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# Biosynthesis of β-carotene (provitamin A) in rice endosperm achieved by genetic engineering

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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 cDNAs coding for (1) phytoene synthase (*psy*) and (2) lycopene β-cyclase (*β-lcy*; both from *Narcissus pseudonarcissus* and both under control of the endosperm-specific glutelin promoter), with (3) a bacterial phytoene desaturase (*crt*I, from *Erwinia uredovora* under constitutive 35S promoter control). This combination covers the requirements for β-carotene synthesis, and yellow, β-carotene-bearing rice endosperm was obtained in the $T_0$ generation. However, further experiments revealed that the presence of *β-lcy* was not necessary, since *psy* and *crt*I alone were able to drive β-carotene synthesis as well as the formation of further downstream xanthophylls. This finding could be explained if these downstream enzymes are either constitutively expressed in rice endosperm or are induced by the transformation, e.g. by products derived therefrom. Based on results in *N. pseudonarcissus* as a model system, a likely hypothesis can be developed that *trans* lycopene or a *trans* lycopene derivative acts as an inductor in a kind of feedback mechanism stimulating endogenous carotenogenic genes.

*2001 Rice biotechnology: improving yield, stress tolerance and grain quality. Wiley, Chichester (Novartis Foundation Symposium 236) p 219–232*

Rice is the major staple food for millions of people. It is generally consumed in its milled form with outer layers (pericarp, tegmen, aleurone layer) 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

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health, such as carotenoids exhibiting provitamin A activity. Thus, rice 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.

The mildest form of vitamin A deficiency is night blindness; however, continuous deficiency causes the symptoms of xerophthalmia and keratomalacia, leading to irreversible blindness. In Southeast Asia it is estimated that 5 million children develop xerophthalmia every year, of which quarter of a million eventually go blind (Sommer 1988). Furthermore, vitamin A deficiency is correlated to fatal afflictions such as diarrhoea, respiratory diseases and childhood diseases such as measles. Vitamin A deficiency negatively alters the incidence of such illnesses, their duration and severity (West et al 1989). According to statistics compiled by the UNICEF, the diets of an estimated 124 million children worldwide are deficient in vitamin A (Humphrey et al 1992). Improved nutrition could prevent 1–2 million deaths annually among children aged 1–4 years and additional 0.25–0.5 million deaths during later childhood (West et al 1989). Since attempts to overcome the problem by oral application of vitamin A are problematic (Pirie 1983, Sommer 1989), mainly due to the lack of infrastructure and educated medical staff, alternatives are urgently required. Such an alternative for highest-risk countries could be to supplement the major staple food, rice, with provitamin A. This can only be achieved by recombinant technologies rather than conventional breeding, due to the lack of rice cultivars producing this provitamin in the endosperm. Since on one hand, the transformation of rice is well established and since, on the other hand, the carotenoid biosynthetic pathway has been molecularly identified during recent years, it appeared feasible to introduce the complete provitamin A ($\beta$-carotene) biosynthetic pathway into rice endosperm by genetic engineering.

We have shown previously that immature rice endosperm synthesizes the early intermediate geranylgeranyl diphosphate. This compound is not solely devoted to carotenogenesis but can be used as a substrate to produce the uncoloured carotene phytoene by expressing the heterologous enzyme phytoene synthase in rice endosperm (Burkhardt et al 1997). We report here on the successful introduction of the complete provitamin A biosynthetic pathway into rice endosperm by genetic engineering, yielding yellow-coloured rice grains to be used to defeat illnesses caused by provitamin A deficiency.

