Golden Rice, Golden Crops, Golden Prospects

Arroz Dorado, cultivos dorados, perspectivas doradas

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SUMMARY

Golden Rice is probably the best known second-generation—nutrition quality enhanced—transgenic crop, but several others are following suit. *Golden Rice* was developed to deal with the problem of vitamin A deficiency (VAD), which affects millions of people all over the world, especially small children in developing countries. VAD not only causes blindness and increased morbidity, but a hitherto almost ignored increase in mortality. While ongoing supplementation and fortification programs are helping to reduce the burden, there is a strong need to develop biofortified crops capable of sustainably providing various micronutrients to target populations. Green biotechnology and progress in the biochemical understanding of the mechanisms of biosynthesis and accumulation pathways of micronutrients have not only paved the way to address VAD but also deficiency of the micronutrients iron, zinc, vitamin E, folate, and essential amino acids. Here I provide an overview of the progress and the political environment of the *Golden Rice* Project as well as the status of a number of related projects in biofortification.

Key words: Biofortification, micronutrient deficiency, vitamin A deficiency, carotenoids, beta-carotene, nutrigenomics

RESUMEN

El *Arroz Dorado* es probablemente el cultivo transgénico de segunda generación –es decir, en lo relacionado con el mejoramiento de la calidad nutricional– mejor conocido en el mundo, aunque varios otros proyectos están siguiendo su ejemplo. El *Arroz Dorado* fue desarrollado para ayudar a resolver el problema de la deficiencia de vitamina A (DVA), la cual afecta a millones de personas a nivel mundial, especialmente a niños pequeños en los países en desarrollo. La DVA no sólo causa ceguera y un incremento de la susceptibilidad a diversas enfermedades, sino que también un hasta ahora ignorado aumento de la mortalidad. Mientras que programas de suplementación y fortificación están contribuyendo a reducir la carga, urge la creación de cultivos biofortificados, capaces de suplir de manera sustentable los micronutrientes necesarios a las poblaciones blanco. La biotecnología verde, y el progreso en el entendimiento de las rutas metabólicas responsables de la biosíntesis y acumulación de los micronutrientes a nivel bioquímico han preparado el camino para no sólo solucionar el problema de la DVA, sino también el de la deficiencia de micronutrientes como hierro, zinc, vitamina E, folato y aminoácidos esenciales. En este documento se da una visión general del progreso del proyecto del *Arroz Dorado*, así como del estado de proyectos afines en biofortificación.

Palabras clave: Biofortificación, deficiencia de micronutrientes, deficiencia de vitamina A, carotenoides, beta-caroteno, nutrigenómica.

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BACKGROUND

As estimated by the UN´s Food and Agriculture Organisation, almost 800 million people living in 98 developing countries did not get enough food to lead a normal, healthy and active life in 2000. Around eleven million children die of malnutrition every year, whereby a vast majority of those deaths are linked to micronutrient deficiencies (Jones et ál., 2003). Nutritional deficiencies with the highest impact on morbidity and mortality are related to the lack of iron, zinc, iodine and vitamin A (Welch and Graham, 2004).

The *Golden Rice* Project was conceived to alleviate the vitamin A deficiency (VAD) problem, because of the magnitude of the problem and the consequent potential impact that a successful intervention would have. Yearly, half a million people—mainly children—become blind as a consequence of VAD, fifty percent of whom may die within a year of becoming blind. VAD severely affects the immune system; hence it is involved in many of these children's deaths in the guise of multiple diseases, like measles and malaria (Caulfield et ál., 2004). It has been calculated that simple measures, like breastfeeding, combined with vitamin A and zinc supplementation, could save 25 percent of those affected children (UNICEF, 2003).

Various public and international programmes for supplementation, industrial fortification and diet diversification have achieved substantial improvements but have difficulty in attaining full coverage of the affected population and, above all, sustainability. Supplementation requires the provision of the needed micronutrients in the form of capsules, for example, while industrial fortification involves the addition of micronutrients to centrally processed foodstuffs, like the addition of vitamin A to oil or butter. Supplementation activities require the involvement of ten thousands of people for the distribution and complicated logistics to reach the target population. Supplementation campaigns are very costly in logistical terms, even though they are often coupled to vaccination campaigns. In small countries, like Nepal or Ghana, the costs amount to approximately $2 million a year (MOST and USAID Micronutrient Program, 2004). Campaigns should preferably be carried out at least twice every year. The reality is that on a global scale ca. 45 percent of children do not get any supplementation (UNICEF, 2003). Both approaches, fortification and supplementation, have difficulties reaching remote rural populations, and are dependent on a continuous flow of funds.

