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**REVIEW**

# RICE CONVENTIONAL AND MOLECULAR BREEDING AT CLRRI (1977-2007)

**Bui Chi Buu[1] and Nguyen Thi Lang[2]**

[1] *Institute of Agricultural Science for Southern Vietnam, Ho Chi Minh City, Vietnam*
[2] *Cuu Long Delta Rice Research Institute, Can Tho, Vietnam*

## ABSTRACT

*The genetic nature of some physiological traits related sink and source was studied through diallel analysis and triple test cross analysis. Fine mapping and physical map were conducted initial steps in an effort to isolate gene Bph-10, which controls the resistance to BPH (biotype 2+3). OM4495 and OM4498 were released through the markers. A sequence tagged site from RG64 on chromosome 6 controlling blast was generated and PCR products were digested with Hae*III*. Some promising high yielding rice genotypes were selected due to STSs and SSRs successfully. RM228 located on chromosome 8, which controls salt stress was recommended to be used to detect the appropriate genotypes. Marker wxF-R on chromosome 6 might be useful in marker-assisted breeding to improve rice genotype with intermediate and low amylose; RM234 on chromosome 7 for grain protein content; and RM223 on chromosome 8 for aroma. Transformation technology of adapted varieties in the Mekong Delta has been studied. The leading rice varieties, which have gained the record of maintaining in large scale areas for more than ten years in Southern regions of Vietnam, are OM576 and OM1490*

**Key words**: gene transformation, marker-assisted selection, quantitative traits

## INTRODUCTION

South Asia is a rich source of diversity for rice and its relatives. Rice is the principal food crop of Vietnam. It is grown on 7.6 million growing ha, and obtained 35-36 million tons. Such increases in production have been due to the adoption of high yielding rice varieties and improved production technologies on water management, fertilizer management and integrated pest management.

The Mekong Delta is the biggest granary of Vietnam. It produces more than 50% of rice in the country. Intensive cultivation of rice in the delta has led to increased threat due to continuous changing disease races and insect biotypes. Moreover, a large part of the delta is severely affected by the acid sulfate soils and saline soils. Thus pests and abiotic stresses lower rice productivity in the Mekong Delta. To overcome these constraints limiting rice production in Vietnam, there is an urgent need to widen the gene pool of rice cultivars cultivated in the Mekong Delta. Fortunately, wild species of rice are an important reservoir of useful genes to meet these challenges. The Mekong Delta is considered as one of important rice gene pools in the country. Genetic divergence analysis could be analyzed through morphological variation among rice accessions or molecular assessment based on polymorphism at several loci.

## GENETIC DIVERGENCE ANALYSIS

Classificatory analysis is proposed by many scientists (Anderson 1957, Ramanujam and Kumar 1964, Mukherjee *et al.* 1971, Venketrao *et al.* 1973, Singh and Chowdhury 1974). The method is as followed: (1) materials consisting of lines, hybrids, cultivars in analyzed in a replicated trial and the measurements on various characters are recorded; (2) two most variable characters are selected, they are used on the X-axis and the Y-axis; (3) all other characters are represented by rays on the glyph, the rays for the same

character having the same position on each glyph; (4) the range of variation in each character is represented by different length of rays, the index value in turn are decided on the basis of range of variability. Using suitable class intervals, the range of variability with regard to a character can be classified into several groups (or genetic clusters). Genetic divergence analysis based on morphological traits often expresses bias due to environmental interaction. So why plant breeders prefer using DNA markers to identify genetic diversity of crop germplasm.

Rice germplasm collection has been done since 1977. The target areas have been identified and investigated as Dong Thap Muoi (Tram Chim sanctuary for in-situ conservation), Ca Mau peninsula (mangrove), Bassac River west side, Long Xuyen tetra-angle (flood prone areas), the Coastal areas. Otherwise, collection has been carried out in other areas not in the Mekong Delta as the High Plateau (upland rice), the Eastern parts of South (rainfed lowland rice), the Central coastal (saline areas and rainfed).

More than 2,200 accessions of traditional rices and 160 accessions of three wild rices species (*Oryza rufipogon*, *O. nivara*, *O. officinalis*) were collected and preserved at the CLRRI's gene bank. Several wild species with lush degree of resistance to pests have been identified. *Oryza rufipogon*, wild rice lushly tolerance to acid sulfate soil occurs in Dong Thap Muoi, Vietnam, has been exploited in our breeding program. Beside that, 30 populations of other wild species from IRRI gene bank were introduced and preserved at CLRRI as exotic germplasm.

