# Transgenic Indica rice to the benefit of Less Developed Countries: Towards pest-resistance, and accumulation of β-carotene in the endosperm

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## 1. Introduction

Indica-type rice feeds more than two billion people, predominantly in developing countries. In humid and semihumid Asia where rice is the basic food, the population is expected to increase by 58 percent over the next 35 years. In thirty years the world will need 70 percent more rice than it requires today. And these ca. 800 million tons of rice will have to be grown with considerable reduction in the input of agrochemicals under sustainable conditions (IRRI, 1993). This immense task requires that traditional plant breeding is supported by every possible contribution from novel technical developments. Genetic engineering, applied with consequence and care, has the potential to contribute to the sustainable production of affordable food for the increasing population in developing countries. For maximum benefit for developing countries, application of gene technology should focus on important problems for which solutions by conventional approaches are not available. For Indica-type rice such problems have been identified and described in a joint study of the International Rice Research Institute (IRRI), Manila, and the Rockefeller Rice Biotechnology Programm, New York (Khush and

Toenniessen, 1991). Among the problems to be solved with high priority are a) resistance to fungal diseases, b) resistance to Tungro virus, c) resistance to Yellow stem borer, d) stable supply of provitamin A, and e) improvement of nutritional quality. The problems mentioned are especially severe for people depending on Indica-type rice. It is, to date, relatively easy to genetically engineer Japonica-type rice and relatively difficult to do the same with Indica-type rice. The tasks mentioned can be solved only by focusing work on Indica-type rice. The International Rice Research Institute, Manila (IRRI) has the world mandate to develop breeding lines to the benefit of small rice farmers, and has already released numerous successful varieties to the rice growing countries (IRRI, 1992). The following research projects are, therefore, performed in collaboration with IRRI and using IRRI breeding lines, to assure direct transfer of experimental success to the target population. Best possible solutions can be found only with the tight involvement of the international scientific community. This is achieved by collaboration with the Rockefeller Rice Biotechnology Program (Toenniessen et al., 1989).

## 2. Gene transfer for integrative transformation

Plant breeding with transgenic characters requires populations of independent transgenic and fertile plants to chose the most stable and best expressing lines for subsequent traditional breeding. This in turn requires routine and efficient gene transfer protocols leading to fertile transgenic plants. Although recovery of transgenic Indica-type rice plants and offspring have been described from our laboratory (Datta et al., 1990, 1992) and fromothers (Christou et al., 1991), gene transfer to IRRI breeding lines is not yet routine and efficient enough, and recovery offertile transgenic plants is still rather inefficient and requires further optimalisation. Gene transfer to Indica-type rice is, to date, possible via direct gene transfer to protoplast (Datta et al., 1990, Ghosh-Biswas et al., 1993) and our group is using this technique still routinely with IR43, IR72 and other Indica rice varieties. With IR72, the at present most advanced breeding line, we still face, however, severe fertility problems; with IR43 we see, so far, better chances to raise sufficient numbers of independent and fertile transgenic plants in the near future. Transgenic Indica rice plants have also been recovered from biolistic treatment of immature embryos (Christou et al., 1991) and from electroporation to split embryos from mature seeds (Xhu and Li, 1994). We have a series of transgenic clones and plants from IR43 and IR72 under investigation and hope to be able at the time of the symposium to present data which allow to judge which of both techniques can be recommend. We also applied electroporation to cells of immature embryos for transformation of cereal cells. Although gene transfer to scutellum cells of wheat was routine and efficient (Klöti et al., 1993), it was not possible, so far, to repeat these results with rice. *Agrobacterium*-mediated transformation of rice has been attempted by other la-

boratories (e.g. Rainieri et al., 1990). However, *Agrobacterium*-mediated transgenic rice plants have, so far, not been recovered from any laboratory.

