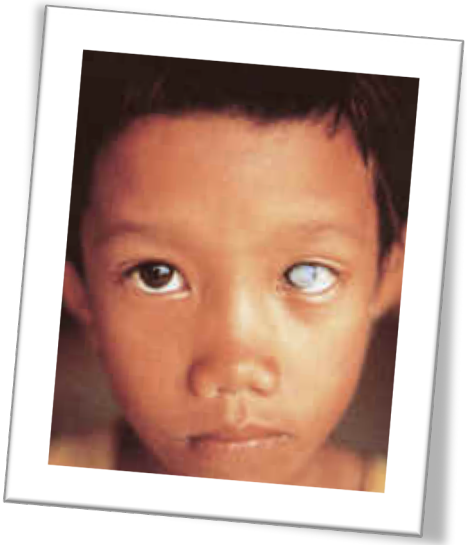
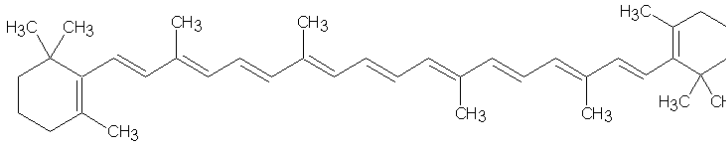
Golden Rice





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1.0 Preface

We chose the topic of Golden Rice, because it becomes more and more a reality. Soon the Golden Rice will already be on the market, and will probably have a big influence, preventing millions of people from vitamin A deficiency. From the first milestones in genetic engineering on, it was clear, that this science would have a big effect on the human being and his environment. Not only was it possible to give plants the ability to protect themselves from insects for example, it generally meant you could manipulate any organisms DNA, to give them the traits it leaked to be as efficient as possible. Of course this also had a big impact on agriculture: it was first possible to increase the amount food that can be produced, that also stays fresh longer and maybe even grows faster. This could mean a huge increase in the quantity of food. Even though this is a big success, it is not the only thing one can. It is also possible to not only grow for example the double amount of tomatoes in a certain time, it is also possible to increase their nutrition levels. This is what Golden Rice is exactly about. In third world countries Golden Rice is one of the best options to counter the vitamin A deficiency, that is causing hundreds of thousands people to become blind, of having a higher vulnerability towards measles and other consequences. The Golden Rice is expected to be used in different developing countries, and the humanitarian "Golden Rice" Network includes already 16 different national institutions in China, India, Indonesia, The Philippines, South Africa and Vietnam. There are also 2 different variations of the Golden Rice: we have the Golden Rice, that was originally developed by Prof. Dr. Ingo Potrykus, and we have the Golden Rice 2, which has up to 23 times more beta-carotene than the original Golden Rice and that was developed by the company "Syngenta". It's very interesting for us, that the science of genetic engineering could take a so direct influence on the whole world in the future. But still, the use of genetically modified food is very disputed, and there are many well educated people who warn us of their use. We still don't know everything that there is to know, especially concerning the long-termed use of that food. How will it change our environment? What about the side effects, things the scientists overlooked? For sure, GMOs (genetically modified organisms) like the Golden Rice are a good thing, but is it really to us to change nature? In our portfolio we try to cover most of the knowledge there is about Golden Rice, to form our own opinion.

