

Potential Benefit of Genetic Engineering in Plant Breeding: Rice, a Case Study

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This paper summarizes recent developments in the field of molecular biology and its application to plant breeding, particularly in rice. Plant breeding in the past mostly depended on the time-consuming crossing of known genomes limited to certain traits. Plant breeding has now benefited from marker-assisted selection and genetic engineering to widen the gene pool, improve plant protection, and increase yield. Future plant breeding will expand based on functional and nutritional genomics, in which gene discovery and high-throughput transformation will accelerate crop design and benefits will accrue to human health, in the form of nutritional food for poor people to reduce malnutrition, or food enriched with antioxidants and with high food value for rich people. Agricultural biotechnology for food is no longer a dream but a reality that will dominate the 21st century for agriculture and human welfare.

Key words: *nutritional genomics, transgenic rice, intellectual property rights.*

The phenotyping of plant genome and the isolation and characterization of genes in conjunction with genetic engineering are envisaged as improving crops for food, feed, and nutrition. Rice is the world's most important food crop. It feeds nearly 2.5 billion people. The current world population of 6 billion is likely to reach 7.5 billion by 2020 and the rice-producing sector will have to produce 40-50% more rice to satisfy the growing demand of the ever-increasing population. Transgenic rice developed by genetic engineering has enormous potential to help produce affordable food for the increasing world population, particularly in developing countries. Tools for gene isolation and characterization and gene technology such as optimization of vector construction and transformation is available. Microarray biochip technology will accelerate the unraveling of functional genomics to move on to the new era of biotech-based plant breeding. I will emphasize here a few such possibilities, which have also been summarized in some other recent articles.¹⁻⁸⁾

Genomics and Rice

Traditionally, structural studies have come near the end of the biological discovery process. Genomics now implies DNA sequencing, the routine use of DNA microarray technology to analyze the gene expression profile at the

mRNA level, and improved informatic tools to organize and analyze such data. Genomics-based strategies for gene discovery, coupled with the high-throughput transformation process, have accelerated the identification of candidate genes. Cultivated rice has evolved through domestication for centuries, which has allowed limited diversity of genetic resources. It is now perceived that many wild species may provide genes that could substantially increase yield.⁹⁾ It is important to characterize wild species and use the genes of interest rather than putting them in the gene bank only. International efforts are in place to sequence the genome from humans to rice.¹⁰⁾

The recent rice genome sequences developed by Monsanto will accelerate gene discovery and crop improvement.^{11,12)} Insertional mutagenesis is now an attractive method for functional analysis by screening the T-DNA insertion in a known gene and recovering sequences flanking the insertions¹³⁾ in addition to transposon tagging using the *Ac/Ds* system.¹⁴⁾

We must address the following questions:

- 1) How do we reorganize rice breeding in the genomic Era?
- 2) How do the international rice genome sequencing project (IRGSP) and Monsanto rice genome help us in such an endeavor?
- 3) How do we present such a knowledge-based intensive technology and obtain public confidence in it?
- 4) How do we convince policymakers of national governments to take advantage of the combined green and gene revolution^{15,16)} to reach most people who depend on agricultural land and all kinds of rice farmers the poor and the rich?

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Abbreviations: ADPGPP, ADP-glucose pyrophosphorylase; ECS, embryogenic cell suspension; EPSP, 5-enolpyruvyl shikimate 3-phosphate, GM, genetically modified; M-6-P, IPR, intellectual property rights; mannose-6-phosphate; PAT, phosphinothricin-N-acetyltransferase; PMI, phosphomannose isomerase; PR, pathogenesis related.