438 accessions of exotic rice cultivars were collected including 400 indica and 38 japonica. Then 160 accessions of improved varieties and four mutants were maintained.

Base collection: 600 accessions sent to National Plant Genetic Resources Centre

Active collection: 1,800 accession stored in deep cold room (- $5^0$C).

Working collection: 2,200 accessions stored in cold room (5-$10^0$C).

One cabinet for breeder seed storage was set up.

Number of accessions to be replanted is 800 every year.

Twenty-four agronomic traits were characterized and catalogued. Late duration genotypes account for 28.0%, medium genotypes 43.9%, early genotypes 7.9%, modern genotype 12.7%, upland genotypes 4.3%, floating genotypes 12.7%.

Tall plant height (>150cm), medium (120-150cm), and normal (<120cm) account for 54.9%, 38.7%, and 5.4%, respectively.

More than 30 g, 20-30 g, and less than 20 g / 1000-grain weight account for 2.4%, 83.7%. and 13.9%, respectively.

Four genetic clusters were identified by Tocher method based on morphological traits. Floating rice cultivars in cluster I are closed to *Oryza rufipogon*. Cluster II included local rices collected from coastal areas with salt tolerance. Cluster III included deep water rices collected from western region of Bassac river with improved plant type. Then cluster IV composed of early-monsoon rice cultivars without photosensitivity.

Selected local rices were analysed through starch gel electrophoresis to ten isozymes representing 19 loci: *Cat-1, Est-1, Est-2, Est-5, Est-9, Amp-1, Amp-2, Amp-3, Amp-4, Enp-1, Sdh-1, Icd-1, Adh-1, Got-1, Got-3, Pgi-1, Pgi-2, Pdg-1, Pdg-2*. Using the algorithm of five diagnostic loci (*Pgi-1, Pgi-2, Amp-3, Amp-2, Amp-1*) to classify rice varieties was done. The genetic polymorphism was analyzed by POPGENE software. All investigated accessions were classified into group 1 (Glaszman 1987). The clustering analysis shows the existence of three main groups of varieties at the level of similarity of 0.64. Cluster I composed of deep water rice from the Mekong Delta, cluster II: upland rice landraces from

the High Plateau, and cluster III: rice cultivars tolerant to acid sulfate. Isozyme analysis showed that average gene diversity index "H" values of landraces from the High Plateau, the Red river delta, the Central coastal, and the Mekong delta are 0.276, 0.259, 0.254, and 0.228, respectively (Buu *et al.* 1997, Buu and Lang 1997, Lang and Buu 1996, Buu 1994, 1996).

Random amplified polymorphism DNA (RAPD) was used as a DNA fingerprinting technique in rice germplasm evaluation. Ten markers of twenty from OPA kit yielded the amplified products as followed: *OPAA11, OPAJ01, OPAA13, OPAB17, OPAC14, OPAG08, OPB06, OPAL09, OPAL08, OPAK12*. Accurate classification of rice germplasm into the two major clusters and many subclusters can provide essential information for selecting parents in the development of intercluster crossing program (Buu and Lang 2001).

Diallel analysis and triple test cross were conventionally applied after selecting materials from clusters having significant genetic distance values to make an orientation of rice breeding program with target traits such as yield, important physiological traits, etc...

Passport data, main files were computerized with standard dBase systems as Fox-Pro, and Excel. Now micro-access is considered to be started.

The software GPM by IPGRI was tried to used but its procedure is slightly complicate and its function is too large in case of only rice species.

Landraces have been used as female parents to overcome "Cina cytoplasm symptom" in CLRRI's hybridization program with 30% of crosses included local rice.

Number of local cultivars from the gene bank used in crossing, however, account for 0.3%. The utilization is still low.

To widen the gene pool, many populations of three wild species were exploited.

NTSYSpc 2.1 was used to identify genetics clusters.

Both phenotyping (eight traits: flowering date, plant height, filled grain %, unfilled grain %, 1000-grain-weight, total dry matter, grain yield per hill) and genotyping (6 SSRs) were implemented.