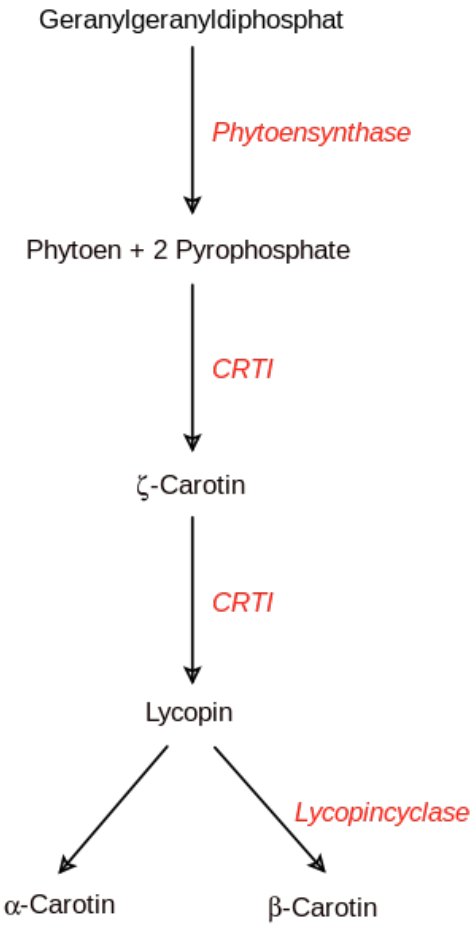
2.0 Introduction

A lot of different organisations and foundations have supported the development of the Golden Rice, by name: the Rockefeller Foundation, United States Agency for International Development, the UN, the EU, the Syngenta Foundation and recently even by the Melinda and Bill Gates Foundation. After more than 20 years of research, it will finally be possible to have a chance against the vitamin A deficiency in third world countries, and at the same time give them a good source of carbohydrates (the nice colour is just a well placed side effect) with the help of the Golden Rice. It would prevent them from blindness and other disease and can even save their lives. Even though this would have an extremely positive effect, we don't know everything about the Golden Rice yet. The method, with which the Golden Rice has been developed works with a special bacteria called Agrobacterium tumefaciens. This special bacteria transfers parts of its own DNA into the plant by a natural process. If you place the genes you want to transfer into a plant into the right place of the bacteria, it will transfer them automatically into the plants cell. To be sure, that we only pick out the plant cells with the right genes, which got transferred into it by the bacteria, we also place something into the bacteria called a "marker-gene". This marker gene will always be transferred with the gene that we really want to have in the plant. Its function is pretty simple: usually the marker gene carries the code for phosphormannose isomerase in it. What you can do now is to expose all the to be infected cells to mannose while they are in a dark room, and you can see that some will die and some won't. Logically, the ones that survived have the marker-gene in them, which automatically means that they also carry the gene we originally wished for. This is about the basic idea how the gene transfer for the Golden Rice worked. Of course this method isn't perfect, but for the case of the manipulation of rice to develop the Golden Rice, this one fits the best. There have been a lot of different studies and there still are some test that are running to make sure that we won't experience a bad surprise. From the first starts of the Golden Rice project in 1982, over the year 1992 when Ingo Potrykus and Peter Beyer, the main researchers for the Golden Rice, met, till 1999 when the first Golden Rice prototype was developed and when the Golden Rice 2 was invented in 2005 by Syngenta. The Golden Rice base then been crossed with different sorts from the regions where it will be used. In 2004 already we have had successful field tests concerning the stability of the transformation events. Also, Golden Rice seeds have been shipped to Vietnam, the Philippines and India for further testing. Syngenta even agreed to hand out the Golden Rice seeds to farms that make less then 10000 dollars a year for free, which would make the whole thing a non-profit act. Recently it has been learned that Golden Rice (GR) is going to be allowed in the Philippines, China and other Asian countries because of new experimental studies financially supported by the Melinda and Bill Gates Foundation. Some still don't agree with the technique of the Golden Rice. There are those, who don't want any genetically modified food to be spread around the world like Greenpeace or WWF, because they don't think that it is up to mankind to change nature. There are some scientists, who warn us of putting too much hope in the Golden Rice project because we still don't know what the exact side effects may be or that we may have missed an important thing in our studies.

