

Symposium: Plant Breeding: A New Tool for Fighting Micronutrient Malnutrition

Golden Rice: Introducing the β -Carotene Biosynthesis Pathway into Rice Endosperm by Genetic Engineering to Defeat Vitamin A Deficiency¹

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ABSTRACT To obtain a functioning provitamin A (β -carotene) biosynthetic pathway in rice endosperm, we introduced in a single, combined transformation effort the cDNA coding for phytoene synthase (*psy*) and lycopene β -cyclase (*β -lcy*) both from *Narcissus pseudonarcissus* and both under the control of the endosperm-specific glutelin promoter together with a bacterial phytoene desaturase (*crtl*, from *Erwinia uredovora* under constitutive 35S promoter control). This combination covers the requirements for β -carotene synthesis and, as hoped, yellow β -carotene-bearing rice endosperm was obtained in the T₀-generation. Additional experiments revealed that the presence of *β -lcy* was not necessary, because *psy* and *crtl* alone were able to drive β -carotene synthesis as well as the formation of further downstream xanthophylls. Plausible explanations for this finding are that these downstream enzymes are constitutively expressed in rice endosperm or are induced by the transformation, e.g., by enzymatically formed products. Results using *N. pseudonarcissus* as a model system led to the development of a hypothesis, our present working model, that *trans*-lycopene or a *trans*-lycopene derivative acts as an inductor in a kind of feedback mechanism stimulating endogenous carotenogenic genes. Various institutional arrangements for disseminating Golden Rice to research institutes in developing countries also are discussed. J. Nutr. 132: 506S–510S, 2002.

KEY WORDS: • provitamin A • transformation • Golden Rice • bioavailability • humanitarian project

Rice is the major staple food for hundreds of millions of people. It is generally consumed in its milled form with outer layers (pericarp, tegmen and aleurone layers) removed. The main reason for milling is to remove the oil-rich aleurone layer, which turns rancid upon storage, especially in tropical and subtropical areas. As a result, the edible part of rice grains consists of the endosperm, filled with starch granules and protein bodies, but it lacks several essential nutrients for the maintenance of health, such as carotenoids exhibiting provitamin A-activity. Thus, reliance on rice as a primary food staple contributes to vitamin A deficiency, a serious public health problem in at least 26 countries including highly populated areas of Asia, Africa and Latin America (2).

A complementary intervention to existing strategies for reducing vitamin A deficiencies in the highest-risk countries is to fortify the major staple food, rice, with provitamin A

through plant breeding. This can only be achieved by recombinant technologies rather than by conventional breeding, due to the lack of any rice cultivars producing this provitamin in the endosperm. Both because the transformation of rice is well-established and because the entire carotenoid biosynthetic pathway has been molecularly identified recently, it seemed feasible to introduce the complete provitamin A (β -carotene) biosynthetic pathway into rice endosperm by genetic engineering.

We have shown previously that immature rice endosperm synthesizes the early intermediate geranylgeranyl diphosphate in the provitamin A biosynthetic pathway. This compound is not solely devoted to carotenogenesis but can be used as a substrate to produce the uncolored carotene phytoene by expressing the heterologous enzyme phytoene synthase in rice endosperm (3). This result prompted further investigations to install the entire pathway (1). Golden Rice, the resulting prototype line, bears the potential—after further improvements and testing—to contribute to the alleviation of vitamin A deficiency, provided that access to the β -carotene-rich seeds by poor farmers in developing countries is possible at the same cost as current popular cultivars. In a novel collaborative agreement between the university-based inventors and the private sector, an agreement has been signed guaranteeing this circumstance. This is discussed further below.

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RESULTS AND DISCUSSION

To engineer the pathway toward β -carotene formation, it is necessary to complement four plant enzymes, namely phytoene synthase, phytoene desaturase, ζ -carotene desaturase (the desaturases catalyzing the introduction of two double bonds, each), and lycopene β -cyclase. Alternatively, the transformation effort can be simplified by reducing the number of enzymes required and using a bacterial carotene desaturases capable of introducing all four double bonds required (Fig. 1).