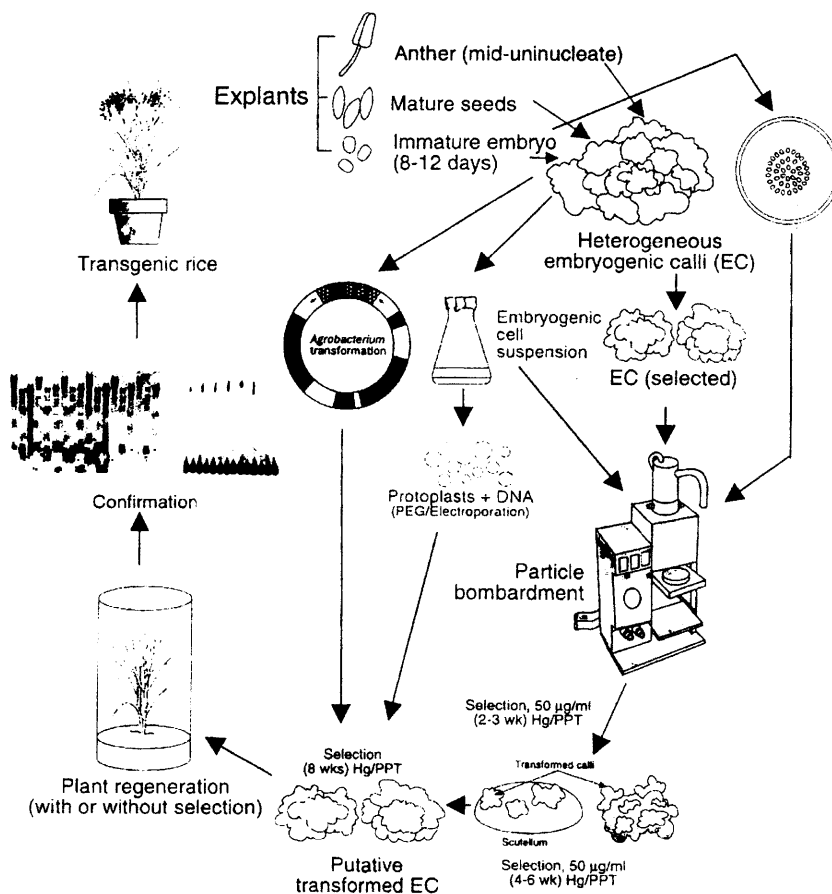


Fig. 1. A schematic protocol for production of transgenic rice plants using biolistic, protoplast, and *Agrobacterium* systems.

This task poses many challenges and rewards for human welfare.

Gene Technology

Major crops such as rice, wheat, maize, cotton, soybean, sunflower, barley, and canola have been transformed using various gene transfer technologies. Methods of transformation that have been used are microinjection, macroinjection, laser beam techniques, pollen tube pathway, dry seed imbibition, and cell/tissue electroporation¹⁷⁾. However, the best reproducible and most unambiguous results have been obtained from the protoplast, biolistic, and *Agrobacterium* methods (Fig. 1). Comparative studies with these systems are also available.^{18,19)}

Protoplast System

The first report of plant regeneration from protoplasts of gramineous species²⁰⁾ opened new possibilities for genetic transformation in cereals such as in japonica rice^{21,22)} and indica rice.²³⁻²⁵⁾ The single-cell origin, non-chimeric nature, and genetic fidelity of plants derived from somatic embryos are very attractive features for any transformation system.

Early success was based on the following:

- 1) Establishment of embryogenic cell suspension (ECS), the key source of regenerable protoplasts.²⁶⁾
- 2) Use of suitable plant transformation vectors with selectable marker genes.
- 3) Continuous efforts to improve tissue culture protocols by different laboratories, e.g., use of 2,4-D, maltose, nurse culture, PEG-MW6000, osmotic adjustment, etc.

Biolistic System

Microprojectile bombardment of immature embryos with high-velocity metal particles to deliver the biologically active DNA into plant cells and recovery of the whole plants from the transformed cells through selection is becoming almost a routine. Genotype-independent transformation may be carried out with the method as there is no biological limitation to DNA delivery. Japonica and indica rice cultivars have been transformed with this system.^{1,27-31)}

