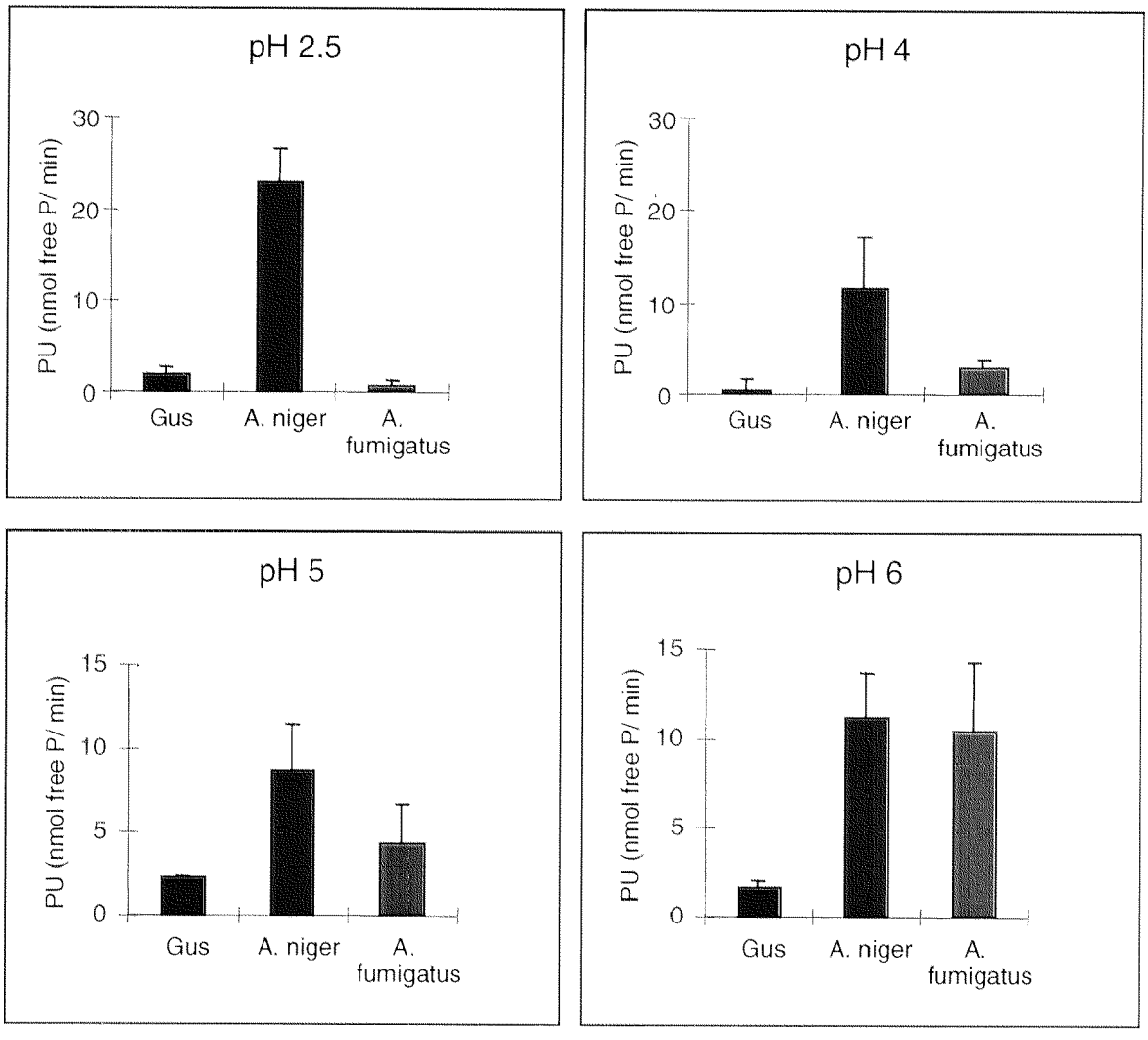


Figure 12: Transient expression of the fungal phytases in rice protoplasts. The phytase activity of the transformed protoplasts was measured at 37°C and different pH condition. Protoplasts transformed with C4GLm2BgNd BGILP (Gus construct) were used as negative controls.

The highest activity of *A. niger* phytase was obtained at pH 2.5, but a significantly increased activity of the transformed protoplasts was observed at all pH tested.

In this experiment *A. fumigatus* phytase showed the optimal activity at pH 6.0, a weak activity, slightly higher as the control protoplasts, at pH 4.0 and 5.0, and no significant activity at pH 2.5. This results correlate well with the pH optimum of the activity of the *A. fumigatus* protein (Wyss, unpubl.).

3.3 Transformation of *Oryza sativa*, Japonica variety Taipei 309

Japonica rice variety Taipei 309, which is known for its good tissue culture properties, was chosen as object for transformation. This variety had been already described by several authors as a suitable model for rice transformation (Christou and Ford, 1995; Rance, *et al.*, 1994).

3.3.1 Biolistic transformation

Four plasmids were constructed for *biolistic* transformation of calli derived from immature embryos and suspension cells.

The genes encoding the ferritin protein from *Phaseolus vulgaris* and the metallothionein-like protein from *Oryza sativa* were separately cloned into a pUC19 derivative plasmid under the control of the endosperm-specific promoter Gt1. The *hpt*IV sequence was inserted in the plasmid resulting in constructs pGt1Fe and pGt1Me, respectively.

Two chimeric phytase genes encoding the barley β-glucanase signal peptide and a cDNA fragment encoding the mature *A. niger* or *fumigatus* phytases driven by the Gt1 promoter were constructed and linked to the *hpt*IV sequence, to yield plasmids pGt1PN and pGt1PF.

Table 5: Biolistic transformation of TP 309. The percentage of fertile plants is related to the number of transgenic independent plants obtained.

Construct	Embryos bombarded	Susp. dishes bombarded	Independ. plants	Transgenic plants	Fertile plants
pGt1Fe		7	12	11	2 (18%)
	200		4	1	0 (0%)
pGt1Me		7	13	12	1 (8.3%)
	220		0	0	0 (0%)
pGt1PN		16	55	49	11 (22%)
	180		5	0	0 (0%)
pGt1PF		12	17	15	4 (27%)
	100		3	0	0 (0%)
Σ		42	97	87	18 (21%)
	700		12	1	0 (0%)

The resulting clones were used for biolistic rice transformation. In total, 700 immature embryos were bombarded. From these experiments 12 independent plants were regenerated and among these plants, only 1 was found to be transgenic. The transgenic plant, however, did not develop normally and was not fertile. Because of the disappointing results obtained after biolistic transformation of immature rice embryos, 42 suspension cell dishes have been transformed. 125 hygromycin-resistant calli were obtained, 97 of which could be regenerated to rice plants. Among these plants, 87 were found by Southern blot analysis to contain the construct, 22 were fertile and exhibited a normal phenotype.

3.3.2 *Agrobacterium*-mediated transformation

The genes encoding the ferritin protein from *Phaseolus vulgaris* and the metallothionein-like protein from *Oryza sativa* were separately cloned into pCAMBIA 1390 under the control of the Glutelin Gt1 promoter. The resulting plasmids pAGt1Fe and pAGt1Me were used for *Agrobacterium*-mediated transformation of 600 precultured mature embryos. 60 independent hygromycin-resistant plants could be regenerated after transformation with either pAGt1Fe or pAGt1Me, 47 of which contained the construct as shown by Southern blot analysis (see table). 38 of these rice plants were fertile, corresponding to a transformation efficiency of 6.3%.

Table 6: *Agrobacterium*-mediated transformation of TP 309 with the genes encoding the ferritin and the metallothionein-like protein. The percentage of transgenic plants is given relative to the number embryos bombarded, whereas the number of fertile plants is relative to the transgenic plants obtained.

Construct	Embryos bombarded	Independent plants	Transgenic plants	Fertile plants
pGt1Fe	300	35	29 (9.7%)	24 (83%)
pGt1Me	300	25	18 (7.2%)	14 (78%)
Σ	600	60	47 (7.8%)	38 (81%)

3.4 Analysis for transgene integration by Southern blot analysis

In order to identify independent lines among the primary transformants and to identify plants that had integrated complete copies of the transgenes, Southern blot analysis was performed with all the plants regenerated.

Genomic plant DNA was isolated from leaves of the primary transformants and was digested to cleave the plasmid-derived sequences at one single site. Differently sized fragments of transgenic DNA were released, depending on the site of integration.

The size of the integrated coding sequences was tested in a second restriction digest. The plant genomic DNA was cut with enzymes that were known to produce a fragment of the encoding genes with the flanking promoter and polyadenylation sequences. Direct comparison of the size of the fragment derived from the plasmid used in transformation with the fragment derived from the genomic plant DNA allowed identification of plants that had integrated complete transgene copies.

3.4.1 Analysis of plants regenerated after biolistic transformation

Analysis of plants transformed with plasmid pGt1Fe

The first plants analysed were those that had been transformed with the ferritin encoding cDNA under the control of a Gt1 promoter. Southern analysis of the plants (see Figure 13) showed that not all of them were transgenic for the ferritin sequence.

To confirm the complete integration of the ferritin sequence, plant DNA samples were digested with *Hinc* II. A signal from a 3400 bp fragment containing the complete sequence, with the flanking glutelin promoter and the 35-S terminator, and part of the CaMV promoter for the *hph* IV gene was expected in the transgenic plants as well as from plasmid pGt1Fe.

Lines Fs1-Fs7, Fs9-Fs12 and Fc4 were clearly transgenic for pfe. They displayed, however, a complicated hybridisation pattern, indicating multiple copy insertion and rearrangement of the transgene. Testing for independence of the transgenic lines was not performed because the hybridisation patterns obtained when testing for the

integration of the ferritin cDNA differed considerably, indicating independence of the transgenic lines.

From a total number of 16 hygromycin-resistant plants recovered from transformation experiments, 7 contained an intact fragment of the expected size when their respective genome were digested with *Hinc* II, suggesting the complete integration of the ferritin sequence. 4 plants contained only fragments with a higher and/or lower molecular weight and 4 plants were not transgenic. Possible explanation for the loss of the new gene of interest could be recombination events within the plasmid during transformation, or truncation of the gene at the site of probe hybridisation.

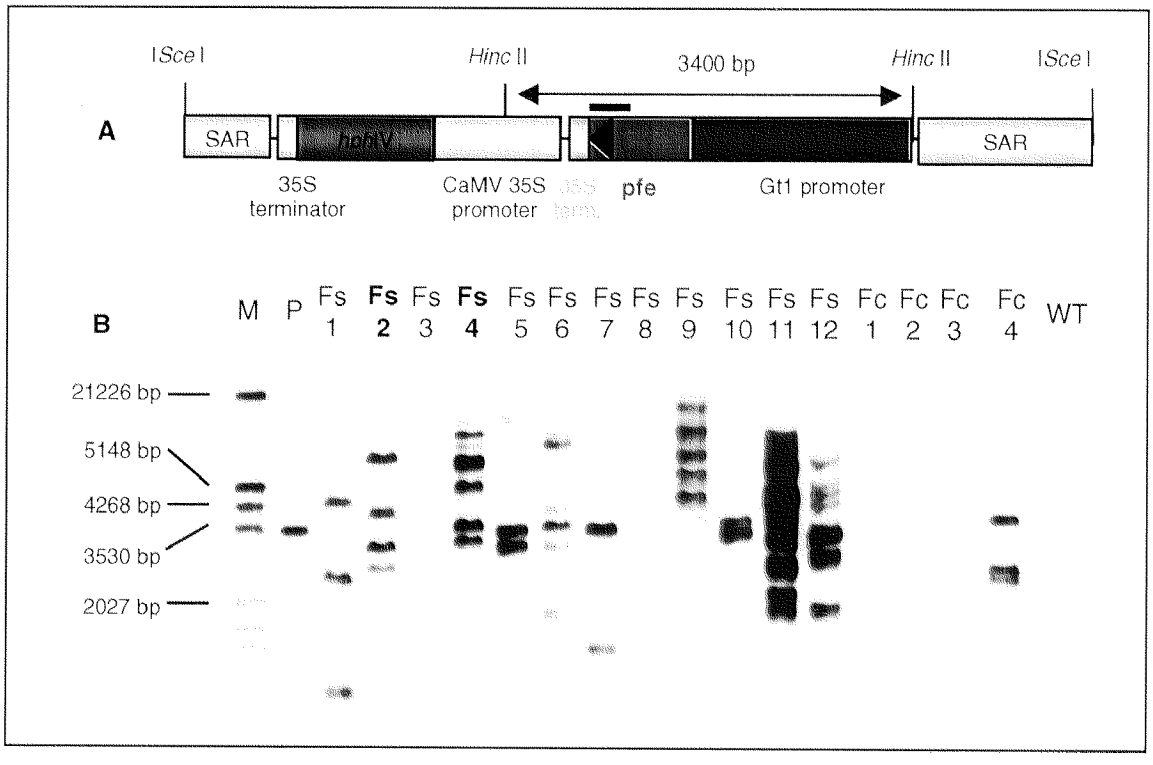


Figure 13: Southern blot analysis of 16 putative pGt1Fe-transgenic plants. A: Schematic drawing of the ferritin cDNA driven by the Glutelin promoter in construct pGt1Fe. B; Analysis for the presence of the ferritin cDNA sequence. The plant genomic DNA was cut with *Hinc* II (releasing a 3.4 kb fragment). Plant genomic DNA from a non transformed plant (WT) was used as negative control, and plasmid pGt1Fe was digested with *Hinc* II as positive control (P). A bar above the pfe gene marks the probe used for hybridisation. Fertile plants are marked in bold.

Analysis of plants transformed with plasmid pGt1Me

Figure 14 shows the analysis of plants obtained after rice transformation with the plasmid pGt1Me. The complete integration of the metallothionein genomic sequence is demonstrated for lines Ms1, Ms2, Ms5, Ms6, Ms9, Ms11, Ms12 and Ms13 by the presence of a 2100 bp fragment containing the complete rgMT sequence, part of the glutelin promoter, the 35-S terminator and part of the *hpt*IV promoter.