## 3. Approach towards Yellow Stemborer resistance

Insect damage is one of the major factors for yield loss in rice farming all over the world. In Southeast Asia alone the value of forgone production caused by insect damage reaches more than 600 million USD per year. More than two third of these losses are caused by two insect species, the rice brown planthopper (BPH; *Delphax oryzae*, Homoptera) and the yellow stemborer (YSB; *Scirpophaga incertulas*, Lepidoptera, Herdt, 1991). Unlike the case of BPH there is no world type rice variety known to contain resistance genes against YSB, which could be used as a source for getting YSB resistant rice by conventional breeding methods.

The entomocidal sporeforming soil-bacterium *Bacillus thuringiensis* (B.t.) offers a promising variation of genes which encode for specific endotoxins. Until now more than 40 nucleotide sequences of such genes have been determined. They are clearly to each other and classified in 17 distinctly different crystal protein genes, the so called *cry*-genes (Peferoen, 1991). These genes encode for proteins either of some 130-140 kDa or some 70 kDa which are first dissolved and then proteolytically cleaved in the midgut of the insects to small toxic fragments of approximately 60 kDa (Faust and Bulla, 1982). After cleavage these toxins bind to specific proteins on the brush-border membranes of the insect gut (Hofmann et al., 1988), followed by disrupting the epithelium, disturbing the ionic balance and thereby paralysing the gut.

Different B.t. formulations have been used as biological insecticides for many years. Disadvantages like poor persistence and short duration of effect under tropical conditions especially during the rainy season, could be overcome by a transgenic approach the expression of B.t. genes in the rice plant itself.

First success has been reported from tobacco (Vaeck et al., 1987), potato (Peferoen et al., 1991), tomato (Fischhoff et al., 1987), cotton (Perlak et al., 1990), maize (Koziel et al., 1993) and Japonica rice (Fujimoto et al., 1993). These plants showed a high level of insect resistance and it is expected that some of these crops will be released on the market in the next few years (Peferoen, 1991).

The aim of our project is the transformation of advanced Indica rice breeding lines with genes conferring resistance to YSB. Therefore we are using the lepidopteran specific *cr*<I A(b)-gene, which has been shown to be effective against YSB as well as rice leaffolder (Lepidoptera, D. Bottrell, personal communication).

Since it is known that the higly conserved C-terminal half of the B.t. toxins is not necessary for the toxicity of the protein, truncated forms of the *cry*-genes have been cloned (Fischhoff et al., 1987). These truncated genes

encode only for the N-terminal half of the crystal proteins and show significantly enhanced expression levels of the B.t. toxins in transgenic plants (Fischhoff et al., 1987; Delannay et al., 1989; Perlak et al., 1990; Koziel et al., 1993; Fujimoto et al., 1993).

As plants in general show a different codon usage than bacteria, a synthetic *cry* I A(b)-gene was constructed (Koziel et al., 1993) which shows a high G-C content in the coding region. As the maize codon usage resembles the codon usage pattern of monocots in general (Murray et al., 1989), this should also lead to a sufficient expression of the *cry* I A(b)-gene in rice.

As a first step towards YSB resistant rice plants we have transformed a truncated version (645 codons) of a wild type and a synthetic *cry* I A(b)-gene to elite Indica rice breeding lines. Resistant clones were analysed by Southern Blotting and showed a clear integration of the *cry* I A(b)-gene in the rice genome. Plants were regenerated, transferred to soil and are growing under greenhouse conditions. For further analysis $R_0$ and $R_1$ plants will be checked by Southern, Northern, and Western Analysis and by insect feeding studies as well.

To minimise the possible development of resistance in insects we put this gene under control of a tissue specific promoter which directs the expression of the *cry* I A(b)-gene only to the leaf sheath, the primary target site of the YSB. Furthermore, we are planning to use a second Bg-gene, the *cry* II A-gene which is known to bind to a different receptor site in the brush-border membrane of the insect gut, as resistance to B.t. toxins could be due to a change in such a receptor site (Ferré et al., 1991).

However, it has to be emphasised that such transgenic rice plants should be planted under field conditions only according to the concept of Integrated Pest Management, IPM, to keep the B.t. toxins as an effective and sustainable tool not only for the next few years (McGaughey et al., 1992).