Initially, we sought to introduce all genes into immature rice embryos (TP 309) stepwise, i.e., singly by particle bombardment, aiming at subsequently unifying all transgenes into a single plant by subsequent crossing. However, this approach was not successful, mainly due to the deleterious integration pattern frequently produced by this transformation technique, as revealed by Southern hybridization analysis. Therefore, *Agrobacterium*-mediated transformation of precultured rice immature embryos was used, designed so as to install the entire β -carotene biosynthetic pathway into rice endosperm in a single transformation effort. Three vectors, schematically depicted in Figure 2, were constructed. pB19hpc combines the sequences for a plant phytoene synthase (*psy*) originating from daffodil (*Narcissus pseudonarcissus*; accession no. X78814) (4) with the sequence coding for a bacterial phytoene desaturase (*crtI*) originating from *Erwinia uredovora* (accession no. D90087), the two being placed under the control of the endosperm-specific glutelin (Gt1) and the constitutive CaMV 35S promoter, respectively. The phytoene synthase cDNA contained a 5'-sequence coding for a functional transit peptide, as was demonstrated previously (5), while the *crtI* gene was fused to the transit peptide sequence of the pea Rubisco small subunit (*tp*), as constructed by Misawa et al. (6). This plasmid, thus, should direct the formation of lycopene in the endosperm plastids, the site of GGPP formation.

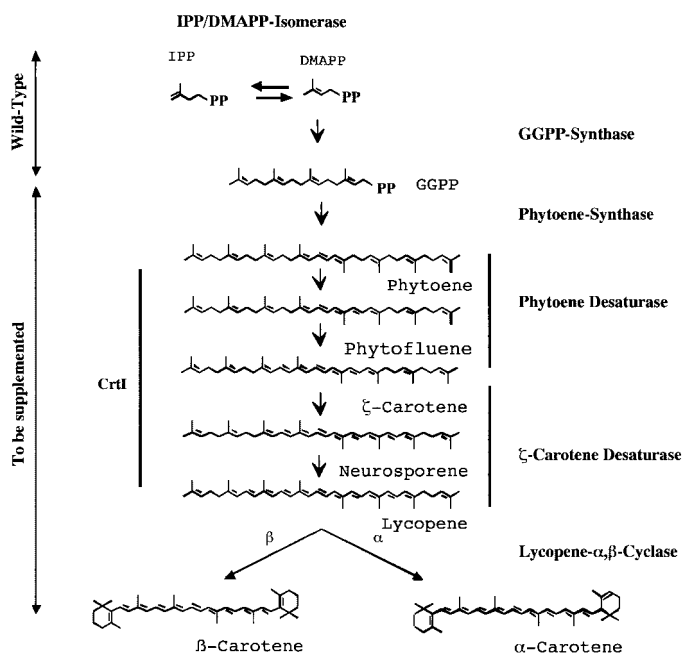


FIGURE 1 Provitamin A biosynthetic pathway. The names of enzymes are given. *CrtI* denotes a bacterial carotene desaturase capable in performing all necessary desaturation reactions for which two enzymes are required in plants. Arrows indicate the prenyllipid biosynthetic capacity of wild-type rice endosperm and the necessary reaction sequence to be completed to yield provitamin A.