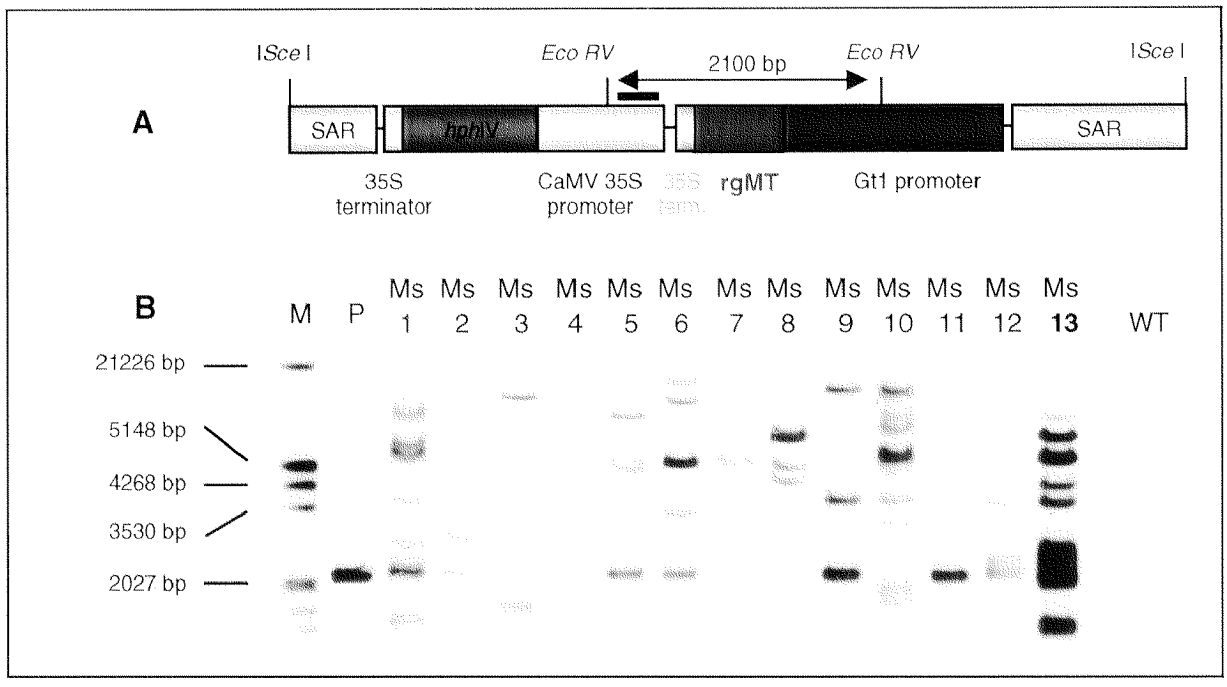


Figure 14: Southern blot analysis of 13 putative pGt1Me-transgenic plants. A: Schematic drawing of the rgMT gene driven by the Glutelin promoter in construct pGt1Me. B: Analysis for the presence of the rgMT genomic sequence. The plant genomic DNA was cut with *Eco* RV (releasing a 2.1 kb fragment). Plant genomic DNA from a non transformed plant (WT) was loaded as negative control, and plasmid pGt1Me was digested with *Eco* RV as positive control (P). The bar above the rgMT gene marks the probe used for hybridisation. Fertile plants are marked in bold.

All lines except for one (Ms4) are transgenic for the metallothionein rice genomic sequence, but the integration patter is complex and most of the integrated fragments were not of the expected size, indicating rearrangement or truncated copies. Lines Ms11 only shows the fragment having the correct size, corresponding to the intact rgMT sequence.

From a total of 13 plants recovered from transformation events with pGt1Me, the DNA of 8 showed an intact fragment of the expected size when digested with *Eco* RV. Only one (line Ms13) from these 13 plants was fertile. All the other rice plants were not fertile and showed abnormal growth.

Analysis of plants transformed with plasmid pGt1PN

The analysis of plants from transformation experiments with the phytase encoding cDNA construct pGt1PN yielded similar results to those obtained for the transformants containing the ferritin and metallothionein construct. Most of the regenerated plants contained fragments that were much larger than expected, suggesting rearrangements of the transgene, or fragments of smaller size, indicating truncated copies.

From a total number of 49 plants regenerated, the DNA of only 22 showed an intact fragment. Out of these, only 11 plants were fertile and showed normal growth, 8 indicating a complete integration of the fungal phytase sequence.

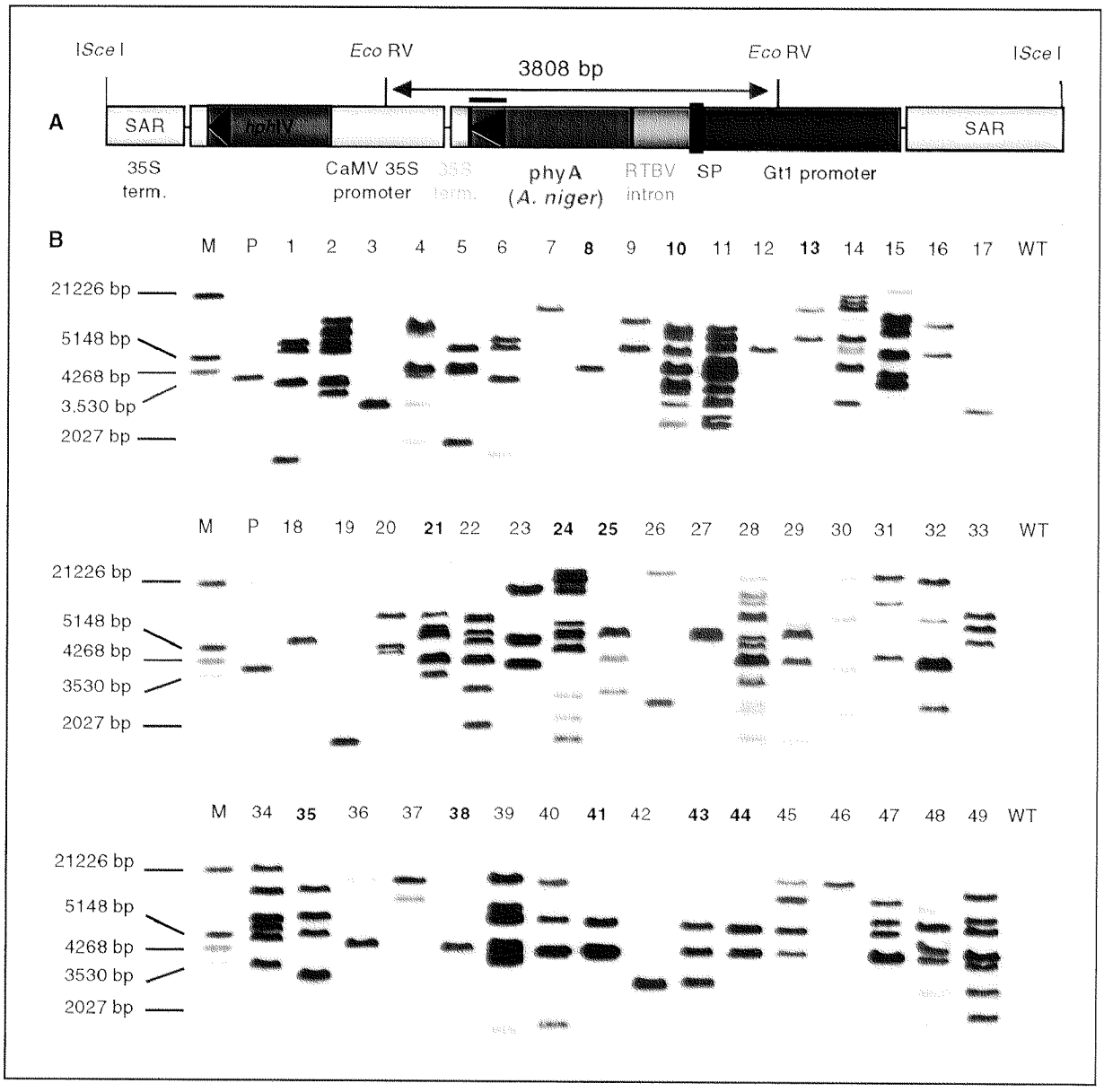


Figure 15: Southern blot analysis of 49 putative pGt1PN-transgenic plants. A: Schematic drawing of the phytase cDNA from *A. niger* driven by the Glutelin promoter in construct pGt1PN. B: Analysis for the presence of the phyA cDNA sequence. The plant genomic DNA was cut with *Eco* RV (releasing a 3.8 kb fragment). Plant genomic DNA from a non transformed plant (WT) was used as negative control and plasmid pGt1PN was digested with *Eco* RV as positive control (P). The bar above the gene of interest marks the probe used for hybridisation. Fertile plants are marked in bold.

Analysis of plants transformed with plasmid pGt1PF

The analysis of plants transformed with the fungal phytase-encoding cDNA from *A. fumigatus* indicated fragment integration of several copies of the transgene. This was evident because of the appearance of multiple bands of different size in the case of DNA digested with restriction enzymes. A signal from 3800 bp fragment containing the complete cDNA, part of the flanking promoter and polyadenylation sequences was expected in transgenic plants, as well as in plasmid pGt1PF.

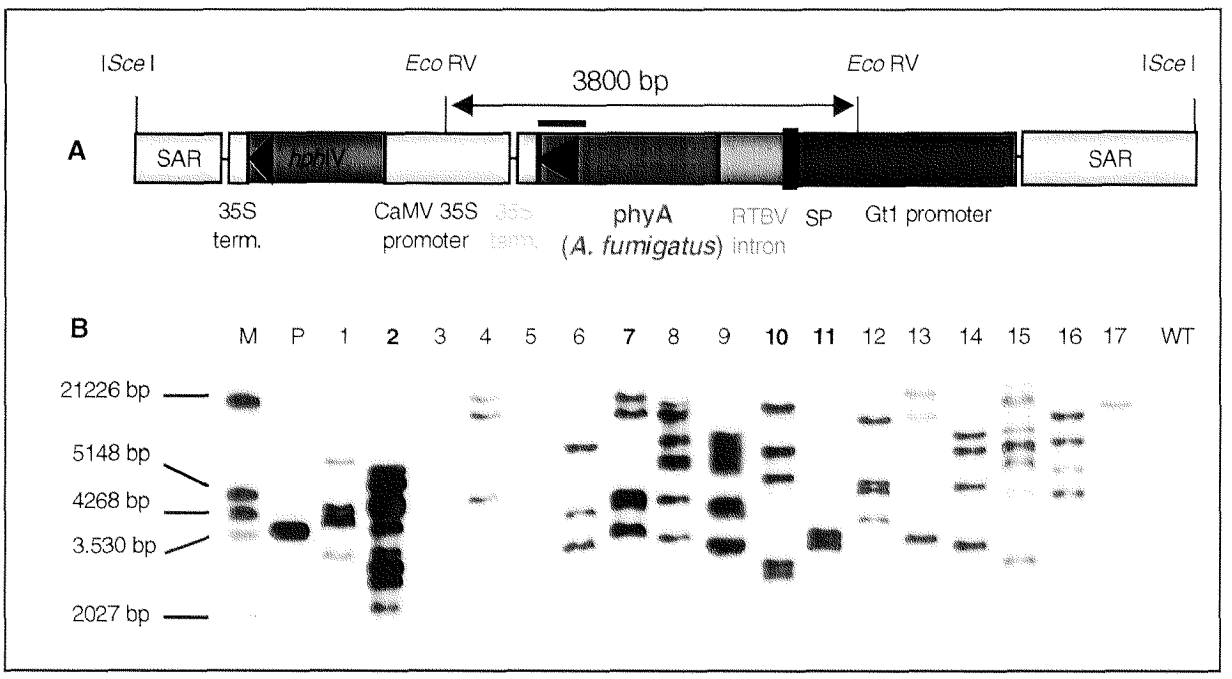


Figure 16: Southern blot analysis of 17 putative pGt1PF-transgenic plants. A: Schematic drawing of the phytase cDNA from *A. fumigatus* driven by the Gt1 promoter in construct pGt1PF. B: Analysis for the presence of the phyA cDNA sequence. The plant genomic DNA was cut with *Eco* RV (releasing a 3.8 kb fragment). Plant genomic DNA from a non transformed plant (WT) was used as negative control and plasmid pGt1PF was digested with *Eco* RV as positive control (P). The bar above the gene of interest marks the probe used for hybridisation. Fertile plants are marked in bold.

The lines analysed in Figure 16 displayed typical hybridisation patterns found for most plants previously analysed.

The few plants possessing the expected fragment contained additional hybridising bands of higher and/or lower molecular weights, indicating integration of multiple copies and rearranged versions of the transgene. Most of the plants did not contain fragments with the correct size and were not fertile. Only 5 plants were fertile and developed normally, one of these (5) was not transgenic.

3.4.2 Analysis of plants regenerated after *Agrobacterium*-mediated transformation

Analysis of plants transformed with pAGt1Fe

The analysis of plants obtained after *Agrobacterium*-mediated transformation with the ferritin-encoding cDNA construct pAGt1Fe yielded far better results them those obtained for the plants regenerated after biolistic transformation of immature embryos and cell suspensions.

It was remarkable that all the transgenic plants showed a complete integration of the ferritin cDNA sequence. A signal from 2500 fragment containing the complete cDNA and flanking promoter and polyadenylation sequences was expected in transgenic plants, as well as in plasmid pAGt1Fe. Only 2 (22, 23) out of 24 plants contained additional hybridising bands of higher molecular weights, indicating rearrangement of the integrated transgene.

To test the independence of DNA integration events and the number of integrated cDNA copies, plant DNA samples were digested with *Eco*RV. On the T-DNA of plasmid pAGt1Fe only one *Eco*RV site is present. Therefore, the number of signals should vary with the number of integrated ferritin cDNA copies. 14 lines had a single copy insertion of the transgene, as shown by the single hybridisation band when the DNA was digested with *Eco*RV. The other lines contained two, three, four or seven copies.