Beside NTSYSpc, a measure for group distance based on multiple characters, which was given by Mahalonobis (1928) was also applied through: (1) collection of data, test of significance, (3) transformation of correlated variables, (4) computation of $D^2$ values, (5) testing of the significance of $D^2$ values, (6) contribution of individual characters towards divergence, (7) grouping of varieties into various clusters.

Rice genome DNA was extracted at miniprep protocol (Kangle *et al.* 1995). The PCR primers were designed based on conserved motifs of BPH resistance genes (Lang *et al.* 1999). Genomic DNA (20ng) from each variety was PCR-amplified using a primer pair as described by Lang (1998). Each 25 mL reaction contains 50 mM KCl, 20 mM Tris HCl, 5mM $MgCl_2$, 0.5 mM of each dNTP, 60 ng of each primer, 20 ng template DNA, and 1 unit of *Taq*. The reaction was conducted using the following conditions: denature 5 min at 95$^o$C, 40 cycles of 1 min at 94$^o$C, 1 min at 45$^o$C, 2 min at 72$^o$C; and a final extension of 7 min at 72$^o$C.

The degree of polymorphism (DP) for each primer combination was calculated based on Falconer (1989) by the following equation

$$\%DF = \frac{\text{Number of varieties with rarer allele}}{\text{Total number of varieties}} x100$$

The highest value for DP in two-allele, three-allele, and four-allele system is 50.0%, 66.7%, and 75.0%, respectively.

## QUANTITATIVE GENETICS

In order to exploit the variation in his source material as efficiently as possible, the breeder needs to have knowledge of the genetic architecture of the characters that he is seeking to improve (Perera *et al.* 1986).

The genetic nature of some physiological traits related sink and source was studied through diallel analysis and triple test cross analysis. Suitable rice materials were chosen to develop high yielding rice varieties adapted to irrigated ecosystem in the Mekong Delta. The results showed that non additive gene action was more important than additive gene action in the inheritance of grain yield, panicle / hill, grains / panicle, biomass yield, harvest index (HI), high density grain index (HDI), leaf area index (LAI) at panicle initiation and heading, net assimilation rate (NAR), crop growth rate (CGR), and leaf area duration (LAD) from initiation to heading (Buu *et al.* 1995).

It revealed the presence of additive gene action and additive x additive type among the parents for grain filling rate (GFR), HDI, HI. It showed the preponderance of additive x dominance for total dry matter at harvest, and dominance x dominance for chlorophyll "b", LAI at heading, GFR, HI and grain yield. The significant differences between grand means of triple test cross families and those of the basis generations in grain yield, high density grain index, expressed a linked digenic interaction (table 1) (Buu *et al.* 1995).

**Table 1.** Testing for equality between the means of triple test cross families and of the basis generations of IR8 / IR46

| Test | Primary branch no. | HDI | GFR | LAI at heading | TDM | Yield |
|---|---|---|---|---|---|---|
| $L_1$- $B_1$ | 0.57ns | -8.44** | 2.80ns | -0.62ns | -13.48** | -7.00** |
| $L_2 - B_2$ | 0.70ns | -15.23** | 1.67ns | 0.67ns | -0.17ns | -5.93** |
| $L_3 - F_2$ | -0.40ns | 4.94* | -2.23ns | -2.23ns | 1.98ns | 4.34** |

Epistatic variance is an orthogonal partitioning of the interaction among loci or interallelic interaction (Hallauer 1979). Assuming an additive-dominance model, it could be demonstrated that the value $L_1 + L_2 - 2L_3$ should not deviate significantly from zero. Duplicate epistasis indicated by the negative [h] and positive [l] was recognized to LAI at heading, GFR and grain yield, may hinder selection. Potential yield estimation based on quantitative physiological characters indicated that exploitation of heterosis maybe useful. The genetic architecture of these characters was related to the genetic basis of the heterosis displayed by the $F_1$'s. The cause of this heterosis may be due to dispersed dominance and interacting genes and due to genes with dominance and epistatic properties that are in linkage disequilibrium (Buu *et al.* 1995).

## MARKER-ASSISTED SELECTION AND MOLECULAR GENETICS APPROACHES TO RICE IMPROVEMENT

Major improvements in rice have been made by plant breeders and geneticists. However, studies on rice at the gene level using molecular biology techniques have been extremely limited in 1980s (Wu *et al.* 1986). They have isolated and determined the DNA sequence of several genes from the nucleus, chloroplast, and mitochondrion of rice.