Biofortification is an alternative, sustainable approach. It is based on the development of crops capable of producing and accumulating the desired micronutrients in the edible portions of the plant. This approach offers an opportunity to attain a more inclusive coverage, especially of the poorest sectors of society who depend primarily on agriculture for their livelihood. Biofortified crops may be obtained through conventional breeding of genetically improved basic staple crops. Genetically improved parental lines can be obtained in various ways, including interbreeding with wild relatives, mutagenesis, or genetic engineering.

VAD is prevalent among the poor who depend mainly on starchy crops for their daily energy intake, because most starchy crops, such as rice, sorghum, cassava or bananas do not contain β-carotene (provitamin A), which our body in turn can convert into vitamin A. Half of the world's population eats rice on a regular basis, and again, for half of those rice is the main dietary energy source (Table 1). The situation is similar for other staple crops like cassava and banana in Africa. Dependence on these crops as the predominant food sources, therefore, necessarily leads to VAD and other micronutrient deficiencies, most severely affecting children and pregnant women.

Staple crops rich in β-carotene could substantially reduce VAD. In rice and in some other crops this can only be achieved using genetic engineering, because the biosynthetic pathway in the edible portions has been turned off as part of the plant's developmental program.

In the case of rice, there are a number of red and brown varieties, but these do not contain β-carotene but other pigments. The unpolished rice grain contains other valuable nutrients, like vitamin B and essential lipids. It is the latter that makes it usually necessary to polish the rice grain, as the lipids may become rancid by oxidation during storage, thus affecting the taste and smell of the grain.

Green plants produce carotenoids in the leaves and often in the flowers, and are the basis of some of the attractive, natural orange, yellow, and red pigments. Among the carotenoids produced in

Table 1. Rice-based societies, vitamin A deficiency status and intake from diet. Countries have been divided into two groups according to rice consumption per capita, i.e. with an approximate consumption of 400 or 200 grams of rice per person per day. SVAD, prevalence of subclinical vitamin A deficiency in children under the age of six; DCI, total daily caloric intake from rice; DVAI, dietary vitamin A intake. Data compiled from FAO, UNICEF and USDA databases. The recommended daily allowance for children is 300 µg per day.

	SVAD %	DCI %	DVAI µg/d
ca. 400 g/d			
Bangladesh	28	71	55.6
Cambodia	42	72	90.3
Indonesia	26	51	289
Lao PDR	42	66	141
Myanmar	35	68	86.8
Viet Nam	12	65	95.8
ca. 200 g/d			
Brunei	n.a.	28	216
Burkina Faso	46	6	65.2
China	12	29	288
Cuba	n.a.	15	125
Guinea	40	33	229
Guinea-Bissau	31	42	163
Guyana	n.a.	31	55.5
India	57	30	124
Korea, DPR	n.a.	34	191
Korea, Rep	n.a.	30	260
Madagascar	42	48	49.7
Malaysia	n.a.	30	330
Nepal	33	38	144
Philippines	23	42	106
Senegal	61	30	106
Sierra Leone	47	45	331
Sri Lanka	n.a.	39	34.9
Suriname	n.a.	27	63.6
Thailand	22	44	114

plants is β-carotene, the best plant source of vitamin A. β-Carotene is converted into retinol (vitamin A) in the body, by central cleavage by an oxygenase, yielding two molecules of retinal. Not all provitamin A sources are equally good sources of vitamin A. α-Carotene, for example, only yields one molecule with vitamin A activity. Bioconversion efficiency can vary, depending on the bioavailability of the provitamin. It can vary from a stoichiometric ratio of 6:1 for fruits like papaya to 24:1 for green leafy vegetables, where carotenoids may be entangled in complex matrices (Yeum and Russell, 2002). Animal sources deliver directly vitamin A, rather than the provitamin, leading to high bioavailability. The most efficient conversion ratio of 2:1 is obtained from β-carotene dissolved in oil.

Carotenoid biosynthesis is often turned off in non-photosynthetic tissues, like in the rice grain. In *Golden Rice*, the carotenoid biosynthetic pathway has been switched back on by transformation with equivalents of the dormant genes (Ye et ál., 2000). This feat was made possible by the advancements in green gene technology made over the last two decades. *Golden Rice* is the result of a collaboration between the laboratories of Profs Ingo Potrykus and Peter Beyer, at the Federal Institute of Technology, Switzerland, and the University of Freiburg, Germany, respectively (Ye et ál., 2000). Improved lines with higher β-carotene content were developed through collaboration with the Swiss agribusiness Syngenta (Paine et ál., 2005). The target of this public-private partnership was to produce enough β-carotene to cover the recommended daily requirements of children and adults alike.