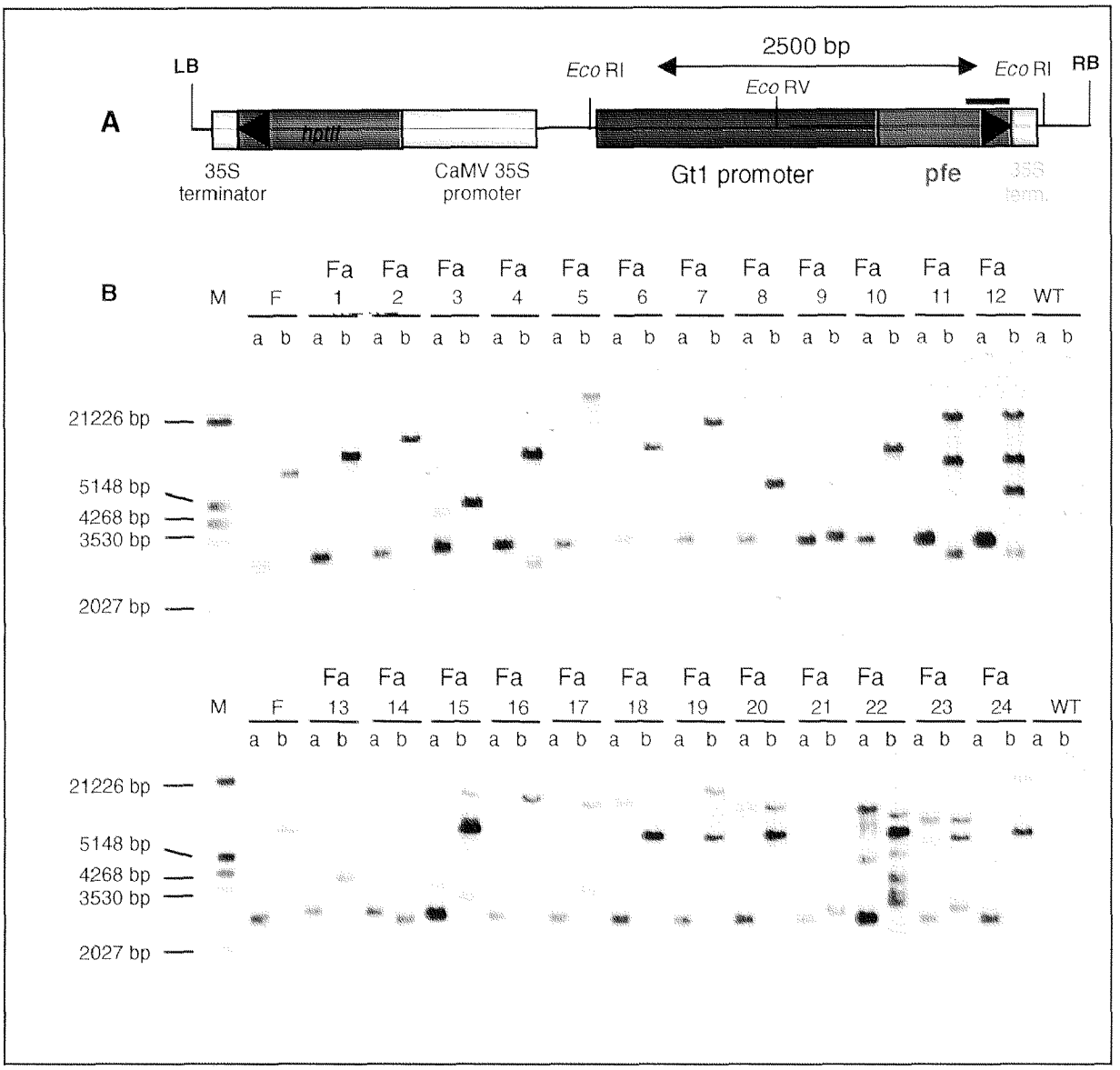


Figure 17: Southern blot analysis of 24 putative transgenic-pAGt1Fe plants. A: Schematic drawing of the ferritin cDNA from *Phaseolus vulgaris* driven by the Gt1 promoter in construct pAGt1Fe. B: Analysis for the presence of the pfe cDNA sequence. The plant genomic DNA was cut with *Eco* RI (releasing a 2.5 kb fragment) and with *Eco* RI to test the independence of the transformation events. Plant genomic DNA from a non transformed plant (WT) was used as negative control and plasmid pAGt1Fe as positive control (F). The bar above the gene of interest marks the probe used for hybridisation. All plants analysed were fertile.

Digestion of the plant DNA with *Eco*RV revealed in several cases (Fa 1, Fa 4, Fa 11, Fa 12, Fa 15, Fa 18, Fa 19, Fa 20, Fa 22, Fa 24) the presence of a band with a strong intensity having almost the same molecular weight as the digested plasmid. This is probably an indication for a contamination with persisting Agrobacteria, which are capable to survive the standard antibiotic treatments of cefotaxime. The integration not only of the T-DNA, but also of the complete pCAMBIA vector into the rice genome, due to an incorrect processing of the T-borders, can also not be excluded.

Analysis of plants transformed with pAGt1Me

The analysis of plants from transformation experiments with the metallothionein rice genomic sequence yielded similar results to those obtained after *Agrobacterium*-mediated transformation with the ferritin construct. All plants transformed with the rgMT-gene (Ma 1-Ma 18) and the wild-type showed the presence of the endogenous gene. The transgenic rice plants showed further a signal from a 2130 bp fragment, indicating the complete integration of the genomic sequence, the flanking glutelin promoter and the polyadenylation sequences. 11 plants out of 18 showed a single copy insertion of the transgene, as shown in Figure 18 by the single hybridisation band obtained when the DNA was digested with *Eco*RV. The other rice lines showed 2 or more inserted copies and rearrangement of the transgene.

An eventual contamination by Agrobacteria can not be excluded in lines, which reveal the presence of a band with almost the same molecular weight as the positive control after *Eco*RV digestion.

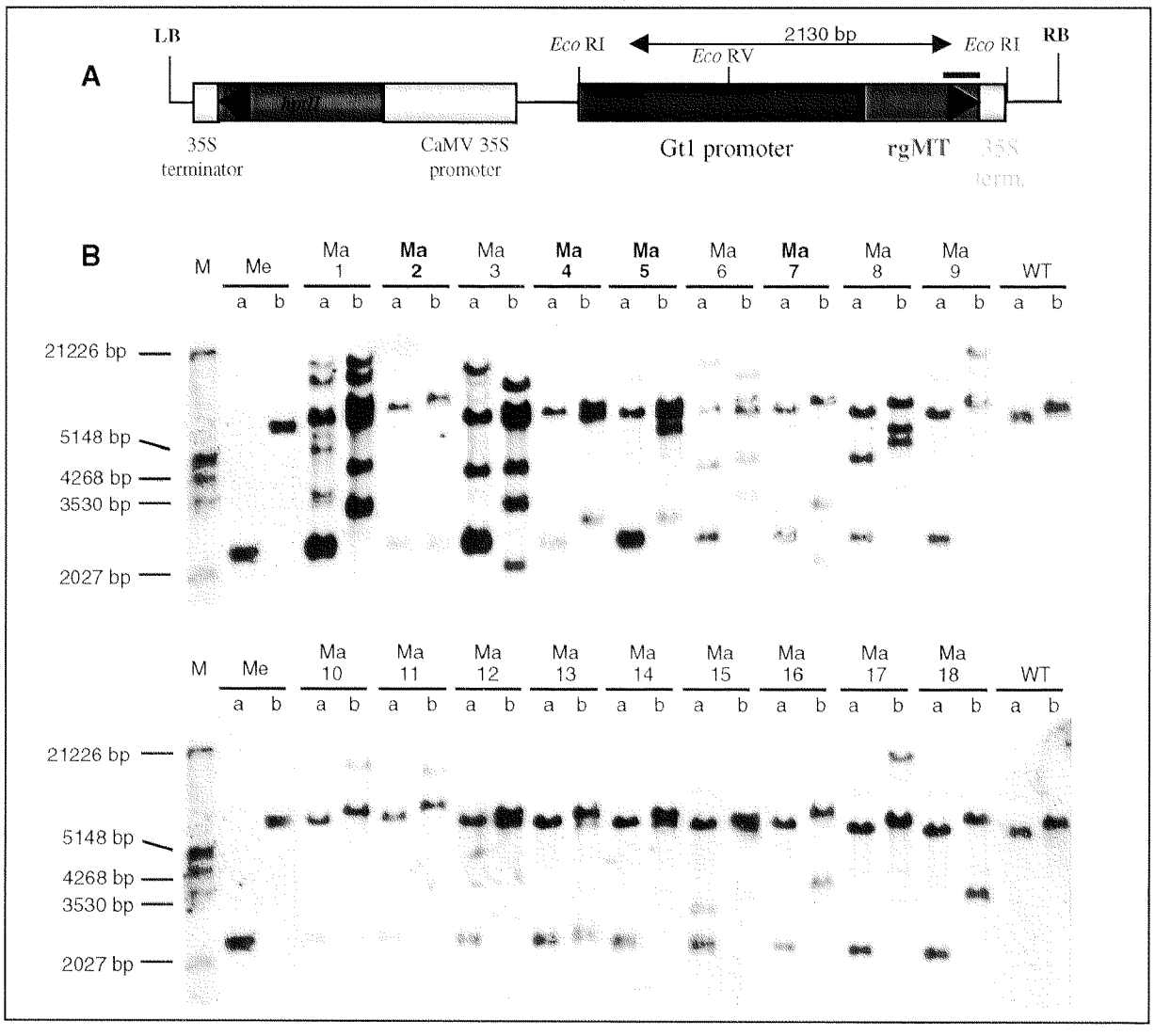


Figure 18: Southern blot analysis of 18 putative pAGt1Me–transgenic plants. A: Schematic drawing of the rgMT gene from *Oryza sativa* driven by the Gt1 promoter in construct pAGt1Me. B: Analysis for the presence of the rgMT genomic sequence. The plant genomic DNA was cut with *Eco* RI (releasing a 2.1 kb fragment) and with *Eco* RI to test the independence of the transformation events. Plant genomic DNA from a non transformed plant (WT) was used as negative control and plasmid pAGt1Me as positive control (M). The bar above the gene of interest marks the probe used for hybridisation. Sterile plants are marked in bold.

Summary of fertile transgenic rice plants.

Those plants obtained from all transformation experiments, which developed normally and were fertile, are summarised in table 7.

Table 7: Summary of transgenic rice plants obtained. The percentage of fertile plants and the percentage of fertile plants containing a fragment of the expected size is given relative to the number of transgenic rice plants obtained.

Construct used for transformation	Total number of transgenic fertile plants	Number of fertile plants containing a fragment of the expected size
Biolistic transformation		
pGt1Fe	2 (16.6%)	1 (8.3%)
pGt1Me	1 (8.3%)	1 (8.3%)
pGt1PN	11 (22.4%)	8 (16.3%)
pGt1PF	4 (26.7%)	3 (20%)
Agrobacterium - mediated transformation		
pAGt1Fe	24 (82.7%)	24 (82.7%)
pAGt1Me	14 (77.8%)	14 (77.8%)

Biolistic transformation of precultured immature embryos and rice cell suspensions yielded plants containing mostly fragments that were much larger than expected, suggesting rearrangment of the transgenes or fragments having a smaller molecular weight, indicating the presence of truncated copies. Moreover, most of the plants regenerated were not fertile and showed abnormal growth.

The rice plants obtained after *Agrobacterium*-mediated transformation of precultured mature embryos contained the fragments of the expected size,

indicating the complete integration of the gene of interest, the flanking glutelin promoter and polyadenylation sequence. Most plants had only one single copy insertion of the transgene, exhibited a normal phenotype and were fully fertile.

3.5 RT-PCR analysis

3.5.1 Metallothionein gene expression

Having established the presence of the metallothionein rice genomic sequence in certain transformed lines, the next question was whether or not the integrated transgene was transcribed. According to the literature, the endosperm is the only tissue in which chimeric genes driven by the Glutelin Gt1 promoter should be expressed. Therefore immature rice endosperm has been used for RNA extraction.

For RT-PCR we used primers designed to recognise gene regions not present in the wild type rice and in the endogenous metallothionein-like protein. With these primers a 310 bp fragment was expected in those lines that showed rgMT transcription, but not in wild type rice. Plasmid pGt1Me was expected to give a 143 bp bigger signal as the transgenic rice plants because of the intron present in the rgMT gene.

For the RT-PCR analysis we used those plants that had been previously tested for the complete integration of the intact metallothionein sequence (see Figures 14 and 18).

As shown in Figure 19, all lines examined except one contained a PCR product of the expected size. The negative control did not give any signal at this size, as the regions recognised by the two primers were not present. The plasmid pGt1Me gave a signal from a fragment having a higher molecular weight as the transgenic lines, indicating the presence of the intron in the rgMT gene.

It was possible to conclude from these results that 11 plants out of 12 not only had the intact metallothionein sequence integrated, but also transcribed the transgene.

