Internat. J. agric. Sci. Vol.2 No.2 July 2006 : (429-432)

Genetic engineering of indica rice in support of sustained production of affordable and high quality food

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ABSTRACT
Genetic upgradation of indica rice is one of the major areas of potential usefulness. Conventional plant breeding programmes have contributed substantially to the improvement of rice, the world over. However, sexual and genetic incompatibility barriers limit conventional breeding techniques. In recent years, there has been a little shift of emphasis in research of crops plants from conventional breeding to genetic engineering under sustainable conditions. To achieve maximum benefit, the application of gene technology should focus on high priority problems for which solutions by conventional approaches are not available.

Key words : Rice, Production, Genetic engineering.

INTRODUCTION

The present world population of 6.1 billion is continually increasing and will probably double by the year 2030 (FAO, 2004). A considerable portion of this increase is likely to occur in the rice consuming natures of Asia. With the ever increasing pressure on arable land due to urban development, more and more food has to be produced from less and less land. Increasing productivity per unit area and time, therefore, remains the challenging task. Furthermore, considering the serious problems of soil health and environment, increased productivity must be accomplished on a sustainable basis. Genetic upgradation of our crop plants is one of the major areas of potential usefulness. Conventional plant breeding has contributed substantially to the crop improvement programme, the world over. However, conventional plant breeding techniques are limited by sexual and genetic incompatibility barriers.

During the recent past, modern biology has undergone significant development specially in the areas of cell and molecular biology. It has been possible to raise plants from single cells, isolated pollens and protoplasts. It has been possible to isolate genes from bacteria and such unrelated species and genera, and their to a wide array of plant species. It is widely recognized that genetic engineering can significantly strengthen plant breeding programmes and help to produce new varieties with desired agronomic traits. It enables plant breeders to achieve results more quickly and efficiently. Biotechnology is taking us from an era of hybrid plants to an era of transgenic plants and we are on the way from a "Green Revolution to a Gene Revolution". Indica-type rice (*Oryza sativa*) feeds more than two and half billion people, predominantly in developing countries followed by Japonica and Javanica types in humid and semi-humid Asia, where rice is the basic food. Rice productivity will have to be increased with considerable reduction in the input of agrochemicals under sustainable conditions (IRRI, 2003). This immerise task requires that traditional plant breeding is supported by every possible contribution from novel technical development. Genetic engineering, applied appropriately and with care, has the potential to contribute to the sustainable production of affordable food for the increasing population. To achieve maximum benefit, the application of gene technology should focus on important problems for which solutions by conventional approaches are not available and indica-type rice has been identified to suffer from such problems. Among the high priority problems to be solved are a) resistance to fungal diseases, b) resistance to Tungro virus, c) resistance to yellow stem borer (Scripophage incentulas), d) stable supply of pro-vitamin A, and e) improvement of nutritional quality. The problems mentioned are especially sever for people depending on Indica-type rice. It is, relatively easy to genetically engineer Japonica-type rice. Methods have been established for gene transfer to Indica rice breeding lines to study possible contributions from genetic engineering.

Methods for introducing genes into indica rice

Early attempts on genetic transformation to obtain transgenic plants focused mainly on the development of efficient and reproductible methods for transformation of major crop plants. However, successful methods are still Ilimited to Agrobacterium-mediated transformation system, direct gene transfer methods with plant protoplasts and biolistic method.

i) Agrobacterium tumefaciens –mediated gene transfer to dicots :

Agrobacterium tumefaciens is the etiological agent of crown gall diseases and produces crown gall tumors on more than one hundred susceptible plant species which commonly have a wound response. To Agrobacterium virulent strains of *Agrobacterium* contain large Ti plasminds, which are responsible for T-DNA transfer and subsequent isease symptoms. Ti plasmids have two sets of sequences necessary for gene transfer to plants. One is the T-DNA region which is flanked by 25-bp direct repeat border sequences responsible for gene (DNA) transfer to plants and other set is comprised of the virulence (Vir) gene sequences. The latter are not transferred during infection but aid excision of T-DNA from Ti plasmid, transfer to an infected plant cell, and insertion into the plant genome.