THE MAKING OF *GOLDEN RICE*

The 1980s saw the cornerstones for the generation of transgenic plants being laid. Similarly, assembly of the carotenoid biosynthetic pathway puzzle relied on extensive research carried out in many laboratories. The generation of the first *Golden Rice* prototype took a concerted effort of seven years, from 1992 to 1999, by the collaborating institutions. Potrykus' and Beyer's ambitious goal was to re-engineer the biosynthetic machinery of the rice grain to produce and accumulate a pigment only found in green tissues and flower petals, against the scepticism of many experts. Carotenogenic tissues not only have active metabolic pathways, including the presence of precursor molecules, but also deposition mechanisms, e.g. carotenoid crystallization, formation of oil droplets, sequestration of carotenoids into membranes or protein-lipid complexes. The non-carotenogenic starchy endosperm of rice is very low in lipids and apparently lacks means for carotenoid deposition. Another unknown in rice endosperm was the supply of precursor molecules.

To make a long story short, *Golden Rice* technology is based on the simple principle that notwithstanding the fact that rice plants synthesise β-carotene in vegetative tissues but not in the grain, most enzymes of the biosynthetic pathway are present in the grain. Addition of only two genes was sufficient to reconstitute the pathway and consequently to accumulate β-carotene in the endosperm. These facts were not at all clear in 1992, and some of the tools to detect low-level gene expression were only being developed at the time. Hence, the genes responsible for β-carotene biosynthesis were introduced one by one, alone and in combination, into rice plants until the breakthrough in 1999, when the first *Golden Rice* prototype was finally obtained (Ye et ál., 2000). Since then carotenoid production in various plant species has been manipulated either by increasing the production of precursor molecules or by blocking the synthesis of enzymes downstream of the desired product (Fraser and Bramley, 2004; Giuliano et ál., 2000).

Geranylgeranyl-diphosphate (GGDP) is a key intermediate in the biosynthesis of isoprenoids, a substance class to which carotenoids also belong. GGDP and its precursor isopentenyl-diphosphate are synthesised from products of glycolysis. Derivatives of GGDP go through a number of isoprenylation and cyclisation reactions, leading to a number of important products, like tocopherols (vitamin E), tocotrienols, carotenoids, abscisic acid (a phytohormone involved in important regulatory processes, like bud and seed dormancy, and various stress responses), gibberellic acid (another phytohormone promoting growth and cell elongation), chlorophyll and other components of the photosynthetic machinery.

As indicated above, genes were introduced into rice one by one or in combination. Phytoene synthase (*Psy*) from daffodil (*Narcissus pseudonarcissus*), which utilises GGDP to form phytoene, a colourless carotene with a triene chromophore (Burkhardt et ál., 1997) alone led to phytoene accumulation but not to desaturated derivatives (Burkhardt et ál., 1997). This indicates that at least one desaturase was absent. Similarly, the expression of carotene desaturase (*CrtI*) from the bacterium *Erwinia uredovora*, which leads to the fully conjugated β-carotene precursor molecule lycopene by adding four double bonds, alone did not produce any colour in rice endosperm. As it turned out, the combination of these two genes led finally to the first *Golden Rice* prototype in 1999 (Ye et ál., 2000) (Figure 1).

Lycopene, a red pigment with an undecaene chromophore, is an intermediate product of PSY and CRTI in transgenic plants, but is virtually absent because it is utilised as a substrate by enzymes down the pathway to generate α- and β-carotene together with variable amounts of oxygenated carotenoids, such as lutein and zeaxanthin. The carotenoid pattern observed in the endosperm revealed that lycopene cyclases (LCY) and α- and β-carotene hydroxylases (HYD) are present in wild-type rice endosperm, while PSY and one or both of the plant carotene desaturases—phytoene desaturase (PDS) and ζ-carotene desaturase (ZDS)—are not (Schaub et ál., 2005).

An alternative pathway activation mechanism, observed in tomato (*Lycopersicon esculentum*) but not in rice, is a feedback induction of endogenous carotenoid biosynthetic genes as a result of the presence of the transgenes. CRTI alone led to an increase of β-carotene rather than lycopene, and endogenous carotenoid biosynthetic genes were upregulated, except for PSY, which was repressed (Romer et ál., 2000). Such CRTI-dependent upregulation may be based on the fact that the plant desaturases, PDS and ZDS, together produce prolycopene, the tetra-*cis* configured form of lycopene (Bartley et ál., 1999), which is subsequently isomerized to the trans form by lycopene *cis-trans* isomerase (CRTISO), a recently identified enzyme of this pathway (Isaacson et ál., 2004; Isaacson et ál., 2002; Park et ál., 2002). In contrast, the bacterial CRTI leads to the exclusive formation of the all-*trans* form of lycopene. The above findings are supported by increased formation of specific carotenogenic mRNAs and proteins in daffodil flowers upon artificial accumulation of all-*trans* lycopene after inhibition of LCY, leading to elevated total carotenoid content (Al-Babili et ál., 1999).

Wild-type rice endosperm displays low levels of all mRNAs required for xanthophyll formation, i.e.