3.0 Description of the engineering technique

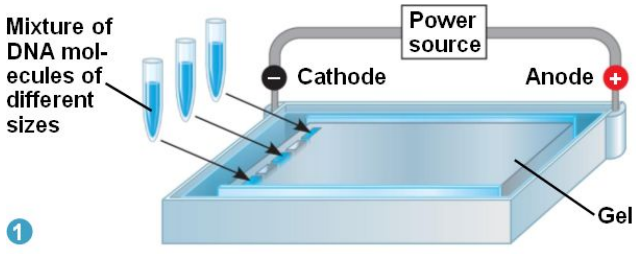
3.1 Introduction

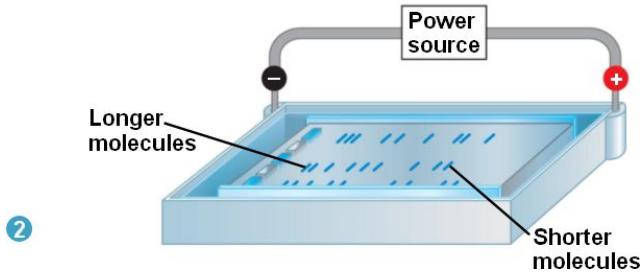
The aim of the Golden Rice project was, to induce a gene for the synthesis of beta-carotene in the endosperm of the rice plant. So we will first of all have a closer look at the biosynthesis of the beta-carotene. This simplified Figure shows the different educts and the products alpha-carotene as well as beta-carotene also known as pro vitamin A. The scientists found out, that all of these educts were already there or will be there, if they could manage to express the gene for the different enzymes to catalyse the reaction.
The gene for the enzyme Phytoensynthase and the enzymes to catalyse the reaction from the phytoen to the lycopin were missing.
Further research showed up, that the endosperm had already included the gene, which produces the enzyme Lycopincyclase to catalyse the synthesis of Lycopin to beta-carotene.
So they had to find 2 matching genes for the expression of the enzymes Phytoensynthase and CRTI. They found one of the desired genes in the Narcissus pseudonarcissus called NpPSY, which was responsible for the synthase of Phytoen. And the other gene CRTI was found in the bacterium Erwina uredovora. When they had all their knowledge and genes together, they finally started the experiments.



3.2 Isolation of the requested genes

For the isolation of the CRTI and the NpPSY genes, they first had to break down the cell walls or membranes (bacteria). This can be accomplished by dissolving the membrane and the cell walls with $NaOH_{(aq)}$. The DNA can be separated from the other substances by adding phenol. Now ethanol is added to cause the precipitation of the DNA. The DNA can be taken out of the test tube for further steps.



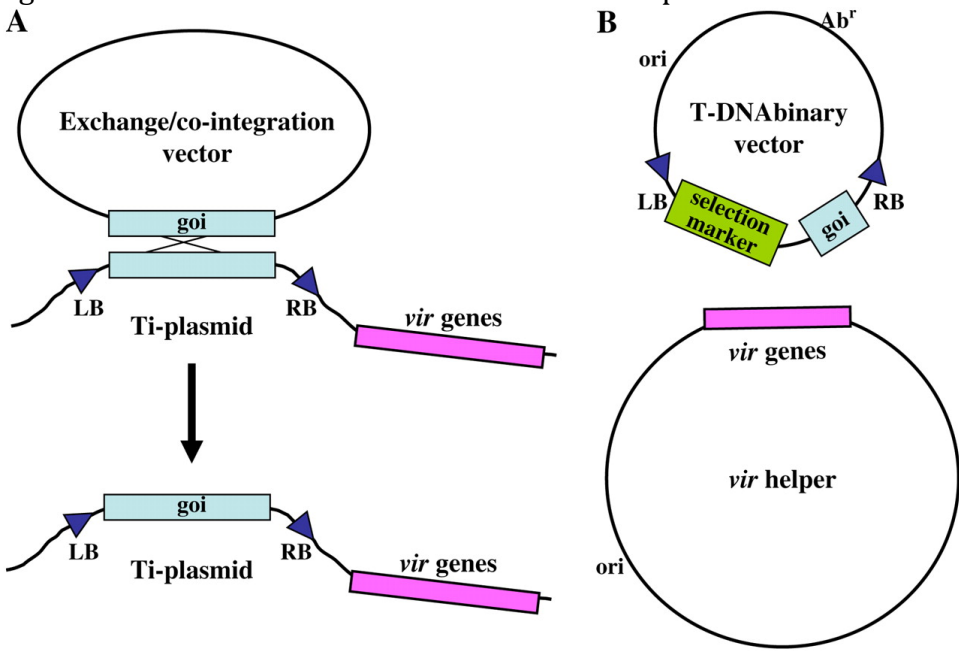


In the next step you have to cut the gained DNA in the desired piece. For this the scientists had to find the suitable restriction enzymes by screening the DNA and using a computer program to locate the right place to cut. Now they add the proper restriction enzymes to cut the sequence at the exact place.
Next they had to separate the bunch of cut DNA sequences. This could be done with a method called gel- electrophoresis. They placed the DNA in a gel tagging the DNA with a fluorescent dye. Now they put the whole thing under currency causing the DNA to

move towards the positively charged side. If the DNA sequence is smaller, it moves faster, so in the end the sequences are ordered by size. Now the scientists can joust cut out the suitable stripes of DNA (CRTI and NpPSY from the gel plate.