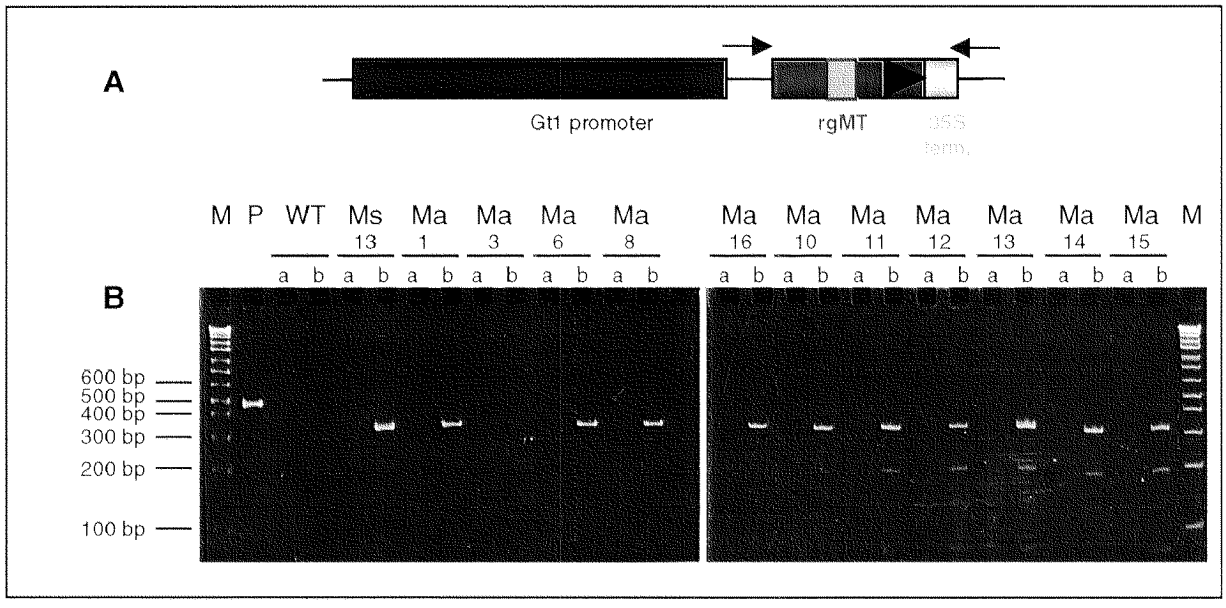


Figure 19: RT-PCR analysis of immature rice seeds to examine rgMT- expression. A: Schematic drawing of the metallothionein-like protein gene driven by the Gt1 promoter in construct pGt1Me. Arrows indicated the two primers used. The striped box in the rgMT gene indicates the intron. B: PCR products were analysed on 5% acrylamide gel. WT: non transformed, wild type rice. Ms: plants obtained after biolistic transformation of rice cell suspensions. Ma: plants obtained after *Agrobacterium*-mediated transformation.

3.5.2 Phytase (*A. fumigatus*) expression

Immature rice seeds from the four transgenic, fertile plants obtained after biolistic transformation with the pGt1PF construct were tested for transcription of the phyA gene. After RT-PCR analysis, a 1380 bp fragment was expected in those lines transcribing the fungal gene, but not in the wild type rice. Because of the presence of the RTBV intron in the construct used for transformation, the positive control, plasmid pGt1PF, was expected to give a signal having a 600 bp bigger molecular weight as the transgenic rice seeds. As shown in Figure 20, seeds from plant 11 gave a signal from the expected size, confirming that the RT-PCR product was the result of the RNA transcription of the rgMT gene and not due to plant DNA contamination. The

negative control did not give any signals at this size, whereas the positive control (plasmid pGt1PF) gave a band at 1980 bp, indicating the presence of the RTBV intron in the plasmid used for rice transformation.

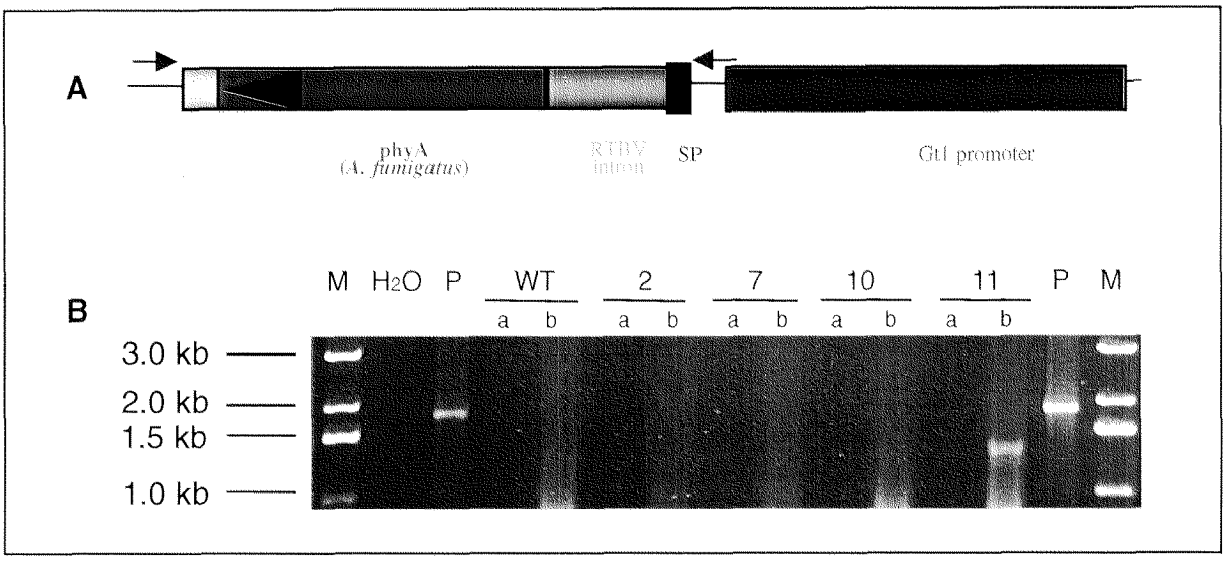


Figure 20: RT-PCR analysis of immature rice seeds to examine phyA- expression. A: Schematic drawing of the *Aspergillus fumigatus* gene driven by the Gt1 promoter in contruct pGt1PF. Arrows indicated the two primers used. B: PCR products were analysed on 0.8% agarose gel. WT: non transformed, wild type rice. PF: plants obtained after biolistic transformation of rice cell suspensions. H_2O: water.

3.6 Northern blot analysis

Northern blot analysis was performed with the lines that had integrated complete copies of the rgMT-gene and that showed a normal phenotype.
Northern blot analysis of seeds from plants transformed with pAGt1Me demonstrated that the rgMT-transgene was clearly overexpressed in 11 out of 12 lines obtained (see Figure 21). Non-transgenic rice as well as line Ma3 showed a weak signal indicating the background expression of the endogenous gene.

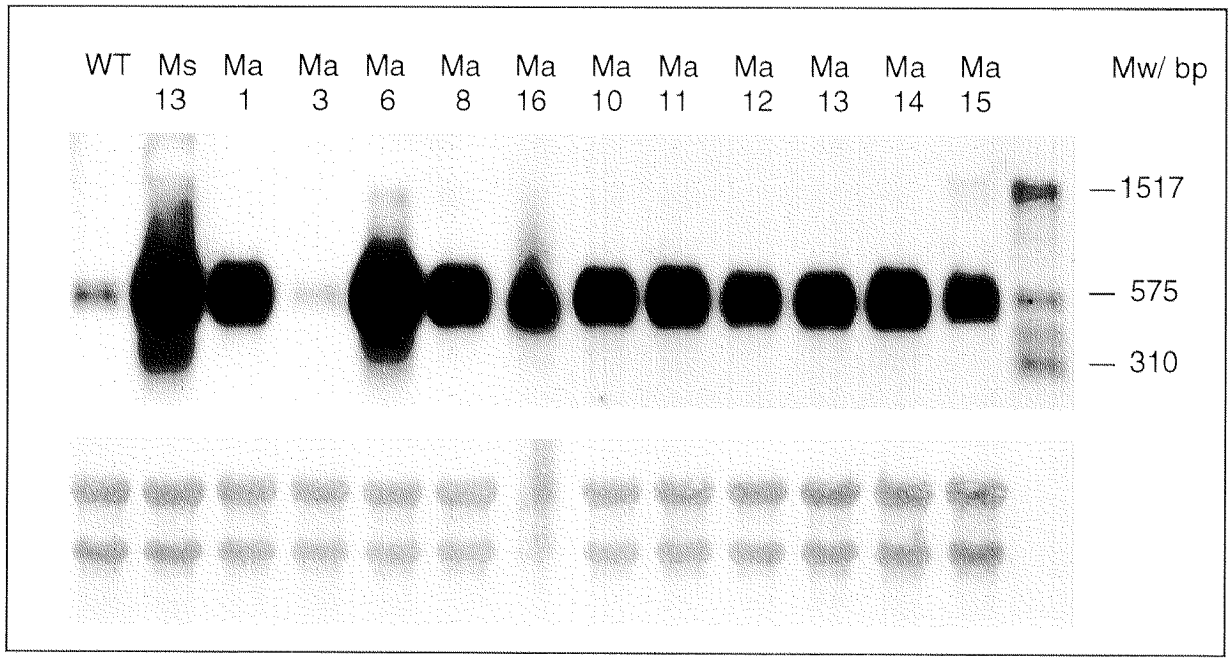


Figure 21: Northern blot analysis of seeds from plants transformed with pAGt1Me. Total RNA (12 µg) from twelve transformants and non transgenic rice (WT) were electrophoresed, transferred onto nitrocellulose membrane, and hybridised with a fragment of the metallothionein-like gene. Ms: plants obtained after biolistic transformation of rice cell suspensions. Ma: plants obtained after *Agrobacterium*-mediated transformation. The membrane after the transfer of RNA, stained with 0.04% methylene blue, is shown in the lower panel.

3.7 Protein analysis

3.7.1 Analysis of seeds from plants transformed with the ferritin gene

In order to assess ferritin protein expression in transgenic lines, immunological studies were performed using antisera raised against the pea ferritin protein.

Total protein extracts of mature rice seeds from transgenic lines obtained after biolistic or *Agrobacterium*-mediated transformations were examined by western blot analysis. A total of 26 pfe transgenic TP 309 lines were analysed, as shown in Figure 22. French bean seeds were used as positive control, as the ferritin introduced into rice had been cloned from *Phaseolus vulgaris*. Untransfromed TP 309 seeds were used as negative control. Equal amounts of total seed protein extracts were separated by SDS-PAGE and subjected to western blot analysis.

The positive control yielded the expected 26.5 kDa signal of the ferritin subunit (Spence, *et al.*, 1991). The immunoreactive polypeptide observed in the transgenic endosperm exhibited the same electrophoretic mobility. The molecular weight of the ferritin subunit extracted from transformants was different to that calculated from the bean ferritin cDNA sequence. Ragland (Ragland, *et al.*, 1990) indicated that the soybean ferritin subunit was synthesised as 32 kDa precursor and convert to 28 kDa by posttranslational processing in the plastid. Sequentially, the 28 kDa subunit could then convert to a 26.5 kDa subunit by cleavage of the extension peptide (EP).

Only the 26.5 kDa subunit was detected in protein extract from the rice seeds and from the French bean. This result shows that the ferritin subunit derived from *Phaseolus* cDNA in transgenic rice plants is cleaved to release the transient peptide and EP in a fashion similar to that of ferritin subunits in the French bean. This can be taken as an indication that the translation product had been imported into the endosperm amyloplasts and had also been processed from its precursor.

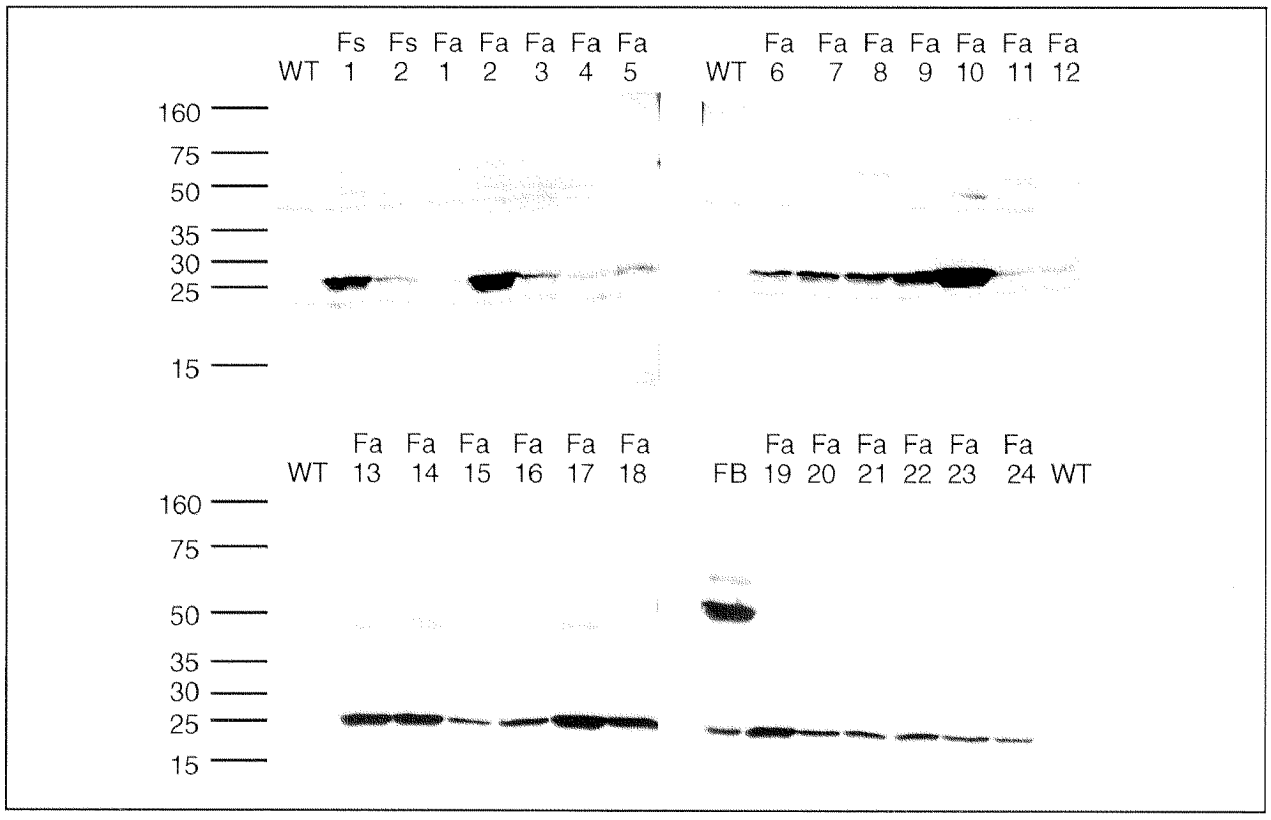


Figure 22: Western blot analysis of 26 transgenic plants. WT= wild-type, non transformed rice seeds, Fs= plants obtained after biolistic transformation of rice suspension cells, Fa= rice obtained after *Agrobacterium*-mediated transformation, FB= French bean as positive control.