## Results and discussion

To engineer the pathway towards $\beta$-carotene formation the complementation of this transgenic plant with three plant enzymes is necessary, namely phytoene desaturase and $\zeta$-carotene desaturase catalysing the introduction of two double bonds each, and lycopene $\beta$-cyclase. Alternatively, to reduce the transformation

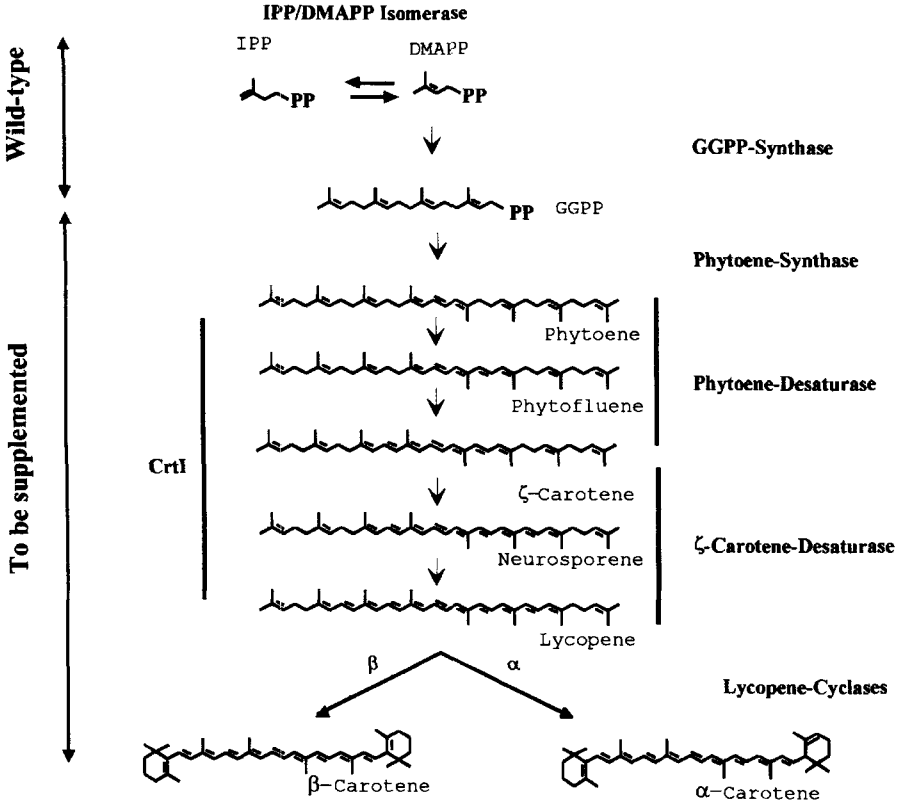


FIG. 1. Provitamin A biosynthetic pathway. The names of enzymes are given. CrtI denotes a bacterial carotene desaturase capable in performing all necessary desaturation reactions for which two enzymes are required in plants. Arrows indicate the prenyllipid biosynthetic capacity of wild-type rice endosperm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

effort by reducing the number of enzymes required, certain bacterial carotene desaturases can be used which are capable of introducing all four double bonds required (see Fig. 1).

Initially, we introduced all genes into immature rice embryos (TP 309) singly by particle bombardment, aiming at 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, in order to install the entire $\beta$-carotene biosynthetic pathway into rice endosperm in a single transformation effort. Three vectors, schematically