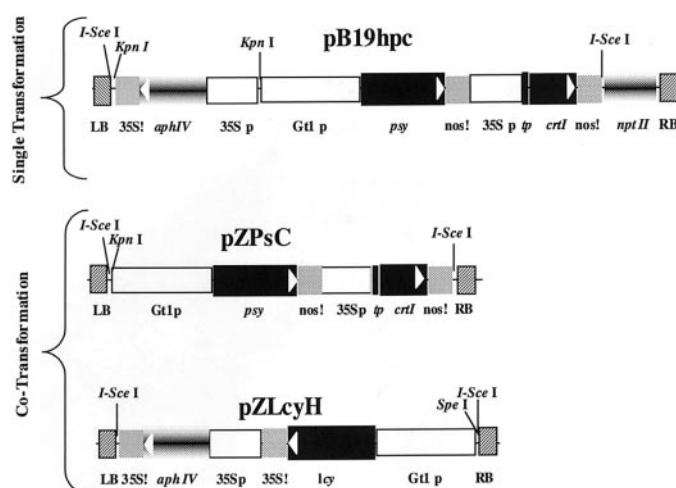


FIGURE 2 DNA constructs used in single transformations and cotransformations. RB, right borders; LB, left borders; *tp*, transit peptide from pea ribulose bis-phosphate carboxylase; *!*, terminator; *p*, promoter; *gt*, glutelin; *psy*, phytoene synthase; *crtI*, bacterial carotene desaturase; *lcy*, lycopene β -cyclase. DNA sequence coding for carotenoid biosynthetic enzymes are given in black.

To complete the β -carotene biosynthetic pathway, cotransformations were carried out employing two vectors, one (pZPsC) carrying *psy* and *crtI*, like in pB19hpc but lacking the expression cassette for the selectable marker *aphIV*, and the other (pZCycH) providing, under glutelin promoter control, the sequence coding for the enzyme lycopene β -cyclase, originating from *N. pseudonarcissus* (accession no. X98796) (7). Like phytoene synthase, lycopene β -cyclase carried a functional transit peptide, allowing plastid-import (5). The combination of both plasmids, thus, should be able to direct β -carotene formation in rice endosperm.

A total of 800 precultured rice immature embryos were inoculated with *Agrobacterium* LBA 4404/pB19hpc. Fifty hygromycin-resistant plants then were analyzed by Southern hybridization analysis (data not shown). All tested lines carried the transgenes and most of the plants showed single insertions, but in some cases multiple insertions were observed.

For cotransformation, ~500 precultured immature embryos were inoculated with an *Agrobacterium* mixture of LBA4404/pZPsC carrying the *psy* and *crtI* genes and LBA4404/pZCycH containing *lcy* together with *aph IV* as the selectable marker. Cotransformed plants were identified by Southern hybridization. All 60 randomly selected regenerated lines were positive for *lcy*, among which 12 plants were cotransformed with pZPsC. Like the transformation above, 1–3 transgene copies were predominant in cotransformed plants. Ten plants harboring all four introduced genes were transferred into the greenhouse for setting seeds. All plants from all transformations described here showed a normal phenotype as well as normal fertility.

Mature F₀ seeds from transformed lines and from control plants were air dried, dehusked and, to isolate the endosperm, polished with emery paper for 8 h on a shaker. In most cases the transformed endosperms exhibited a clearly notable yellow color, indicating carotenoid formation.

The pB19hpc single transformants showed a clear 3:1 (colored:noncolored) segregation pattern, whereas the pZPsC/pZCycH double transformants showed a wider deviation in segregation, as expected. To our surprise, the pB19hpc single transformants, although equipped for lycopene (red) synthesis,

were not distinguished in color compared with the pZPsC/pZCycH double transformants equipped for β -carotene (yellow) synthesis.

Seeds from individual lines (1 g of each) were ground to a fine powder and extracted to complete decolorization with acetone. The combined extracts were quantified photometrically and analyzed qualitatively by HPLC. The carotenoid pattern observed with the pB19hpc single transformants explained the phenotype that we noted visually. None of these lines accumulated detectable amounts of lycopene. Instead, the pathway was completed to form β -carotene, and even lutein and zeaxanthin were formed to some extent, resulting in a carotenoid pattern that is qualitatively quite similar to the one present in green leaves. This suggests that the lycopene $\alpha(\epsilon)$ - and β -cyclases as well as the hydroxylase are either constitutively expressed in rice endosperm or that the expression of these downstream enzymes is induced by lycopene formation or by products derived there from (see below).