The Ti plasmid of *Agrobacterium fumefaciens* has been genetically engineered to create "disarmed" plasmids or vectors which can carry any DNA sequence of interest into an infected plant, without tumerous growth of the host plant. Ti plasmid derived cloning vectors are capable of replication in *Eacherichis coil* as well as in *Agrobacterium,* allowing for convenient manipulations. The vector is then transferred to an *Agrobacterium tumefeciens* host strain which is "disarmed" but still carrier the virulence functions necessary for infection of plant tissue. The final *Agrobacterium tumefaciens* strain crying the DNA of interest is grown in a overnight culture of use in co-cultivation experiments the very next day. Two selectable marker genes are usually carried on the plasmid, one for selection in bacteria and the other in plants. Introduction of foreign genes, into plants tissues by A. *tumefsciens* co-cultivation is at present the most widely used method for the study of plant gene expression I dicots.

Plants viruses as vecter for DNA (gene) transfer :

Techniques are being developed to use certain DNA and RNA plant viruses, e.g. caulimo viruses, Gemini viruses etc. The culiflower mosaic virus (C_aMV; a caulmo virus) is a DNA virus. A DNA segment

may be integrated into the viral DNA, which is suitably modified to serve as a vector, and the host plants may than be infected with the especially contributed virus following infection, the virus spreads systemically into the host plant, plant cells usually have a high copy number of the virus. Therefore, each cell will also have a very high copy number of the transferred gene.

Direct gene transfer to monocots through plant protoplast

To overcome the limitation posed by the *A tumefactions mediated* transformation system, a variety of DNA delivery methods have been developed for the transformation of menocote like rice, corn and wheat. Transformation has been achieved in plant protoplasts through facilitation of DNA uptake by polyethylene glycel (PEG) treatment, electroporation or a combination of these methods (Datta *et al.* 1992).

Significant advance have been made during the past decade in plant regeneration from plant protoplasts of monocots. This process has not only allowed realization of several somatic cell hybrids, but also made it possible to transfer foreign genes into plants that are not amenable to *A tumefactions* system. Plants protoplasts are isolated single cells, stipped of their cell wall by enzymatic treatment, thus facilitating the transfer of foreign genes through the plasmamenbrane. The other advantage is that all derived cell of a transformed protoplast will contain the foreign genes of interest, thus the regenerated transgenic plants will have a uniform genetic makeup. The capability of protoplasts for cell division, proliferation and plant regeneration is referred to as competence, such as heat shack (Abdullah *et al.* 1986), low dose irradiation etc. when competent protoplasts with high viability are available methods such as PEG treatment or electroperation can be used to transfer genes.

(a) PEG – medicated gene transfer :

PEG acts by increasing the permeability of cell memberane memberanes, and it has been identified as an efficient agent for protoplast fusion in somatic cell hybridization studies involving various plants species. PEG-mediated direct transformation of protoplasts is relatively simple various parameters important for gene transfer with PEG have been identified. For example, the optimal conc. Of PEG is 15-20%. If the PEG conc. Is too high, cell viability will decrease, if too low, the gene transfer efficiency will decrease.

(b) Electropration-mediated gene transfer :

The electroperation method is based on the use of short electrical pulses of high field strength to increase the permeability of protoplast membranes to facilitate DNA uptake. This method was successfully used by several investigation to introduce DNA into rice protoplasts (Toriyama *et al.* 1988). Shimamoto *et al.* (1989) used this method to introduce a bacterial HPH gene, encoding hygromycin B-resistance into rice protoplasts and developed 14 transgenic plants.

Biolistic gene transfer

The biolistic process (also known as the particle gun bombardment method) was invented by John Sangord and his colleagues at Cornell University. The term 'biolistic' (biological bollistics) was coined to described the nature of the delivery of foreign DNA into living cells or tissues through bombardment with a biolistic device. The process involves high velocity acceleration of micro-projectiles carrying foreign carrying foreign DNA to penetrate the cell wall and membrane, and to deliver the DNA inside plant cell. The invention of the biolistic process provides an important additional alternative means for transforming higher plants those not amenable to DNA system.

