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FINAL TECHNICAL REPORT

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Marker Assisted Selection (MAS) for Participatory Plant Breeding (PPB) in Rice

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Executive Summary (500 words max)

The first major component of this project was to test a novel breeding approach, termed marker-evaluated selection (MES). MES shares the principles of association analysis and quantitative trait locus (QTL) mapping, but it has fundamental differences. The key is that the selection for stress tolerance is maximised in a cross between carefully chosen parents and changes in allele frequencies are then detected. It was tested using progeny derived from the upland rice variety Kalinga III that was crossed to IR64, IR36, Vandana and Radha 32. The progeny were selected over a number of generations across a range of environments in India and Nepal. Selected and non-selected lines were evaluated with different types of molecular markers (SSR-microsatellites, SNPs and transposon-display markers) from throughout the genome in order to follow the effects of selection on marker frequency. Significant allele shifts towards the most well adapted parent were found in both upland and lowland conditions. The most extreme shifts were seen in the varieties Ashoka 228 and Ashoka 200F derived from the cross IR64/Kalinga III. They had inherited a very small proportion of their alleles (less than 5%) from the upland-adapted parent IR64. It is highly probable that these 5% IR64 alleles are linked to QTLs that contribute to their increased yield compared with Kalinga III. We recommend further studies to confirm this hypothesis.

A second major component of the project was to continue the marker-assisted backcrossing (MABC) programme and test lines with different target introgressions for their field performance, and expression of phenotypic traits under controlled conditions. This was successfully completed and we confirmed experimentally that at least one root QTL retains its phenotypic effect by increasing root length when expressed in the Kalinga III genetic background. Near-isogenic lines (NILs) were shown to produce a greater biomass in the field, and some out-performed Kalinga III for grain and straw yield in drought trials in India. This is, to our knowledge, the first time that utilisation and validation of rice root QTLs has been carried out using introgression into a novel genetic background was used in the initial QTL mapping.

The partnerships developed in this project have enhanced the capacity of Nepalese and Indian scientists to carry out marker-assisted breeding in rice. Novel varieties for drought prone areas have been developed and thoroughly evaluated. They are highly appropriate for dissemination to farmers in India, Nepal and Bangladesh and we recommend that DFID should prioritise work to enhance and monitor their uptake.

Background

Around 20% of the total rice area in Asia, a minimum of 23 million ha, is drought-prone (Pandey *et al*., 2000). More than half of this total is located in eastern India where rice is the most important crop and the economic and social costs of drought can be devastating. Poor farmers in marginal areas have benefited little from high yielding, ‘green revolution’ varieties that have transformed the productivity of more favourable areas. They continue to grow landraces that are characterised by low productivity and susceptibility to diseases and pests.

Client oriented breeding (COB) methods can help overcome many constraints by more rapidly producing and placing in farmers’ hands new varieties with greater utility to farmers (Witcombe *et al*., 2006 a and b). It offers a rapid, cost effective strategy for developing farmer-preferred, superior varieties. New varieties can give farmers higher, more stable yields without the need to apply more purchased inputs or labour.

The first aim of this project was to establish the novel strategy, marker-evaluated selection (MES). Rice lines and bulks selected through COB were evaluated. MES can identify regions of the genome that, because they determine useful traits, have been selected during the breeding programme. These may be found across environments or be season- or niche-specific. Once identified these markers can be used for marker-assisted selection (MAS) to select ideotypes for specific seasons and ecosystems. Crosses were made with the upland variety Kalinga III and the products have been selected using COB in India and Nepal in other PSP projects. Many varieties were selected for a range of ecosystems and they show significant improvements over Kalinga III for grain yield, straw strength and disease resistance. It has been estimated by GVT that their introduction in the GVT project area could increase productivity by over 50%. There is potential to further increase the yield of these varieties by making crosses between the best lines.

