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Review in Advance first posted online on November 24, 2014. (Changes may still occur before final publication online and in print.)

From the Concept of Totipotency to Biofortified Cereals

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Annu. Rev. Plant Biol. 2015. 66:2.1–2.22

The *Annual Review of Plant Biology* is online at plant.annualreviews.org

This article's doi: 10.1146/annurev-arplant-043014-114734

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Keywords

Golden Rice, biofortification, genetic engineering, public good, GMO regulation

Abstract

I was a college teacher when opportunity opened a path into academia. A fascination with totipotency channeled me into research on tissue culture. As I was more interested in contributions to food security than in scientific novelty, I turned my attention to the development of genetic modification technology for cereals. From my cell culture experience, I had reasons not to trust *Agrobacterium* for that purpose, and I developed direct gene transfer instead. In the early 1990s, I became aware of the problem of micronutrient deficiency, particularly vitamin A deficiency in rice-eating populations. Golden Rice, which contains increased amounts of provitamin A, was probably instrumental for the concept of biofortification to take off. I realized that this rice would remain an academic exercise if product development and product registration were not addressed, and this is what I focused on after my retirement. Although progress is slowly being made, had I known what this pursuit would entail, perhaps I would not have started. Hopefully Golden Rice will reach the needy during my lifetime.

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PROLOGUE

My life has followed a series of serendipities. I lost my home, but gained my wife in turn. I was an outdoor naturalist, but a PhD opportunity and a research scientist position converted me into a genetic engineer. I was determined to develop genetic modification technology for food security, and met PhD students, coworkers, and colleagues who had or developed the necessary expertise. I was promoted into positions with increasing opportunities and responsibilities, culminating in a full professorship at ETH Zurich. However, I would have been lost in my academic and teaching duties if my colleague and friend Nikolaus Amrhein had not coached me. Fortunately, I also met Peter Beyer, without whom I would not have achieved the highlight of my career. The key scientific breakthrough came just one month before my unwelcome retirement from ETH Zurich, which in turn gave me the time needed to advance Golden Rice toward its deployment, and through circumstance I met Adrian Dubock, the engine behind Golden Rice product development. Finally, with the anti–genetically modified organism (GMO) lobby, I was not short of enemies, but I gained enthusiastic supporters in turn.

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Figure 1

(*a*,*b*) Ingo and Inge, 1954; (*c*) birding, 1980; (*d*) our VW T2 Camper, 1978; and (*e*) the family at our 50th wedding anniversary.

THE RECREATIONAL BASIS: MY FAMILY, VW CAMPERS, AND BIRDS

I was born in Hirschberg, Germany (now Jelenia Góra, Poland), in 1933; my father was a physician and directed a military hospital in the nearby mountains, and my mother was one of the first German sports students. I had two brothers and a normal life until we escaped in February 1945 from the Russian army and reached northern Bavaria. We had lost our home, our property, our father, and the financial basis of our life, and had gained a sister (born the day of my father's death). My mother was a hero. She not only kept us alive, but also transferred to me her love of nature, culture, and classical music and her social conscience. And she managed to support us through higher education. These were tough but valuable times; hunger was the predominant experience.

Bamberg became our new home. During my spare time from college, I watched wildlife with my brother. In 1953, I fell in love with the woman I am still married to. As one could not marry without a profession, my prime task was to get one without detour, and I studied biology and sports for a college teacher's career. We lived the traditional family life, with the wife taking care of all family problems and the husband focusing on his career. Two changes cast a heavy shadow on my wife's life: She had to accept that her outdoor naturalist became a laboratory scientist, and that her genetic engineer was under continual stress from the anti-GMO lobby and, therefore, seldom in good spirits. Our best times were (and are!) on our VW camper tours, beginning with a self-furnished T1 in 1974, followed by all subsequent models, and continuing now with a comfortable custom-made T5. The most effective recreation from professional stress was birding, a hobby my wife fortunately shares (see **Figure 1**). A collection of freeze frames from my bird films is available at **http://www.birdphoto-potrykus.ch**.

THE MOTIVATION TO ENTER A CAREER IN SCIENCE

I had completed my exams, finished my three years of teacher's training, and was an established college teacher in Cologne when the director of the Max Planck Institute for Plant Breeding

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Research, Prof. J. Straub, invited me to work for a PhD. The topic was plastid inheritance in *Petunia* (55). After completion, I went back to the college to continue teaching. In 1970, Prof. D. Hess, whom I knew from the Max Planck Institute, offered me a research scientist position at his Institute of Plant Physiology in Stuttgart-Hohenheim. I was already motivated by the challenge of food security, and throughout my academic life I was more of an engineer (wishing to solve concrete problems) than a scientist (working for scientific novelty). I was fascinated by the phenomenon of totipotency in plant cells and the hypothetical opportunity to base plant breeding on large populations of single cells. This brought me into contact with the emerging area of plant tissue culture, my scientific base for decades.

INITIAL YEARS OF FUN WITH THE MODEL PLANT *PETUNIA*

My first goal was to regenerate plants from protoplasts of *Petunia*. Nagata & Takebe (50) had just reported on the first case with tobacco, and my goal was soon reached and rewarded with the publication of two papers, one in *Nature* (21, 74). As my vision was to breed plants from a single somatic cell, the next task was changing the genetic makeup of single cells. Because I had been focused on organelles, my first question was whether it would be possible to introduce them into protoplasts. This was indeed possible: We introduced isolated nuclei, chloroplasts, and single-celled blue-green algae (57, 77). We also fused protoplasts and subprotoplasts, and combined subprotoplasts with protoplasts (31, 32, 56).