The pZPsC/pZCycH double transformants exhibited a more variable carotenoid pattern. This ranged from phenotypes that are similar to the ones from the single transformations to others that contain β -carotene as almost the only carotenoid. Our line z11b is an example of the latter also representing up to now the winner in quantitative terms. A carotenoid content of 1.6 $\mu\text{g/g}$ dry rice endosperm was determined. From a nutritional point of view, it is not yet clear whether lines producing provitamin A (β -carotene) or lines possessing additionally zeaxanthin and lutein are to be selected, because it has been discovered during recent years that these xanthophylls are present in the eye's macula and, hence, their deficiency may contribute to macular degeneration, leading to blindness (8). In this respect, lutein and zeaxanthin, therefore, may represent valuable compounds for human health.

As stated above, there is an unexpected carotenoid pattern in the transgenic rice seeds exhibiting an active carotenoid biosynthetic pathway that proceeds beyond the point allowed by the enzymes introduced by the transformation. Currently, it cannot be ruled out that the transformation using the bacterial *crtI* -gene promotes a hitherto unknown feedback mechanism enabling the transcriptional activation of carotenogenic genes. The effector may be lycopene itself (or products derived therefrom) that is all *trans* configured when being formed by the bacterial enzyme, while the two plant desaturases yield a poly-*cis* configured lycopene, termed prolycopene (9). A working hypothesis is that prolycopene represents a biosynthetic intermediate, while *trans*-lycopene may act as an initiator of this feedback mechanism. To test this, we took advantage of the chemical compound CPTA [2-(chlorophenylthio)triethylamine hydrochloride] that acts as a lycopene cyclase inhibitor and leads to the accumulation of *trans*-lycopene (10). CPTA treatment, thus, mimics with respect to *trans*-lycopene formation our rice single transformation using plasmid pB19hpc. When CPTA was administered to daffodil flowers, they then turned reddish within 8 h due to lycopene accumulation. However, concomitantly, the carotenoid content was increased two to three times over the untreated controls. Northern blots conducted with probes directed against four carotenogenic mRNA as well as Western blots conducted with the corresponding specific antibodies showed that both the levels of specific mRNA examined as well as the amounts of the specific proteins were markedly increased over the untreated controls (11) (Fig. 3).

This result cannot be explained by the well-known action of CPTA as a lycopene cyclase inhibitor but indicates the presence of a novel regulatory mechanism. In fact, RNA subtraction between CPTA-treated and untreated tissue re-

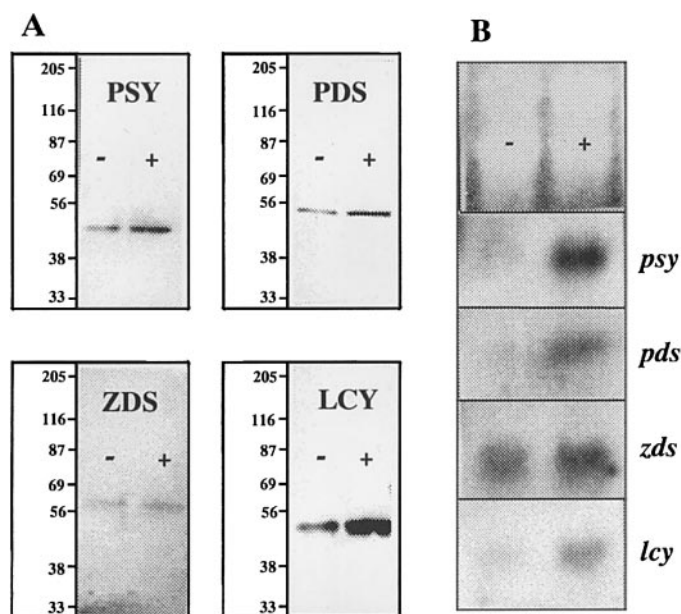


FIGURE 3 Northern and Western blot analysis of wild-type (-) and of *trans*-lycopene-accumulating, CPTA-treated petals (+).

vealed the induction of mRNA accumulation for at least two carotenoid biosynthetic genes, coding for deoxyxylulose phosphate synthase and β -carotene hydroxylase (unpublished data). Utilizing the transgenic rice in hand, further work is in progress to clarify molecularly whether this mechanism works here as well.

