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RICE BREEDING AND INHERITANCE OF HERBICIDE RESISTANCE IN CLEARFIELD RICE (*Oryza sativa* L.)

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ABSTRACT

An ethyl methane sulfonate–induced mutation of the acetolactate synthase. The first two herbicide-resistant rice varieties available for commercial production in southern United States were the imidazolinone-resistant varieties 'CL 121' and 'CL 141' by Clear Field. Six modern varieties as OM2717, AS996, OM2395, OMCS2000, IR64, OM1490 from CLRRI and seven lines from Clear Field Company (B1, B2, B3, B4, B5, B6, B7 carrying herbicide resistance gene) were crossed in all possible combinations without reciprocals. These crosses segregated into 3:1 resistant to susceptible, suggesting that a dominant gene controls resistance to herbicide. This was confirmed by reaction of F_3 progenies, which segregated into 1:2:1 homozygous resistant to segregating to homozygous susceptible. Polymorphic marker RM267, RM262, RM285, RM256, RM271, RM44, RM261 detected target gene in the progenies. Five lines namely OM5712, OM5727, OM5728, OM5749, OM5755 exhibited survival ability when herbicide applied. Forty-three lines have reduced to survive under imazapyr stress (40-95% died). OMCF9 (OM 2395 / B2) and OMCF6 (IR64 / B1) obtained the highest yield. They exhibited their tolerance to imazapyr herbicide.

Key words: Clearfield rice, herbicide resistance, imidazolinone

INTRODUCTION

The use of resistance varieties is the most logical and economical way of reducing herbicide in rice. Rice varieties with resistance to herbicides representing three separate modes of action are currently in advanced breeding programs and certified seed production. Commercial development is under way for three herbicide resistant rice varieties: Clearfieldt4 (imidazolinone resistant), Roundup Readyt (glyphosate resistant), and LibertyLinkt (glufosinate resistant). The respective varieties will be resistant to the imidazolinone, glycine, and phosphinic acid chemical families (Schmidt 1997). The transgenes for glyphosate resistance are derived from a plant or a bacterial source and encode for an alternative enzyme with an active site that is not inhibited by the herbicide (OECD 1999a). The transgene for glufosinate resistance was derived from a soil bacterium (Michiels and Johnson 2001; OECD 1999b). An ethyl methane sulfonate–induced mutation of the acetolactate synthase. Some new varieties' development: the first herbicide-resistant rice available for commercial production in southern United States was the imidazolinone-resistant varieties 'CL 121' and 'CL 141', which were planted in 2002 (Carlson *et al.* 2002; DC Mazour, personal communication). New imidazolinone resistant varieties ('CL 161' and 'Clearfield XL8') based on the second mutation will be introduced in 2003. Newpatht herbicide (imazethapyr) has been approved by U.S. EPA for use on these varieties. Glufosinate-resistant rice varieties with similar adaptations may be available in subsequent years. Imidazolinone- and glufosinate-resistant rice varieties are also in advanced stages of development in Brazil. Glyphosate-resistant rice varieties are under development, but anticipated availability dates glufosinate- and glyphosate-resistant rice varieties are transgenic plants deriving their resistance from DNA insertions that originated in foreign species. These also fall under the broader term genetically modified organism (GMO). Because DNA in Clearfield rice varieties was modified by chemical mutation breeding, it is not classified as a GMO. Thus, under the current regulatory system, it requires fewer domestic and international approvals than do its transgenic counterparts (David *et al.* 2003)

Therefore, one of the major objectives of rice improvement program at CLRRI is development of improved germplasm with resistance to these herbicides. At present, a number of varieties and breeding lines with resistance to the more important herbicide have already been developed. The objective of this study is to develop new varieties with significant resistance to herbicide.

MATERIALS AND METHODS

The data used in this study were obtained with six modern varieties (OM2717, AS996 OM2395, OMCS2000, IR64, and OM1490) from CLRRI and seven lines from Clear field Company (B1, B2, B3, B4, B5, B6, B7 carry gene with herbicide resistance) that were crossed in all possible combinations without reciprocals. Field experiments were conducted at the Cuu Long Delta Rice Research Institute. The experimental design was a randomized complete block design with five replications. Each plot, 2 m long and 10 m wide, was transplanted with spacing of 20x15 cm. Plot sites were located outside the commercial rice production areas of the respective states. Soil preparation and irrigation management were typical for the rice agricultural systems of CLRRI. Evaluation of population lines and the parents of rice was done under imazapyr stress (210 kg ai/ha).The following variables were measured such as plant height, date of first and 50% tillering, date of first, 50%, and last heading (heading defined as date of panicle emergence from the boot). Five panicles per plant and five plants per plot were harvested at physiological maturity. After collection, panicles were carefully transported to a laboratory and rapped ten times against a plastic bucket. The percentage of seeds remaining on the panicle was used to give a seed. Total seed weight, 1000-seed weight, and total seed number were also determined. Panicles were stripped by hand into a bucket; all seed returned to the sample envelope and allowed to dry for 3 days at 50^0C. The seeds were then passed through a small seed lot thresher to separate the blank florets from the seed. Both empty florets and total seed weights were obtained. A sample of 1000 seeds was weighed and the total number of seeds was calculated.