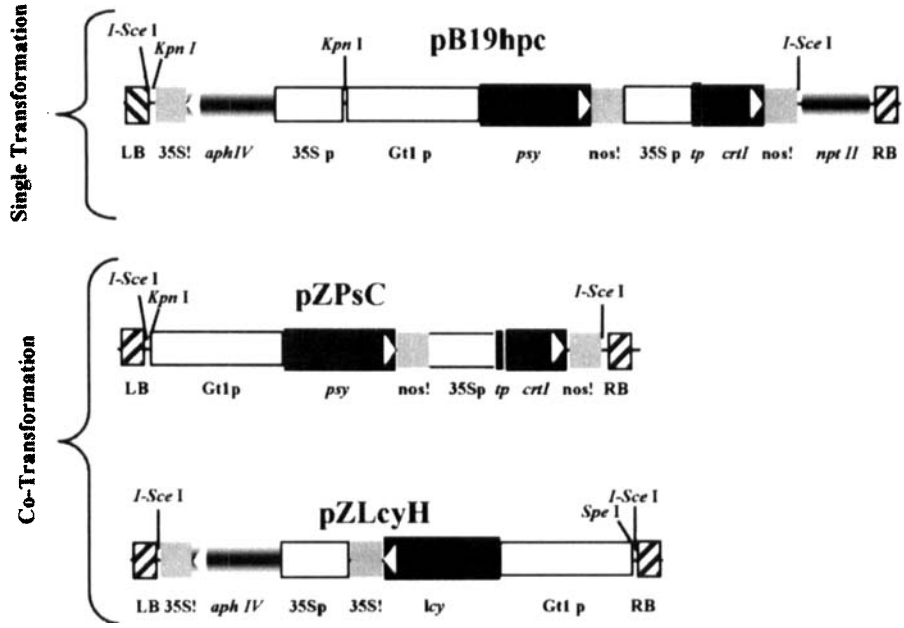


FIG. 2. DNA constructs used in single transformations and co-transformations. RB, LB, right and left borders; tp, transit peptide from pea ribulose bisphosphate carboxylase; !, terminator; p, promoter; gt, glutelin. DNA sequences coding for carotenoid biosynthetic enzymes are given in black; *psy*, phytoene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

depicted in Fig. 2, were constructed. pB19hpc combining the sequences for a plant phytoene synthase (*psy*) originating from daffodil (*Narcissus pseudonarcissus*; Acc. No. X78814; Schledz et al 1996) with the sequence coding for a bacterial phytoene desaturase (*crt*I) originating from *Erwinia uredovora* (Acc. 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 earlier (Bonk et al 1997), while the *crt*I gene was fused to the transit peptide sequence of the pea Rubisco small subunit (*tp*), as constructed by Misawa et al (1993). This plasmid should thus direct the formation of lycopene in the endosperm plastids, the site of geranylgeranyl diphosphate (GGPP) formation.

To complete the β-carotene biosynthetic pathway, co-transformations were carried out employing two vectors, one (pZPsC) carrying *psy* and *crt*I, like in pB19hpc but lacking the selectable marker *aph*IV expression cassette, the other (pZCycH) providing under glutelin promoter control the sequence coding for the enzyme lycopene β-cyclase, originating from *N. pseudonarcissus* (Acc.

No. X98796, Al-Babili et al 1996). As with phytoene synthase, lycopene β-cyclase carried a functional transit peptide allowing plastid-import (Bonk et al 1997). The combination of both plasmids should thus 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 were then analysed by Southern blot (not shown) using DIG-labelled internal fragments from *psy* and *crt*I DNA as probes. Meganuclease I–*Sce*I digest released a *c.* 10 kb insertion comprising the *aph*IV, *psy* and *crt*I expression cassettes. *Kpn*I was used to estimate the insertion copy number. All tested lines carried the transgenes and most of the plants revealed single insertions, but in some cases multiple insertions were observed.

For co-transformation about 500 precultured immature embryos were inoculated with an *Agrobacterium* mixture of LBA4404/pZPsC carrying *psy* and *crt*I genes and LBA4404/pZCycH containing *lcy* and *aph*IV as the selectable marker. Co-transformed plants were identified by Southern hybridization. To probe for the presence pZPsC, the same restriction was applied as above using the respective probes, whereas the presence of the pZCycH expression cassettes was screened separately by probing I-*Sce*I and *Spe*I-digested genomic DNA with internal *lcy* fragments. All 60 randomly selected regenerated lines were positive for *lcy,* among which 12 plants were co-transformed with pZPsC as shown by the presence of the expected fragments; 6.6 kb for the I-*Sce*I-excised *psy* and *crt*I expression cassettes from pZPsC and 9.5 kb for the *lcy* and *aph*IV genes from pZCycH. Like the transformation from above, 1–3 transgene copies were predominant in co-transformed plants. Ten plants harbouring 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 $F_0$ seeds from transformed lines and from control plants were air dried, dehusked and, in order to isolate the endosperm, treated with emery paper for 8 h on a shaker. In most cases the transformed endosperms exhibited clearly notable yellow colour, indicating carotenoid formation. The pB19hpc single transformants showed a clear 3:1 (coloured/non-coloured) segregation pattern, whereas the pZPsC/pZCycH double transformants (Fig. 3B) 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 colour compared to the pZPsC/pZCycH double transformants equipped for β-carotene (yellow) synthesis.