3.3 Insertion of the gene in the agrobacterium tumefaciens

To transfer the gene into a plant cell, the scientists often use nature itself to simplify the process. The agrobacterium tumefaciens is a naturally occurring vector, which attacks dicotyledonous plants. In this process it goes through the cell wall with the so-called vir-plasmid (virulence) and inserts the Ti-plasmid (tumour inducing) into the nucleus of the plant cell. Finally the plant is forced to built cells causing a tumour.
Now they used the agrobacterium tumefaciens with a disarmed (removed) Ti-plasmid, because they didn't want the plant to produce tumour cells in the end.
Now they look at the correct cutting side left border and right border in the agrobacteria and add the matching sequences on the isolated DNA, to make sure that it will match. This can be done with the usage of ligase in a test tube. Today the scientists are kind of lazy and just add a multiple cloning site, which helps to attach the DNA afterwards in the suited place. Next they have to add a marker gene in the DNA to distinguish later on, if the transport of the gene was succesfully or not. If everything went how it should, they now have a test tube full of DNA , which ha to be transmitted first into a e. coli bacteria to create another plasmid ring. This is reached by the application of the electroporation. The electroporation is nothing else than opening the cell membrane of the e. coli bacteria with electrical tension. In this moment, the added DNA sequence with the NpPSY, CRTI, the marker gene and the multiple cloning sides enters the bacteria.
Now the plasmid of the e. coli has to have the origin of the agrobacterium tumefaciens, which can also just be added through electroporation. The DNA sequence is taken up by the plasmid and now the bacterias are settled on a growing media.
After a while a lot of replication has occured and the whole plasmid of the e. coli is taken out with the same method used in 3.2 the isolation of genes.
The isolated plasmids are then inserted in the agrobacterium tumefaciens, by electroporation. Now contains two plasmid rings: one of the e. coli bacterium with the genes NpPSY and CRTI as well as the origin of the agrobacterium tumefaciens and the marker gene (phosphomanose isomerase) and the one with the vir-genes of the agrobacterium tumefaciens and the disarmed ti-plasmid.



The CRTI and NpPSY genes are inserted in the agrobacterium with the whole Plasmid, like in Figure B. The Boarders of the new ti-Plasmid just have to fit to one of the boarders of the vir-gene to be transferred later on.

The figure A represents a direct integration of the ti-plasmid withot the e. choli, but the scientists used the method in B to produce Golden Rice.

3.4 Integration of the genes into the rice cell with the agrobacterium tumefaciens

As next they had to put the agrobacterium in a culture medium to replicate it.
Then they used embryotic sativa rice cells and added the agrobacterium.
The reason they used the embryotic cells is because they probably have a thinner cell wall. Naturally the agrobacterium don`t attacks monocytodelons but they can in the embryotic state. To enter the cell the agrobacterium uses it`s vir-genes. In the cell, it activates the promoters and several enzymes help to cut out the NpPsy and the CRTI and the marker gene and include it into the nucleus of the plant cell. If everything went right, the genes and the marker gene are correctly inserted in the DNA.