Uptake of DNA—a hot topic in the early 1970s, but one for which there was no conclusive evidence (47)—was the next challenge (34). Let me briefly report on an experiment conducted ten years before the first proof of plant transformation with naked DNA (53) and nine years before the first *Agrobacterium*-mediated transgenic plant (4, 6, 23, 33). We felt the need for a genetic marker, and Prof. Hess had a monogenic, dominant, red-flowering petunia and a monogenic, recessive, white-flowering petunia. Would total DNA from the red-flowering mutant—in analogy to sexual hybridization—lead to pink-flowering transgenics when adding DNA to protoplasts from the white-flowering mutant? Together with PhD student Franz Hoffmann, we regenerated numerous plants, and the first flower turned up pink, exactly like a sexual hybrid. However, one by one, only pink flowers came up, and it was soon obvious that we were faced with an artifact. We had probably used leaves from young hybrid plantlets growing side-by-side with ours for an anther culture experiment, instead of those from the white-flowering plants, as the protoplast source for the experiment.

This was my first lesson on artifacts and controls. I have seen much of this during the next 30 years, and my critical interventions at conferences were not too welcome. Of course, there was lots of room for improvement of this first experiment, but I had other plans.

FIFTEEN YEARS OF TOUGH WORK: TECHNOLOGY DEVELOPMENT FOR CEREALS

Working with model plants was fun and led to publications and recognition. But it was not the objective: I wanted to develop plant breeding technology as a contribution to food security. Somehow this had become a fixed idea of mine in the mid-1960s, when I taught special courses on food security to my college students. I was convinced that a real impact on food security would require a shift away from models to direct work with crop plants.

In 1974, Prof. G. Melchers offered me a position as a junior group leader at the Max Planck Institute for Genetics in Ladenburg/Heidelberg, which opened the opportunity to focus, with excellent conditions, on cereals. Two PhD students joined me and supported this work on tissue,

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microspore, and protoplast culture. One of them, Horst Lörz, later continued work on crop biotechnology as a full professor in Hamburg.

Two years later, I received an offer from the Friedrich Miescher Institute (FMI) in Basel to establish three groups in plant biotechnology. I decided that the groups should focus on protoplasts (led by myself), haploidy (led by Emrys Thomas), and mutagenesis (led by Patrick King). Two additional groups were soon added in molecular biology (led by Thomas Hohn and Barbara Hohn). Fred Meins, an epigenetics researcher, later replaced Emrys Thomas. This added up to a powerful plant team that benefited from a fantastic research environment and many outstanding colleagues in basic medical research. These years put the plant work at the FMI on the map.

Cereal Mesophyll Protoplasts Refuse to Be Totipotent

Altogether, I spent 15 years trying to convince cereal mesophyll protoplasts and protoplasts from other differentiated tissues to behave as could be expected for totipotent cells. To that end, I developed a specific technology: the hanging microdrop array culture (30, 76). The experimental unit is a 40-μL droplet containing approximately 200 protoplasts; the droplets hang from the lid of a 9-cm petri dish and are arranged in a regular 7 × 7 pattern. The protoplasts assemble in a monolayer at the droplet surface, allowing for swift microscopic inspection of the entire population. I studied the effect of all plant tissue culture factors ever considered in up to seven-factor gradient combinations—probably the most extensive approach to a tissue culture problem ever undertaken. I recorded numerous promising dedifferentiation reactions and cell divisions up to a maximum of 64-cell proembryo-like stages. In no case, however, did this lead to sustained development. After having tested more than 120,000 different cell culture variations, I finally gave up. There were more important tasks than to try to continue to force these protoplasts into a regenerative pathway. In parallel, we also worked with meristems and somatic embryos (58, 75, 97, 103–107) without finding a viable experimental basis for our genetic engineering plans.

I had learned the hard way that differentiated graminaceous cells are probably terminally differentiated. The typical wound response—responding to mechanical wounding with dedifferentiation and cell division, which is the basis for all plant tissue culture systems except those based on adventitious meristems—is not present in graminaceous species. Instead, wounding in these species leads to the accumulation of phenols in wound-adjacent cells and likely to programmed cell death. If I were a few decades younger, I would still be intrigued enough to work on solving this problem of differentiated cereal protoplasts.

For someone interested in developing genetic engineering technology for cereals, the T-DNA obviously had no chance to complete its infection cycle in cells soaked with phenols and determined to die. Now, of course, it is possible to transform cereals using *Agrobacterium*, but the underlying biology is entirely different and was not known at that time. Nevertheless, protoplasts offered the opportunity to approach a vector-independent gene transfer protocol along the lines of our earlier red/white *Petunia* transformation approach.

Direct Gene Transfer Leads to Mendelian Inheritance of the Transgene

Compared with the *Petunia* experiment from 1973, the scientific and technological base for direct gene transfer was a major improvement. Microbe molecular biology and genetic engineering were far advanced, gene isolation was nearly routine, selectable marker genes were available, and we had with Barbara and Thomas Hohn two hard-core molecular genetics partners. For the purpose of testing vector-free uptake, integration, and expression of a single gene in plant cells, it was, of course, appropriate to shift again to a model system. Our breakthrough came the same year as the

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first *Agrobacterium* transformation. Our paper on the regeneration of transgenic tobacco plants from protoplasts treated with a vector-free gene was published a year later (53), providing the first definite proof for uptake and integration of naked DNA into a plant genome (47). A year after that, we demonstrated Mendelian inheritance of the transgene by documenting stable hemizygous integration into the nuclear genome (78), later providing a cytological demonstration (49). We further showed that cotransformation of unlinked genes was possible (87) and that direct gene transfer works not only with herbaceous dicots, which are responsive to *Agrobacterium*, but also with graminaceous monocots (79).