To confirm the presence of the mature ferritin protein, which would indicate that the transcribed ferritin subunits assembled together forming the mature protein, equal amount of total seeds protein extracts were separated by a native gel. This preserved the intact ferritin protein structure preventing the denaturation of the protein to its subunits.

After separation of the proteins, the gels were stained by silver-staining procedure.

Untransformed TP 309 seeds were used as negative control, whereas French bean seeds and purified horse spleen ferritin (Sigma) as positive control.

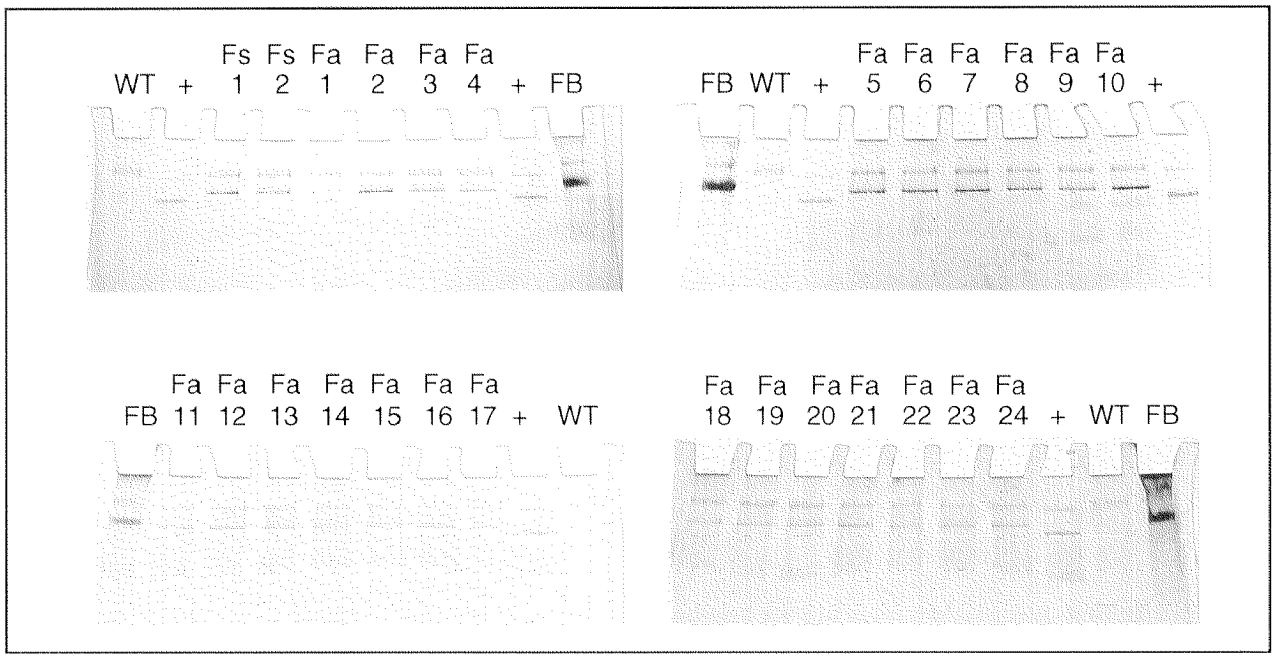


Figure 23: Acrylamide gels of protein extract from seeds expressing the ferritin subunit, stained by silver-staining procedure. WT= wild-type, non transformed rice seeds, Fs= plants obtained after biolistic transformation of rice suspension cells, Fa= rice obtained after *Agrobacterium*-mediated transformation, += purified horse spleen ferritin, FB= French bean.

Because of the strong gradient of the native gel (4-20%), big proteins found in the upper part of the gel stained much quicker than small proteins present in the thick lower part of the gel.

Total protein extracts of mature rice seeds from those transgenic lines that showed the expression of the ferritin subunits gave a high molecular weight band, additional to wild type rice, indicating that the ferritin subunits were not only expressed, but, after transport into plastid, assembled together forming the mature protein.

3.7.2 Analysis of seeds from plants transformed with the phyA gene (*A. niger*)

The fungal protein expression was tested in all transgenic, fertile plants obtained after biolistic transformation of rice suspension cells with pGt1PN. Using antiserum raised against the *A. niger* phytase only two proteins could be detected in all transgenic rice seeds and in the negative control. These two proteins had a molecular weight of 65 and 58 kDa, corresponding to the two endogenous phytases present in the rice grains [Hayakawa, 1989 #176]. No further immunoreactive proteins could be detected in the transgenic rice grains, indicating that none of the transgenic plants obtained expressed the fungal protein.

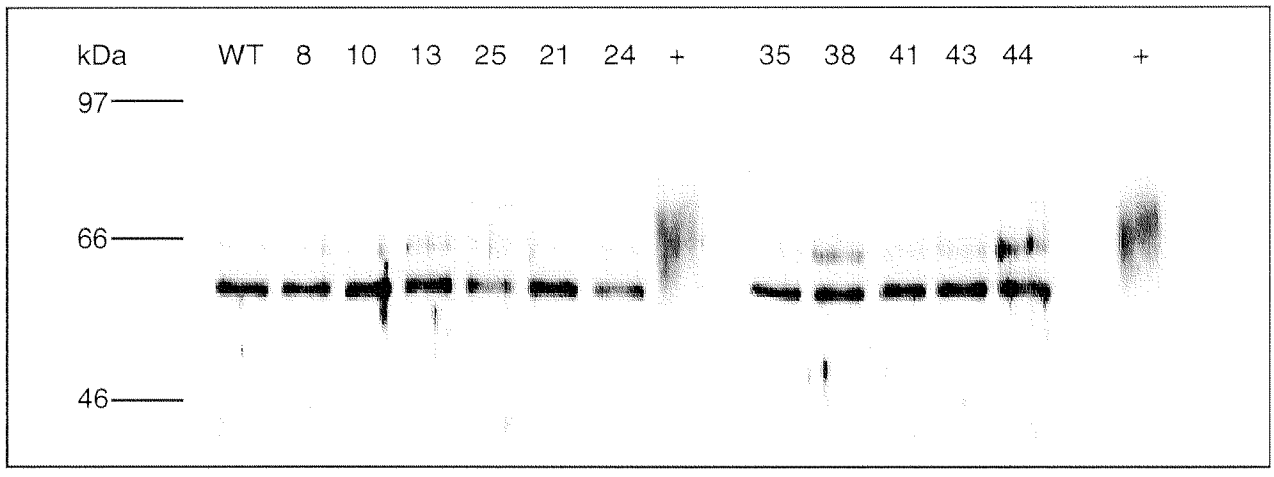


Figure 24: Western blot analysis of 11 transgenic rice plants. WT= wild-type seeds, non transformed rice seeds, += purified fungal phytase from *A. niger.*

3.7.3 Analysis of seeds from plants transformed with the phyA gene (*A. fumigatus*)

In order to assess the expression of the phytase from *A. fumigatus* in the transgenic rice seeds, proteins were extract from 4 fertile transgenic plants to perform western blot analysis. 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 [Hayakawa, 1989 #176]. Lines 2, 7 and 11 showed an additional band at 55 kDa not present in line 10 and 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 the fungal phytase in plants [Verwoerd, 1995 #144]. Two transformed plants (2 and 11) 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.

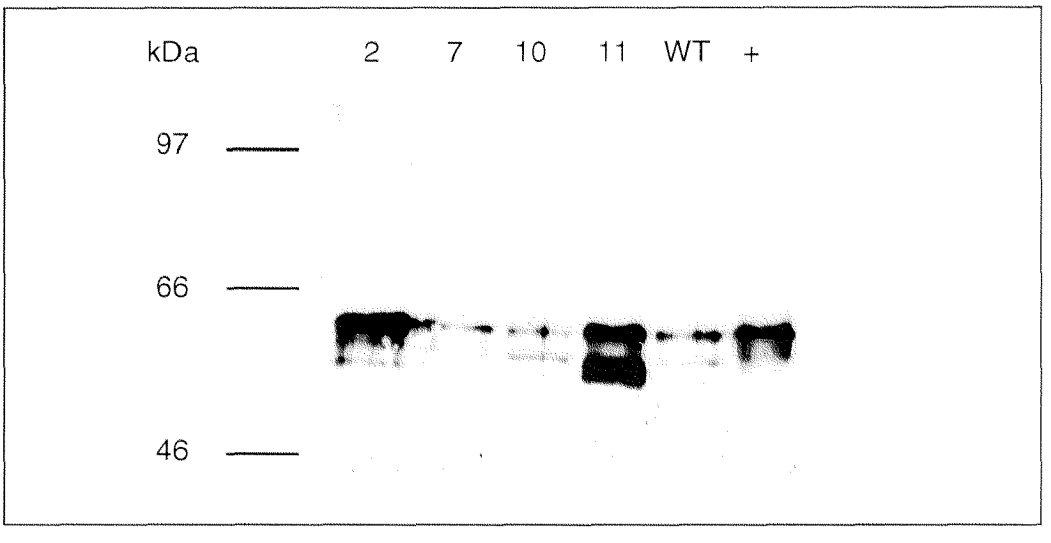


Figure 25: Western blot analysis of 4 transgenic rice plants. WT= wild-type seeds, non transformed rice seeds, += purified fungal phytase from *A. fumigatus*.

3.8 Biochemical analysis

3.8.1 Iron content of rice seeds expressing the ferritin

To determine whether the introduction of the ferritin from *Phaseolus vulgaris* led to a higher iron content of the transgenic rice seeds, the iron content was measured by graphite furnance atomic absorption spectroscopy after mineralisation of the rice samples by microwave digestion.

Regenerated plants expressing the ferritin protein from *Phaseolus vulgaris* showed an improved iron accumulation in seeds (see Figure 26). The amount of iron present in the transgenic grains varied considerably between different lines. Several plants did not show any significant increase of the iron content, whereas plants Fs 4, Fa 2, Fa 3, Fa 10, Fa 16 and Fa 23 contained between 35 and 73% more iron atoms. In line Fs2 a remarkable augmentation of 117% of the iron amount was measured compared to the mean value of the controls.

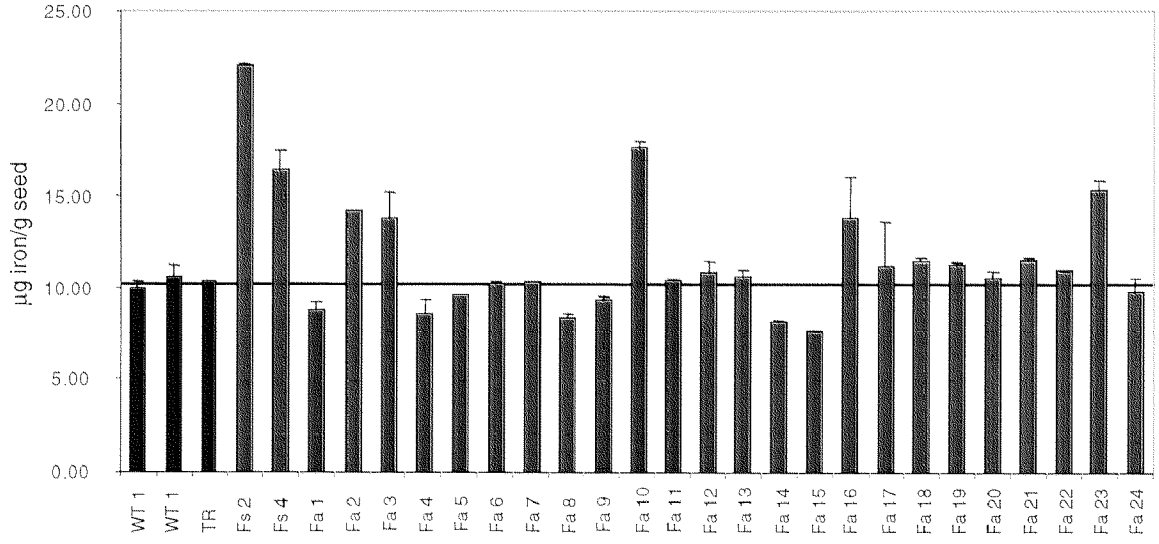


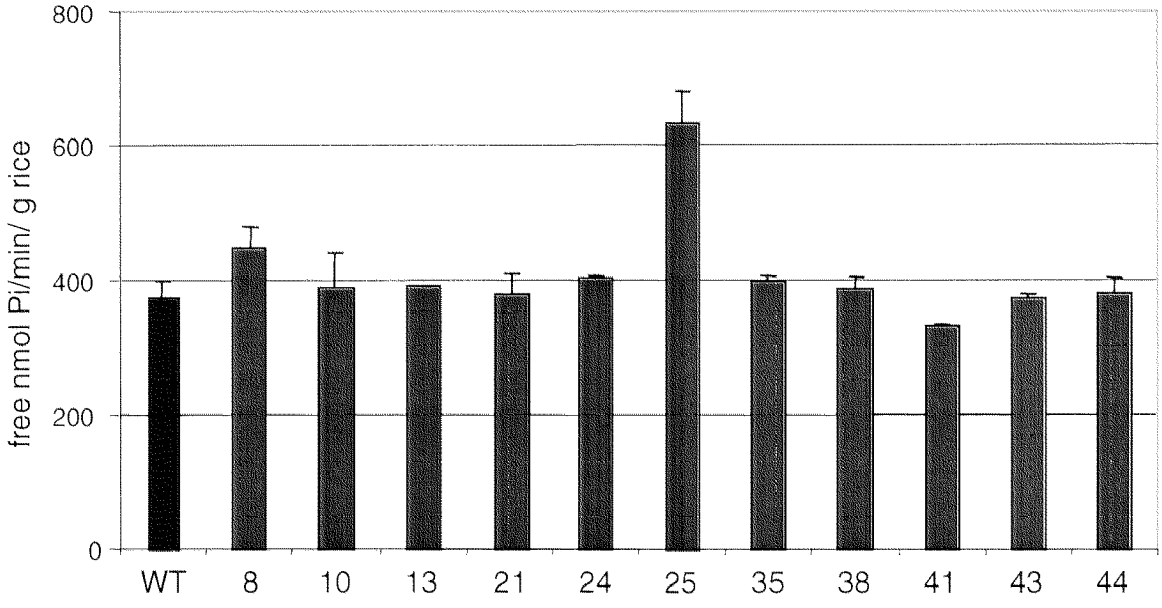
Figure 26: Iron content of seeds from 26 transgenic rice lines was measured by Graphite Furnace Atomic Absorption Spectrometry. Two untransformed plants (WT1, WT2) and a plant transformed with the rgMT-gene (TR) were used as controls. Fs= plants obtained after biolistic transformation of rice suspension cells, Fa= rice obtained after *Agrobacterium*-mediated transformation. The line indicates the mean value of the iron content of the control seeds.