3.5 Segregation of the successfully modified cells.

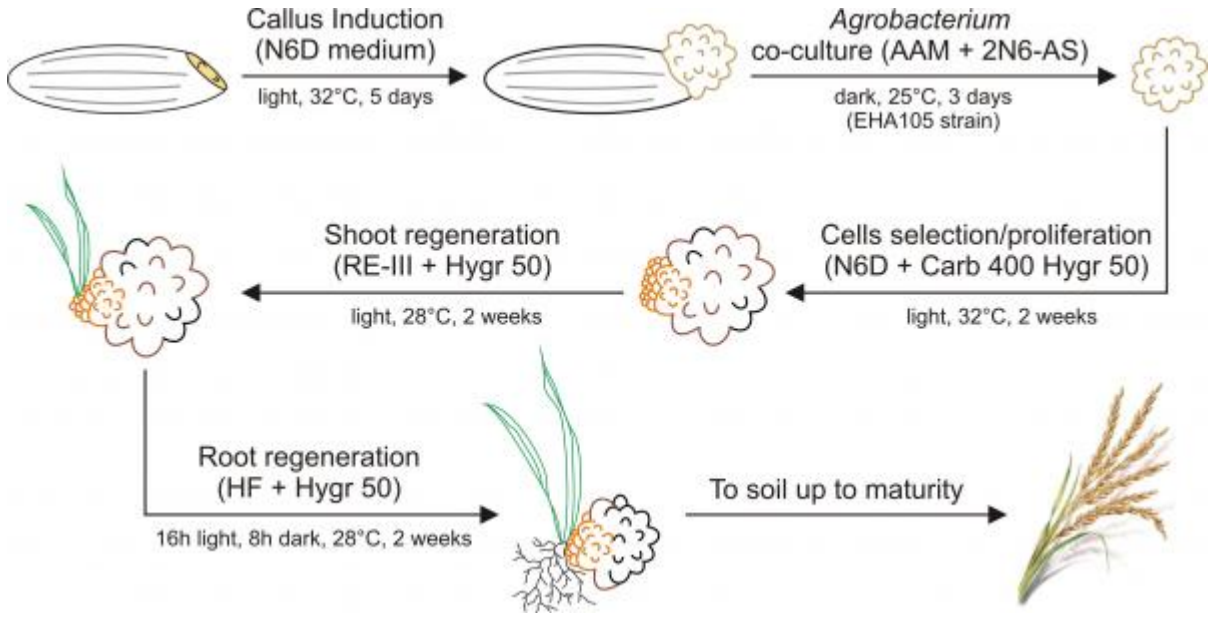
To identify whether the genes were successfully inserted, they used the marker gene (phosphomanose isomerase). This gene allows the plant cells to digest sugar (mannose). Then the plant cells are placed on a mannose enriched grow media in a dark place.
The plant cells with successfully transmitted genes will use the mannose to built up their cells and separate themselves The plant cells which don't have the marker gene and therefore mostly no other transmitted genes will simply die because they cant take up the mannose and they'll starve to death. The successfully transmitted plants will build a callus structure, which means a lot of cells unarranged in a petri dish. Then the scientists will give phytohormones to the cells and put them under light. The plant will grow over time to a fertile plant, which now can interbreed with another modified plant producing fertile offspring and the endosperm with beta-carotene.

4.0 Pictures of research institutions

Unexpectedly - Basel being the research headquarters of Syngenta - we couldn't find a research institution working on Golden Rice (GR) to visit. Instead, we interviewed Dr. Jan Lucht who had previously done research for eight years on GMO-food. His answers to our written questions can be found in the appendix. Dr. Lucht told us in the live interview that practically all research on Golden Rice has stopped in Europe due to the lack of acceptance of GMO-food.

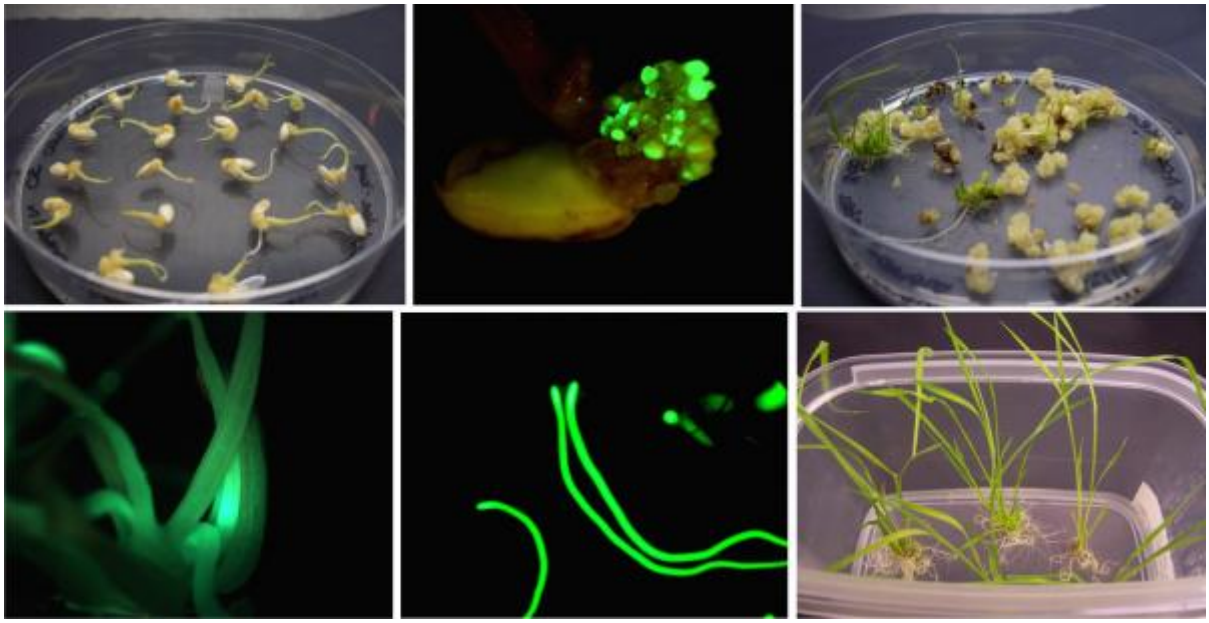
In order to obtain photographs of labs and procedures of GR-research from earlier days we contacted Dr. Potrykus, the inventor of GR, who had been Professor at the Institute for Plant Sciences, Swiss Federal Institute of Technology (ETH, Zurich); after trying to reach him for over a month we finally succeeded one day before due date of this paper. He couldn't supply us with pictures.