While the majority of labs advanced *Agrobacterium* technology and my group was focusing on direct gene transfer, further gene transfer protocols were also being explored (47, pp. 96–100), including biolistics and microinjection. I published several assessments (59–62) and stayed focused on direct gene transfer, but also followed in parallel the concept of in situ precision transformation via microtargeting and microinjection (see below).

TWELVE YEARS ON CONTRIBUTIONS TO FOOD SECURITY

In 1986, I received the call offering a full professorship at ETH Zurich along with the task of establishing, together with Prof. J. Nösberger (who worked on crop physiology), a new Institute of Plant Sciences that would combine applied research in agronomy with basic research in plant biology. For basic research, ETH provided positions and infrastructure for three groups, which ultimately focused on plant biotechnology (myself), plant physiology and biochemistry (Prof. N. Amrhein), and plant genetics (Prof. K. Apel). This concept of close collaboration between basic and applied researchers in the interest of agriculture was ideal for my vision of developing plant biotechnology in support of food security and involved a decade of education in agricultural science. The intimate association with the state of the art in agronomic research opened my eyes to the naïveté of the concepts of many of the molecular biology colleagues, who tend to believe that a proof-of-concept case in a model plant is the solution to a problem in a crop plant—or, worse,

Figure 2

My group in the first year at ETH Zurich (1987).

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the ideas of urban greens with no educational background in biology and/or agriculture, defended with missionary ideology, on how to change agriculture to solve the food security problem.

From a Group Leader to a Professor with Junior Groups

The ETH position of full professor created the opportunity to support advanced coworkers in forming interdependent groups. Jerszy Paszkowski, who had joined me at the FMI as a fresh PhD, was interested in studying the flexibility of the genome and built a group working toward gene targeting and homologous recombination (5, 52). He later returned to the FMI as group leader, became a full professor in Geneva, and is now in Cambridge. Swapan Datta, who had joined me at the FMI with his wife, Karabi, was interested in protoplasts and developed rice transformation technology for both *japonica* and *indica* rice on the basis of his anther-derived embryogenic suspensions (14–18). He left for a group leader position at the International Rice Research Institute (IRRI) in the Philippines, became a full professor in Calcutta, and is now a deputy director general (crop science) in the Indian Council of Agricultural Research. Gunther Neuhaus optimized microinjection for transformation of precursor cells of somatic embryos (40, 41, 48, 85, 86), moved to a full professor position at the University of Freiburg, and is currently the vice rector for research there. German Spangenberg, a fresh PhD when he joined, was so impressive that I promoted him early on to develop his own group, and within a few years he became the world leader in forage grass biotechnology (54, 89–91, 92, 94, 101, 102); since the mid-1990s, he has been a full professor in Melbourne, Australia.

Other group leaders stayed until my retirement. Christof Sautter invented microtargeting technology, enabling precise biolistic treatment of native shoot meristems (9, 13, 27, 28, 35, 42, 63, 81–84) reaching the cell layer of the meristem (LII), which forms the macro- and microspores and thus transfers genetic changes to the offspring. With Monika Clausen, he developed a fungus-resistant wheat (13), which kept him busy long beyond my retirement and led to him becoming the focal point of anti-GMO activists, who repeatedly destroyed his test fields. His fight for the completion of the experiment and his engagement in the GMO discourse ended his academic career on a bitter note (22). Johannes Fütterer achieved great competence in plant virus research (10, 12, 24–26, 39). Johanna Puonti-Kaerlas did pioneering work on cassava biotechnology but left ETH Zurich before she could harvest the fruits of ten years of investment in technology development (43, 44, 80, 98, 99, 110, 111).

Some Wild Ideas I Could Not Follow Because of My Retirement

Roland Bilang performed excellent work toward rice plastid transformation, a problem that remains unsolved (8). We approached this problem in context with the wild idea of transferring the *nif* regulon into rice—not to make rice nitrogen autonomous (a task I do not judge realistic), but rather to activate the regulon (from a regulatory gene placed into the nucleus) during the grain-filling period, to protect the photosynthetic apparatus of the flag leaf for higher yield.

I never considered C_4 engineering a feasible idea until I discovered that rice leaves have the typical Kranz anatomy of C_4 plants but do not use it. What is missing are the chloroplasts in the bundle sheath cells and, of course, the enzymes and their cell-specific regulation. If a rice accession could be found with chloroplasts in the bundle sheath cells instead of proplastids, then the engineering of the handful of genes should not be too big a problem. To initiate a screen for bundle sheath chloroplasts, I requested a collection of diverse rice lines from IRRI, but I could not use them before my retirement. To my knowledge, the Bill and Melinda Gates Foundation–funded

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program on C_4 engineering in rice misses the great opportunity of making use of the existing C_4 histology.

On the Work of a Few Selected PhD Students from That Time

While my coworkers developed their junior groups, I continued my work toward food security with PhD students. Here, I restrict my remarks to those who worked on genetic engineering of rice. Joachim Wünn produced the first Bt rice seeds (108), which were kidnapped by Greenpeace on the way to IRRI in the Philippines for field testing. (Radical GMO opposition in Switzerland dates back to as early as 1984; when I joined ETH Zurich in 1987, the university built a "hand-grenade-proof glasshouse" for our transgenic research.) Andreas Klöti worked on the development of rice tungro bacilliform virus resistance, a task not achieved to date. Paola Lucca researched high-iron rice in collaboration with Prof. R. Hurrell from the ETH Zurich Laboratory of Human Nutrition. I am especially fond of this work of Paola's because she took a threefold approach to a complex problem: Rice contains very little iron in conjunction with high amounts of phytate (an efficient inhibitor of iron resorption), and a vegetarian diet generally leads to poor iron resorption. Paola created a sink for iron in the endosperm by expressing a ferritin gene, leading to a threefold increase. She expressed a metallothionin-like gene from *Oryza* and achieved a sevenfold increase in iron resorption–enhancing cysteine. She approached phytate degradation during cooking (so as not to interfere with the phosphate storage phytate prior to germination) using a thermotolerant phytase from *Aspergillus fumigatus*. To prevent interference with germination, the enzyme was excreted into the extracellular space—the cell wall. One transgenic line expressed the phytase to high levels. In small intestine simulation experiments, the phytase degraded the phytate to zero. However, within the cell wall, the enzyme could not refold and lost its thermotolerance (45, 46).