Agrobacterium System

In nature, the Gram-negative soil bacterium *Agrobacterium tumefaciens* causes crown gall formation in dicots by a multi-step transformation process. At present, *Agro*-transformation has been extensively used for both dicot and

monocot species.³²⁾ During *Agro*-infection, a small segment of one extra chromosomal plasmid Ti (tumor inducing), the T-DNA, surrounded by a 24-bp border repeat, is transferred to the plant cell and integrated into the host chromosome in the nucleus. The T-DNA transfer system is determined by the *vir* genes, whereas the flanking 24-bp direct repeats are essential as recognition signals for the transfer apparatus. For plant transformation, wild-type T-DNA was removed to create disarmed strains such as LBA4404³³⁾ and C58C1.³⁴⁾ Various genes, including selectable marker genes and genes of interest, can be placed in the vector and introduced into the disarmed strains. The binary vector system consists of a plasmid providing the virulence functions needed for transfer and a small vector carrying an artificial T-DNA. The binary vectors replicate in *Escherichia coli* as well as in *A. tumefaciens* and allow easy cloning of genes of interest between the T-DNA borders. Besides the vectors pBin19,³⁵⁾ pGA482,³⁶⁾ and pB1121³⁷⁾ usually used in dicots, a new binary vector, pGA1611, for rice has been constructed.³⁸⁾ This vector contains the unique *Hind*III, *Sac*I, *Hpa*I, and *Kpn*I for cloning of a gene between the maize *Ubi* promoter and *nos* terminator.

Chloroplast Transformation

In some crops, such as strawberry and sunflower, gene flow or outcrossing is very common and causes serious concern to environmentalists. Maternal inheritance of foreign genes through chloroplast genetic engineering is highly desirable for those crops in which potential outcrossing is possible.

This technology is emerging as an alternative to overcome concerns regarding nuclear genetic engineering.^{39,41)} However, whether this system works for cereals remains to be studied.

Defense Genes and Plant Protection

A large number of PR protein genes are now reported⁴²⁾ along with a few resistance genes.^{43,44)} It is still a challenge to engineer rice against fungal pathogens, particularly sheath blight and blast. Considerable progress has been made in recent years in understanding host-pathogen interactions and the coordinated functioning of plant defense mechanisms against pathogenic fungi.^{45,46)} Transgenic rice with several PR-genes (*Chi*11, *RC*7, and *tlp*5) has been developed and enhanced resistance against the fungi *Rhizoctonia solani* and *Pyricularia oryzae* that cause sheath blight and blast disease, respectively has been reported.^{29,42,47-52)}

Table 1. Development and use of transgenic rice with agronomically important genes.

Rice	Method Used	Genes Transferred	Traits
Indica (IR72)	Protoplast (PEG)	<i>bar</i> * ⁸	Resistant to herbicide ²⁴⁾
Japonica	Protoplast (electroporation) virus	cp-stripe	Resistant to stripe virus ⁵³⁾
Japonica	Protoplast (electroporation)	<i>Bt</i>	Resistant to insects ⁵⁴⁾
Japonica	<i>Agrobacterium</i>	<i>Bt</i>	YSB & SSB ⁵⁵⁾
Indica	Protoplast (PEG)	<i>chi</i> 11	Resistant to sheath blight ⁴⁷⁾
Japonica	Protoplast	cc	Resistant to insects ⁵⁶⁾
Japonica	Biolistic	<i>Xa</i> -21	Resistant to bacterial blight ⁵⁷⁾
Indica	Protoplast and biolistic	<i>Bt</i> (<i>DWR</i>)	Resistant to stem borer ⁵⁸⁾
Japonica	Biolistic and protoplast	<i>pin</i> II	Resistant to insects ⁵⁹⁾
Indica	Biolistic	<i>Bt</i>	Resistant to stem borer ⁶⁰⁾
Indica/Japonica	Biolistic and protoplast	early nodulin	Biological N ₂ fixation ⁶¹⁾
Indica/Japonica	Biolistic and protoplast	early nodulin	Biological N ₂ fixation ⁶²⁾
Indica	Biolistic	<i>Bt</i>	Resistant to stem borer ⁶³⁾
Indica/Japonica	Biolistic and protoplast	<i>Bt</i> (tissue-specific)	Resistant to stem borer ²⁹⁾
Indica (IR72)	Biolistic	<i>Xa</i> -21	Resistant to bacterial blight ⁶⁴⁾
Indica	Biolistic	<i>Bt</i> ML for hybrid rice	Resistant to stem borer ⁶⁵⁾
Indica	Biolistic	<i>Bt</i> restorer for hybrid rice	Resistant to stem borer/leaffolder field evaluated ⁶⁶⁾
Indica	Biolistic	PR genes	Resistant to sheath blight ^{42,48)}
Japonica	<i>Agrobacterium</i>	Ferritin	High iron storage ⁶⁷⁾
Japonica	<i>Agrobacterium</i>	<i>pepc</i>	Higher photosynthesis ⁶⁸⁾
Indica	Biolistic	viral replicase CP1, CP2, CP3	Resistance to RTSV and RTBV ⁶⁹⁾
Indica	Biolistic	PR-genes	Resistance to sheath blight ⁵²⁾
Indica	<i>Agrobacterium</i>	PR-genes	Resistance to sheath blight ⁴⁹⁾
Indica	Biolistic	PR-genes	Resistance to sheath blight ⁵⁰⁾
Japonica	<i>Agrobacterium</i>	<i>psy</i> , <i>lyc</i> , <i>crt</i> I	β-carotene synthesis ⁷⁰⁾
Japonica	Biolistic	<i>adh</i> , <i>pd</i> c	Submergence tolerance ⁷¹⁾
Japonica	Biolistic	waxy gene	Amylose content ⁷²⁾