The use marker-assisted selection to improve the rooting quality of the variety Kalinga III was a second aim of this project. The development of a deep and thick root system in rice has been associated with improved drought resistance (Fukai and Cooper, 1995). Previous PSP projects identified quantitative trait loci (QTLs) contributing to root traits in rice (Price *et al*., 2000; and Price *et al*., 2002) and initiated a backcross programme to introduce the most useful QTLs from Azucena to Kalinga III. This project advanced and tested near isogenic lines (NILs) from this programme using client oriented selection with farmers in eastern India. In a parallel experiment, modified single-large-scale MAS (Ribaut J-M. and Betrán, 1999) was used to create a set of segregating bulks that contained target QTLs. These were selected by farmers in eastern India and the resultant bulks compared for root traits and yield in multi-location drought trials.

References

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Ribaut J-M. and Betrán J. (1999). Single large-scale marker-assisted selection SLS-MAS. Molecular Breeding 5, 531-541.

Witcombe J.R., Joshi K.D., Gyawali, S. Musa, A.M., Johansen C., Virk D.S. and Sthapit B.R. (2005) Participatory plant breeding is better described as highly client-oriented plant breeding I. Four indicators of client-orientation in plant breeding Experimental Agriculture, 41: 299-319.

Witcombe J.R., Gyawali, S. Sunwar, S. Sthapit B.R. and Joshi K.D. (2006) Participatory plant breeding is better described as highly client-oriented plant breeding II. Optional farmer collaboration in the segregating generations. Experimental Agriculture 42: 79-90.

Project Purpose

Novel methods of aiding conventional plant breeding to overcome biotic or abiotic constraints developed, tested, piloted and promoted.

Research Activities

Output 1: Marker-evaluated selection strategy established

1.1. *A*nalyse MES data to identify farmer-preferred regions for varying ecosystems and seasons in best PPB lines from four crosses (Kalinga III with Radha 32, IR64, IR36 and Vandana).

1.2. Intra-cultivar diversity in Ashoka 200F and Ashoka 228 analysed with molecular markers.

1.3. MES to evaluate successful PPB material from other crosses.

Output 2: Ideotype rice genotypes developed for specific seasons and ecosystems

2.1. Plan ideotypes and select and make crosses that can give rise to them.

Output 3: NILs developed for fine mapping of root QTLs and aroma

3.1. Pyramids and NILs genotyped.

3.2. Root phenotype screens of advanced backcross and pyramid lines carried out to fine-map QTLs; also compare effects of different genetic backgrounds on QTLs.

Output 4:Effectivenss of incorporating MAS with PPB validated

4.1. Screen advanced bulks and pyramid lines in field trials for drought resistance.

4.2. Three different strategies for combining MAS and PPB compared.

4.3. Enter in official trials advanced MAS bulks and lines selected by farmers in PPB.

4.4. PPB and MAS bulks tested for root phenotype.

Output 5: A range of useful SNP markers developed

Activity 5.1. SNPs tested for polymorphism and method optimised for high throughput genotyping.

Output 6: MAS methods transferred to breeders in India, Nepal and Bangladesh

Activity 6.1. Workshops and training.

Outputs

Output 1

We have tested an alternative approach for the location of QTLs – marker-evaluated selection (MES) – that makes no prior assumptions as to which traits are important. This was tested on the products of selection in three populations that all shared Kalinga III as a common parent (Table 1).