The project with the greatest impact, however, started with the PhD thesis of Peter Burckhardt.

THE GOLDEN RICE PROJECT: PROOF OF CONCEPT

From the Idea to Engineer the Provitamin A Pathway to the Proof of Concept

I began the Golden Rice project in 1991 with ETH funding. While I was searching for additional funds, the Rockefeller Foundation responded with a brainstorming meeting late in 1992 in New York. I went with my PhD student Peter Burckhardt, who on the plane introduced me to Peter Beyer from the University of Freiburg. The other experts at the meeting considered our engineering concept unachievable, but they nevertheless recommended the financing of one PhD student each in my and Peter Beyer's labs. This was the beginning of the most fruitful, pleasant, and productive collaboration in my career, for reasons of personal chemistry and complementary expertise.

Our strategy was to use direct gene transfer for single genes that were subsequently combined by crossing. Peter Burckhardt completed his PhD work by demonstrating that the phytoene synthase from daffodil channeled the pathway in the desired direction, leading to fertile plants (11). When he left, we continued with various group members and investigated the other genes. In 1998, I asked Xudong Ye, who had just finished his PhD with German Spangenberg, to take over. In collaboration with Peter Beyer's group, we decided to shift to *Agrobacterium* because of its simpler integration pattern, and to transfer all genes in one cotransformation experiment. This was by now expected to be achievable because with the *Erwinia* double desaturase, we needed "only"

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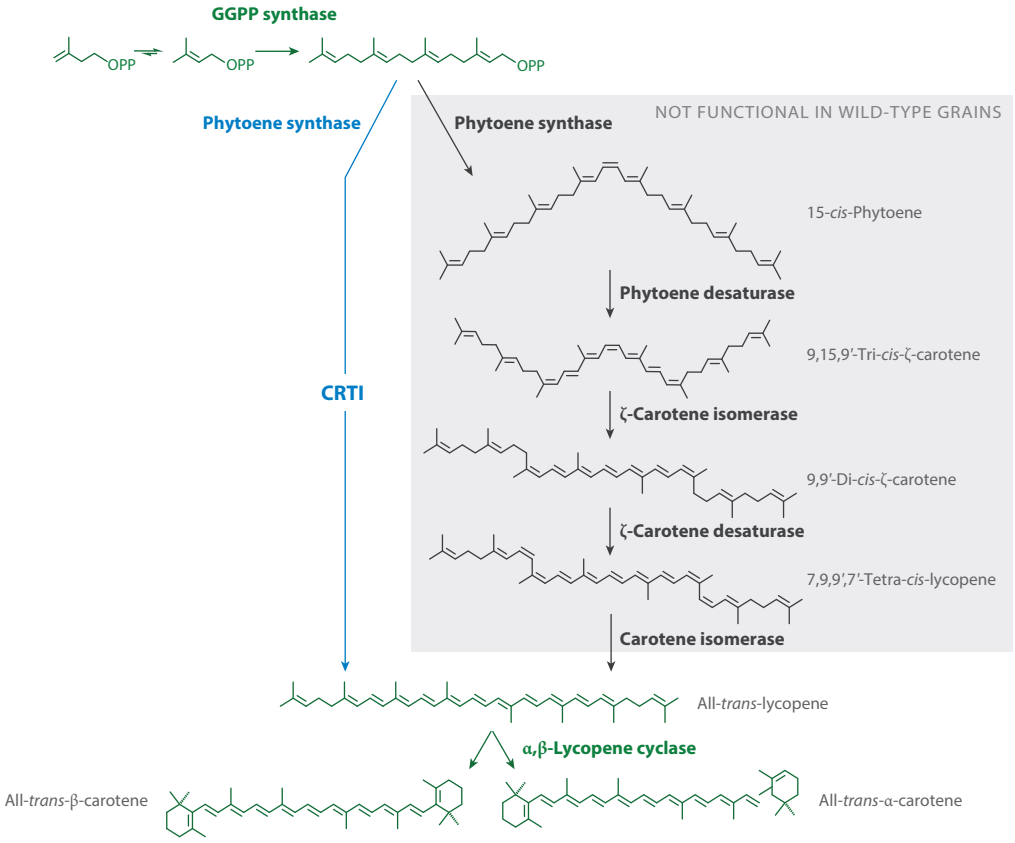

Figure 3

The engineering task: Green structures denote activities in the wild-type endosperm. The latest precursor available is geranylgeranyl diphosphate (GGPP). The subsequent steps are not functional (*gray*), and two transgenic proteins (*blue*), phytoene synthase and CRTI, need to be provided to bridge the gap toward lycopene beta-cyclase, which is active in the wild type and receives the lycopene substrate upon transgene expression.

four genes—those for the phytoene synthase, the double desaturase, and lycopene cyclase—and a selectable marker (**Figure 3**).

Xudong Ye grew the 50 best-looking lines to maturity. Peter Beyer polished the seeds and analyzed them with high-performance liquid chromatography. In late February 1999, early in the evening, Peter Beyer phoned me in my lab: "Ingo, open your computer. I am sending you a picture you will enjoy!"