*Used as selectable marker gene; ML = maintainer line; DWR = deepwater rice; YSB = yellow stem borer; SSB = striped stem borer.

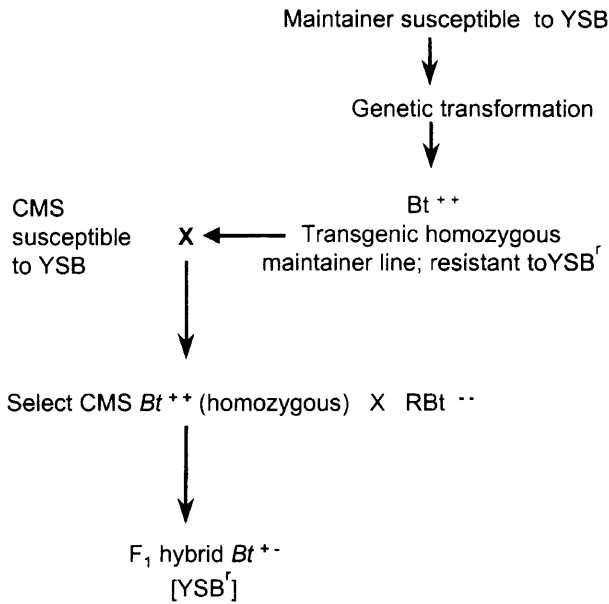


Fig. 2. Schematic figure showing use of a transgenic breeding approach for developing yellow stem borer resistant (YSB) rice hybrids. CMS = cytoplasmic male sterility.

Most transgenic crops that are grown on a production scale and are commercially available are engineered with insect and herbicide resistance (Table 1). *Bt* rice has shown great promise and potential for environment-friendly plants that can be grown in the field without application of costly pesticides. *Bt* maize and cotton have now been extensively planted in many countries including the USA, Europe, and Asia.^{73,74} A hybrid commercial *Bt* rice, Shan You 63, has now been grown in China on a large scale. It has shown resistance to two insect pests, leaffolder and stem borer,⁶⁶ based on a concept published earlier (Fig. 2).⁶⁵ *Xa21* rice (transgenic IR72) has been developed with enhanced resistance against bacterial blight (BB) disease caused by *Xanthomonas oryzae* pv. *oryzae*.⁶⁴ A dominant gene for resistance to BB, designated as *Xa21*, was transferred conventionally from the wild species *O. longistaminata* to the cultivated IR24.⁷⁵ The resulting line, IRBB21, confers resistance to all the known races of *Xoo* in India and the Philippines.⁷⁶ *Xa21* supports a role for cellular signaling in plant disease resistance.⁷⁷ Map-based cloning of *Xa21* located on chromosome 11 and transfer of this gene to commercial rice IR72 and field evaluation have been demonstrated.⁷⁸

Nutritional Genomics, Water Stress and Grain Quality of Rice

Plant foods provide almost all the essential vitamins and minerals and several other health-promoting phytochemicals. Unfortunately, most people, particularly poor people, are unable to obtain such varieties of food and staple food contains very low micronutrients. Nutritional genomics

offers novel approaches for identifying plant biosynthetic genes of nutritional value.⁷⁹

Transgenic rice with enhanced iron⁶⁷) and β -carotene-enriched rice have been reported.⁸⁰ Ferritin is an iron-storage protein found in animals, plants, and bacteria. This gene has been isolated and sequenced in plants, including soybean, French bean, pea, and maize. The introduced ferritin gene was expressed under the control of a rice seed-storage protein glutelin promoter to accumulate iron in the rice grain.