After explants are transformed with suitable vectors having gene of interest, we need to select the transformed cells/tissues. This is facilitated by the presence of selectable marker genes available in the vector. Two kinds of marker genes play a crucial role in plant genetic transformation one, consists of the selectable markers which make it possible to select transformed cells, tissues or whole plants under conditions which prevent the growth of untransformed tissue e.g. Nemycin hosphotransferase (npt. II). Hygromycin phosphotransferase etc. The other class of marker genes comprises of the assayable markers commonly referred to as reporter genes e.g. B-glucuronidase (GUS), Luciferase (LUS) etc. the selectable marker genes enable the transformed cell to survive in media containing toxic levels of the selection agent, which is usually an antibiotic or a herbicide. Any cells, which are into transformed, die due to the presence of antibiotic or herbicide. Other antibiotics and herbicides may require more judicious use, since even low conc. Can cause rapid cell death. In some cases, selection is exercised only after the regeneration is achieved, because adventious root formation is sensitive to antibiotics.

Molecular analysis of transgenic plants

Antibiotic primary regenerants should be analysed by the method of Southern blot analysis (Southern, 1975). Hybridisation with a radioactively labeled probe specific for the protein coding region of the antibiotic/herebicide gene demonstrate integration of this gene into the genome. Enzyme essay helps in the identification of that the integrated foreign gene is functional. Resistance to any antibiotic/ herbicide is based on inactivation of the antibiotic. Then it will observe that the plant not only carry the transgence but also express it.

Breeding with transgenes required independent populations of fertile, transgenic plants to chose the most stable and best expressing lines for subsequent traditional breeding. Although recovery of transgenic indica-type rice plants and their offsprings has been deseribed (Datta *et al.* 1990), gene transfer to IRRI breeding lines is not yet routine and efficient, and recovery of fertile, transgenic plants is still also rather inefficient and requires further optimization.

Possible contributions from genetic engineering

1) Approach towards yellow stem borer resistance :

Insect damage is one of the major causes of yield loss in rice farming all over the world. In Southest Asia, the value of lost production caused by insect damage reaches more than 600 million dollars per year. More than two thirds of these losses are caused by two insect species, the rice brown plant hoper (*Delphax oryzeos*), order Homopterra and yellow stem borer (*Scirpophage incerpulas*). Brown plant hopper for which resistance genes are known in the gene pool, no such gene are available for resistance genes are known in the gene pool, no such gene are available for resistance against yellow stem borer.

The entomocidal spore forming, soil bacterium *Racillus thuringiensis* offers a promising range of genes which encodes specific endotoxin. To date, more than 50 different nucleotide sequences of such gene have been determined. They are clearly related to each other, and have been classified into 17 distinctly different crystal protein genes, the so-called cry genes (Perferoen, 1991). These genes encode proteins which are first dissolve and then proteolytically cleaved in the midgut of the larvae of lepidepteran insects to toxic fragments. Tehse toxins kind to specific protein memberance of the insect gut, resulting in disruption of the epithelium and thereby causes paralysis.

Different Bt 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 Bt-genes in the rice plant itself.

The transformation of advanced indica rice breeding lines with gene confirming resistance to yellow stem borer. The use of specific cry 1A (b)-gene, which has been shown to be effective against yellow stem borer and rice leaf folder Resistance clones were analysed by Southern Blotting and showed a clear integration of the cry IA (b) – gene in the rice genome. Plants were regenerated transferred to soil and are growing under green house conditions. To minimize the possible development of resistance in insects, we put this gene under the control of a tissue specific promoter which directs the expressions of the cry 1A(b)-gene only to the leaf sheath, the primary target site of the yellow stem borer.

It has to be emphasized that such transgenic rice plants should be planted under field conditions only, according to the concept of Integrate Pest Management (IPM), to keep the Bt-toxins as an effective, sustainable and durable tool (Mc Gaughey *et al.* 1972).

Rice transformation and expression of the modified Cry 1A(b) gene in transgenic rice plant

Funjimoto et al. (1993) introduced the chemeric cry 1A(b) into embryogenic rice protoplasts by co-transformation with hygromycin resistance genes. A large number of hygromycin resistant calli were obtained and 623 out of 1327 contained the sequence of the cry 1A (b) gene. DNA gel plot analysis of the cry 1A(b) containing calli revealed one to several copies of the expected fragment with a few cases of rearranged copies. A total of 51 fertile plants were regenerated from 26 independent callus lines. Ten transgenic plants were selected and their selfed R_1 and R_2 progeny seed obtained.

Examine expression of the cry 1A(b) gene in transgenic rice plants poly (A) RN As isolated from laves of R_1 and R_2 generation transgenic plants were hybridized with the ciding region of the modified cry 1 A(b) gene. The results indicated that high levels of cry 1A(b) transcripts were present in leaves or transgenic rice plants, and no degradation of the m RNA was detected.