And this is the picture (**Figure 4**)! I do not recall how many polished endosperms we had looked at over the years, and now there were some golden ones. Don't they look like gems? And how much more precious they are!

At my farewell symposium on March 31, 1999, on the occasion of my retirement from ETH Zurich, we presented these results—together with the high-iron rice—for the first time to the public. The endosperm contained up to 1.6 μg of provitamin A per gram of rice, and the best line had approximately 85% beta-carotene. In addition to Xudong, a key role was played by Salim Al-Babili from Peter Beyer's lab, the *ex aequo* lead author of the publication (109). As there are already numerous publications about Golden Rice (e.g., 1, 7, 19, 20, 64–69, 71–73) and several websites

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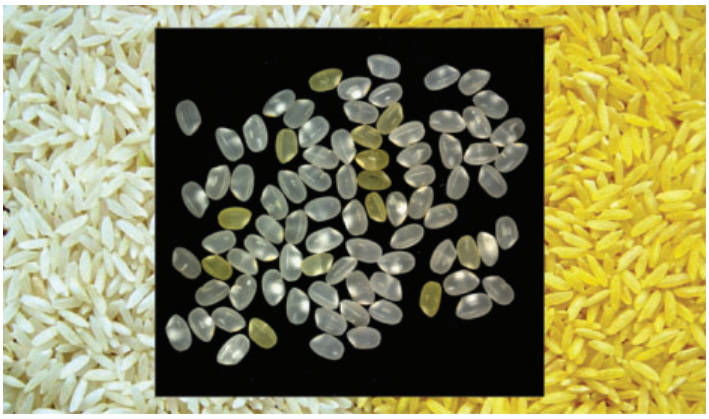

Figure 4

Eureka! The first golden endosperms, polished, segregating T1 in *Oryza sativa* ssp. *japonica* rice, on a background of the present technology.

providing detailed information (**http://www.goldenrice.org**, **http://www.irri.org/goldenrice**), here I focus on only a few aspects.

Attaining Recognition Is Fine but Does Not Solve the Problem

The concept of using the potential of genetic engineering technology in a public sector project for the public health of poor societies was welcomed with sympathy, and even enthusiasm, by the scientific community, the media, and the public. Golden Rice experienced an overwhelming recognition, even making it to the cover of *Time* magazine in the US and Asian editions, provoking the opposition of anti-GMO activists.

It was expected in the fields "soon." Based on experience with such single-locus traits and marker-assisted breeding, fewer than eight backcross generations were expected to be needed to introgress the trait into elite public germplasm. According to Gurdev Khush—IRRI's outstanding rice breeder, a member of the Golden Rice Humanitarian Board (see below), and a World Food Prize laureate—Golden Rice was expected in the farmers' fields in Asia by 2002. We are now more than a decade past that, and it will take at least until 2017 before Golden Rice can be handed over to the farmers—15 years later than expected! Why? The simple answer: Golden Rice is a GMO! These 15 years are not a specific "time penalty" for Golden Rice; they apply more or less to all GMO products.

PRODUCT DEVELOPMENT UNDER THE HUMANITARIAN BOARD

The Vision, the Core Board, and the Board

Having achieved a proof of concept for the science, we realized that Golden Rice would remain only an academic achievement if we ourselves did not complete the technological development necessary to assist vitamin A–deficiency sufferers in rice-eating populations. We wanted the micronutrition in Golden Rice to be available in developing countries, as a public good, free of charge, to subsistence farmers and urban poor, in their preferred rice varieties, and with enough provitamin A to protect against vitamin A deficiency. As academics, we had no idea what that entailed, and we realized that we needed help from someone who knew. Adrian Dubock had the necessary competence, shared our vision, and invested enormously to help with the project (see

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Figure 5

The core board advancing Golden Rice toward deployment (*left to right*): Adrian Dubock, Peter Beyer, and myself.

Figure 5). He designed a public-private partnership with Syngenta, became project leader and executive secretary, and was the engine for all that had to follow, including work that continued after his retirement from Syngenta at the end of 2007.

We established a Golden Rice Humanitarian Board of complementary competencies (29) as the governing body and legal entity that guided product development. Decisions are made and recorded at board meetings. Public sector licensees in the Philippines, India, Bangladesh, Indonesia, Vietnam, and China develop varieties adjusted to their national needs and collaborate in the Humanitarian Golden Rice Network. Participation requires the signing of license agreements with the inventors. IRRI in the Philippines and the Indian Department of Biotechnology also have the right to issue licenses.

Financial Support

The public domain provides funding for scientific novelty; financial support for product development is beyond the scope of academia. Our vision was to reduce vitamin A deficiency with a public-good product, and there is little doubt that this lies within the responsibility of the public sector. Of course, no private enterprise can develop a product and give it away freely. Financial support was fortunately received from altruistic organizations such as the Rockefeller Foundation, USAID, and the Syngenta Foundation. After 1999, there was no support from the European Commission, and at no time was there support from any UN institution, such as the World Health Organization or the Food and Agriculture Organization. The Gates Foundation started to fund the project at the end of 2010 (see below).

The Syngenta Agreement and Intellectual Property Rights

Fortunately, the private sector had an interest in commercial exploitation of our invention, which opened the opportunity for a deal. Syngenta was interested in a commercial product, whereas we supported a humanitarian one. Adrian was the architect of a solution for these conflicting interests.

We transferred our rights to Syngenta to allow their commercial exploitation of the technology and licensed back the rights for carefully defined humanitarian use (in developing countries, for

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resource-poor farmers, in public germplasm, with no charge for the technology and local sales and replanting allowed). The project undertook to follow regulatory imperatives and respect national sovereignty. Export of the technology between licensees is allowed only for humanitarian research and use. Improvements by either party are included in the cross-licensing. Syngenta legally obligated themselves to provide specific regulatory and improvement assistance to the humanitarian project. The public sector project also benefited in more general ways from private sector know-how and "in-kind" support, including solutions to patent problems.