## 4. Approach towards tungro virus resistance

The rice tungro disease by a complex of two viruses, rice tungro spherical virus (RTSV) and rice tungro bacilliform virus (RTBV, Hibino et al., 1978). The severe symptoms are caused by RTBV (Dasgupta et al., 1991). RTBV is a member of the newly assigned group of badnaviruses (Hay et al., 1991; Qu et al., 1991) and is related to the better studied caulimoviruses in its life cycle and genome organisation (Hohn and Fütterer, 1992). While engineered virus resistance has been achieved for a great number of RNA plant viruses, for the DNA containing badna- and caulimoviruses no successful strategy has been reported so far (Wilson, 1993). Since these viruses have very different replication cycles it is not clear whether approaches that worked with RNA viruses (i.e. constitutive expression of viral coat proteins or wild-type or mutated replicases) will also work for RTBV. It is, however, to be expected that also for RTBV, expression of functional viral proteins already

at the onset of virus infection could interfere with an ordered progression through the viral life cycle and that expression of mutated viral proteins might interfere with the function of normal viral proteins by competition. We therefore have introduced into rice constructs designed to express RTBV proteins 1, 3 and 4. The protein is a precursor from which by proteolytic processing the viral coat protein, reverse transcriptase and probably a variety of other proteins are generated. The locations of these proteins within the precursor can at present be deduced only from sequence homologies to related viruses. A variety of constructs for direct expression of the coat protein or the reverse transcriptase have been prepared on the basis of such estimations.

In the coat protein region, a sequence motif containing three invariable cysteines and one histidine is conserved between almost all viruses using reverse transcriptase in their replication cycle (retro- and plant pararetroviruses; Covey, 1986). This motif, which is involved in several RNA binding steps, has been mutated to glycine since such a mutation has been shown to preserve the structure (and thus part of the function) of the remaining part of the coat protein but to abolish infectivity of a retrovirus (De Rocquigny et al., 1992; Morellet et al., 1992).

In the reverse transcriptase region, a mutation was introduced into a higly conserved sequence motif containing two aspartates (Argos, 1988). In addition, subfragments of the polyfunctional reverse transcriptase have been cloned in analogy to results from some RNA viruses where subfragments of the polymerase gene produced high levels of protection (Wilson, 1993).

Since functions for the remaining RTBV proteins are unknown, sensible mutations are difficult to design. In the protein 4 we localised a leucine zipper similar to those that are involved in protein-protein interactions in many other proteins (Gruissem, 1990). In a yeast system (Fields and Song, 1989) we found that the protein 4 indeed has the capacity to dimerise but the dimerisation domain resides outside the leucine zipper which, however, may interact with another protein. We have cloned subfragments of the protein 4 coding region containing either the leucine zipper or the dimerisation domain to express proteins that lack one of the interaction domains and thus probably will not have a complete function but still will interact with one of the original partners and therefore will act as competitive inhibitor.

In addition to these approaches involving expression of a protein, we also have introduced a construct expressing antisense RNA against the leader sequence of the RTBV pregenomic RNA. Antisense RNAs had little effect on RNA viruses (Wilson, 1993), but viruses like RTBV with a nuclear phase might be more susceptible.

Most of these strategies follow the work performed with RNA viruses. Approaches that would more specifically use the particular molecular biology of RTBV require a detailed knowledge of the viral life cycle. Therefore, we also study viral gene expression mechanisms and the potential function of viral gene products in transient expression systems (Fütterer et al., 1993).

Transgenic plants from most of the constructs have been recovered from hygromycin selection and grown to maturity in the greenhose. Seeds have been harvested and transferred, with the permission of the Philippine Biosafety Committee to the International Rice Research Institute, Manila, where they are screened for resistance.

## 5. Approach towards provitamin A accumulation in rice endosperm

According to UNICEF statistics world-wide, over 124 million children are estimated to be vitamin A deficient (Humphrey et al., 1992). Improved vitamin A nutrition would be expected to prevent approximately 1-2 million deaths annually among children aged 1-4 years. An additional 0.25-0.5 million deaths may be avoided if improved vitamin A nutriture can be achieved during the later childhood. Improved vitamin A nutriture alone therefore could prevent 1.3-2.5 million of nearly 8 million late infancy and preschool-age child deaths that occur each year in the highest-risk countries (West Jr. et al., 1989).