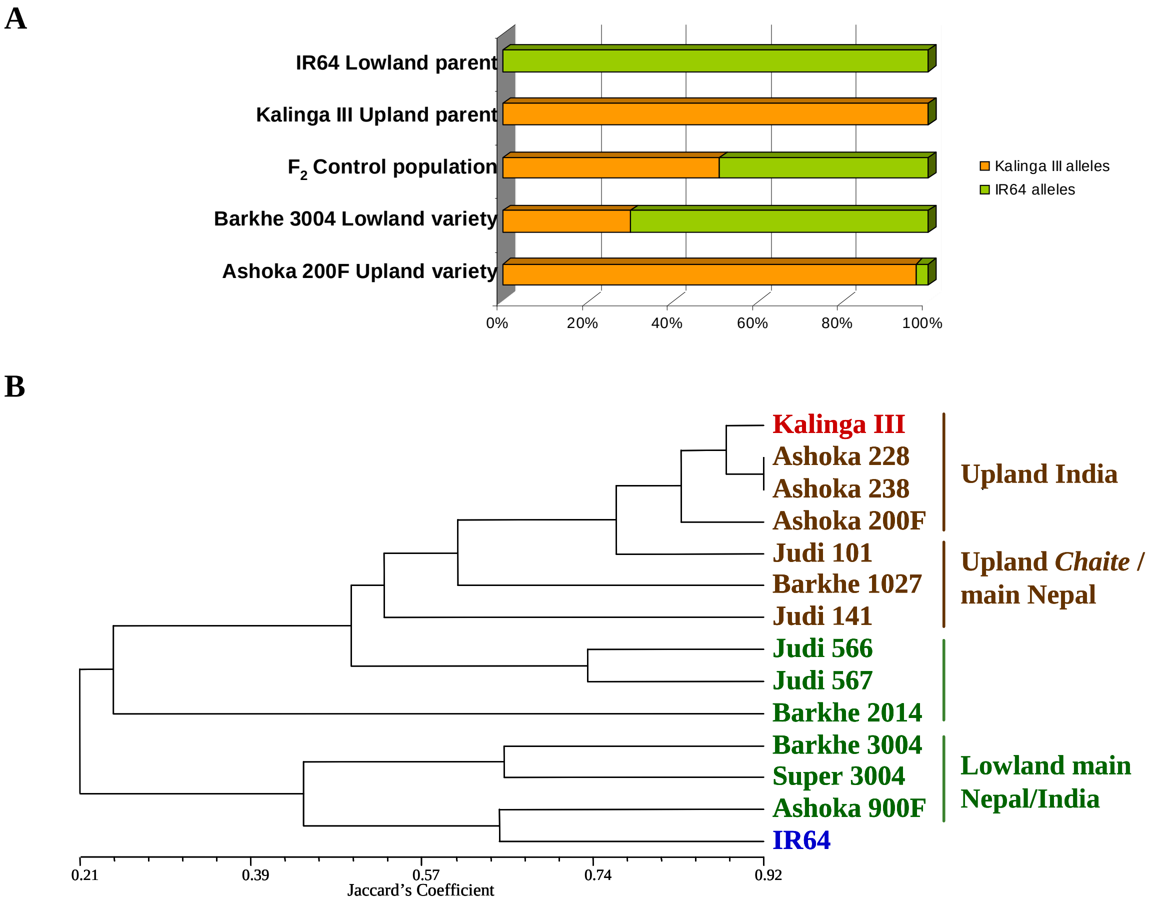
Table 1. Selected varieties from COB that were evaluated for allele frequency from three populations derived from crosses with Kalinga III.

	Variety crossed to Kalinga III		
	IR64 (n = 15)	Radha 32 (n = 10)	IR36 (n = 5)
Target environment			
India upland	4 Ashoka		5 Sudha
India lowland	1 Ashoka		
Nepal upland *Chaite* season	2 Judi	2 Judi	
Nepal lowland *Chaite* season	2 Judi	8 Judi	
Nepal upland main season	1 Barkhe		
Nepal medium/lowland main season	5 Barkhe		
Number of selected lines or bulks	15 (+ 12)	10	5
Total number of markers tested	155 SSR 12 SNP	115 SSR	115 SSR
Percentage polymorphic SSR markers	45	50	42

Marker loci linked to important traits for adaptation to specific environments were identified without making any prior assumptions about which traits might be important. Genomic regions from Kalinga III were strongly selected in the upland environments and regions from IR64 in the lowland ones (Figure 1). However, exceptions occurred where the upland parent contributed positively to lowland adaptation and *vice versa*.

The results can be used as a basis for the development of second cycle varieties using marker-assisted selection to produce genotypic ideotypes for specific target environments. The very strong selection for genomic regions from the adapted parents of the wide (upland x lowland) cross indicates that, in non marker-assisted breeding, where genetically distant parents have been used, modified backcross breeding should be efficient. A single backcross to the adapted parent for a specific ecosystem will result in a higher frequency of segregants with the desired high genetic contribution from the adapted parent.