Scheme of the step involved of selecting the genetically modified cells and their growth



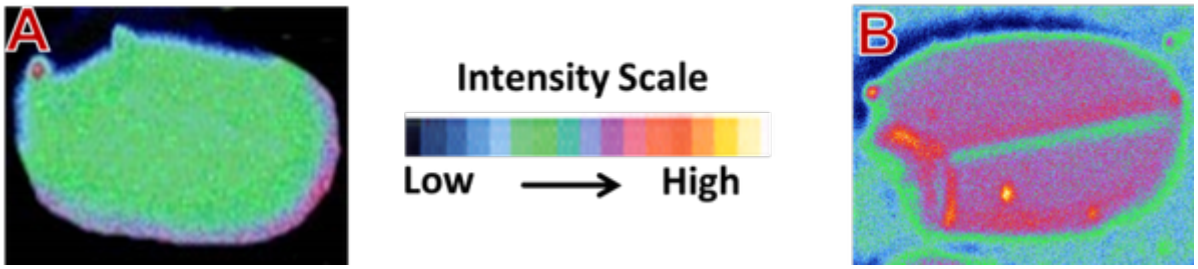
Below some pictures related to the generation of transgenic rice plants are reported.

You can see the different calli on the grow media.



Resource of text and pictures: http://www.plantlab.sssup.it/RiceTransformation

In this figure you see the measures of the iron content in two rice grains by micro-X-ray fluorescence spectrometry. They want to increase iron level genetically in plants. (Also as food supliment)



A) Rice growing at the rice greenhouse facility of the ETH Zurich

B) Experiments with rice grown on hydroponic system being conducted at the rice greenhouse facility of the ETH Zurich

Resource: http://www.pb.ethz.ch/research/cerealbiotech1

Here one can see the change in colors due to progress in increasing the beta-carotene level, which has usually a yellow appearance.

Resource: http://www.goldenrice.org/Content2-How/how1_sci.html

5.0 Discussion

The methods applied developing Golden Rice (GR) are to a large extent still in use today in comparable projects. Agrobacterium tumefaciens is still being used. There are small differences in the marker genes used today, in that marker genes which are expressing antibiotic residents are cut out again. Alternatively the pmi (phosphomannose isomerase) gene method is available. With these marker genes the successfully modified cells grow better and faster allowing a distinction between gene-modified cells and unmodified ones (explained in point 3). Another progress is that the Ti-plasmids are already defused and the E.Coli bacterium plasmids are already prepared with the marker genes and the wanted T-DNA, thus eliminating the repetition of these steps in every cloning experiment.

There are plans for future research to further enhance advantageous features of rice, e.g. by inserting genes that enable rice to take up zinc and iron; or to cross different basic rice plants used in individual regions with Golden Rice. The goal is to increase the availability of β-Carotene. E.g. alone in Japan different rice types are used depending on where they are cultivated (North or South). So, different countries in Asia have to take the kinds of rice growing in the specific region. Until today we have already a big variety of crossed rice types.

In the discussion on whether Golden Rice should be used or not opinions are sharply divided. The proponents argue are that Vitamin A deficiency (VAD) which can lead to life threatening illnesses occurs mainly in poor Asian countries where people eat a lot of rice and can't afford a balanced diet supplying enough Vitamin A. Golden Rice proponents - like Bill Gates and his foundation - say that GR can help a lot of poor people and they think that the risk for man and nature is insignificant.