The Rockefeller Foundation established the international program on rice biotechnology in 1985-1999, which has contributed greatly in moving the frontiers of knowledge on rice cellular and molecular genetics. The Rice Genome Research Program (RGRP) in Japan has moved the frontiers of our knowledge on the rice genome far beyond anyone's expectations. A high density map of more 2,200 molecular markers is now under way, facilitating the cloning of importance genes not only from rice but also from other cereals because of the small size of the rice genome and its synteny with those of other cereals (Rothschild 1996).

Gene symbols were accepted till the establishment of the Rice Genetics Cooperatives in 1985, and an international network on rice genome sequence was established in 1998 under the leadership of the RGRP (Khush and Brar 2001). Projects on functional genomics began in 1999.

The availability of comprehensive molecular linkage maps, tight linkage of target genes with molecular markers, and rapid development of polymerase chain reaction (PCR)-based markers have facilitated the employment of marker-assisted selection (MAS) in rice breeding. MAS increases the efficiency of a breeding program by selecting for markers linked to target traits or QTLs (Khush and Brar 2001). Protocols for PCR-based MAS have been developed (Zheng *et al.* 1995)

New high-throughput methods are being developed for expression analysis. Biochips are being used to follow changes in gene expression in response to abiotic stresses. Using gene chips or microarrays, the representative genes of rice can be analyzed on glass slide and used in RNA hybridization to reveal gene expression patterns and identify pathways by association (Khush and Brar 2001).

***MAS on brown plant hopper resistance (BPH)***

An informative RFLP marker RG457 showed the closest linkage to gene *Bph-10* from *Oryza australiensis*. Two primers designed from RG457 as STS markers allow heterozygous to be distinguished from the two homozygotes. An STS was generated and PCR products digested with *Hin*fI and *Alu*I. The banding pattern of the 37 $F_2$ individuals could be classified into homozygote for the IR54742-type marker. The accuracy of predicting homologous resistant genotypes based on flanking marker data was 100% for BPH using single marker (Lang *et al.* 1999). Fine mapping and physical map were conducted in initial steps with an effort to isolate gene *Bph-10,* which controls the resistance to BPH (biotype 2+3) (Lang and Buu 2003). OM4495 and OM4498 were released through the markers.

***MAS on bacterial leaf blight (BB) resistance***

Genotypes with stable resistance *e.g.* Nep Hoa Vang, Bong Trang, and Ba Le have been developed in Vietnam from such phenotyping, and others have often expressed no stability for the resistance (Buu *et al.* 1997). The collected *Xoo* isolates were divided into seven groups based on their pathogenicity. MAS could simply the process of combining the recessive gene *xa-5* with other dominant BB resistance genes that have overlapping effects or race specificities (Blair and McCouch 1997). Trang Lun, Trang Phuoc, Trang Mua contained the genes *xa-5* and *xa-13* are recommended using as donors in CLRRI rice breeding program. Released varieties such as OM997, OM1490, OM1723 are resistant to most isolates collected from the South (Buu and Lang 2003). Microsatellite markers were recommended to be in MAS as RM21 and RM190 on chromosomes 11 and 6, respectively (Pha and Lang 2004).

***MAS on blast resistance***

Blast is one of the most destructive diseases of rice in the Mekong Delta. Breeding varieties resistant to blast is the most effective way to control the disease. The selected RILs (C101A51) from a cross between 5173 (gene *Pi-2*) and CO39 linked to RG64 were added to the set for blast rsistance, with a genetic distance of 2.8 cM, located on chromosome 6. A sequence tagged site was generated and PCR products were digested with *Hae*III. The resistant genotypes had a 750 bp-band and the susceptiple genotypes had a 650 bp-band. Phenotypically, collected rice blast isolates were classified into four pathogenic races in 1996. The highest virulence was recognized in race 106.4. Some promising high yielding rice genotypes were selected due to STSs and SSRs successfully (Buu and Lang 2003). But the current resistant genotypes were not stable in large scale areas of the Delta.