Figure 1. A. Summary of MES results in two selected lines, their parents and the unselected control. Barkhe 3004 was selected for lowlands and Ashoka 200F was selected for uplands. B Dendrogram based on Jaccards similarity of varieties from the IR6f4/Kalinga III cross shows that variation between selected lines perfectly matches the target environment.



A putative region where IR64 alleles contribute significantly in upland varieties Ashoka 228 and Ashoka 200F was identified on chromosome 8 using the transposon display marker RIM2A. Although not fixed in either variety, it has been conserved in both at a higher level than any other IR64 allele detected with 50 SSR markers and 5 RIM2 markers (amplifying approximately 30 alleles).

Table 2. Proportion of IR64 alleles at RIM2A and the nearest SSR marker on chromosome 8 in two upland varieties and IR64.

Variety	RIM2A	Number of lines tested	RM25	Number of lines tested
Ashoka 200F	12%	107	2%	62
Ashoka 228	42%	125	0%	62
IR64	92%	13	94%	16

Variation within COB varieties

IRRI tested 50 lines of A200F and 50 of A228 with 35 SSR loci. The results revealed mean frequencies of IR64 alleles as 11% for A200F and 63% for A900F and confirmed shifts in allele frequency in selections towards the parent most adapted to the target environment.

We compared intra-varietal variation in Ashoka 200F and Ashoka 900F produced by a very simple bulk breeding method (mass selection with no line selection at any stage) with one line-selected variety, Ashoka 228. Their parents, the upland variety Kalinga III and the irrigated transplanted medium-lowland variety IR64, both originated through line-selection and were used as control varieties.

At 43 SSR loci there was more genetic variation between lines within the bulk-selected varieties than within Ashoka 228. Kalinga III was the least variable variety while IR64 had less heterozygosity but greater heterogeneity than the two bulk-selected varieties (Table 3). Despite the greater simplicity of the method, mass-selection in bulk populations produced varieties that met the distinctness, uniformity and stability (DUS) criteria for seed certification in India. Such uniformity was achieved because of the high selection pressures applied to the bulk. We conclude that this very simple bulk-population breeding approach is highly cost-effective and produces sufficient seed for wide testing earlier than any alternative method.

Table 3. Proportion of Kalinga III alleles detected in three varieties derived through COB methods from the cross Kalinga III and IR64. Ashoka 200F and Ashoka 228 were selected for upland conditions and Ashoka 900F was selected for medium-lowlands in eastern India.

class	Kalinga III	Ashoka 200F	Ashoka 228	Ashoka 900F	IR64
100%	16	19	48	0	0
96-99%	0	23	11	0	0
90-95%	0	8	3	0	0
80-89%	0	8	0	0	0
70-79%	0	1	0	0	0
60-69%	0	1	0	0	0
50-59%	0	0	0	0	0
40-49%	0	0	0	9	0
30-39%	0	1	0	46	3
20-29%	0	0	0	7	0
0-19%	0	0	0	0	13
	16	61	62	62	16

Marker-analysis of COB varieties produced from other crosses

Fifty SSR markers were tested on two varieties bred using COB methods in Nepal from a cross between Fuji 102, a high yielding modern variety and a local Nepalese variety, Chhomrong Dhan (cold tolerant, responsive to higher nutrient levels, adapted to limited water or upland conditions, red grains, early maturing, high yielding). The varieties, Machhapuchhre-3 (M-3) and Machhapuchhre-9 (M-9) are being widely adopted by farmers in high-altitude sites in Nepal. There were more alleles from Chhomrong Dhan in both selected varieties (64% in M-3 and 60% in M-9) than would be expected if there had been no selection, however no control population was tested to confirm this conclusion.

Output 2

New crosses were made using the best performing varieties to come from COB as parents (Appendix 1). Progeny were supplied to collaborators for testing in specific ecosystems.

For Output 3

This work was a continuation of the marker-assisted backcrossing that was started in project R6673 to introduce QTLs from Azucena for aroma and root growth (and hence drought resistance) into Kalinga III.

Twenty-two near-isogenic lines (NILs) were evaluated for root traits in five field experiments in Bangalore, India. The target segment on chromosome 9 (RM242-RM201) significantly increased root length under both irrigated and drought stress treatments, confirming that this root length QTL functions in a novel genetic background (Table 5). No significant effects on root length were found at the other four targets.