A lot of politicians and people seem to be afraid that gene modified food is dangerous for people's health (vitamin A in high concentrations could be harmful), nature and the independence of farmers. Although these are three completely separate lines of argumentation, they are frequently mixed together in public argumentation of opponents. Groups on the contra side like Green Peace are afraid that if you allow GR other GM foods would be permitted. They call GR a Trojan horse. Therefore the administration barrier is from a scientific point of view relatively high. Some people furthermore think that GMO is unnatural and men would play god an influence the nature in a way no meant to be. Others think that men would influence evolution. ms. Therefore they are against changing the DNA of a plant. They want restrictions which have the consequence that is more expensive to produce GMO-food.

In an "open letter" in 2009 twenty American scientists criticized that the tests made with humans which ate GR were dangerous and against the ethics code of Nuremberg. They complain that there weren't any tests with animals before the tests with humans. They said also that derivates of β-Carotene could cause birth defects. In response pro-scientists also wrote an open letter in which they stated that the criticisms are unjustified and that it was written by a radical anti-genetics group of scientists. They also wrote that there weren't any offences against the ethics code of Nuremberg. They also accused the other group of wanting an agriculture without any genetic modifications and of accepting therefore millions of people getting blind and dying. According to them more people died because of VAD than people died during the holocaust. The reaction of the contra side was that they accused the supporters of the

Golden Rice Project to handle this conflict in an aggressive and disrespectful way. They called the Golden Rice Project a Trojan horse.

Other opponents of the Golden Rice project stated that Golden Rice doesn't contain enough β-Carotene to heal VAD. Proponents answer that Golden Rice 2 produces much more β-Carotene than its antecessor (GR 1 produces 4 times more than the prototype and GR 2 produces 23 times more than GR 1); and that the Golden Rice humanitarian project doesn't claim to heal all the VAD but to reduce it. In case of Golden Rice the Asian population is affected mostly. States in Asia didn't have to deal with genetics in the past. So they looked how states in America and Europe handle this topic. After they had seen that politics in Europe is really careful with that they took this opinion over. Another criticism at Golden Rice is that the used marker genes could harm people if dangerous bacteria and viruses would take them over and could then be immune to antibiotics. However, today the completely harmless pmi method is used, so there aren't any antibiotic resistances in GR genes.

The fear of a general health risk from GM food like Golden Rice or is scientifically unfounded. While Vitamin A affords no health risks to children or adults it can cause damage to a fetus when taken by the mother in very high concentration early in pregnancy (e.g. from vitamin pills or large amounts of liver eaten). Experiments have shown that it's unlikely that people would consume enough rice to obtain a dangerous concentration. Until today no catastrophe happened with GM food like Golden Rice. There is no evidence that it is seriously harmful to health. So the fear of it is not justified.

Another question is a possible danger to the environment. GR is in this respect different from GM Monsanto-plants, that are made highly resistant to all kinds of pesticides and call for the application of excessive amounts of such pesticides which then contaminate the soil and water.

If we compare the dangers and advantages of GR we have on one hand millions of adults and children dying and getting blind because of VAD, who can profit from GR, and on the other hand the scientifically unjustified fear that GM food is harmful.

While opponents propose that you could help the people which have VAD by giving them a balanced died or invest in storage so they can store e.g. the cans of rice containing provitamin A (beta-carotene), in our view this offers no solution. The problem is that that these people are poor and these methods are far too expensive. In developed countries it's maybe not necessary to have GM food but in poor countries where the people don't have access to a balanced diet Golden Rice is a good thing.

In our opinion considering all the arguments we think that contra activists and politicians in Europe shouldn't prevent the use of Golden Rice in Asia, because it could help to save thousands of lives each year.

6.0 Summary

The whole Golden Rice project started at 1982, was and still is a fully humanitarian project with no expectations concerning profit. The first Golden Rice prototype was developed 1999, the Golden Rice 1, which had 4 times more beta-carotene then the prototype, and the Golden Rice 2, which even had 23 times more beta-carotene then the Golden Rice 1, followed. The method used was the Agrobacterium tumefaciens-method which shows more success then other methods, that were tried out to create Golden Rice. Our interest in this topic bases on the thought, that this rice is simply supposed to help the world. It's a humanitarian project, which means that no profit will be made, and the seeds will be given away to small farms for free! Of course there are some controversies, like with any other GMO, but what we always must keep in mind is, how much this will help developing countries, especially their population, to fight vitamin A deficiency, which causes blindness and several other health damages and can even lead to death. The interview with Dr. Jan Lucht confirmed our assumption that the Golden Rice project is a realistic option to fight VAD. The question may appear, why it still isn't allowed and distributed. This is because a lot of politicians and bureaucracy make it very difficult for GMOs to arrive at the world market. Recently news broke, that Golden Rice will be permitted to be sold or given away in some Asian countries (China, the Philippines etc.). This breakthrough is the result of new scientific studies.