Intellectual property rights were the first hurdle. While doing basic research, scientists tend to freely exploit all published knowledge. With product development, this changes dramatically: Intellectual property rights must be respected. The number of patents initially thought (erroneously) to be involved was shocking; however, for our humanitarian project, we required and received free licenses for humanitarian use for all relevant intellectual property, thanks to the cooperation (facilitated through Adrian) of Bayer, Mogen, Monsanto, Novartis, and Zeneca, as well as an anonymous Japanese company.

Increased Provitamin A Content and SGR2 Events

It was not clear how much provitamin A Golden Rice needed to deliver, because we had no bioavailability data. According to a hypothetically possible 2:1 conversion ratio [confirmed in 2012 (95, 96)], our 1.6 μg of provitamin A per gram of rice would have been sufficient. The lack of bioavailability data, however, allowed Greenpeace in February 2001 to launch its very effective "fool's gold" campaign, in which they claimed that meeting an adult's daily vitamin A requirement by eating Golden Rice would require consuming 3.7 kg of dry-weight rice, or approximately 9 kg of cooked rice.

Because we were not yet in a position to understand the bioavailability of Golden Rice's beta-carotene in the human body, increasing the amount accumulated became important. In a joint program, Peter Beyer's lab and the Syngenta lab analyzed the pathway for rate-limiting steps. Syngenta worked on phytoene synthase, and Peter's lab worked on all other enzymes. Phytoene synthase turned out to be rate limiting (51). It would perhaps have been reasonable for Peter to be a coauthor in this publication, but it nevertheless reinforced that the improved materials were available to be managed by the Humanitarian Board for our humanitarian project. The apparent increase was impressive, and doubts about insufficient amounts were silenced. Later, it turned out that useful data concerning beta-carotene levels can be obtained only after three months of storage, by which time degradation—common to all food plant sources of beta-carotene—has stabilized. All subsequent calculations took these losses as well as cooking losses into account, and all data now available suggest that, despite Greenpeace's 2001 claims, about 40 g of dry Golden Rice (corresponding to 150 g of cooked Golden Rice) per day is likely to prevent death and blindness (**Figure 6**).

From 2005 on, Syngenta transformation events—the SGR2 events—were considered the best for the project. After discussion, six different transformation events in an *Oryza sativa* ssp. *javanica* background were supplied to IRRI in the Philippines and to the Indian Agricultural Research Institute for introgression into publicly owned *Oryza sativa* ssp. *indica* germplasm (which is preferred by both growers and consumers in Asia) and for evaluation in Asian soils and climatic conditions. To minimize the risk of "unintended presence" of unregistered transformation events as seeds in future trade, all previous material from the Golden Rice network was destroyed—an extremely tough decision for our partners, but necessitated by the regulations governing GMO crops, even though polished rice is not a living modified organism (and so not covered by the Cartagena Protocol of the Convention on Biological Diversity), every polished grain of Golden Rice is easily

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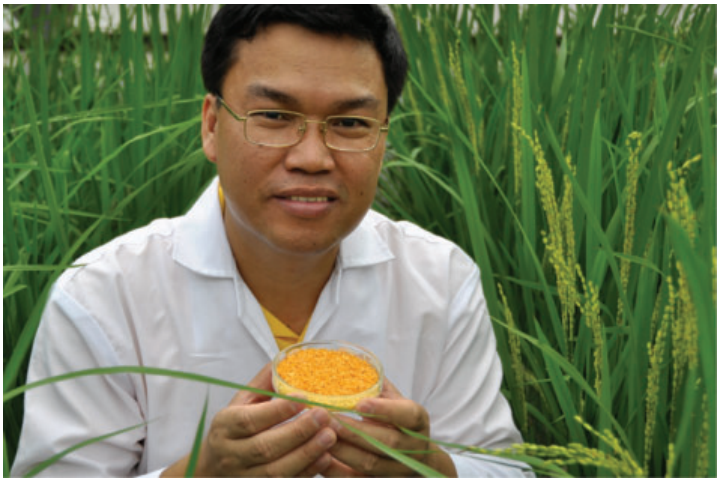

Figure 6

Forty grams of Golden Rice—the daily amount expected to be sufficient to prevent death or blindness resulting from vitamin A deficiency—in the hands of Dr. Tony Alfonso of the Philippine Rice Research Institute. Photo courtesy Tony Alfonso.

identifiable by its distinctive golden color, and unintentional export of Golden Rice seed is most unlikely.

Breeding the Trait into *Indica* Rice Varieties: The Lead Event

The rice breeders of the Golden Rice network explored which *indica* varieties in their respective countries were expected to be the most important, and they each took responsibility for the transfer, by conventional breeding, of the trait into four of those varieties. Successful seed breeding requires numerous repetitions and careful observation and selection of phenotype in response to environmental conditions. Just imagine the impossibility of trying to do this job without permission to sow seed in open fields. The first completely open field trial of Golden Rice occurred in the United States in 2005. The necessary backcrossing in adapted cultivars in confined field trials in Asia was not possible until 2008. The multilocation field trials in the Philippines were not in place until 2012. All previous work in Asia was in the artificial environments of greenhouses or screen houses. As agronomic data are critical, this limitation was an extremely severe impediment to progress.