Inhertance of the cry 1A(b) gene in transgenic rice plants

Inhitance of the introduced cry 1 A(b) gene from R0 transgenic plants to the next R1 generation was examined by PCR analysis of selfed progeny derived from transgenic lines. The lines examined exhibited a ratio close to 3:1, suggesting that cry 1A(b) gene was integrated in a single chromosome and stably inherited.

Approach towards fungal disease resistance

Rice blast (*Magnophorthe grisea)* and sheath blight (*Rhizoctonia solani)* are fungal diseases of rice that cause significant losses. Important productivity gains would be possible if these problems may be solved in the case of rice blast. The development of gene transfer system for indica and japenica rice opens up possibilities for treating the effects of expression these candidate antifungal genes as strategies to overcome sheath blight and rice blast constraints. Multiple natural host response mechanisms, including the accumulation of defensive enzymes (e.g. chitinoses, B-1, 3-glucanases, etc.) are involved in plant resistance to phytopathogenic fungi (Boller, 1988). Chitnase preparations, especially in combination with B-1, 3-glucanases inhibit fungal growth *in vitro.*

Transformation and regeneration of various rice varieties has been reported by a number of researchers in the last few years and, marker or reporter genes such as npt, hpn, gus A and bar were used in these experiments.

Stilbene synthase is an enzyume found in a few plant species which synthesis the phytoalexin trans-reservetol using substrates commonly present in plants trans-resvertrol. Seems to have the potential for playing role in the early protection of plants gainst fungal pathogens. Stark-Lorenzen *et al.* (1997) demonstrated the transcription of the grapevine stilbane synthese gene in the R2 progeny of transgenic rice after biotic and biotic stresses and present first results which indicate enhanced resistance of transgenic rice against the rice pathogens *Phyricularia oryzae.* Transcription of stilbene-synthase-specific mRNA was investigated in young leaves of plants of the R2 generation. Formation and accumulation of stilbene-synthase-specific mRNA could be detected in response to inoculation of leaves with the pathogen of the rice blast disease.

The strongest response, however, was achieved after inoculation with P. oryzae and first clear signal of stilbene synthase-specific mRNA already accrued just 1 hr. after inoculation. The transcription of stilbene synthase mRNA after pathogen induction was transient and increased rapidly for at least 5 hours followed by a decrease. A repeated slight increase occurred after 48 hrs. and stilbene synthase mRNA synthesis disappeared completely 72 hrs. inoculation.

Approach towards herbicide-resistant indices

Rathore *et al.* (1993) used the *bar* gene in combination with the herbicide-resistant rice plants. Protoplants, obtained from regenerable suspension cultures established from immature embryo callus, were transformed using PEG-mediated DNA uptake transferred calli could be selected 2-4 Weeks after placing the protoplast-derived calli on medium containing the selective agent, phosphinathricin (PPT), the active component of Basata. Calli resistant to PPT were capable of regenerating plants. Phosphinathricin acetyltransferase (PAT) essay confirmed the expression of the bar gene in plants obtained from PPT – resistant calli. The only exceptions were two plants obtained from the same callus that had multiple copies of the bar gene integrated into their genomes. The transgenic status of the plants was verified by Southern blot analysis. The efficiency of co-transformation with a reporter gene gusA, was 30%. The plants were self-fertile. Both the *bar* and *gus*A genes were transmitted to progeny as confirmed by Southern analysis.

Similarly, Datta *et al.* (1992) transformed the commercially important Indica rice cultivar 1 R72 using direct gene transfer to protoplasts PEG-mediated transformation was done with two plasmid constructs containing either CaMv-355 promoter/HPH chimaeric gene conferring resistance to hygromycin (Hg) or CaMv 355 promoter/BAR chimaeric gene conferring resistance to a commercial herbicide (Basta) containing phosphinothricin (PPT). Data from Southern analysis and enzyme assays proved that the transgene was stably integrated into the host genome and expressed. Transgenic plants showed complete resistance to high doses of the commercial formulation of PPT.