β -carotene, a precursor of vitamin A (retinol), does not occur naturally in the endosperm of rice. Ye *et al.*⁸⁰ have reported transgenic japonica rice (T309) that produces grain with yellow-colored endosperm. Biochemical analysis confirmed that the color represents β -carotene (provitamin A). Three genes, *psy*, *lyc* (cloned from *Narcissus pseudonarcissus*), and *crt1* (cloned from *Erwinia uredovora*), driven by an endosperm-specific promoter (*gt1*), have been introduced into rice to lead to the biosynthesis of β -carotene. Food with β -carotene is converted to vitamin A in the human body and is released when needed. Children and women suffer from iron and vitamin A deficiency more than men. Rice with carotenoid may help protect their lives. Transgenic tomato has been shown to contain a very high amount of β -carotene using the *crt1* gene described earlier.^{81,82}

Several studies have also demonstrated that a high intake of dietary oxidants in the form of vitamin A, C, and E or carotenoids was correlated with a lower incidence of so-called aging diseases and the prevention of photo-oxidation of retinol photo-receptors, resulting in a recovery of retinol.⁸³

Now, the following questions remain to be answered:

- 1) How will the public accept yellow rice?
- 2) Is there any trade-off with nutritional quality as a result of high β -carotene biosynthesis?
- 3) How will end-users (people below the poverty level) gain access to such yellow rice?

Lysine, an important essential but limiting amino acid in rice that might promote the uptake of trace minerals, can be improved by genetic engineering. The introduction of two bacterial enzymes *DHDPS* (dihydrodipicolinic acid synthase) and *AK* (aspartokinase), encoded by the *Corynebacterium dapA* gene and a mutant *E. coli lysC* gene, has enhanced lysine about fivefold in canola, maize, and soybean seeds.^{84,85} The introduction of this gene is a realistic approach for improving the lysine content in rice. Nutritional genomics will have a tremendous impact on the improvement of food for human health.⁷⁹ It is now possible to develop micronutrient-enriched rice with iron, β -carotene, and lysine by genetic engineering.³

Water Stress

Drought, water stress, salinity, and submergence together

cause considerable damage to crop yield. Scientists are now beginning to make progress in developing new strains of crop plants, including rice, more capable of withstanding such stresses.⁸⁶⁾ Transgenic canola was obtained with protection against frost and salinity and a single regulatory gene when introduced in *Arabidopsis* showed tolerance for cold, drought, and salt, all of which exert their deleterious effects by dehydrating cells by generating a variety of enzymes that cells use to produce chemicals and proteins that can protect against dehydration.⁸⁷⁾ A several reports on developing transgenic crops including rice with resistance to water stress have been highlighted.⁸⁸⁻⁹⁰⁾ Biotechnological research in improving rice for abiotic stress could help farmers tremendously in fragile ecosystem.⁶¹⁾

Quality and Yield Improvement

Starch polysaccharides, a major storage metabolite in plants, may positively affect the yield of several crops.⁹²⁾ ADP-glucose pyrophosphorylase (ADPGPP) is a critical enzyme for regulating starch biosynthesis in plant tissues. It should be possible to improve starch production in storage tissues by the regulated expression of the gene encoding this enzyme.⁹²⁾ Starch levels and dry matter accumulation were enhanced in potato tubers of plants transformed with the *glgC* gene from *E. coli* encoding ADPGPP.⁹²⁾ The nature of starch produced by the transgenic tubers was similar to that of the control. At IRRI, we have introduced the *glgC* gene (kindly provided by Dr. Tom Okita, USA) into rice and its expression is now being investigated in the homozygous transgenic line.