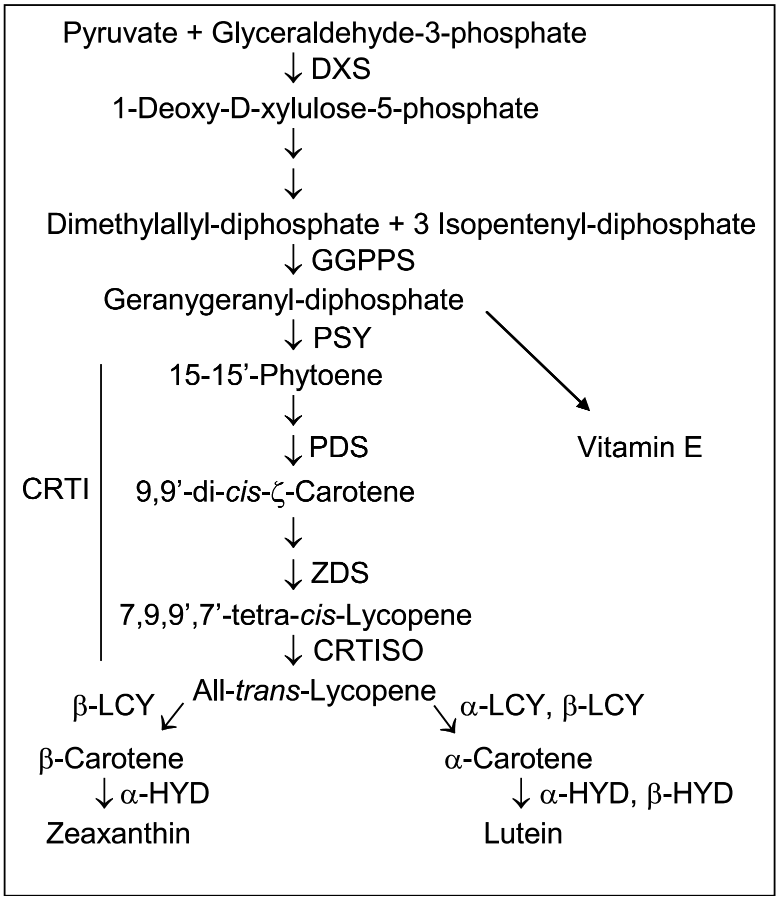

Figure 1. Carotene biosynthesis and re-engineering of the pathway in *Golden Rice*. DXS, 1-deoxy-D-xylulose-5-phosphate synthase; GGPPS, geranylgeranyl-diphosphate synthase; PSY, phytoene synthase; CRTI, bacterial carotene desaturase; PDS, phytoene desaturase; ZDS, ζ-carotene desaturase; CRTISO, carotene *cis-trans*-isomerase; LCY, lycopene cyclase; HYD, carotene hydroxylase. In *Golden Rice* two transgenes have been introduced to reconstitute the complete pathway, all other enzymes are present and active in the rice grain. The two genes are Psy, from daffodil (*Narcissus pseudonarcissus*) in GR1 or maize (*Zea mays*) in GR2, and *CrtI*, from the soil bacterium *Erwinia uredovora*. CRTI replaces PDS, ZDS and CRTISO. Desaturation by CRTI procedes via all-*trans* intermediates, as opposed to the plant enzymes.

PSY, PDS, ZDS, CRTISO, β-LCY, ε-LCY, β-HYD, and ε-HYD, although considering the sensitivity of the PCR method used, the PSY transcript was effectively absent, which is consistent with the fact that introduction of PSY is required—but not sufficient—to produce the *golden* phenotype. Also PDS and ZDS are probably expressed at too low levels to confer the *golden* phenotype. The endosperm-specific expression of the PDS/ZDS system—instead of CRTI—in rice endosperm resulted in the formation of comparable levels of coloured carotenoids. This shows that the rice endosperm provides the complex requirements for the activity of the plant desaturases. PDS requires namely a redox chain, employing quinones, a quinone reductase, and molecular oxygen as a terminal electron acceptor (Beyer et ál., 1989; Mayer et ál., 1990; Nievelstein et ál., 1995) to which it is linked via an oxidase (for a review see Kuntz, 2004). This redox pathway is especially important in non-green carotenoid-bearing tissues like endosperm, while the photosynthetic electron transport is thought to play an analogous role in chloroplasts. Nevertheless, CRTI was the preferred transgene over the plant-own desaturases due to its multifunctionality and minimal requirements for co-factors.