Seeds from individual lines (1 g, each) were ground to a fine powder and extracted to complete decolourization with acetone. The combined extracts were quantified photometrically and analysed qualitatively by HPLC (Fig. 3). The

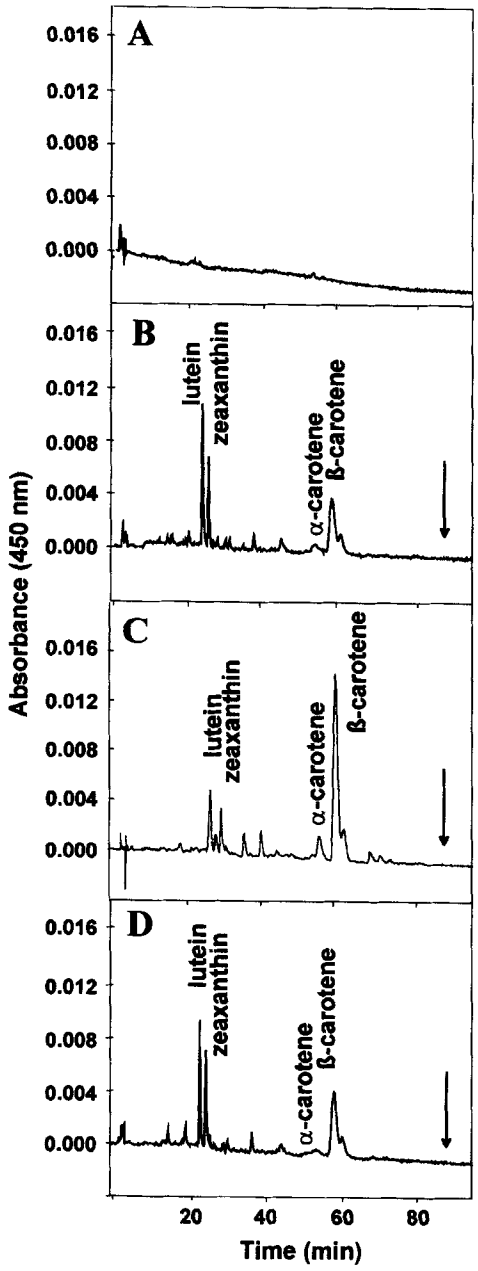


FIG. 3. HPLC analysis of wild-type and transformed rice endosperm on a $C_{30}$ reversed phase column. (A) untransformed control which is carotenoid-free. (B) single transformant, (C, D) co-transformants. The arrow indicates the position of lycopene in the system used.

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 α(ε)- 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 therefrom (see below).

The pZPsC/pZCycH double transformants exhibited a more variable carotenoid pattern (Fig. 3). 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 such an example (see Fig. 3B) also representing up to now the 'winner' in quantitative terms. A carotenoid content of 1.6 μg/g endosperm was determined. Considering the fact that the $F_0$-generation of seeds now analysed is not homozygous with respect to the transgenes (so that a segregation between colourless, intermediate-coloured and strongly-coloured grains takes place), it can be assumed that this line as well as several others will safely reach our anticipated goal of 2 μmole/g endosperm (Burkhardt et al 1997). 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, since it turns out during recent years that these xanthophylls are present in the eye's macula and their deficiency may contribute to macular degeneration, leading to blindness (Landrum et al 1997). In this respect lutein and zeaxanthin may therefore represent valuable compounds exhibiting vitamin character. Work has been initiated to cover these aspects as well as safety considerations that will begin as soon as there is sufficient homogeneous material available.

Preliminary evidence has accumulated to provide an explanation for the unexpected carotenoid pattern in the transgenic rice seeds. Currently it cannot be excluded that the transformation using the bacterial *crt*I-gene promotes a hitherto unknown feedback mechanism enabling the transcriptional activation of carotenogenic genes. The effector may be the 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 (Bartley et al 1999). In a working hypothesis, 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 (Beyer et al 1991). CPTA-treatment thus mimics with respect to *trans*