***QTL resistance of sheath blight***

Sheath blight is also a severe disease in the Mekong Delta. 266 NILs with randomly introgressed Lemont segment of a cross between Lemont x Teqing were used. 15 M-QTLs detected for lesion height (LH) and actual lesion length (ALL) were mapped on seven chromosomes (1, 2, 3, 4, 5, 9, and 12), explaining 35.8%-93-8% of the phenotypic variation. Four QTLs that were found not associated with plant morphology or heading date are potentially useful in breeding programs for sheath blight resistance (Loan *et al.* 2004).

***MAS for salt tolerance***

Salt tolerant varieties have generally been considered as the most economical and effective way of increasing crop production in saline soils. The RILs derived from the cross Tenasai 2 / CB were evaluated for survival days, dry root weight, dry shoot weight, $Na^+$, $K^+$, $Na^+/K^+$ ratio in culture solution (EC = 12dS/m). A linkage map was constructed using 108 RFLPs and SSRs covering 2,340.50 cM, with an average interval of 21.68 cM between marker loci. RM228 located on chromosome 8 was recommended to be used to detect the appropriate genotypes (Lang *et al.* 2003a). Some were released as OM4498, OM5636 in 2006.

***MAS for aluminum tolerance***

We found one accession of *Oryza rufipogon* collected in Tram Chim, Dong Thap Muoi is strongly tolerant to acid sulfate soils. A population of 171 $F_6$ recombinant inbred lines derived from the cross of IR64 x *O. rufipogon*. A genetic map, consisting of 151 molecular markers covering 1,755 cM with an average distance of 11.6 cM between loci, was constructed. A major QTL for relative root length (RRL), which explained 24.9% of the phenotypic variation, was found on chromosome 3 of rice. These results indicated the possibilities to use MAS and pyramiding QTLs for enhancing AL tolerance in rice (Bay *et al.* 2003). AS997 was officially released and has become a leading variety adapted to acid sulfate soil areas in the Mekong Delta so far.

***MAS for grain quality properties***

Due to the increasing demand of rice with good grain quality for export and domestic markets, there is a need to breed varieties possessing good grain quality (Bong 2000)

Amylose content is one of the important traits of grain quality properties of rice. One primer pair for the locus *wxF-R* showed association with amylose content. The co-segregation data of the marker and amylose content on 120 $BC_2F_2$ of IR64 x Hoa Lai were analyzed by means of a single marker linear regression approach. This marker might be useful in marker-assisted breeding to improve rice genotype with intermediate and low amylose (Lang and Buu 2004).

Protein content in rice grain (GPC) is an important trait. The study was undertaken with a view to tag gene(s) controlling GPC using molecular marker in rice. Genotype IR64 with low GPC (7.5%) was crossed with genotype Nang Thom Cho Dao with high GPC (10.62%). Genetic map was set up in $BC_3F_2$ of the cross. Microsatellite marker RM234 on chromosome 7 was recommended as a useful tool for MAS (Lang and Buu 2005).

The aromatic rice is preferred over non-aromatic rice during special occasion and for export, and thus they command a higher market price. RG28 on chromosome 8 was converted by sequencing into SSR and used as primers for PCR amplification of genomic DNA from progenies. Microsatellite marker RM223 closely linked to *fgr* gene is recommended as a useful tool for MAS (Lang and Buu 2002).