It has been shown that, in contrast to PSY, CRTI is not rate-limiting and is capable of desaturating large amounts of phytoene, thereby increasing β-carotene accumulation (Paine et ál., 2005). The primary sequence of CRTI is unrelated to the plant-type desaturases. This might explain its simpler co-factor requirements and may therefore be more effective in rice endosperm than the plant-type desaturases. On the other hand, CRTISO, which is also active in rice endosperm seems to have evolved from CRTI (Isaacson et ál., 2002; Park et ál., 2002).

Even though PSY, PDS, and ZDS expression in rice endosperm established the poly-*cis* pathway of carotene desaturation, the β-carotene formed was predominantly in the all-*trans* form. Furthermore, the endosperm from CRTI transgenic plants yielded the identical isomer ratio of β-carotenes, indicating that rice CRTISO determines the isomeric ratio (Isaacson et ál., 2004). The activity of CRTISO is also responsible for the formation of cyclic end groups. In its absence, cyclic carotenoids and derived xanthophylls would not form in non-green tissues, as has been shown with the *tangerine* mutation in tomato fruit and in etioplasts from the Arabidopsis *ccr2* mutant, both lacking functional CRTISO (Isaacson et ál., 2002; Park et ál., 2002).

The activity of rice the LCYs never proved to be rate limiting, since lycopene did not accumulate. Thus, yellow pigment formation in *Golden Rice* is the result of active intrinsic cyclases. No feedback regulatory loop seems to affect the expression of rice carotenoid biosynthetic genes present in rice, as indicated by the fact that all the corresponding mRNA levels remained unchanged as compared to the wild-type.

The flux of substrate into either branch of xanthophyll formation is controlled by the two LCYs, which convert lycopene into β-carotene (β,β-carotene) and α-carotene (β, ε-carotene). However, the transcripts of the cyclases remained unchanged. This strongly indicates that the isomer ratio in lycopene determines the probability of β- or ε-ring formation, in this case leading to a preferential formation of β-carotene and the concomitant decrease in lutein.

PRODUCT DEVELOPMENT

It was recognised that at 1.6 μg/g of β-carotene and in the absence of a more varied diet the GR prototype would not be able to fully cover the daily provitamin A requirements of the target population. Some rural populations in Bangladesh, for example, eat more than 80 percent of their daily caloric intake in the form of rice.

The first improved *Golden Rice*, called GR1, contained the phytoene synthase (*Psy*) gene from daffodil and the carotene desaturase (*Crt*I) gene from the bacterium *Erwinia uredovora*, as in the prototype *Golden Rice*. In GR1 both genes were expressed only in the rice endosperm under the control of the rice grain storage protein glutelin promoter, rather than constitutively as in the prototype (Figure 2). One reason for this modification was to avoid any interference with light-harvesting capacity in leaves caused by a potential decrease in lutein, as mentioned above (Al-Babili and Beyer, 2005). The levels of carotenoids obtained with GR1 in the field were 6 μg/g on average, an almost fourfold increase over the prototype. This β-carotenoid content is expected to be able to cover the recommended daily allowance (RDA) values for children if we factor in a modest intake of vegetables and fish or other animal sources, as is usually the case in the rural diets of Southeast Asian countries.

In complex biosynthetic pathways there is usually a rate-limiting step, which in this case is PSY. Therefore, *Psy* genes from different plant sources were compared in separate transformation experiments. As it turned out, the maize and rice genes gave the best results when transformed into rice, which led to the development of GR2, using the maize gene (Paine et ál., 2005). In the process, *GR2* lines accumulating high amounts of carotenoids were obtained. One regulatory clean line contained 37 μg/g carotenoid content, 80 percent of which was β-carotene. This represents an astounding 23-fold increase over the prototype.

The recommended daily allowance (RDA) of vitamin A for 1-3 year-old children is 300 μg (half the RDA is enough to maintain vitamin A blood levels at a normal, healthy level) (Institute of Medicine, 2001). Based on a retinol equivalency ratio for β-carotene of 12:1, half the RDA would be provided in 72 g of GR2. This is perfectly compatible with rice consumption levels in target countries, which lie at 100-200 g of rice per child per day.

The *golden* trait can in principle be directly engineered into many different rice varieties, but be-

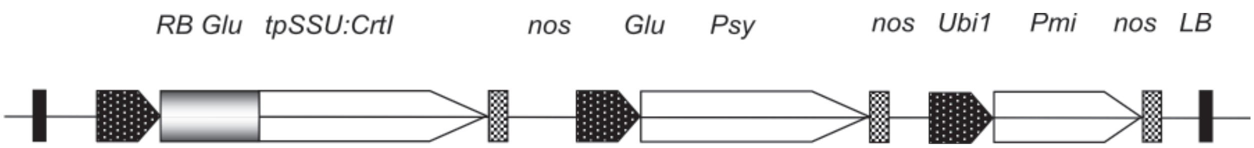

Figure 2. Gene construct used for the generation of *Golden Rice*. RB, T-DNA right border sequence; *Glu*, rice endosperm-specific glutelin promoter; *tpSSU*, pea ribulose bis-phosphate carboxylase small subunit transit peptide for chloroplast localisation; *nos*, nopaline synthase terminator; *Psy*, phytoene synthase gene from *Narcissus pseudonarcissus* (GR1) or *Zea mays* (GR2); *Ubi1*, maize polyubiquitin promoter; *Pmi*, phosphomannose isomerase gene from *E. coli* for positive selection; LB, T-DNA left border sequence.

cause of the stringent regulatory requirements, the aim is to have only one regulatory clean event as the starter seed for introgression in multiple breeding programmes (Hoa et ál., 2003).