Approach towards tungro virus resistance

The rice tungro disease is caused by a complex of two viruses, rice fungro spherical virus (RTSV) and rice fungro baclliform virus (RTBV). It is, however, to be expected that for RTBV, expression of functional viral proteins at the onset of virus infection could interfere with an ordered progression through the viral life cycle, and that expression of mulated viral proteins by competition. We have therefore introduced into indica rice breeding lines (IR43 IR72) constructs designed to express RTBV proteins 1,3 and 4 Protein 3 is a precursor from which the viral coat protein, reverse transenptase and probably a variety of other proteins are generated by proteolytic processing.

It has been reported that introduction of CP(Coat-protein) gene is a promising approach for introducing viral resistance in cereals. This strategy in applicable to other viruses, such as tungro virus, which is causing serve damage to indica rice in several Asian countries.

Coet-protein-mediated (CP-moderated) protein against virus disease has been applied to control rice stripe virus (RSV). RSV is transmitted by small brown plants hoppers and causes serious damage to rice. The CP expression vector used in the experiment consisted of the 355 promoter, the first intron of *Cat,* coding sequence of the CP gene, and the polyadenylation site from the nopaline synthese gene. The amount of the CP produce in the rice leaves was estimated to be to 0.5% of the total soluble protein. In the essay for viral resistance, transgenic plants expressing CP did not exhibit disease symptoms, whereas the non-transferred control plants and the transformed plants not expressing the CP showed clear disease symptoms, indicating, that the resistance to RSV depended on the expression of the introduced, CP gene. The CP gene was stably transmitted to the progency of primary transgenic plants.

Approach towards pro vitamin A accumulation in rice endosperm

According to UNICEF statistics world-wide, over 124 million children an estimated to Vitamin A- deficient, improved vitamin A nutrition could present approximately 1-2 million deaths annually among children aged 1-4 years. Rice in its milled form, as it is consumed by most people in South East Asia, is characterised by the complete absence of pro-vitamin A. The million rice kernel consists exclusively of the endosperm. The embryo and the aleurone layer have been

removed during processing of the rice grain. The main objective of this study is to initiate carotenoid biosynthesis in the rice endosperm tissue to increase the daily vitamin A uptake of people predominantlyliving on rice. To provide the minimum requirements relevant carotenoids to young infants, and assuming rice as the role dietary source, 1-2 ug B-carotene per gram uncooked rice would be need in rice ecdosperm.

The carotenoid pathway is a branch of the central isoprenoid pathway which is characterized by four key enzymes necessary for carotenoid biosynthesis. Thesis are phytoene synthase, phytocene desaturase, carotene desturase and lycopene cylclase. The genes encoding for these enzyme are available from both higher plants (Fray & Greison, 1993) Furthermore scientists went to develop a trasient expression system which allow us to assess the function and activity of genes and pronotase which we will use for transformation. The first construct to be tested will be phytoene synthese under the control of a C_aMV 358 promoter and two different rice glutelin promoters (Dkita *et al.* 1989). Today success has been made towards the development of some transgenic breeding lines which are rich in vitamin A.

Approach towards improvement of nutritional quality

Milled rice not only lacks vitamins, it is also deficient is essential amino acids such as cysteine, methionine and lysine. In higher plants, lysine is synthesized from aspartate via the aspartate biosynthetic pathway (Bryan, 1980) which also leads to the synthesis of methionine and threonine. The biosynthesis of lysine in plants is regulated by several feedback loops. Di hydre-dipicolinate synthease (DHPS) from *E. coil* is far less sensitive to feed-back inhibition than is DHPS from higher plants To study whether expression of the feed-back inhibition than is DHPS from higher plants. To study whether expression of the feed-back insensitive enzyme in rice endosperm might lead to an increase in free lysine, we have focused endosperm specific promoters from rice (Okite *et al.* 1989) to the *E. Coil deep* A gene conding to DHPS. In addition an approach aimed at increasing the content of the essential sulphur containing amino acids cysteine and methionine, in milled rice will be undertaken.

CONCLUSIONS

To achieve the goal of food security for all by 2025, productivity of rice which is the staple food for 1/3rd of world population, need much more efforts. Genetic engineering offers the opportunity for the same where conventional approaches are not possible. So far, many transgenic rice plants have been developed but their commerlization is are probably due to constraits posed by the registration of these plants and other environmental safely considerations. Transgenic plants in creats other than rice are available in the World market, so, we can hope the commercialization of transgenic rice in near future. Potential seems to be more promising if these genetic engineering approaches are supplemented with the conventional breeding methods.

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Received : August, 2005; Accepted : March, 2006