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9.0 Appendix

Interview Golden Rice:

Jan Lucht, scienceindustries

1. **Which type of gene transfer methods did you apply to transfer the genes into the DNA of rice?**

Agrobacterium-mediated transformation of rice embryogenic calli.

2. **Are there better/easier methods today?**

Agrobacterium-mediated transformation is still the most widely used method, the protocol is constantly improved but the basics remain the same. Alternative: biolistics (gene gun), but this has drawbacks (too high transgene copy numer, rearrangements).

3. **How did you modify Golden Rice 1 to Golden Rice 2?**

Golden Rice 2 was a completely new construction, based on the transformation of a different rice variety with a different vector. The same crtI gene from the bacterium Erwinia was used, but instead of the original daffodil psy gene a psy gene from maize was introduced. Also, a different selection procedure was used (see Q7).

4. **How did you isolate the genes of interest from their plants of origin?**

e.g. Maize psy from GR2: obtained from different scientist (maize c-DNA library screened with probe from transposon-tagged mutant). Rice psy gene cloned by locating candidate gene in genomic sequence; cloning by PCR.

5. **How did you exchange the wild type DNA in the Ti-Plasmid of Agrobacterium tumefaciens with the wanted T-DNA sequences?**

For the creation of transgenic rice plants, "disarmend" Agrobacteriae were used that lack the tumor-forming genes on the Ti Plasmid. These bacterial strains have a large Ti plasmid with the virulence genes (but without transferrable DNA) and a small T-DNA binary vector that can replicate in E. coli and in Agrobacterium. The gene of interest is cloned into the binary vector between the border sequences, and then introduced to A. tumefaciens (see graphic from Lee&Gelvin 2008).

6. **Are there alternative vectors to Agrobacterium tumefaciens? If yes: Which ones?**

No alternative biological vectors are used for plant transformation (but other transformation methods without vectors are available; see Q 2)

7. **How did you distinguish between the successfully modified cells and the unchanged cells in detail, e.g.: Which was the antibiotic used?**

*For GR1, transformed cells were identified with the **aphIV hygromycin antibiotic resistance gene.***

*GR2 transformants were identified with the help of the **pmi marker gene** that allows plant cells to use mannose sugar as carbon source.*

8. **How did you select for plant cells, in which the inserted genes are expressed and make the right products?**

GR1: visual selection of transgenic plants with yellow grain endosperm, and molecular analysis of presence of transgenes

GR2: first test of different psy genes by transformation of rice and visual inspection of calli (yellow?). Then production of transgenic plants with maize psy gene, visual inspection of grains.

9. **Is there any need and possibility to improve Golden Rice 2?**

The Provitamin A trait has to be introduced into locally adapted rice varieties, suitable to different climatic regions. In addition, rice can be further improved with micronutrients ("biofortification") like iron or zinc by genetic engineering.

10. **I found in the literature an approximate description of the reaction steps of the synthesis of β-Carotin from GGPP, but little details. Could you help with the specific details of the reactions?**

See Al-Babili & Beyer 2005

Email from Prof. Ingo Potrykus

Sehr geehrter Herr Stähli,

ich habe keine Bilder von meinem Labor. Die wissenschaftliche Arbeit wurde an der ETH Zürich, Institut für Pflanzenwissenschaften und in der Universität Freiburg/Breisgau im Institut für Zellbiologie durchgeführt. Die Produktentwicklung erfolgt seit 12 Jahren in 16 Partnerinstituten in den Philipinen, Bangladesh, Vietnam, Indien, China, Indonesien. Die Genehmigung zur Freisetzung ist keine politische Entscheidung sondern die Enscheidung der nationalen Biosicherheitsinstitutionen aufgrund der Beurteilung eines Dossiers in dem alle Daten zu Sicherheitsfragen dokumentiert sind. Die experimentelle Arbeit für dieses Dossier beanspruchte mehr als 6 Jahre! Im attachment finden Sie ein paar Illustrationen unserer Partner in den Philippinen. Dort werden die ersten Freigaben Ende 2013 erfolgen.

Mit freundlichem Gruss,

Ingo Potrykus