3.8.2 Phytase activity in *A niger*-transgenic rice seeds

Analysis of the phytase activity was measured at pH 5.0 and 58°C, which are the optimal conditions for the activity of the phytase from *A. niger* (Ullah and Gibson, 1987).

All lines except for one did not show any significant increase in phytase activity. Line 25 showed a 50% higher activity as the wild type and the other transgenic lines.

Figure 27: The phytase activity of seed extract from plants transformed with the fungal phytase from *A. niger* and a untransformed control (WT) was measured at pH 5.0 and 58°C.



The plant showing a higher phytase activity, line 25, produced only seeds exhibiting an abnormal phenotype. The seeds were opaque and thin (see Figure 28). These characteristics were maintained even in the second generation, indicating that these different qualities were not due only to the transformation stress and in vitro growth conditions. 6 seeds of a non transformed plants weight 132 mg, whereas 6

transgenic seeds (2nd generation) from line 25 weigh 83 mg, representing 63% of the normal weight.

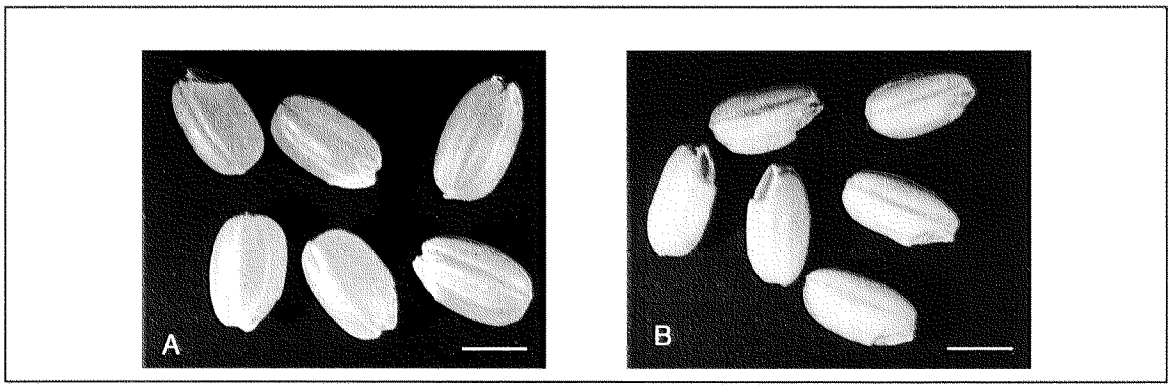


Figure 28: Seeds from a non-transformed rice plants (A) and seeds from the second generation of plant 25 (B).

3.8.3 *A. fumigatus* phytase activity in transgenic rice seeds

Analysis of the phytase activity in seed extracts was performed at 37°C and pH 6.0, which are the optimal condition for the activity of the fungal phytase. Three transgenic plants showed an increased in phytase activity in the grains. Compared to the non-transformed grains, two plants produced seeds with the double wild-type phytase activity, whereas the third plant (line 11, Figure 29) had an increased phytase activity of its grains of more than 100 fold, from 66 to 7260 phytase units/ g rice. Phytase activity correlated well with the amount of the 55 kDa protein detected by phytase antibodies (see Figure 25).

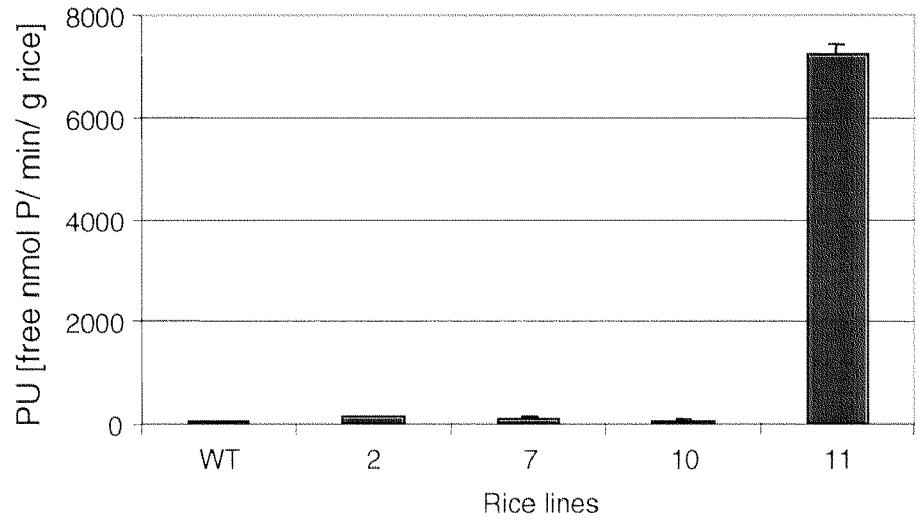


Figure 29: The phytase activity of seed extracts from plants transformed with the fungal phytase from *A. fumigatus* and a untransformed control (WT) was measured at pH 6.0 and 37°C.

3.8.4 Phytate content in transgenic rice seeds

To determine whether the expression of the fungal phytase from *Aspergillus fumigatus* affected the normal metabolism of the seeds by altering the level of the phytic acid, the content of the inositol tri-, tetra-, penta- and hexaphosphate was measured.

After extraction of the inositol phosphates from the seeds, inositol phosphates were separated from the crude extract by ion-exchange chromatography and analysed by ion-pair reverse phase HPLC.

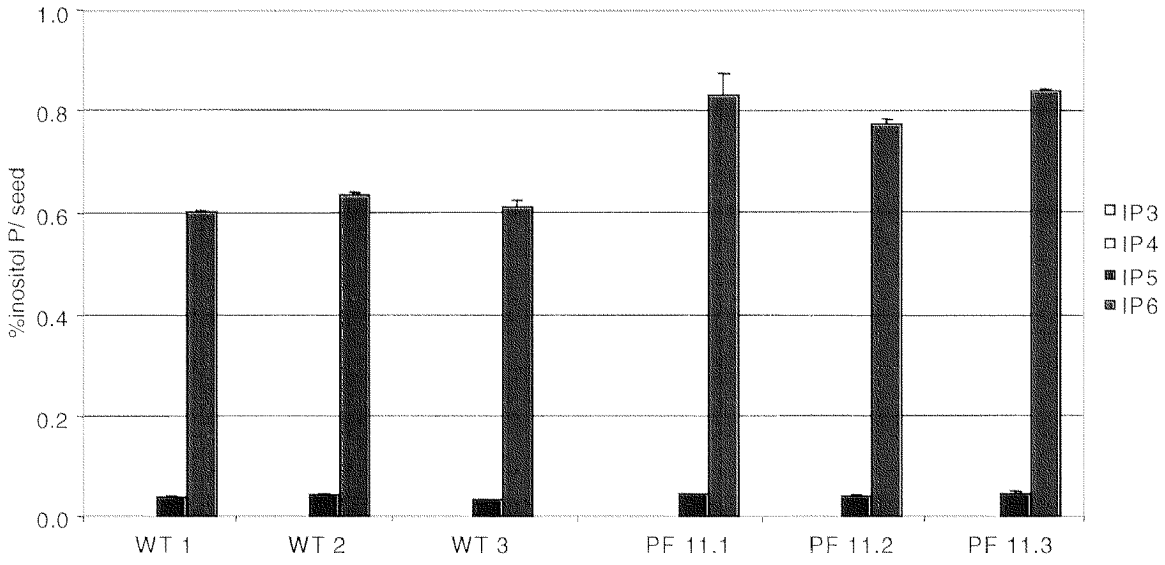


Figure 30: Inositol-phosphate content of seeds expressing the phytase from *A. fumigatus*. WT= untransformed control seeds, 11.1, 11.2, 11.3= second generation of seeds from line 11.

The inositol content of the seeds from the second generation of plant 11 strongly expressing the fungal phytase was measured and compared to the non transformed rice plants. The phytic acid content of seeds from 3 non transformed plants ranged from 0.60 to 0.63%, whereas the content of the seeds from 3 transgenic plants ranged from 0.77 to 0.84%. No inositol tri- and tetraphosphate could be detected in the rice seeds.

Phytic acid level after treatment of the seeds

To determine if the expressed fungal protein can have an influence on the phytic acid level in the rice seeds during cooking or digestion, rice seeds were subjected to heat treatment at different pH conditions. Seeds were heated in water at 100°C for 20 minutes to simulate the cooking process. To feign digestion condition, rice seeds were suspended in 0.4M glycin-HCl buffer at pH 2.5 and shaken at 280 rpm for 2h or in 0.4M imidazol-HCl buffer at pH 6.5 and shaken for 1 h at 37°C.

No significant differences could be detected after the simulated cooking process and stomach digestion in non transformed seeds and seeds from rice plant 11.1. The amount of inositol-phosphates slightly decreased after 2 h at pH 2.5 (about 10% decrease), but no major decrease in inositol hexa- (IP6) and pentaphospahte (IP5) could be noticed. Since IP6 and IP5 are responsible for iron chelation, no improvement of iron bioavailability is expected after such treatments.

However, after 1 hour treatment at pH 6.5, the difference between transgenic and non transformed seeds was remarkable. The level of inositol phosphates in the transgenic seeds strongly decreased (80% decrease) and only IP3 could be detected by HPLC analysis. Traces of IP6 were still present in the digested transgenic rice sample (about 0.15 µmol/ g rice), which do not have any influence on iron absorption (Sandberg, *et al.*, 1989). In contrast, the same treatment to wild type rice led to a small decrease of phytic acid only (10%), with a relative increase in inositol penta- and tetraphosphate.

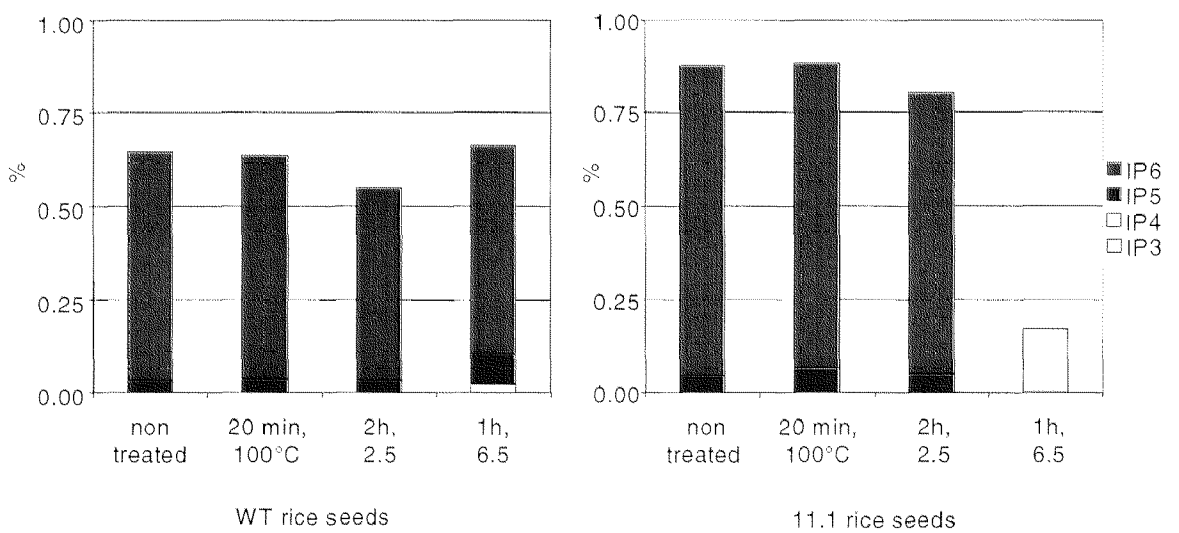


Figure 31: Inositol-phosphate content of seeds after treatment at different pH and temperatures conditions. WT= Wild-type rice seeds, 11.1= seeds from line 11.1 expressing the fungal phytase from *A. fumigatus*. 20 min, 100°C = cooking condition, 2h, pH 2.5, 37°C = stomach conditions, 1h, pH 6, 37°C = intestinal conditions.

3.8.5 Cysteine content in metallothionein-transgenic rice seeds

The cysteine content of seeds from lines overexpressing the rgMT-gene was determined after separation of the amino acids by ion exchange chromatography and a post-column reaction with ninhydrin. Amino acid analysis demonstrated that the cysteine content was increased in transgenic plants (see Figure 32). The increase in cysteine content in seeds overexpressing the rgMT gene was in the range of 20%. The cysteine content in the control seeds varied from 10.4 to 13.1 mg/ g amino acid and in the transgenic seeds from 13.1 to 14.9 mg/ g amino acid. The mean value of the controls was 1.17%, whereas the mean value of the metallothionein-like protein overexpressing seeds reached a level of 1.47%. There was no increase in cysteine content when expressed per 100 g seeds (see Figure 33).