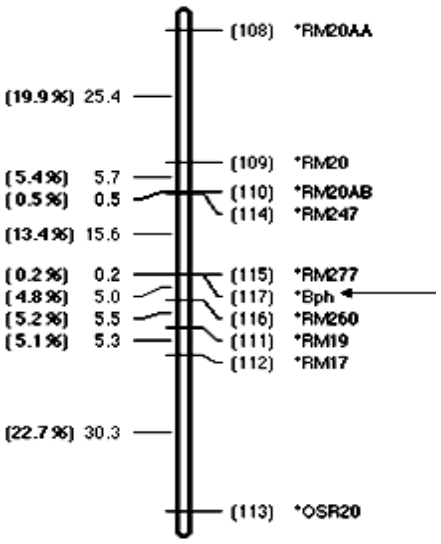


**Figure 1.** *Bph-10* gene closely linked to RM277 and RM260 on chromosome 12

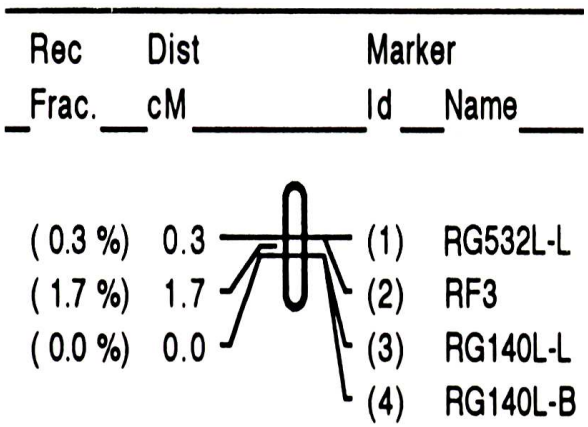


**Figure 2.** *Rf-3* gene closely linked to RG140L-L and RG140L-B

***Sequence tagged site marker diagnostics for fertility-restoring gene and tms-3***

Several genes for male sterility and fertility restoration linked with DNA markers have been identified. Of these genes, *tms-1* was mapped on chromosome 8, *tms-3*(t) on chromosome 6 (Subudhi *et al.* 1997, Lang *et al.* 1997) and *Rf-3*, the nuclear fertility-restoring gene for wild abortive cytoplasmic male sterility (WA-CMS), on chromosome 1 (Zhang *et al.* 1996). STS markers designed from RG140 and RG532 were closely linked to *Rf-3* (Figure 2), F18F/RM and F18FM/RM linked to *tms-3*. The utility of STS markers for the transfer of *Rf-3* and *tms-3* through marker-assisted selection was demonstrated (Lang *et al.* 2003b, Lang *et al.* 2000).

***Gene transfer to rice genotypes***

*Agrobacterium tumefaciens* provides a routine and efficient gene transfer system for a variety of plant species, but it apparently does not function with cereals (Potrykus 1991). CLRRI scientists has overcome the event through using appropriate plasmids and successfully transformed target genes into indica rice cultivars. Transformation technology of adapted varieties in the Mekong Delta has been studied. KDML 105 was engineered with GNA gene for brown plant hopper resistance and *cryIAc* for stemborer resistance (Bong 1998, Bong *et al.* 1999). The function of *Cre/lox* site-specific recombination system to excise a gene from rice genome was studied (Cuc Hoa *et al.* 2000). Approaches to improve *CrtI* in rice endosperm for increasing β-carotene content in golden rice was interestingly reported (Cuc Hoa *et al.* 2005) with the construction of pCarNew, pFun3, pTOK233, gene *pmi* (phosphomannose isomerase) and *Agrobacterium tumefaciens* LBA 4404.

**FUTURE APPROACHES**

The leading rice varieties coming from CLRRI, which have gained the record of maintaining in large scale areas for more than ten years in the Southern regions of Vietnam, are OM576 (Ham Trau) and OM1490.

Looking to the future, we have to (1) broaden the genetic background of rice varieties from both landraces and wild rice species, (2) break yield ceiling and stabilize productivity with the emphasis of brown plant hopper and blast resistance, (3) improve grain quality and nutrition value of rice, (4) meet the demand of climate changing and water deficit to have drought tolerant genotypes and others tolerant to abiotic stress

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## Chọn tạo giống lúa và nghiên cứu cơ sở di truyền cây lúa trong 30 năm qua (1977-2007) ở Đồng bằng sông Cửu Long

Viện Lúa ĐBSCL đã bắt đầu bằng công việc sưu tập nguồn tài nguyên di truyền và đánh giá tính đa dạng của chúng trong ngân hàng gen. Phân tích di truyền số lượng nhằm xác định một chiến lược lai tạo và chọn lọc hợp lý trên cơ sở phân tích các tính trạng liên quan đến nguồn và sức chứa. Viện đã ứng dụng dấu chuẩn phân tử để chọn tạo giống lúa (MAS) kháng rầy nâu, kháng bệnh đạo ôn, chống chịu mặn, chống chịu phèn, hàm lượng protein trong hạt, hàm lượng amylose, mùi thơm. Giống lúa đầu tiên thông qua MAS là OM4498 và OM4495. Ngoài ra Viện đã thành công bước đầu trong chuyển nạp gen mục tiêu vào cây lúa indica bằng *Agrobacterium* với nội dung tiếp cận promoter đa chức năng, marker chọn lọc trên đường mannose, plasmid thích ứng. Giống lúa đạt kỷ lục phát triển trên diện rộng ở phía Nam trên 10 năm là OM576 và OM1490.