Azucena alleles at the locus RM248 (below the target root QTL on chromosome 7) delayed flowering. Tiller number was greatest in the NIL that had Kalinga III alleles at all the five target regions and it was significantly higher tillering than Kalinga III. This NIL was predicted to contain approximately 15% Azucena alleles at non-target regions, so Azucena alleles at non-target regions may contribute positively to tillering.

Table 5. Multi-factor analysis of variance for root length for 5 experiments conducted at UAS, Bangalore on 19 NILs with the effect of Azucena alleles at 6 chromosome segments in the Kalinga III genetic background.

Factor	DF	F	P
Chromosome 1 RM5 (A/H or K)	1	0.00	0.952
Chromosome 2 RM221-RM213 (A/H or K)	1	0.21	0.649
Chromosome 7 RM351-RM234 (A/H or K)	1	0.04	0.847
Chromosome 8 RM223 (A/H or K)	1	1.36	0.244
Chromosome 9 RM242-RM201 (A/H or K)	1	21.72	0.000***
Chromosome 11 RM229-RM206 (A/H or K)	1	2.23	0.137
Stage at analysis (<56d, <76d or maturity)	2	34.58	0.000***
Treatment (DT or WW)	1	6.22	0.013*
Experiment	4	3.61	0.007**
Error	434		
Total	447		

Output 4

Lines and bulks developed from the marker-assisted backcrossing programme were tested by breeders and farmers in COB in eastern India.

Pyramids

Advanced pyramid lines were tested for their suitability for upland environments. From approximately 30 tested on a small plot basis in observational trial in 2001, four were selected, including the control line (PY 81) with no QTLs. NILs with introgressed QTLs (PY 82, PY 83 and PY 84) were tested in further observational trials in 2002 and 2003 and PY 82 and PY 84 were selected in both years but PY 83 only in 2002 (Table 6).

Table 6. Near-isogenic lines (NILs) derived from the BC3 generation showing the genotype (+ Azucena, - Kalinga III, S segregating) at four root QTLs and results of selection in observational trials at GVT research farm, Ranchi, India. NT = Not tested.

Field ID	QTL 2	QTL 7	QTL 9	QTL 11	2001	2002	2003
PY 83	+	-	+	+	Selected	Selected	Rejected
PY 82	+	-	-	+	Selected	Selected	Selected
PY 81	-	-	-	-	Selected	NT	NT
PY 85	-	+	+	-	Rejected	Rejected	NT
PY 84	+	S	S	+	Selected	Selected	Selected

Data from seven on-station trials were combined and these showed that the NILs produced between 7 to 20% more grain than Kalinga III and 5 – 24 % more straw than Kalinga III. PY 82 had the highest grain yield. The presence of Azucena alleles (at either target QTLs or non-target regions) was associated with increased grain and straw yield. The target root QTLs were associated with increased straw yield (18%) and taller plants (5%) but there was no effect on grain yield.

Bulks

Field data from six bulks derived from marker-assisted selection for root QTLs and selected by farmers in COB since 2000 were used to draw similar conclusions to the results from pyramids (above). The bulks were purified and three have been tested in AICRIP trials since 2002. They are highly suited to medium-upland conditions in eastern India. Bulk 5 (Richa 5) is aromatic and is particularly liked by farmers for its grain quality. We are hopeful that Bulk 5 will be released in eastern India.

Output 5

Single Nucleotide Polymorphisms (SNPs)

Rice genetics and breeding are entering a new era where genetic variation can be evaluated to the single letter (base pair) of the genetic code. Several versions of the rice genome are published and this has enabled us to examine polymorphism at single nucleotides. Twelve SNP markers that were developed from polymorphism between *Indica* and *Japonica* were tested as part of the MES study (output 1). The level of variation within the Kalinga III crosses was disappointing, but this was likely to be due to the fact that all crosses tested in this study were from *Indica*/*Indica* crosses. They were not tested in the Kalinga III/Azucena material. Two SNP markers were optimised for multiplex testing on the CEQ8000 sequencer.