REACHING OUT

Golden Rice will be made available to developing countries within the framework of a humanitarian project. This was, from the onset, a public research project designed to reduce malnutrition in developing countries. The hurdle of intellectual property rights attached to the technologies, mainly in the form of patents (Kryder et ál., 2000) used in the production of *Golden Rice* was overcome thanks to strong support from the private sector in the form of free licences for humanitarian use. This arrangement opened the way to collaborations with public rice research institutions in developing countries, providing freedom to operate to develop locally adapted *Golden Rice* varieties.

The *Golden Rice* approach does not interfere with a continued use of traditional varieties. The *golden* trait can be bred into any local variety by conventional means within four to six back-crosses or two years of breeding with the help of marker-assisted selection. The locally adapted *golden* varieties will become the property of the farmers and they will also be able to use part of their harvest to sow in the next growing season, without restrictions. Furthermore, *Golden Rice* is compatible with the use of traditional farming systems; it does not require more agronomic inputs than the parental variety.

Moving *Golden Rice* from the laboratory to the field is not a trivial exercise. A group of international experts from reputed institutions provides strategic guidance to the project. At the next level, a network of national institutions participates in the development and distribution of locally adapted varieties. To date, the *Golden Rice* Network includes 16 national institutions in Bangladesh, China, India, Indonesia, Nepal, the Philippines, Vietnam, and South Africa. The Network is under the strategic guidance of the *Golden Rice* Humanitarian Board and under the management of a network coordinator, based at the International Rice Research Institute (IRRI), in the Philippines.

If we put aside the research investment by Syngenta so far, investment in the public sector has been relatively modest thus far ($2.4 million over nine years). Product development, however, is time-consuming and requires substantial additional funding. Funds for product development are normally not provided by the public sector, since the work involved is not academic in nature. In universities researchers are evaluated based on scientific productivity, and not marketable products. The latter is the realm of industry.

Expenses increase even more dramatically when it comes to biosafety assessment as required for regulatory purposes. But once a novel, biofortified variety has been approved and handed over to farmers, the system can develop its full potential. From this point on, the technology is built into each and every seed and does not require additional investment.

GOLDEN CROPS

The *Golden Rice* achievement demonstrates that sometimes even complex metabolic pathways can be reactivated or reconstructed, even in tissues that are not expected to be able to accumulate the desired metabolites. This finding can also be of importance to researchers working on the production of plant-derived pharmaceuticals (Ma et ál., 2005) but in the context of the present review it is relevant for those who want to improve the nutritional quality of foodstuffs, especially with regards to enrichment with micronutrients. Some of the best starchy caloric sources in the world lack adequate amounts of micronutrients, not only carotenoids and other vitamins but also minerals and essential amino acids. *Golden Rice* has served as a proof of concept for biofortification obtained through genetic engineering and has thus inspired other projects along the same vein. But while the concept as such is transferable, still each crop requires its own fix, depending on the presence or absence of active enzymes along the metabolic and catabolic pathways involved.

Potato is one of the major staple crops in the world, but in most cultivars β-carotene is present only in trace amounts. While the wild potato species *Solanum phureja* can contain high levels of carotenoids, only a small fraction consists of β-carotene. This fact calls for a transgenic solution to improve the nutritional quality of this important crop. This feat has been meanwhile accomplished with the participation of Peter Beyer's lab, one of the inventors of *Golden Rice*. In one approach, increase of β-carotene in potato was obtained by blocking the expression of ε-lycopene cyclase (ε-LCY), which diverts lycopene into the α-carotene and lutein branch of the pathway, using a tuber-specific RNA-silencing approach (Diretto et ál., 2006). Using this approach β-carotene content increased up to 14-fold. Remarkably, lutein levels were not reduced in these tubers, indicating that ε-LCY is not rate-limiting. An even greater increase in carotenoid production in potato was obtained by introducing a mini-pathway consisting of three genes from Erwinia: phytoene synthase (*CrtB*), phytoene desaturase (*CrtI*), and lycopene β-cyclase (*CrtY*) (Diretto et ál., 2007). Total carotenoid content increased 20-fold while β-carotene increased 3600-fold, up to 47 µg/g dry weight. Unexpectedly, phytoene also stays high in these lines, suggesting that desaturation is still rate limiting, as opposed to canola, where expression of *CrtI* and *CrtB* leaves only trace amounts of phytoene (Ravanello et ál., 2003; Shewmaker et ál., 1999). Another difference is that expression of *CrtY* does not lead to increased production of total carotenoids.