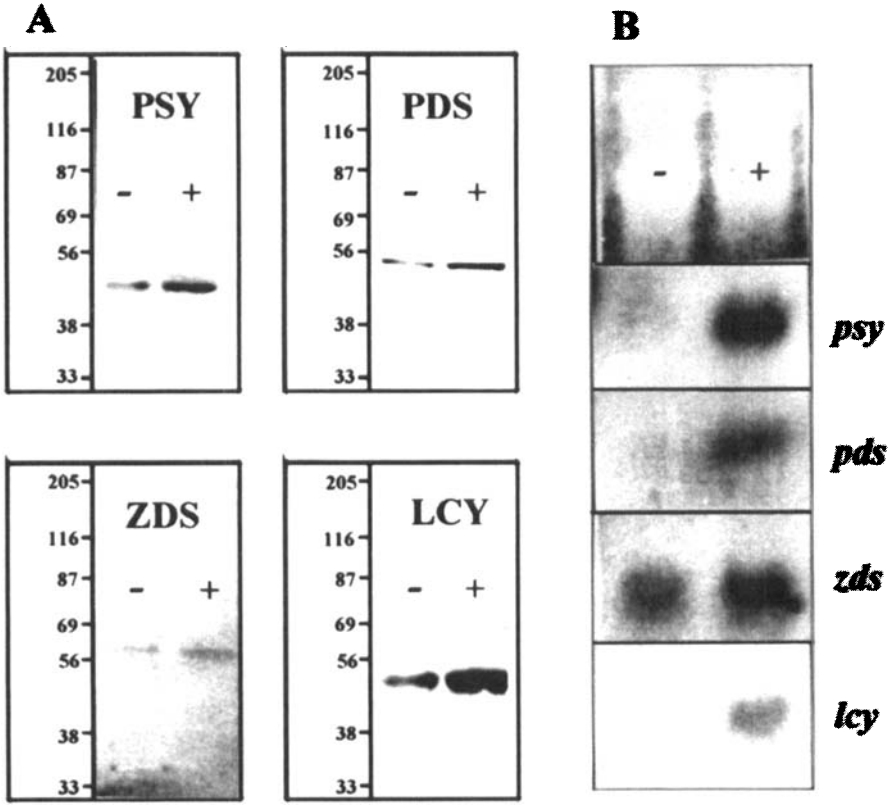


FIG. 4. Northern and western blot analysis of CPTA-treated and untreated daffodil flowers. (A) Western Blot. Total protein (10 $\mu$g) was isolated from treated (+) and untreated (−) flowers and after SDS-PAGE transferred to nitrocellulose membranes. The blots were then developed using homologous antibodies directed against the carotenogenic enzymes as given. (B) Northern blot. Total RNA (20 $\mu$g) was isolated from CPTA-treated (+) and untreated (−) flowers. The immobilized RNA was repeatedly stripped to allow subsequent hybridization with different probes. PSY, phytoene synthase; PDS, phytoene desaturase; ZDS, $\zeta$-carotene desaturase; LCY, lycopene cyclase.

lycopene formation our rice single transformation using plasmid pB19hpc. CPTA was administered to daffodil flowers, which then turn 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 mRNAs as well as Western blots conducted with the respective homologous antibodies showed that both the specific mRNAs examined as well as the specific protein amounts were markedly increased over the untreated controls (Al-Babili et al 1999; Fig. 4).

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. Utilizing the transgenic rice in hand, further work is in progress to clarify molecularly as to whether this mechanism works here as well.

## Conclusions

In a 'proof-of concept', 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 the applied technology appear now feasible and work is in progress to optimize the yellow rice now in our hands. This relates to e.g. the use of different structural genes or the use of different selectable marker genes. With the observation that the lycopene $\beta$-cyclase is not necessary to achieve provitamin A synthesis it may even be possible to remove the selectable marker from co-transformants by breeding techniques (see Fig. 2). The observation that a regulatory pathway may be involved calls for in-depth biochemical and molecular biological analyses, which will be carried out as soon as sufficient material becomes available. Then, studies on the bioavailability of the provitamin, transfer of the trait into elite varieties, and risk assessments etc. will be carried out in collaboration with other research institutes.

*Acknowledgements*

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## DISCUSSION

*Parker:* Do you know why your original crossing strategy didn't work?

*Beyer:* I have no idea. There have been problems with expression when using the ballistic approach mainly due to deleterious integration patterns. The change to *Agrobacterium*-mediated transformation was a good choice, because this gave us nice integration patterns and good expression.