Additionally, starting with its first meeting, in August 2001, the Humanitarian Board adopted a strategy, based on private sector advice (itself driven by the impediments of the regulatory system for GMO crops), of "one transformation event everywhere." All variety development worldwide must be based on one lead event. This strategy has the advantage that one set of regulatory data is valid for the regulation of all varieties in most national regulatory systems in place for GMO crops, which has potentially important (especially for a public sector project) cost reduction implications. For Golden Rice, the lead event selection decision in 2009 therefore had to be made in the absence of sufficient field data concerning phenotype. The selection was based on consideration of morphological data, data on carotenoid accumulation and degradation, and data from the human bioavailability studies that had been recently completed and for which we had been critically waiting. Syngenta had provided molecular data to IRRI, which regrettably

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were not available to the board when selecting the lead event, and time was wasted until field trials demonstrated that agronomic performance was impaired in the lead event previously selected (36), requiring a shift to another event that had been carried along in case of an eventual emergency.

Regulation Delayed Variety Development by More Than Ten Years

We followed advice from Syngenta, with the consequence that we adopted the private sector strategy: everything "regulatory clean" from the beginning. Regulation thus affects the work on a GMO product long before the collection of data for a regulatory dossier. The necessary steps include deleting the antibiotic resistance marker (>2 years), screening for regulatory-clean events (>2 years), carrying out the transboundary movement of seeds (<2 years), carrying out the obligatory sequence from greenhouse to screen house to field (>2 years), obtaining permission for working in the field (>6 years), meeting the requirements for one-event selection (>2 years), performing experiments for the regulatory dossier (>4 years), and completing the deregulation procedure (<1 year). Owing to space restrictions, I refer readers to Reference 68 for a further discussion of this topic.

Bioavailability and Putative Impact

The lack of bioavailability data was a severe handicap until the first results came in; data from US adults became available in 2009, followed by data from children in 2012. Animal studies do not provide valid data for provitamin A carotenoids because animals metabolize them differently than humans do. Sophisticated labeling technology was necessary, and it took three years to grow enough expensive deuterium-labeled material for small-scale experiments (96) because the Golden Rice in the growth chamber in Houston was eaten first by aphids and then by mites. The results from a further study with Chinese children were very exciting—close to a 2:1 conversion, with 60 g of Golden Rice providing 60% of the Chinese recommended daily allowance (95). This study, which involved measurements from a single consumption of Golden Rice, demonstrated clearly that Golden Rice has the potential for an effective intervention. Not unexpectedly, given its 2001 position, Greenpeace condemned the research as soon as it was published (and four years after it was completed!), cynically trying to detract from the excellence of the results. The entirely political echoes of this criticism are still playing out, having already unjustly impacted the lives of several extremely dedicated and ethical clinical scientists. We have repeatedly attempted to obtain financial support for more and larger nutrition studies, especially when the Gates Foundation became involved in supporting the project, but so far have not been successful. Although the funds for efficacy studies have been secured from the Gates Foundation, these studies have not been possible owing to the regulatory requirements for food and feed approval.

The putative impact of Golden Rice has been examined in socioeconomic ex ante studies. The best data available are for India. According to Qaim and colleagues (93), the annual burden in India amounts to a loss of 71,600 lives, or 2,328,000 disability-adjusted life years (DALYs); Golden Rice could annually save about 40,000 lives, or 1,382,000 DALYs. In terms of cost effectiveness, the actual cost for the most effective traditional intervention—vitamin A capsules—amounts to US$134–599 per DALY saved. Golden Rice, by comparison, would cost US$3–19 per DALY saved (calculated at that time on the assumption of a 12:1 rather than 2:1 bioconversion of the beta-carotene to vitamin A). These costs include all money spent in ten years of proof-of-concept work plus all money spent on product development, deregulation, and variety registration. Given these unprecedented low costs and the lack of recurrent costs after adoption, this intervention is highly sustainable.

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Biofortification, Other Golden Crops, and Further Nutrition-Enhanced Traits

Golden Rice was the first purposefully created biofortified crop, and it attracted substantial support to the concept. The term biofortification describes the idea of using genetics to enhance the micronutrient content of staple crops. This is possible using traditional breeding, where the natural variation provides a basis for improving the micronutrient content. Examples of such staples obtained through breeding include vitamin A–biofortified orange sweet potatoes, maize, and cassava varieties; high-iron beans and pearl millets; and high-zinc rice and wheat lines, all of which have been developed and are currently being released under the HarvestPlus program (**http://www.harvestplus.org**). Biofortified crops created using traditional approaches have been able to advance substantially faster than Golden Rice because they are not burdened by regulation.

Transgenic approaches are advantageous when the nutrient does not naturally exist in a crop (e.g., provitamin A in rice), where variation is insufficient, and where breeding is difficult. The BioCassava Plus program of the Donald Danforth Plant Science Center (**http://www.danforthcenter.org**) is using genetic modification to develop, among other things, crops that are high in iron and provitamin A. The Queensland University of Technology and the National Agricultural Research Organisation of Uganda are working toward transgenic provitamin A and iron in cooking bananas; bananas with up to 20 ppm provitamin A have been developed, and trials have commenced in Uganda (**http://www.banana21.org**). A transgenic high-iron rice variety containing 14 ppm iron in the polished grain (38), under development at IRRI, is now moving beyond the proof-of concept phase. Additionally, transgenic approaches are being used to improve the nutritional quality of sorghum (**http://www.biosorghum.org**). The GMO-specific "penalty" has the consequence that granting agencies and researchers tend to avoid GMO approaches.

Numerous further projects on nutrition-enhanced GMO traits in the research and development pipeline are faced with the same research-stifling environment as Golden Rice. This work includes protein modifications to improve content in essential amino acids, engineering of antioxidants (e.g., lycopene and astaxanthin), engineering of carbohydrates (e.g., inulin and amylose), modification of fatty acids (e.g., omega-3 and polyunsaturated fatty acids), reduction of plant allergens (e.g., in rice, soybean, and wheat), engineering of vitamins beyond provitamin A (e.g., E, C, B_6, and B_9), and reduction of toxins (e.g., linamarin in cassava) (100).