Rice in its milled form, as it is consumed by most people, in South East Asia is characterised by the complete absence of provitamin A. The milled rice kemel consists exclusively of the endosperm. The embryo and the aleuron layer have been removed during processing of the rice grain.

The aim of this project is to initiate the carotenoid biosynthesis in the rice endosperm tissue to increase the daily vitamin A uptake of people predominantly feeding on rice.

It is known for maize and sorghum that cereal endosperm cells can produce and accumulate carotenoids (Buckner et al., 1991). Furthermore the starch storage tissues of potato and cassava (Pentedao and Almeida, 1988), accumulate carotenoids in considerable amounts. To provide the minimum requirements of relevant carotenoids to young infants, and assuming rice as the sole dietary source, 1-2 μg β-carotene per gram uncooked rice would be needed in rice endosperm (The Rockefeller Foundation, 1993). This is roughly 1/4 – 1/2 of the amount produced in maize endosperm, and enough to turn the rice noticeably but not dark yellow.

The carotenoid pathway is a branch of the central isoprenoid pathway which is characterised by 4 key enzymes that are necessary for carotenoid biosynthesis. These are the phytoene synthase, the phytoene desaturase, the ζ-carotene desaturase and the lycopene cyclase. The genes encoding for these enzymes are available both from higher plants (Fray and Grierson, 1993; Ray et al., 1987; Linden et al., 1993; Bartley et al., 1991) and bacteria (Armstrong et al., 1989).

Our strategy is to produce transgene indica rice varieties which contain either single genes or several genes in combination. So far, we have regenerated transgenic plants containing a phytoene synthase cDNA from daffodil

(Beyer et al., unpublished results) under the control of endosperm specific promoters (Okita et al., 1989) to ensure exclusive expression in the endosperm. The analysis of these plants, done in close collaboration with Dr. P. Beyer, Freiburg (FRG), revealed, that it is indeed possible to shift the basic isoprenoid pathway such that good quantities of phytoene are accumulated in the endosperm. On this basis it will, hopefully, be possible to complete the pathway towards provitamin A by adding genes for phytoene desaturase, ζ-carotene desaturase and, if necessary, lycopene cyclase.

## 6. Approach towards fungal disease resistance

Rice blast (*Magnophorthe grisea*) and sheath blight (*Rhizoctonia solani*) are fungal diseases of rice that cause significant yield losses (Reissig et al., 1986; Toenniessen, 1991). It has been estimated that important productivity gains could be possible if these challenges — in the case of rice blast particularly in association with upland drought — would be overcome (Herdt, 1991). In addition, conventional approaches to improve these traits in rice have been scored as ineffective even with substantial research (Herdt, 1991).

Multiple natural host response mechanism, including the accumulation of defensive enzymes (e.g. chitinases, β-1,3-glucanases, etc.) are involved in plant resistance to phytopathogenic fungi (Boller, 1988). Chitinase preparations, expecially in combination with β-1,3-glucanases, inhibit fungal growth *in vitro* (Mauch et al., 1988; Arlorio et al., 1992; Sela-Buurlage et al., 1993). Chitinases have also been shown to accumulate around invading hyphae *in planta* (Benhamou et al., 1990; Collinge et al., 1993). Transgenic approaches based on the constitutive expression of a bean endochitinase gene in tobacco (Broglie et al., 1991) and canola (Benhamou et al., 1993), or based on the wound-inducible expression of a barley seed ribosome inactivating protein (RIP) in tobacco (Logemann et al., 1992) have been reported to lead to encreased protection over *Thizoctonia solani*. In addition, osmotin-like proteins inducible by osmotic stress, such as tobacco AP24 (Melchers et al., 1993), have been shown to be pathogen-induced proteins with inhibitory activity toward fungal pathogens (Woloshuk et al., 1991). The development of tene transfer systems for Indica and Japonica rice opened up possibilities for testing the effects of expression of these candidate anti-fungal (and stress-response) genes as strategies to overcome sheath blight and upland drought/blast constraints. Putative transgenic rice clones are under selection for: a) barley RIP cDNA under control of the inducible rice chitinase promoter RCH10 (Zhu et al., 1993), and b) β-1,3-glucanase and chitinase driven by the Ti-1'2' dual promoter (collaboration J. Mundy, Copenhagen, Denmark). In addition, research aimed at expressing in transgenic rice plants: a) tobacco osmotin-like AP24, b) bean endochitinase, and c) tobacco β-1,3-glucanase, individually and in a concerted manner, has recently been initiated.