*Leach:* Do you find an increase in carotenoids in green parts of the plants?

*Beyer:* We looked at that. We see no change in the green parts of our plants, although one of the transferred genes is under constitutive promoter control.

*Okita:* Have you ever sectioned one of the seeds to see whether there is uniform synthesis of β-carotene?

*Beyer:* It is hard to tell: I have the impression that it is quite uniform, although the GT1 promoter is more active in the outer layers of the endosperm.

*Khush:* Could you see any patches on the grain samples?

*Beyer:* No. The colour is not just restricted to some layers of the endosperm.

*Presting:* What is the nutritional significance? Is it enough vitamin A to make a difference? Are you planning to look at some of the interactions that Robin Graham described between zinc, iron and vitamin A?

*Beyer:* Keep in mind that provitamin A is not the vitamin. The vitamin the result of a dioxygenase-cleavage which occurs in animals. Vitamin A is then normally converted into an ester, which represents the effective compound. The amount required is measured in retinyl equivalents (RE). The dosage required to avoid

the problems of provitamin A deficiency is 100 μg RE per day. This would necessitate 2 μg β-carotene/g uncooked rice. Our most successful rice lines contain the provitamin in that range. We get 1.4–1.8 μg/g, which is almost the 2 μg that we wanted. But you have to consider that these are numbers from analyses carried out with the segregating lines so, according to Mendelian laws, we should exceed that value in the homozygous lines. However, bioavailability is still an issue that remains to be investigated as soon as there is sufficient material available.

*Ku:* What is the efficiency of co-transformation in the last experiment you did?

*Beyer:* It was surprisingly high. Among the resistant plants, they were 20% co-transformed.

*Ku:* Earlier, you said something about segregation: what was the problem there? In our case when we crossed two lines with the two genes, we only needed to look at about 50 seedlings to find one homozygous for both genes.

*Beyer:* We have the 3:1 segregation pattern in the single transformant. The co-transformants are simply unpredictable, as one may imagine. This awaits further analysis.

*Leach:* Is there any β-carotene in the plastids in the roots?

*Beyer:* No.

*Gale:* Peter Bayer, your work is tremendously important for public perception of genetic modifications. What is your deployment strategy for the golden rice?

*Beyer:* Everything is looking very encouraging at the moment. Most important is to keep the rice on the beneficial track on which it begun. This is not an easy task to do. We must, for instance care for the intellectual property rights (IPR) issue. We tried to settle this ourselves, but this has not been possible because of our lack of expertise in this field. Luckily we received help from the ISAAA, a humanitarian organization that is involved in the transfer of technology from developed countries to developing countries. They are carrying out an IPR audit as a first step, revealing that about 70 patents must be taken into consideration. Meanwhile we have received considerable assistance from other parties but no contracts have been signed as yet. Because of this I can't disclose much. However, I can read out a few statements which explain our joint position. 'Agreement has been reached between the inventors of golden rice and a number of private sector organizations. Contractual agreements will be completed shortly. Central to the agreement is that the investors are able to honour all their original concepts of making the technology free to resource-poor farmers in developing countries. This technology is a gift to these people from the inventors and the Rockefeller Foundation. The principal partner company will assist by providing specialist technical support to the investors and recipient country institutions. The Rockefeller Foundation which initiated this project almost 10 years ago sees this as

a good example of public–private partnership in making available the technology to contribute to the elevation of vitamin A deficiency, induced blindness and malnutrition. Other not-for-profit organisations have been and will be invited to support the project too, to further enhance the efforts in this latest stage of co-operation.'

*Mazur:* This shows that freedom to operate can be a messy issue. How many different parties are represented in those 70 patents?

*Beyer:* There are several parties involved. But the number of parties is reducing with all the mergers that are taking place!

*Leach:* What is the acceptance going to be of rice that is yellow? I remember that the Rockefeller Foundation did a study many years ago where they tried to get people in Asia to eat yellow rice, and they refused because they associated yellow rice with tainted rice.