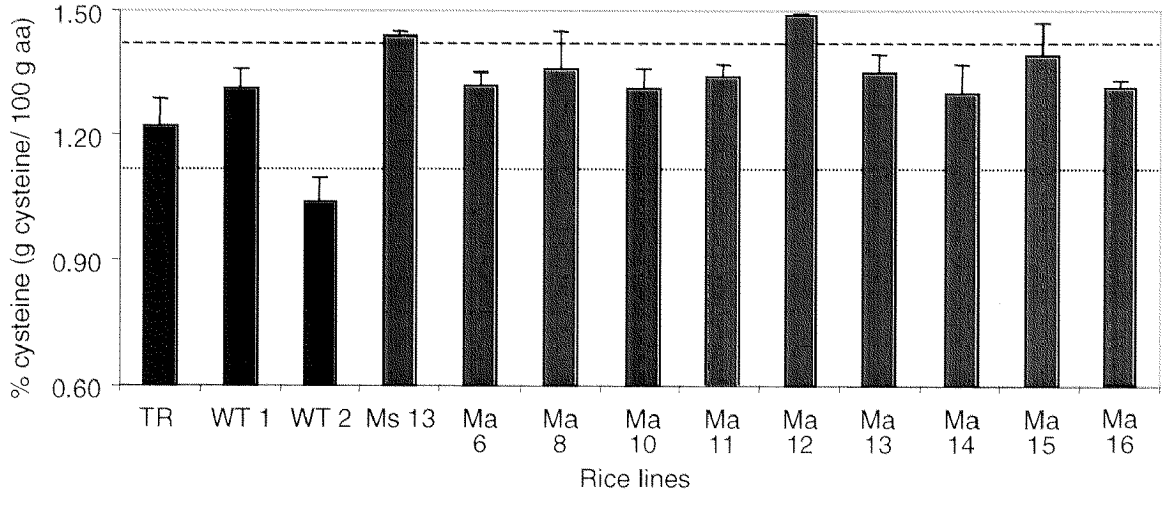


Figure 32: Cysteine content of rice seeds overexpressing the rgMT-gene. The amount of cysteine is given as gram cysteine pro 100 gram amino acid (aa). Untransformed plants (WT1, WT2) and a plant transformed with the pfe-gene (TR) were used as controls. The dotted line indicates the mean value of the controls, whereas the dushed line the mean value of the transgenic seeds. Ms= plants obtained after biolistic transformation of rice suspension cells, Ma= rice obtained after *Agrobacterium*-mediated transformation.

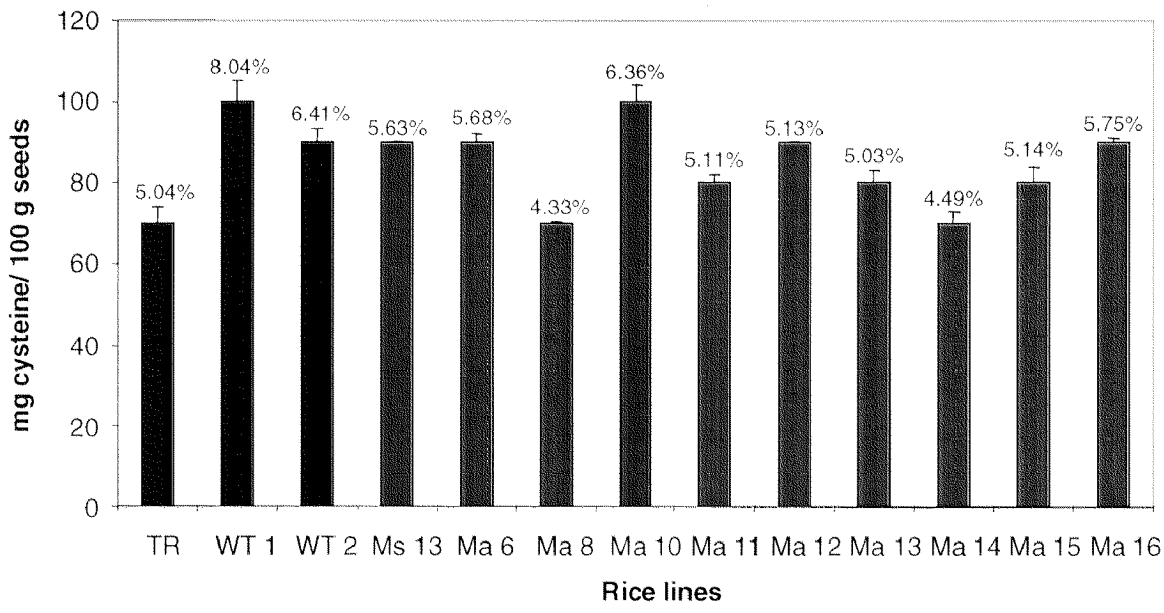


Figure 33: Cysteine content of rice seeds overexpressing the rgMT-gene. The amount of cysteine is given as mg cysteine pro gram rice seeds. Untransformed plants (WT1, WT2) and a plant transformed with the pfe-gene (TR) were used as controls. Ms= plants obtained after biolistic transformation of rice suspension cells, Ma= rice obtained after *Agrobacterium*-mediated transformation. The percentage of protein present in the rice seeds is indicated over the columns.

4 DISCUSSION

Three possibilities for increasing the amount of absorbable iron from rice-based meals have been explored. First a ferritin gene from *Phaseolus vulgaris* was introduced into rice grains increasing the iron content up to two fold. Then in an attempt to increase iron bioavailability, fungal phytases were introduced into the rice endosperm. Before using the phytase genes (from *Aspergillus niger* and *A. fumigatus*) for stable transformation of rice, the phytase activity of the respective proteins was measured in a transient protoplast assay. The protoplasts obtained showed a higher phytase activity as the controls, indicating that the fungal genes were suitable for rice transformation. Furthermore, the cysteine-rich metallothionein-like protein of rice was overexpressed in the endosperm tissue. While the cysteine level of the total rice protein changed only slightly, the phytase level of the grains transformed with the *A. fumigatus* phytase increased more than 100 fold. The thermotolerant phytase should resist destruction during cooking and was present at a concentration high enough to degrade most of the meal phytic acid during digestion. In contrast, no phytase activity could be detected in the grains from plants transformed with the *A. niger* gene.

4.1 Transient phytase expression in rice protoplasts

During the long processes of rice transformation and plant regeneration it would be difficult to obtain information on the functionality of the chimeric constructs encoding the two fungal genes, as the proteins are expressed only in the rice endosperm. Therefore, a transient expression system to receive information on the functionality of the transgene constructs has been used.

The constructs pPhyNC and pPhyFC, containing the chimeric phytase gene encoding the glucanase signal peptide and a cDNA fragment encoding the mature *A. niger* or *A. fumigatus* protein driven by the CaMV 35S promoter, were tested in

the transient protoplast expression system. Protoplasts transformed with the *A. niger* protein revealed a higher phytase activity compared to the control at all pH-values tested (see Figure 12). The activity observed was even higher than that of protoplasts transformed with the *A. fumigatus* protein. Only at pH 6.0 the level of the phytase activity was similar between the two fungal enzymes. This was quite surprising because the thermotolerant mutant Q27L phytase from *A. fumigatus* was supposed to have a much higher specific activity compared to that from A *niger* (see Figure 11) (Tomschy, *et al.*, unpubl., Ullah and Gibson, 1987). Results from transient expression of the two fungal proteins indicated that the *A. niger* phytase was probably more suitable for rice transformation as its activity was detectable over a broader pH rangen

Stable, biolistic transformation of rice suspension cells with the pGt1PN containing the chimeric phytase gene under the control of the glutelin Gt1 promoter led to 11 transgenic and fertile plants. The promoter is known to provide a high level of specific expression in the rice endosperm (Kim, *et al.*, 1993; Zheng, 1993), Southern blot analysis of these rice plants revealed that 8 out of 11 plants tested contained a fragment of the expected size, indicating the presence of the complete phytase gene. The fungal protein could not be detected in any plant (see Figure 24). As the immundetection of the protein by western blot was very sensitive (about 1 ng protein), it can be excluded that the expression of the transgenic protein was below the detection level. RNA analysis also confirmed that no gene transcription occurred (data not shown). It is unclear why the fungal protein was not transcribed in the rice endosperm despite the positive results obtained with the transient expression in protoplasts. Possible reasons for this result might be RNA unstability, which could explain the negative results by RT-PCR, or a wrong processing of the chimeric phytase gene.

4.2 Evaluation of rice transformation methods

The first transgenic rice plants were recovered by Toriyama, *et al.*, 1988; Zhang and Wu, 1988 and Zhang, *et al.*, 1988 using protoplast technology and direct DNA transfer. Subsequently, many reports describing recovery of transgenic rice plants appeared in the literature. The range of transformed genotypes, transformation methods and efficiencies expanded and improved significantly since these original reports.

Direct, biolistic gene transfer to calli derived from immature embryos and rice suspension cells of Japonica variety, followed by regeneration of trangenic plants, was a well established system in our laboratory when this work started (Burkhardt, *et al.*, 1997; Wunn, *et al.*, 1996). Bombardment-based methodology is responsible for the effective genetic manipulation of major cereals including rice. Many groups reported significant advances on various aspects of rice molecular biology and genetic engineering using procedures based on bombardment technology. Stability of expression was found to be dependent on the nature of the promoter and the transgene, and in specific cases on gene copy number.

It has been demonstrated that *Japonica* rice variety Taipei 309 is a suitable target for transformation (Christou and Ford, 1995; Li, *et al.*, 1993). The major advantages of this variety are its excellent tissue culture properties and good response to hygromycin as selective agent. Transformation efficiencies using immature embryos of up to 30% has been reported(Christou and Ford, 1995; Li, *et al.*, 1993) and TP309 is generally considered the model rice variety for transformation.

The evaluation of different strategies, which could lead to a higher iron content of the rice grains and to a potentially better bioavailability, was the main aim of this work. Therefore, the best model variety had to be used. Later, when transgenes have been identified which confer the desired quality improvements, these can be transferred to Indica varieties, the most important rice varieties for food security in Southeast Asia. This transfer can be achieved either by transformation of Indica varieties or by classical backcrossing from the transgenic Japonica varieties.

In our experiments, plants obtained after biolistic transformation often showed abnormal growth and sterility. This was a major problem, as only 21% of the plants obtained after direct transfer to rice suspension cells developed normal fertile plants, and none of the regenerated plants after transformation of immature embryos was fertile. Such results are much worse than previously reported for biolistic transformation and it is unclear how these differences came about. However, other experiments performed in our laboratories with biolistic transformation of rice suspension cells led to similar results, with an extremely low transformation efficiency and a very high level (80%) of plants exhibiting an abnormal phenotype like stunted growth and sterility (Werthmüller, Bourdon, pers. comm.). This experience indicates that the extremely low fertility was neither due to the introduced genes, nor to the transformation system per se, but rather to unreproducible circumstances.

Southern blot analysis of the plants obtained revealed that almost all plants contained multiple copies of the transgenes, truncated copies and rearrangment of the gene of interest. This is actually a typical pattern after biolistic transformation and was obtained with all plasmids used.

Segregation analysis from these plants (data not shown) showed that the vast majority of loci of multiple integration events are genetically linked. Direct gene transfer system often produce fragmented and rearranged multiple trangenic integrations at a single genetic locus (Christou and Swain, 1990; Saul and Potrykus, 1990).

Further plants containing the *Phaseolus* ferritin and overexpressing the rice metallothionein-like protein have been regenerated using *Agrobacterium*-mediated transformation of calli derived from mature embryos. *Agrobacterium tumefaciens* had been routinely used in gene transfer experiments to dicotyledonous plants, but monocotyledonous plants including important cereals were considered recalcitrant to this technology as they are outside the biological host range of grown gall.

Numerous approaches towards the infection of monocotyledonous plants with *Agrobacterium* had been undertaken in many laboratories, but the results were not conclusive until recently. Efficient transformation protocols for *Agrobacterium*-

mediated transformation were reported for rice between 1994 and 1997 (Aldemita and Hodges, 1996; Dong, *et al.*, 1996; Hiei, *et al.*, 1994; Toki, 1997). A key point in the protocols was the fact that tissues consisting of actively dividing, embryogenic cells, such as precultures immature and mature embryos, were co-cultivated with *Agrobacterium* in the presence of acetosyringone, which is a potent inducer of the virulence genes. Advantages of the *Agrobacterium*-mediated transformation of rice, like with dicotyledons, include the transfer of pieces of DNA with defined ends with minimal rearrangements, the transfer of relatively large segments of DNA, the integration of small numbers of copies of genes into plants chromosomes, and high quality and fertility of transgenic plants.

Plants obtained after *Agrobacterium* infection showed the characteristics described above. Eighty-one percent of the rice plants obtained were fertile and showed a normal phenotype, representing a considerable improvement compared to the results obtained after biolistic transformation (21% with rice suspension cells, 0% with immature embryos). Furthermore, most of the plants contained only 1-3 copies of the transgenes. Since an increased copy number may result in reduced expression levels of the transgenic protein, the presence of only a few copies of the transgene is a clear advantage.

Southern blot analysis, after DNA digestion with an enzyme that cut once into the T-DNA, revealed the presence of a band with almost the same molecular weight as the digested plasmid. This indicated probably a contamination with persisting agrobacteria, which are capable to survive the standard antibiotic treatments of cefotaxime. After co-cultivation of agrobacteria with plant tissue, selection of the transformed cells takes place on a medium containing high level of antibiotics intended to eliminate the agrobacteria. However, the complete elimination of agrobacteria from transgenic tissue was difficult in many cases (Mogilner, *et al.*, 1993; Landsmann, *et al.*, 1995), possibly because the bacteria are capable of forming covert relationships with plants as observed with *Enterobacteriaceae* in a number of plant tissue culture system (Holland and Polacco, 1994). The persistence of *Agrobacterium tumefaciens* cells in transformed tobacco plants despite the presence of 300 mg/ l cefotaxime in the tissue culture medium was assessed over a

period of 12 months post-transformation *in vitro*, and 3 to 6 months thereafter *ex vitro* (Matzk, *et al.*, 1996). The accumulation of persisting bacteria in the basal parts of tobacco plants would indicate that there is a low probability of agrobacteria contaminating the progeny of seed-propagated plants like tobacco and rice.