All these genes have been, or will be combined with different promoters

and target sequences. Numerous transgenic plants have been regenerated and seeds harvested for infection analysis. The first success we can report upon is with a rice chitnase gene regulated by the constitutive 35S promoter from CaMV. Infection assays with mature plants performed in the containment greenhouse of IRRI revealed an increased tolerance to sheath blirght (*Rhizoctonia solani*) (Lin et al., 1995).

## 7. Concluding remarks

The goal of our scientific work is to contribute to future sustained production of affordable and high quality food in developing countries. Fungal pests destroy in the range between 20-40 million metric tons of rice harvest per year. Sheath blight resistance may have the potential to safe 10-20 million tons. Insect pests are responsible for the loss of ca 10-20 million metric tons. Yellow stem borer is the major pest for which no resistance excists. The engineered resistance provided by our group may contribute to safe around 5 million tons of rice. If our anti-tungro virus strategies are successful, this will be the basis for another 5 million tons of rice, not lost to pests. We tried to organise this step out of the ivory tower of pure science into application in such a way it will not end in an academic exercise. We tried to make sure to work on problems which are a heavy burden on a great number of poor people and we are trying to organise our science in such a way that it complements traditional plant breeding. We can reach our goal only, if the novel characters we introduce into Indica rice will be used in breeding programmes; this is guaranteed through our collaboration with IRRI. The novel characters will be successful only in breeding if they are stable and effective. This requires that we can provide the breeders with a collection of many transgenic plants for every novel character to select the best possible case for his breeding programme. This in turn requires more efficient gene transfer protocols than those available to date.

Success or failure of our goal will, however, not only depend on success or failure of our experiments and subsequent breeding programmes. It will also depend on political, social and psychological circumstances in those countries in which the novel, genetically engineered varieties are supposed to help solving problems. Risk assessment will be an integral part of the projects, however, the judgement of scientists and national biosafety committees on the security of transgenic plants or food gained from transgenic plants will not necessarily lead to an acceptance of these plants or food by the local population. If we were e.g. able to recover a transgenic rice which accumulates sufficient provitamin A to stop vitamin A deficiencies, there is no guarantee, that people would be willing to eat this rice. Therefore, there is much educational and political work ahead of us in addition to what we are trying to achieve.

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## Transgenic Indica rice to the benefit of Less Developed Countries: Towards pest-resistance, and accumulation of β-carotene in the endosperm

Summary

Indica-type rice provides the staple food for 2 billion people in Third World Countries. We have established gene transfer to IRRI breeding lines to explore the contributions of genetic engineering to sustained and stable production of high quality food. Experiments are in progress on the development of resistance towards Yellow Stem Borer, towards Rice Tungro Virus, towards fungal pests, and towards accumulation of provitamin A in the endosperm. So far we have recovered the first transgenic Indica rice with elevated resistance to sheath blight (*Rhizoctonia solani*) and with 100% resistance against yellow stem borer (*Scirpophaga incertulas*). We have also harvested seed from a series of transgenic rice plants harbouring a number of different transgenic DNA sequences representing different anti tungro virus disease strategies. And we have, so far, been able to initiated the β-carotene pathway in the endosperm to the accumulation of phytogene, hoping, that it will be possible to subsequently complete the pathway towards provitamin A.

**Key words:**
Oryza sativa, Indica-type rice, genetic engineering, vitamin A endosperm, insect-resistance, virus-resistance, fungus-resistance.

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