In a collaboration between the Tata Energy Research Institute (TERI) and Monsanto in India, Indian mustard (*Brassica juncea*), a popular source of cooking oil in India, was transformed with bacterial *Psy* and *CrtI* genes using Monsanto technology developed for the transformation of canola (*Brassica napus*). The idea behind this approach is to exploit the high bioavailability of β-carotene in oil as a means of supplying it to the target population in a highly effective carrier. This project is now awaiting an *ex-ante* socio-economic assessment before going ahead (Agricultural Biotechnology Support Project, 2003).

Similarly, in an USAID funded project for Africa, Iowa State University is developing "*Golden Maize*" (*Zea mays*) by introducing the bacterial *CrtB* (phytoene synthase) and *CrtI* (carotene desaturase) genes (Rodermel et ál., 2006). Lines accumulating 4-14 µg/g dry weight have been obtained, which meets the 50 percent estimated average requirement (EAR) of vitamin A recommended for children by USAID and HarvestPlus. While in rice options are restricted because the embryo is lost during polishing, in maize carotene deposition can be directed to the endosperm or the embryo. The latter option deals with the fact that there would be less of a problem with the adoption of yellow maize in regions where white varieties are preferred and also that the embryo has a higher oil concentration, thus offering a favourable environment for carotenoids and making it also more bioavailable.

In 2005 the Bill & Melinda Gates Foundation launched their Grand Challenges in Global Health initiative. Out of the 44 projects being funded within that initiative four are in the agricultural sector, and all are about crop biofortification. The four projects address the challenge to 'Improve nutrition to promote health by creating a full range of optimal, bioavailable nutrients in a single staple plant species.' The gauntlet has been taken up by four international consortia, led by the Queensland University of Technology for banana (*Musa spp*) (http://www.ihbi.qut.edu.au/research/tropical/biofort/), Ohio State University for cassava (*Manihot esculenta*) (http://www.biocassavaplus.org/), the Africa Biofortified Project for sorghum (*Sorghum bicolor*) (http://www.supersorghum.org/), and the University of Freiburg for rice (*Oryza sativa*) (http://www.goldenrice.org/Content5-GCGH/GCGH1.html). The grant awarded to the University of Freiburg is being utilized to further improve *Golden Rice* by adding genes that will promote the accumulation of iron, zinc, vitamin E and high-quality protein in rice grains. The other consortia mentioned are pursuing similar goals in addition to combinations with genetic traits for biotic and abiotic stress resistance and adaptations. Recently, the re-engineering of the folate biosynthetic pathway in tomato was reported (Diaz de la Garza et ál., 2007), and there are already ongoing discussions about including this trait into the crops mentioned above.

Again, for the establishment of β-carotene biosynthesis in these very different crops and tissues (grains, roots and fruits) it will be necessary to determine the presence or absence of biosynthetic, downstream and catabolic genes case by case. While cassava and banana varieties with high β-carotene content are available (Chaves et ál., 2000; Englberger et ál., 2003; Englberger et ál., 2006; Iglesias et ál., 1997), the problem in these specific cases is that these crops are not amenable to breeding because of lack of sexuality in the case of banana, and because of the agricultural use of vegetative propagation for the highly heterozygous cassava plants, for the purpose of genotype preservation.

As mentioned above, all these crops constitute the main caloric intake in many developing countries where rice is not the main food source, and they all lack essential micronutrients. It is very encouraging to see that the *Golden Rice* approach has played a catalytic role in inspiring these activities, and it can only be hoped that people in need will soon have access to better nutrition thanks to these projects and the support of people with a vision and understanding.

NATURAL GAMES WITH COLOUR

Sometimes, in nature, mutations lead to the activation of carotenoid biosynthesis in a usually non-carotenogenic organ. This was the case for carrots (*Daucus carota*), a plant originating in Afghanistan and brought initially to Europe in various colours except for orange. Carrots containing purple-red anthocyanins were first grown in the Middle and Far East, along with white, red, yellow, green and black versions. The Dutch then introduced the orange mutant in honour of Dutch Royal House of Orange in the 16th century. The mutation, which leads to the accumulation of β-carotene to levels of up to 6 mg/g dry weight in root tissues, seems to be due to the segregation of a yet unknown dominant inhibitor (Buishand and Gabelman, 1979). These mutants accumulate large amounts of β-carotene, although its bioavailability is rather low, because it is deposited in crystalline form. Bioavailability can be raised by cooking and serving with some oil though (Jayarajan et ál., 1980; Roodenburg et ál., 2000). Cooking generally has a positive effect on bioavailability, while retention of carotenoids after heat treatment is very high, as was shown for orange-fleshed sweepotato (van Jaarsveld et ál., 2005).