Several observations seem to confirm that the plants obtained were really transgenic even thought the possible bacteria contamination and that the results obtained by Southern blot were not "false positive" results. Northern blot analysis of seeds containing the transgenic rgMT-gene revealed that the endogenous protein was clearly overexpressed in the transformed seeds. Because of the presence of one intron in the rgMT sequence, the higher amount of metallothionein-like RNA can not be attributed to bacterial transcription. Localisation analysis of persisting agrobacteria in transgenic tobacco plants (Matzk, *et al.*, 1996) revealed that no bacteria could be isolated from seeds, flowers, and young leaves. Moreover, it is quite improbable that the glutelin promoter, highly specific for the protein expression in the rice endosperm tissue (Zheng, 1993; Zheng and Murai, 1997), is active in procaryotic bacterial cells. Therefore, the positive results obtained after western blot analysis of seeds containing the *Phaseolus* gene confirms the integration of the ferritin gene into the rice genome and its transcription.

A major concern is the problem of transferring genetically modified Agrobacteria into the environment. This could cause an undesirable spread of transgenes in the ecosystem. Therefore, it would be judicious to eliminate agrobacteria before releasing plants into the field. Because of the low probability of agrobacteria persisting in the upper part of the plants and therefore in the progeny (Matzk, *et al.*, 1996), the second generation should not have problems concerning contamination.

4.3 Protein expression analysis

Western blot analysis of seeds from plants obtained after transformation with the pfe gene and Northern blot analysis of seeds containing the metallothionein-like gene plasmid revealed that almost all the transgenic seeds expressed the gene

introduced. The expression level varied from plant to plant, what is generally true for plants obtained from different transformation events.

As the results published are often incomplete, and only the plants showing the best qualities are reported, it is quite difficult to interpret these results and place them in the context of other transformation events. Burkhardt (Burkhardt, *et al.*, 1997) reported the expression of a phytoene synthase cDNA from *Narcissus pseudonarcissus* under the control of either a constitutive or an endosperm-specific promoter (Gt1) in the rice seeds. Only 2 out of 27 plants showed immunoreactive an signal in western blot analysis. Those two plants expressing the transgenic protein contained the gene driven by the glutelin promoter. Rice seeds harboring the soybean ferritin gene under the control of the glutelin GluB-1 promoter are reported by Goto (Goto, *et al.*, 1999). Thirty independent transgenic rice lines were obtained by *Agrobacterium*-mediated transformation. Three lines were further analysed and were western positive. Results of the other 27 rice lines were not specified. Therefore it would seem that the expression obtained with the pfe and rgMT gene (100% of the plants harboring the pfe gene and 91% of the plants transformed with the rgMT gene express the transgenic protein) is highly.

Western blot analysis of plants containing the fungal phytase genes (from *A. niger* or *A. fumigatus*) did not give uniform results. The transgenic fungal protein from A. *niger* could not be detected in any of the 8 plants containing the entire expression cassette (promoter/ phyA gene/ terminator). The same negative results were obtained after RT-PCR analysis (data not shown). This result was quite surprising as the transient expression of the phyA from *A. niger* in rice protoplasts led to an increased phytase activity in the transformed protoplasts. It is unclear why none of the transgenic plants harboring the phyA gene expressed the fungal protein in the rice endosperm. In contrast, 3 plants out of 3 containing the phyA gene from *A. fumigatus* revealed the presence of the fungal protein. Two plants displayed a very weak immunoreactive signal in western analysis, whereas one plant showed a strong signal indicating a high expression level of the transgenic protein. Transgenic tobacco seeds harboring the phytase from *A. niger* have been engineered to increased the phosphourus utilisation when used as animal feed

(Pen, *et al.*, 1993). Seeds of 23 independent transgenic plant lines showed accumulation of the enzyme to 1% of the total soluble protein. Even if the total number of plants obtained is not reported, it is clear that the fungal gene was suitable for tobacco transformation and led to a good expression level. Stable accumulation of *A. niger* phytase was also reported in transgenic tobacco leaves (Verwoerd, *et al.*, 1995). A total of 72 independent antibiotica-resistant plants were tested for phytase activity. The average expression level in all 72 plants was 0.2%. The percentage of plants expressing the fungal protein was not given.

4.4 Phytase activity from seeds harboring the phytase gene (*A. niger*)

Of some concern is the activity improvement of seeds from rice plant 25 harboring the fungal phytase gene from *A. niger* although no fungal proteins could be detected in the grains from this line by western blot and the same negative results were obtained after RT-PCR analysis of RNA from immature seeds (data not shown). The increased activity can therefore not be explained with the presence of the transgenic protein. However, it has to be considered that seeds from line 25 did not exhibit a normal phenotype. They were smaller, thin and opaque. Moreover, due to the different consistence of the transformed rice grains, the transgenic seeds weighted only 63% of normal, non transgenic seeds. This means that for the analysis of the phytase activity, more transgenic seeds than control seeds have been used. This could explain the activity increase of line 25 despite the absence of the fungal protein.

4.5 Phytase and seed germination

Since phytic acid is the storage form of phosphorus in plants for an efficient germination of the seedlings (Pernollet, 1978), it is important that the introduced genes do not degrade the phytate already during the seed formation. For this reason,

no enzymes were introduced into the rice endosperm, which interfere with the biosynthesis of phytic acid. Constructs that produce antisense RNA of genes which are responsible for the subsequent phosphorylation of glucose-6-P to phytic acid, such as *myo*-inositol-1-P synthase and phosphoinositol kinase, could be one possibility to decrease the level of phytic acid. However, this would decrease the concentration of phytate already in the seeds during their formation, leading eventually to problems during germination.

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* (11.1, 11.2, 11.3) 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 secrets the protein to the extracellular fluid (Fuetterer, pers. communication).

Mutations that reduce phytic acid content of grains, termed "*low phytic acid*" mutations (*lpa*), have been isolated by chemical mutagenesis from maize (Raboy and Gerbasi, 1996) and barley (Larson, *et al.*, 1998). Two types of biochemical phenotypes have been detected in barley and maize. The first, and most common, type of mutation (designated *lpa1*) shows a decrease in phytic acid P and a molar-equivalent increase in inorganic P. The second type of mutation (designated *lpa2*) also shows a decrease in phytic acid P, but does not show molar-equivalent increases in inorganic P (relative to loss of phytic acid P). Instead, a significant portion of the total seed P remains bound in lower inositol polyphosphates (e.g., *myo*-inositol pentaphosphate, IP5) in the *lpa2* mutants. Problems in germination due to the decrease in phytic acid have not been reported, probably because the total amount of phosphorous in the grains did not vary.

Iron absorption from tortillas prepared with the low-phytic acid maize (*lpa1* mutant) was reported to be 49% greater than from wild type maize tortillas, indicating that consumption of genetically modified, low phytic acid strains of maize may improve

iron absorption in human populations that consume maize-based diets (Mendoza, *et al.*, 1998). Improvement of the iron nutrition with the *lpa2* mutant was not tested and, actually, not expected, as IP5 still strongly binds iron, thus inhibiting its absorption.

4.6 Ferritin versus haem-protein

Haem iron enters the mucosal cell through a pathway different from that for non-haem iron (Foundation, 1995). It is transferred into the cell as intact haem moiety (Figure 1). Haem iron is highly available (20 to 30 percent absorbed) and its absorption is relatively little affected by dietary factors (FAO, 1988). Once within the cell, iron is released from haem and it joins an absorption pathway common to both haem and non-haem iron (Foundation, 1995).

Because of the good absorbability of the iron atom contained, haem-proteins such as peroxidases, leghemoglobin and cytochromes would be an ideal tool to increase the amount of highly bioavailable iron in the food. Unfortunately, these molecules only contain one Fe atom. To reach the daily iron requirement, Fe proteins would be needed in an unphysiological high concentration. With a hypothetical expression level of the transgenic proteins in plants of 1%, it would be impossible to obtain any significant improvement in the iron supply of populations, where rice is the staple food. A clear advantage is offered from the ferritin molecule, which can store up to 4500 iron atoms in the central cavity (Harrison and Arosio, 1996; Korcz and Twardowski, 1992).

4.7 Ascorbic acid versus metallothionein-like protein

Ascorbic acid is the best known and most potent enhancer of iron absorption, both in its natural form in fruits and vegetables (Ballot, *et al.*, 1987) and when added as the free compound (Cook and Monsen, 1977). The enhancing effect is dose related over the range from 25 to 1000 mg (Cook and Monsen, 1977). At high enough

concentrations, ascorbic acid can overcome the inhibitory effect of phytic acid in cereals (Hallberg, *et al.*, 1989) and in soy formula (Davidsson, *et al.*, 1994), and it can partially overcome the effect of polyphenols from tea (Disler, *et al.*, 1975). It is possible that the marked promoting effect of ascorbic acid on the iron absorption is due to two different but concurrent mechanisms. One way of action would be due to the formation of iron ascorbate complexes with suitable stability constants in the gastrointestinal lumen. In this way, ascorbic acid would counteract the influence of other competing ligands such as phytate phosphates. Another mechanism would be related to the properties of ascorbic acid to reduce ferric to ferrous iron at low pH (Conrad and Schade, 1968).

However, ascorbic acid in the rice endosperm would have several disadvantages.

The expression of ascorbic acid in rice endosperm would require the introduction of several enzymes (different dehydrogenases and a 2,5-diketo-D-gluconic acid reductase) (Anderson, *et al.*, 1985), for which the genes have not been isolated and cloned, so far. Moreover, ascorbic acid would be sensitive to losses during storage and cooking and it would probably alter the taste of the rice in an unacceptable way. Such relevant consequences made it impossible to choose ascorbic acid as a tool for improving the iron bioavailability from a rice meal. The overexpression of the endogenous metallothionein-like protein seemed to be more appropriate and without such disadvantages in the rice grains.

4.8 Nutritional concerns of the transgenic rice grains

The increased iron content in our transgenic plants is assumed to be as a result of the expression of the *Phaseolus vulgaris* ferritin gene. This assumes that either there is already sufficient iron in the plant, which can be transferred to the seed ferritin, or that more iron can be absorbed via the root system, transported through the plant and deposited in the seeds. As the amount of iron in the rice seed is only a small part (ca. 4%) of the total shoot iron (Grusak, *et al.*, 1999), it would seem that transfer of iron and accumulation in the seed might be the limiting step.

Recently Goto (Goto, *et al.*, 1999) has similarly reported the expression of a soybean ferritin in rice seeds increasing the iron content of up to 3 fold. The 2-3 fold extra iron consumed by eating the transgenic rice grains would appear to be of nutritional significance. The iron intake from a daily consumption of about 300 g rice by an adult (IRRI, 1993) 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 of an adult women of child-bearing age (Council, 1989), one of the population groups most at risk of iron deficiency. Thus, while providing a very useful increase in iron intake, there is still room for further improvement.

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 gastro-intestinal tract during digestion and thus is not available for absorption. Human studies with intrinsically radiolabelled animal ferritin indicated that the iron contained within molecule ferritin added to a meal is only about half as well absorbed as vegetable iron (Martinez Torres, *et al.*, 1976; Martinez Torres, *et al.*, 1986) and as ferrous sulphate (Skikne, *et al.*, 1997). There are no human studies with intrinsically labelled plant ferritins. Beard (Beard, *et al.*, 1996) however suggested that intrinsically labelled animal ferritin might not be typical of the ferritin found in normal animal tissues. They recommended a re-evaluation of ferritin bioavailability and showed that iron content in horse spleen ferritin was as bioavailable as ferrous sulphate in anaemic rats. The anaemic rat has been shown to predict well the relative bioavailability in man of different fortification compounds (Forbes, *et al.*, 1989) although it is not a good model to predict the magnitude of enhancers and inhibitors of iron absorption (Reddy, *et al.*, 1996). It remains to be shown therefore to what extent iron contained in plant ferritin can be utilised in man.

The insertion of the phytase gene from *Aspergillus fumigatus* into rice however has a great potential to improve iron nutrition in rice-eating populations. The phytase activity of the transgenic rice is extremely high (7260 units/g). It is as high as the natural phytase activity in rye and much higher than in other cereal grains and legume seeds, which we have analysed using the same methodology (Egli,

Davidsson, Hurrell, unpublished). Unlike normal cereal and legume phytases, the enzyme from *Aspergillus fumigatus* is reported to be thermotolerant (only 10% loss after 20 min at 100°C) and to have a broad pH optimum from pH 2.5 to 7.5 (Pasamontes, *et al.*, 1997). It is probable therefore that it would withstand the normal cooking of rice and still be sufficiently active to degrade a substantial amount of phytate in the gastrointestinal tract during digestion of the meal. When a similar microbial phytase from *Aspergillus niger* was added to a high phytate bread roll prior to consumption, iron absorption by human subjects increased from 14.3 to 26.1% (Sandberg, *et al.*, 1996). An equivalent phytase activity would be contained in about 30 g of the transgenic rice. It can be calculated that 100 g of transgenic rice would be sufficient to degrade 1 g phytic acid in 10 to 15 minutes. Such an activity would have the potential to improve iron absorption considerably from rice meals alone or from rice meal combined with high-phytate legumes such as soy. HPLC analysis of the transgenic rice seeds (line 11.1) confirmed that after 1 hour at 37°C and pH 6.5 (intestinal conditions during digestion) inositol 6, 5 and 4 phosphates were completely hydrolysed to lower inositol polyphosphates (e.g., *myo*-inositol triphosphate or lower), which do not bind iron and do not have any influence on iron absorption (Sandberg, *et al.*, 1989).

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 cysteine, or the equivalent amount of cysteine as the tri-peptide glutathione, were added to a maize meal, iron absorption approximately doubled. By overexpressing metallothionein in rice, we increased the cysteine content of the total seed protein of about 20%, but due to a reduction in protein level in the transformed seeds, the cysteine content remained unchanged. Even if it were possible to engineer a more substantial increase in cysteine in homozygous lines, it remains to be demonstrated that the cysteine-containing peptides formed during the digestion of metallothionein, enhance iron absorption in man in a similar way to free cysteine, glutathione or the cysteine-containing peptides of meat.

4.9 Conclusion and future aspects of the project

In conclusion, 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 man of iron from *Phaseolus* ferritin, to confirm that the phytase from *Aspergillus fumigatus* is not destroyed during cooking and remains active in the gastrointestinal tract, and to measure the influence of metallothionein on iron absorption. Of the three genes introduced into rice, the phytase would appear at this stage to have by far the greatest potential for improving iron nutrition in rice eating populations.

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