Output 6

Throughout this project there was dialogue and interaction between the molecular researchers in the UK and project collaborators in India and Nepal. Workshops took place in Ranchi in Sept 2002 and March 2004. A new biotechnology centre was opened at BAU, Ranchi in 2003. Presentations on marker-assisted breeding were given at the centre by K.A. Steele in March 2003 and December 2004. There now exists the capacity at BAU, Ranchi and NARC, Nepal to conduct out marker-assisted breeding in rice.

Contribution of Outputs

- Marker Evaluated Selection has been tested, validated and published.

- High selection pressures applied during selection under droughted upland conditions lead to allele shifts towards the upland parent. Ashoka 228 and Ashoka 200F, both derived from the cross IR64/Kalinga III, inherited less than 5% of their alleles from IR64. We hypothesise that these 5% IR64 alleles contribute to the increased yield of Ashoka 228 and Ashoka 200F over Kalinga III.

- Three varieties, produced using COB methods (Ashoka 200F and Ashoka 900F selected by bulk breeding and Ashoka 228 selected by line selection), meet the distinctness, uniformity and stability criteria for seed certification in India.

- Advanced bulks derived through modified single large-scale MAS for root and aroma QTLs have been selected, tested and purified in upland ecosystems in eastern India. These varieties now require multiplication and dissemination.

- Root screens have confirmed the function of a root length QTL from Azucena (chromosome 9) in a Kalinga III background.

- Some NILs with several QTLs from Azucena in different combinations and genetic backgrounds out-performed Kalinga III in drought trials in India.

- Testing of NILs with introgressed regions showed that some of the introgressed regions carried genes for delayed flowering. The overall effect of target introgressions was to improve biomass but this did not translate into increased yield across all environments.

- Novel crosses have been made between the most outstanding material from a range of DFID PSP projects. The progeny from some of these are already undergoing field trials, and others have shown much promise in the field.

- Poor people in marginal rice-growing areas of India, Nepal and Bangladesh will benefit greatly from the production and seed dissemination of novel rice cultivars generated in this project.

- Further selection and development of the segregating material developed in this project by DFID is strongly recommended.

Dissemination

Project web page: **http://www.cazs.bangor.ac.uk/ricemas/index.htm**

Refereed publications

Steele KA, Price AH, Shashidhar HE, Witcombe JR. (2006) Marker-assisted selection to introgress rice QTLs controlling root traits into an Indian upland rice variety Theoretical and Applied Genetics 112: 208-221.

Bajracharya, J. Steele, K.A., Jarvis D.I., Sthapit B.R. and Witcombe J.R. (2006) Rice landrace diversity in Nepal: Variability of agro-morphological traits and SSR markers in landraces from a high-altitude site. Field Crops Research 95: 327-335.

Steele K.A., Edwards G., Zhu J and Witcombe J.R. (2004) Marker evaluated selection in rice: shifts in allele frequency among bulks selected in contrasting agricultural environments identify genomic regions of importance to rice adaptation and breeding. Theoretical and Applied Genetics 109: 1247-1260

Other Dissemination of Results

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Journalists in India interviewed D.S. Virk and K.A. Steele and articles appeared in the Ranchi local press in December 2004.

Amar Prasad, J.S. Gangwar, V. Singh, S.C. Prasad, D.S. Virk, J.R. Witcombe and K.A. Steele. "The State of art in drought tolerance breeding and seed dissemination strategies" Paper presented at the Rockefeller Workshop, Bangalore, July 23rd-25th, 2003.

K.A. Steele, D.N.Singh, R. Kumar, S.C. Prasad, M. Billore, D.S. Virk, H. E. Shashidhar and J.R. Witcombe (2004) Marker-assisted selection of four root QTL and aroma to improve Kalinga III: Evaluation of pyramid lines and advanced bulks from backcrossing. Paper presented at the National Conference on Increasing Rice Production under Water Limited Environment, 3-4 December 2004, Birsa Agricultural University, Ranchi, Jharkhand, India.

Steele K.A. & Witcombe J.R. Marker evaluated selection: evaluating allele frequencies in rice selected under extreme environments to find agronomically important loci. Oral presentation presented at Interdrought II, Rome, Italy 24-28 September 2005.