Consequences, new lines and further developments

One implication of the transformations described above is that the cotransformation is not necessary in rice endosperm, but that a construct containing *psy* and *crtI* might be sufficient to install the entire pathway. Accordingly, we reconstructed the plasmid pB19hpc with the following modifications. First, the hygromycin-selectable marker gene was exchanged against the *PMI* gene; concomitantly, the selection procedure for *PMI* was established for rice (12). Second, the *nptII* gene, left unnecessarily within the border sequences of the old construct (Fig. 2), was removed. New single lines have been produced recently showing again yellow color (Fig. 4). Carotenoid quantification showed again in the best performing segregating F_0 line, a carotenoid content of 1.6 μg carotenoid/g dry rice endosperm.

Further work now in progress aims to increase the provitamin A amount by identifying the metabolic rate-limiting bottlenecks in Golden Rice. New transformations are underway using different endosperm-specific promoters, a codon-optimized *crtI*-gene and early pathway genes of the so-called nonmevalonate pathway of isoprenoid biosynthesis (13). Further proof-of-concept work aiming to measure and enhance the bioavailability and bioefficacy of provitamin A are underway. One further approach aims to unify high-iron rice lines with provitamin A lines because it is known that provitamin A is capable of increasing the bioavailability of iron.

Golden Rice is not expected to provide 100% of vitamin A in the diet but to add to present intakes to reach vitamin A sufficiency. As stated above, the current lines are only prototypes and efforts are underway to triple the amount of the provitamin in the endosperm at minimum. Certainly, a high

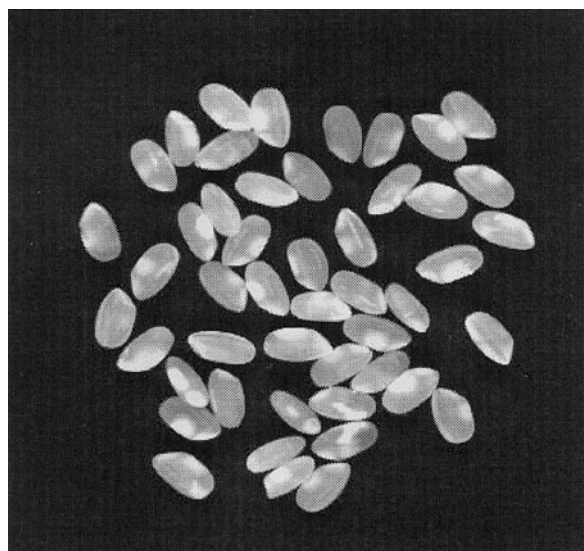


FIGURE 4 Examples of a new yellow rice line obtained using *PMI* as a selectable marker gene.

priority for research is an evaluation of the bioavailability and bioefficacy of the pro-vitamin A contained in Golden Rice. This research has been severely hampered in the past by the necessity of producing a sufficient quantity of grain (multiple kilograms) to be used in feeding trials in accepted model systems (pig, preruminant calves and ferrets) in safety greenhouses in Europe and restrictions prohibiting field trials outside of greenhouses. However, novel analytical methods have become available (utilizing HPLC-linked electrochemical detection or deuterium labeling in combination with HPLC and mass spectrometry) significantly lower amounts of rice. Efforts are currently underway to allow Golden Rice to be imported into the United States, where bioavailability investigations using these techniques can be conducted.

Legal situation and first steps for eventual dissemination

The development of Golden Rice has been made possible by sequential funding (3 y each) by two agencies: first the Rockefeller Foundation and then a research program of the European Community. Although funding from the Rockefeller Foundation was free of obligations, European Community funding required the participation of an industrial partner that would hold rights to inventions developed during the research. In this case, the industrial partner was Zeneca (merged recently with Novartis to form Syngenta). This European Community funding obligation has affected the current legal status of the Golden Rice project, which continues on two tracks, one being noncommercial (or humanitarian) and the other commercial (Fig. 5).