Opposition and Supporters

Golden Rice is a prime target of the anti-GMO lobby, with Greenpeace having taken the lead for decades. The media and Internet campaigns against Golden Rice peaked in two recent actions: (*a*) the destruction of a test field in the Philippines and (*b*) the blackmailing of the scientists involved in the bioavailability study with Chinese schoolchildren (95). The vandalism act in the Philippines was followed by an overwhelming response from the scientific community: An editorial in *Science* (2) elicited a protest against such actions signed by approximately 6,500 scientists from around the world.

Pressures on the Humanitarian Golden Rice Project

The inventors' vision for Golden Rice is that it should reach the needy as soon and in as many countries as possible. Every delay leads to unjustifiable blindness and death (**Figure 7**). Since the paper by Tang et al. (95), it has been obvious that Golden Rice has great potential as an effective additional intervention for vitamin A deficiency. Public sector rice breeders in all countries with a high prevalence of vitamin A deficiency and rice consumption should collaborate and pool their

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Figure 7

A girl who lost one eye to vitamin A deficiency. Millions have not been as fortunate and instead have been blinded or died—despite the potential of Golden Rice. It is time to "lift the shadows of death" (88).

skills to achieve this objective as quickly as possible. Each government, within its own sovereignty, should then make its own decisions on when to approve Golden Rice for adoption.

Regrettably, the suspicion caused by the campaigning of the anti-GMO activists has a stultifying effect on such scientific cooperation. This campaigning, along with related continual harassment in the press, has the effect of making all institutions—public, private, and philanthropic—nervous about proper engagement in and discussion of data and the normal ebb and flow of scientific research advances and setbacks. Unjustified though it is in terms of scientific merit, the regulatory and political environment also causes institutions to be wary of controversy and reputational entanglement and nervous about possible claims for "liability" redress.

In recent years, we in the Golden Rice project have struggled with the many, many ramifications of these issues, which have sternly tested the commitment of all involved to the higher goals of the project. These problems have undoubtedly delayed the project, not least in the ways recently described by IRRI (36). It is too soon to be certain, but we hope that the worst of these trials are behind us.

When Will Golden Rice Reach the Needy?

We have experienced a continuous slippage of timelines and refrain from any further predictions. Golden Rice will, most probably, not reach the needy before 2017 (**Figure 8**). The first country will be the Philippines, in conjunction with Bangladesh and Indonesia. IRRI is fully committed and works in close collaboration with the Philippine Rice Research Institute, and reliable answers to the question in the heading above will be available from their websites (**http://www.irri.org/goldenrice**, **http://www.philrice.gov.ph**). India, Vietnam, and China are, so far, lagging behind.

EPILOGUE

The vision of the early 1970s—to develop a new tool for plant breeding by combining the "miracle of totipotency" of somatic cells with the potential of genetic engineering—has come true. The

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Figure 8

With the blessing of Pope Francis I, Golden Rice will reach the poor! Photo courtesy Robert Paarlberg.

progress in tissue culture, the exploding knowledge in molecular biology, and the unprecedented precision and versatility of genetic engineering offer breathtaking perspectives. Despite many motivated colleagues and a host of proof-of-concept cases, regulated transgenic products are widespread (37) but limited to very few cases. Why?

A well-financed anti-GMO lobby, the media amplification of their message, and the short, election-driven horizons of politicians are all huge hurdles for effective application of genetic engineering, especially in the public sector. One problem, however, outweighs all the others: regulation based on an extreme interpretation of the "precautionary principle." Golden Rice exemplifies what it entails, and with what effect, to follow these regulations and also how such hurdles to sensible science prevent the use of the technology for public good. Without science-based regulation, the potential of the technology will remain restricted to a few projects of multinational companies, paradoxically ensuring the self-fulfillment of one of the major arguments of the anti-GMO lobby: that the technology only benefits company profits. Therefore, "regulation must be revolutionized" (70).

DISCLOSURE STATEMENT

The author is not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

ACKNOWLEDGMENTS

These acknowledgments are limited to individuals not mentioned in the text. I feel responsible for all of the PhDs and coworkers who were motivated to support the food security concept and sacrificed their possible academic careers by working on cereals and cassava instead of model plants. I am grateful to my technician at the FMI, Ijrina Petruska, who so well took care of the group, and to my greenhouse manager at ETH, Katalyn Konja, who kept our sophisticated greenhouse program in perfect shape. The members of the Golden Rice Humanitarian Board have supported the project for more than ten years without expecting (or receiving) any reward. Dr. Jorge Mayer

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was a highly efficient project leader until he accepted a job in Australia, and he is still managing our website.

Special thanks go to all the public sector rice breeders in the Golden Rice network, of whom there are too many to name individually, who invested an enormous effort under extremely restrictive conditions to develop varieties for the needy in their countries, and who repeatedly had to destroy their work and stoically start with another transgenic event when circumstance required.

Vociferous support by Greenpeace cofounder Dr. Patrick Moore (**http://www.allowgoldenricenow.org**), who brought his campaigning experience to bear on educating the public at large about the immoral stance of anti–Golden Rice activists, especially Greenpeace, and the engagement in the public debate of Prof. Klaus Ammann (3) and the Forum Grüne Vernunft (**http://www.gruenevernunft.de**) are gratefully acknowledged.

Finally, a word to the regulatory authorities: I am strongly critical of the politically required yet scientifically unjustified regulations currently applying to GMO crops. Please understand that this is not a criticism of the regulatory authorities or individual regulators who fulfill their statutory obligations professionally, and who also suffer the burden of the unscientific attitudes expressed by some members of the public and some politicians.

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