The grain quality of rice is a complex character comprising several components such as appearance, cooking and eating, and nutritional quality. Grain quality is now a serious problem in many rice-producing countries of the world. These problems seem to be severe in hybrid rice. Poor cooking and eating quality is directly related to the following factors: the amount of amylose content (AC) of starch, gel consistency (GC), and gelatinization temperature (GT). Fortunately, all three traits characterized in Shan You 63 (a commercial hybrid rice grown in China) are controlled by a single locus, characterized by a molecular marker-based QTL (quantitative trait locus) and by whole-genome scanning with MAPMAKER/QTL.⁹³⁾ Further studies revealed that all three traits were either controlled by the *Wx* locus or by a genomic region tightly linked to this locus.^{93,94)} This finding may accelerate the achievement of two objectives:

As the *Wx* locus has already been characterized⁹⁵⁾ and the sequence of the *Wx* gene is available,⁹⁶⁾ transfer of this gene to a desirable cultivar is possible, as shown by Terada *et al.*⁷²⁾

Marker assisted selection in improvement of the quality of hybrid rice.

Transgenic Rice with Agronomically Important Genes

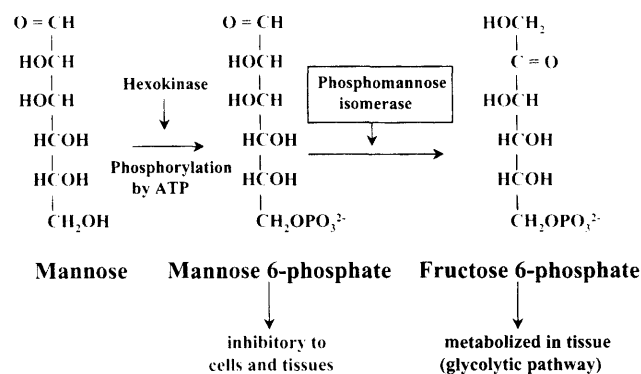


Fig. 3. Selection system using phosphomannose isomerase gene and mannose as a selectable agent.

A large number of rice cultivars (japonica, indica, and javanica) have been transformed with many genes of agronomic interest (Table 1). Transgenic crops have now been field tested in many countries covering 39.9 million hectares globally. Seven transgenic crops such as maize, cotton, canola, rapeseed, potato, squash, and papaya are grown commercially in 12 countries.⁷²⁾ Two important traits have been incorporated: herbicide resistance for weed control and plant protection, particularly with the *Bt* gene. Future prospects involve novel genes that can be introduced to generate plant lines useful for producing materials ranging from pharmaceuticals⁹⁷⁾ to biodegradable plastics.⁹⁸⁾ A few transgenic rice lines have been field evaluated and found to have an excellent performance.^{66,78,80)} It is important to mention that field evaluation is not field release, rather, it is a confirmation of research evaluation under natural environmental conditions. A more extensive field evaluation of transgenic crops is required to develop the deployment strategy for different transgenic rice cultivars and lines suitable for specific or diverse ecosystems.

Selectable Marker Genes, *nptII*, *hph*, *bar*, and *mpi*

Several selectable marker genes have been used in cereal and rice transformation.¹⁾ Though there is no evidence of any toxic or adverse effect of the use of antibiotic resistance genes in plant transformation, a negative perception exists for using such selectable marker genes in crops. Alternatively, a new selection strategy involves the use of mannose, which cannot be metabolized by many plant species. A schematic presentation shows the conversion of mannose to mannose-6-phosphate (M-6-P) by a hexokinase, resulting in severe growth inhibition (Fig. 3). The transgene product phosphomannose isomerase (PMI) converts M-6-P to fructose 6-phosphate, which is readily metabolized, giving the transformed cells a metabolic advantage.^{2,99)} Mannose-6-phosphate is toxic to non-transgenic cells/tissues, which supports the mechanism of mannose selection. We are working on this Positech™ selection in rice, aiming at more acceptability by the public as an