Syngenta received through the involvement of the German startup company Greenovations an exclusive license for the commercial use of the technology in developed nations. Simultaneously, Syngenta granted back to the inventors (Potrykus/Beyer) the exclusive license and the right to grant sub-licenses for its noncommercial use. Agreement has been reached between the two tracks that we believe serves our mutual interests. For example, all knowledge derived from research on the industrial marketing track will be made available free of charge to the humanitarian track of the project. Moreover, a severe intellectual property rights problem in the

humanitarian project that could not be dealt with by private persons or by their universities has been solved thanks to the input of Dr. Adrian Dubock of Syngenta. Development of Golden Rice required the use of various technologies that are properties of several industrial companies and some universities. The noncommercial use of Golden Rice in the developing world required the written consent of the respective intellectual property rights holders. The necessary multi-lateral negotiations required—as it turned out—interindustrial interaction involving the respective expertise of various parties involved.

The humanitarian project has established an advisory panel, called the Humanitarian Board, which meets regularly. Syngenta is represented on the Board along with scientists of various disciplines, some working for international and other agencies involved with assistance programs to developing countries. The complementary expertise of the individuals involved ensures the flow of information between the two tracks and ensures that all steps taken are in accordance with the intraproject legal requirements and with respect to the laws of various countries interested in receiving the technology. Through the humanitarian board, a noncommercial license can be obtained by national and international research institutes. It is at these institutes that further development, such as the transfer of the β -carotene trait into local varieties by classical breeding or by transformation or the breeding of provitamin A varieties with stable and high yields, will be carried out.

In January 2001, the first transfer of the technology took place to the International Rice Research Institute based in the Philippines, a member institute of the Consultative Group on International Agricultural Research with a long-standing and proven expertise in breeding improved rice varieties for dissemination to developing countries, primarily in but not restricted to Asia. In addition, facilitated by the Indo-Swiss Collaboration on Biotechnology, further research and development of Golden Rice in India is being pursued in collaboration with national research institutes. Dr. Hoa, a Vietnamese visiting scientist, has transformed several local varieties. She will take these seeds back to Vietnam to conduct further

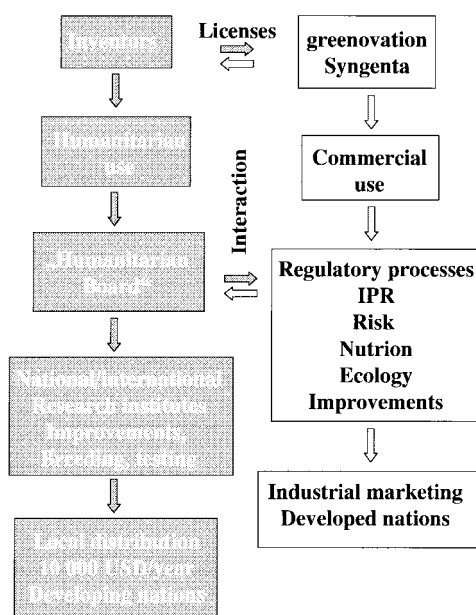


FIGURE 5 The legal situation of the Golden Rice project (see text for explanations).

research there, in accordance with a sublicense agreement with the Qcuu Long Delta Rice Research Institute. A possible transfer to China is currently being discussed with the Chinese Ministry of Science and Technology; the Minister of Agriculture of Indonesia also has expressed interest in entering into similar discussions.

CONCLUSION

In a proof-of-concept study, we have shown that it is possible to establish a biosynthetic pathway de novo in rice endosperm, enabling the accumulation of provitamin A. Many variations of this applied technology appear feasible and work is in progress to optimize the yellow rice lines now in our hands. In part, this involves the use of different structural genes and the use of different selectable marker genes. With the discovery that the lycopene β -cyclase is not necessary to achieve provitamin A synthesis, it may even be possible to remove the selectable marker from cotransformants by breeding techniques (Fig. 2). The observation that a regulatory pathway may be involved calls for in-depth biochemical and molecular biological analyses, currently being undertaken. Studies on the bioavailability of the provitamin A, transfer of the trait into agronomically important varieties, and risk assessments will be carried out in collaboration with other research institutes.

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