Glossary

UAS	University of Agricultural Sciences
BAU	Birsa Agricultural University
BC	Backcross Population
CAZS-NR	CAZS Natural resources
Chaite	February sown rice (Nepal)
COB	Client oriented breeding
DUS	Distinctness, uniformity and stability
DFID	Department for International Development
GVT	Gramin Vikas Trust (India)
IRRI	International Rice Research Institute
JIC	John Innes Centre
LI-BIRD	Local Initiatives for Biodiversity, Research and Development (Nepal)
Main	Main rice growing season (Nepal)
MABC	Marker-assisted backcross
MAS	Marker-assisted selection
MES	Marker-evaluated selection
NIL	Near-isogenic (introgression) line
PCR	DNA amplification via polymerase chain reaction
PPB	Participatory Plant Breeding
PY	Pyramid (multiple targets introgressed in the same line)
QTL	Quantitative Trait Loci
RM	Random Mating Population
SSR	Simple sequence repeats or microsatellites
SNP	Single nucleotide polymorphism

Appendix 1. Rice crosses made in this project and their current status.

Cross ID	**Parent 1**	**Parent 2**	**F_2 Produced**	**Generation 2006**	**Location of field testing**	**Current status**
CAZS01-004	KIII/Azucena //KIII///KIII	Ashoka 200F	2002	F_8	Eastern India	Pure line: K224
CAZS01-005	KIII/Azucena //KIII///KIII	Ashoka 200F	2002	F_8	Eastern India	Pure lines: K225 K228, K229
CAZS02-007	Judi 582	Barkhe 2026	2003	F_3	Nepal	F_2 sent to Nepal in Jan 2004 - dropped
CAZS02-008	Judi 582	Barkhe 2027	2003	F_5	Nepal	F_2 sent to Nepal in Jan 2004
CAZS02-009	PBRC78	Ashoka 228	2004	F_5	Eastern India	Selections made
CAZS02-010	Komal 13	Ashoka 200F	2003	F_5	Eastern India	Selection made
CAZS02-011	RRU95001	Ashoka 200F	2003	F_7	Eastern India	Selections made
CAZS02-012	Sathi 34-36/Kalinga III	Ashoka 228	2003	F_5	Eastern India	Selections made
CAZS02-019	IR55423-01	Barkhe 2027	2003	?	Eastern India	
CAZS02-020	CT6510-24-1-2	Barkhe 2027	2003	F_4	Eastern India	Rejected in 2005
CAZS02-025	Ashoka 200F	Judi 582	2003	F_4	Nepal	Dropped in Nepal but suggested for India
CAZS02-026	Judi 582	Ashoka 200F	2003	F_4	Nepal	Dropped in Nepal but suggested for India
CAZS02-027	Komal 13	Barkhe 1027	2004	?	Eastern India	
CAZS02-028	Judi 582	Judi 101	2004	?	Nepal	F_2 sent to Nepal in Jan 2004
CAZS02-030	Judi 582	Sugandha-1	2004	?	Nepal	F_2 sent to Nepal in Jan 2004
CAZS02-043	Sugandha 1	Py2F2 #1, Py2F2 #8 BC 21-01-03-03-46	2003	F_5	Nepal	Bulk and line selections made
CAZS02-047	Ashoka 228	CT6510-24-12	2004	Cross failed		Dropped
CAZS02-048	IR55423-01	Ashoka 228	2004	Cross failed		Dropped
CAZS030-57	Ashoka 200F	Ashoka 238	2004	F_4		Population advanced in glasshouse: 700 lines
CAZS030-58	Ashoka 238	Ashoka 228	2005	F_2	India	130 F_2 available
CAZS040-62	Sugandha 1/ PY2F_2 #1	Sugandha 1	2005	F_2	India	110g F_2 available
CAZS040-63	Jire Chobo	Barkhe 2045	2005	F_2	Nepal	110g F_2 available
CAZS040-64	Gutra local	Ashoka 228	2006	F_2	India	35g F_2 available
CAZS040-65	Gutra local	Ashoka 200F	2006	F_2	India	130g F_2 available