*Beyer:* There may be some lack of acceptance when people do not see a benefit. But this yellow rice will be available to people who urgently need improved nutrition. Certainly acceptance is also linked to the perception of potential risks. I would like to add that environmental and risk assessment studies will be carried out. Metabolic profiling studies will also be essential. Potentially, interfering with signal pathways could have unforeseen results, which may even have a positive impact, but we need to double check the content of the seeds.

*Bouis:* With regard to the dissemination strategy, I have spent a good part of the last five years trying to push this idea of breeding for nutrition. I think the problems can be overcome, but we have to be clear about the different steps once the breeding is done. The first step in the dissemination strategy is to incorporate the nutritional characteristics in the highest-yielding and most profitable varieties. The farmers will not adopt these varieties because they are nutritious, but because they are profitable. We have some evidence that the trace minerals are compatible with high yields. We have no reason to believe that the $\beta$-carotene is going to harm yields, but it will not promote yields either. The second step is ensuring consumer acceptance. There are many strategies that communications experts have for trying to get people to adopt things that they are not used to. This will be a cost that will have to be borne by governments, convincing consumers to eat yellow rice. With the trace minerals, we don't think it is going to change the consumer characteristics of the rice, so we are not as worried about consumer acceptance. The third and most difficult issue is that of bioavailability. We have to establish that these things are really going to help peoples' nutrition. On the trace minerals, people are consuming perhaps 50% of the RDAs, and we think we can raise this by another 25% with the parameters we currently have. The data on vitamin A rice from Bangladesh suggest that at the present levels of $\beta$-carotene, this would increase the vitamin A intakes by 25%. The final problem is getting the money together to try these strategies. We have been making these arguments about the trace

minerals for the last five years and have only been able to raise US$1.5 million for this strategy. It has been difficult.

*Graham:* On this acceptance issue, in Australia we have a wheat variety registration process, which involves bakers and millers. They are very conservative. They want white flour and say that this is what the market demands. We also have a maverick breeder who came up with a rather creamy-floured wheat of a beautiful agronomic type. He found a market for yellow alkaline noodles in Malaysia, and got this wheat past the registration board in this way. We have found that the creamy-coloured bread made from this wheat has a sweeter smell than the white and appears to hold its freshness, so there is now a boutique industry developing around this variety. A more sophisticated market is evolving.

*Khush:* I should add that in the Indian subcontinent, in certain preparations saffron colour is added to rice. This natural yellow rice would have much higher acceptance there. There is one problem in consumer acceptance of golden rice: when rice is spoiled by poor storage it becomes yellowish. Consumers might therefore equate golden rice with spoiled rice. However, I feel that these problems can be addressed through public education.

*Presting:* You showed fairly high co-transformation efficiency in your system. I am interested in getting feedback from anyone who is doing transformations with the intent of public release on whether it would be a good strategy to use co-transformation and eventually cross-out the selectable marker. Is this something we should worry about when we make these transgenic organisms?

*Beyer:* This is certainly an issue. I am told by people more experienced than myself in transformation that in most cases the integration is in one locus, so we can't cross this out. It is probably a matter of how many lines we look at. It would be taking such an effort to result in selectable marker-free plants. In addition, we have a visible phenotype, which assists us greatly in doing these kind of things.

*Presting:* Which selectable marker did you use in your experiments?

*Beyer:* Hygromycin, but we are aiming to use the mannose-phosphate isomerase systems in new transformations.

*Ku:* I would add that the antibiotics we use for selection of transformed plants are not commonly used in human disease treatments. However, there is public concern about their use.

*Elliott:* I particularly appreciate the sensitivity of Maurice Ku's last comment. The anti-GM crops campaigns have raised public awareness of our use of selectable markers by suggesting that such markers do threaten the health of animal and human populations that eat GM crops. While we may believe that this is nonsense, we should not respond to accusations of sloppy molecular biology with reflex negatives. The commercial crops do not need the markers, so we should exploit transformation systems for generating marker-free transgenic

plants (Joersbo & Okkels 1996, McCormac et al 1999, Yolder & Goldsbrough 1994). Our own pBECKs 2000 vectors have been designed to address the need for a 'clean gene' system which allows the redundant marker genes to be removed from the genome of the transgenic plant after selection (McCormac et al 1999).

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