A more recent discovery is the case of the already commercially available orange-coloured cauliflower (*Brassica oleracea* var *botrytis*). This semi-dominant mutation is due to the insertion of a retrotransposon into the so-called Or allele (Or for orange) (Lu et ál., 2006). Although its role is not completely understood, Or is known to be involved in proplastid differentiation and other non-coloured plastids into chromoplasts for carotenoid accumulation. Thus, rather than exerting an influence on carotenoid biosynthesis, Or most likely acts by increasing sink strength and thus sequestration of carotenoids. The principle is transferable, as was shown through the generation of yellow-fleshed potatoes by expressing Or in transgenic potatoes (Lu et ál., 2006). The downside of this approach thus far is that in homozygous plants growth is stunted, probably due to not well understood pleiotropic effects.

OUTLOOK

There is plenty of goodwill in the public and in the private sectors worldwide to exploit the potential of green biotechnology for the benefit of the poor. However, without a realistic risk assessment approach, funds for public research will not suffice to deliver on the promise of biotechnology. Under circumstances like the extreme use of the precautionary principle, scientific progress will become detached from product development and the population at large would not benefit from the great potential of the technology.

Hopefully some countries affected by VAD will be able to release locally adapted *Golden Rice* varieties within a couple of years, pending regulatory approval. Considering that *Golden Rice* could substantially reduce morbidity and mortality, it is not easily understandable why the project is not getting wider international support to carry it through the regulatory process following the fast track. Notwithstanding the fact that during the last 20 years a vast knowledge base on the production and commercialisation of genetically modified plants has accumulated, a substantial amount of time and money will have to be spent on carrying out the required biosafety assessments. Approval of a new line might cost in the range of $4 million to comply with all requirements of toxicology and allergenicity testing, biochemical equivalency, environmental effects and other tests (Ramaswami and Pray, 2007).

Golden Rice has already gone through many greenhouse tests and two field trials that were carried out in Louisiana, because at the time target countries had no biosafety regulations in place. Meanwhile introgression of selected *Golden Rice* lines into locally adapted varieties is very advanced in the Philippines and in India. First field trials are expected for the end of 2007. Preliminary results from bioavailability trials indicate that β-carotene from *Golden Rice* is converted into vitamin A at even better ratios than expected. The institutions involved are working hand in hand with regulators towards approval of *Golden Rice* for widespread use. But distribution to farmers may still lie several years away, as details of the required tests and the costs attached are not yet known.

In any case, it is hoped that pragmatism will prevail and that the costs will stay sensibly below those of commercial crops, like Bt cotton (Ramaswami and Pray, 2007). After all, more than 20 years of experience with genetically modified plants covering more than 100 million hectares worldwide in 2006 (Clive, 2006) have not resulted in one single mishap causing damage to health or the environment. Thousands of carefully conducted biosafety experiments have been carried out by prestigious institutions, and all results have led to the conclusion that there is no specific risk associated with the technology beyond that inherent to traditional plant breeding or natural evolution. Furthermore, ecologists have concluded that the *golden* trait does not pose any conceivable risk to the environment as it will not provide any additional selective advantage to the crop.

Calls for experience and science-based regulatory regimes worldwide get further support from a number of socio-economic *ex-ante* studies carried out to predict the impact of *Golden Rice* adoption in selected target countries. A recent World Bank report concluded that the potential welfare improvement for Southeast Asian countries, derived from the adoption of *Golden Rice*, would be in the range of billions of dollars annually (Anderson et ál., 2004). This gain in terms of health improvement of the population and the economic benefit to the country would dwarf the negative impact that the adoption of transgenic technology could have on trade because of import bans on transgenic commodities. As applicable to most developing countries, the largest share of the production of rice and most other crops is consumed locally (Binenbaum et ál., 2000). As it turns our for India and Bangladesh, more than 50 percent of deaths of children under five years of age would be preventable if *Golden Rice* were adopted on a wide scale (Stein et ál., 2007; Stein et ál., 2006; Zimmermann and Ahmed, 2006; Zimmermann and Qaim, 2004). The recovery on investment for this intervention would be very fast and maintenance costs minimal compared to present interventions.

The whole issue has an important ethical component to it, as stated by the Nuffield Council on Bioethics, which concluded in their 2004 report that *'the European Union is ignoring a moral imperative to promote genetically modified crops for their great potential for helping the developing world'*, and that they believed EU regulators had not paid enough attention to the impact of EU regulations on agriculture in developing countries (Nuffield, 2003).

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