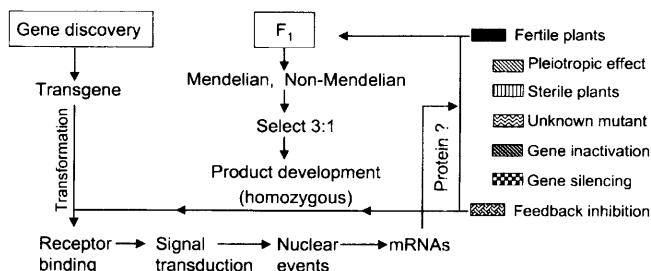


Fig. 4. Transgene to product development.

environmentally friendly system (*mpi* was available from Novartis). It seems that Positech™ selection is an efficient and direct system as an alternative to complex systems such as *Cre/lox*¹⁰⁰ and MAT vectors.¹⁰¹

Herbicide Resistance Technology and Relevance to Rice

Herbicide resistance genes that originate from various organisms including bacteria and plants are frequently used to select transformants.^{24,102} This is now one of the most important traits that has been incorporated into crops such as cotton, soybean, and maize for commercial use against weeds.

Resistance to herbicide is conferred by phosphinothricin-N-acetyltransferase (PAT), which inactivates PPT by acetylation using acetyl coenzyme A as a cofactor. Two similar genes encode PAT, *bar* (isolated from *Streptomyces hygroscopicus*) and *pat* (from *S. viridochromogenes*) which confers resistance to BASTA. In the field, BASTA is used as a weed control. It has been field evaluated and found to be environmentally friendly as it is metabolizable in nature.¹⁰³

Glyphosate (N-phosphonomethylglycine) inhibits the enzyme 5-enolpyruvyl shikimate 3-phosphate (EPSP) synthase, which is involved in the shikimate pathway. Glyphosate is the active compound in the well-known herbicide Roundup™, produced by Monsanto. Resistance to glyphosate is not based on inactivation of the herbicide, rather, the gene inhibits EPSP synthase.¹⁰² This is a single dominant gene stably inherited and shown to work in many crops, and it has been evaluated in the field. The possibility of unintended harm to crops and human food seems remote. Currently, among the transgenic crops, there have been some noteworthy developments in traits: herbicide tolerance contributed the most (69% or 8.3 million hectares) in 1998-99 and the stacked genes of insect resistance and herbicide tolerance in both maize and cotton contributed 21% (2.6 million hectares) as reported by James.⁷⁴

Weeds cause severe problems in rice and reduce yield considerably. Transplanting is the traditional method of rice cultivation in Asia and it can partially control weeds. Direct seeding, however is becoming more inevitable because of increased mechanization, which reduces labor costs. Direct seeding now covers about 29 million ha in Asia (21% of the

total rice area in the region). However, the chemical costs of weed control in direct-seeded rice tend to be higher than those of transplanted rice and these chemicals threaten human health and the environment.¹⁰⁴ Direct seeding will expand in Asia because of water shortages, high labor costs and efforts to increase productivity. Herbicide technology combined with the transgenic approach may give farmers an excellent opportunity to modernize agricultural-technology with reduced labor in an environmentally friendly system.

Ethical, Theological, and Intellectual Property Rights (IPR)

The technology of gene discovery and its application to crop improvement has developed faster than the public perception. The understanding and acceptance of any new technology by the public takes time and is far from uniform. GM foods are no exception in a heterogeneous society. Concerns about the following issues need to be addressed:

- 1) Horizontal gene flow to weeds by outcrossing?
- 2) Food safety of GM foods
- 3) Conflict between plant varietal protection (PVP) and IPR of gene discovery
- 4) Beneficiaries of the technology: farmers, consumers, the private sector, and public institutions

Many of these scientific issues, including successful product development need extensive study and public understanding (Fig. 4). A few good examples of field evaluation have to be demonstrated for public understanding and appreciation of this new technology. The private sector has invested an enormous amount of money in biotechnology, which revolutionized interest and progress in crop improvement. Consumers particularly in the developing world did not see much benefit. Asia will require more food in the coming decades with less land, water, and agrochemicals. GM foods based on precise plant breeding and efficient crop management may provide the benefits people need. Unlike any other industrial development and IP protection for luxury goods, benefits from this potential technology should be available to the people who need them most. Once the benefits of genetic engineering in agriculture reach small farms and the industrial level, everyone will benefit from such development. The market economy can function well in parallel with welfare economics, at least for rice.

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