STATISTICAL ANALYSES

Plot means were used for all analysis. In variance analysis, the source of variation involved replications, entries and entry replication interaction that was used as error term. Replications and the entry replication interaction were considered as random effects. The difference between the means was performed using the Duncan's Multiple Range Test.

Data were subjected to analysis of variance (Table 1). Variance components and their standard errors were estimated by equating observed mean squares to their expectations.

Estimates of broad-sense heritability (h^2_B) based on progeny means were computed from variance components.

Genotypic coefficient of variation (GCV%) was estimated according to the method suggested by Singh and Chaudhary (1977).

Table 1. ANOVA for all variables of the progenies.

Source of variation	df	MS	E(MS)
Blocks	b-1	M_b	
Progenies	g-1	M_g	$\sigma^2 + b\sigma^2_G$
Error	(b-1)(g-1)	M_e	σ^2

b: number of blocks; g; number of progenies; M_b: block mean squares; M_g: progeny mean squares; M_e: error mean squares; σ^2_G: genetic variance; and σ^2: error variance.

Performance evaluation

For the performance test, agronomic characteristics such as plant height, panicle length, tillers per hill, spikelets per panicle, spikelets per hill, and grain yield were investigated and compared with those of the original plant in the herbicide resistance and non-herbicide field trials, with three replications per lines. Evaluations of lines were done at maturity. Analysis of variance and mean comparisons of the data from R- S fields were carried out. The t- test value at 5% and 1 % level of significance determined the superiority of the tested lines over.

DNA extraction

DNA suitable for PCR analysis was prepared using a simplified miniscale procedure. A piece of young rice leaf (2 cm) was collected and placed in a labeled 1.5 ml centrifuge tube in ice. The leaf was ground using a polished glass rod in a well of a Spot Test Plate (Thomas Scientific) after adding 400 µl of extraction buffer (50 mM Tris-HCl pH 8.0, 25mM EDTA, 300mM NaCl and 1% SDS). Grinding was done until the buffer turned green, which is an indication of cell breakage and release of chloroplasts and cell contents. Another 400 µl of the extraction buffer was added and mixed into the well by pipetting. Amount 400 µl of the lysate was transferred to the original tube of the leaf sample. The lysate was deproteinized using 400 µl of chloroform. The aqueous supernatant was transferred to a new 1.5 ml tube and DNA precipitated using absolute ethanol. DNA was air-dried and resuspended in 50 µl of TE buffer (10mM Tris-HCl pH 8.0, 1mM EDTA pH 8.0). An aliquot of 1 µl is sufficient for PCR analysis. The remaining DNA was stored in -20^{0}C for any later use.

For the microsatellite assay: PCR amplification was performed in 10mM Tris-HCl (pH 8), 50mM KCl, 1.5mM $MgCl_2$,1 unit of TAKARA Taq, 4 nmol dNTP, 10 pmol primer and 50 ng genomic DNA. The PCR reactions were denatured at 95^{o}C for 5 min, followed by 35 cycles of 94^{0}C for 60 seconds, 55^{0}C for 30 seconds and 72^{0}C for 60 seconds. The final extension was at 72^{o}C for 5 min. After PCR, 13µl of loading buffer (98% formamide, 10mm EDTA, 0.025% bromophenol blue, 0.025% xylene cyanol) were added. Polymorphism in PCR products were detected by ethidium bromide staining after electrophoresis on 5% agarose gels.

RESULTS

Reactions of parents to imazapyr

Almost OMCS2000, IR64, OM2395, OM1490, OM2517, AS996, OM4495 from CLRRI were grown and seven lines from Clear field Company as B1, B2, B3, B4, B5, B6, B7 and PWC 16 as check for herbicide resistance. The results of seven improved varieties from CLRRI plants showed that all were susceptible to imazapyr as expected (Table 1). Plants from B1, B2, B3, B4, B5, B6, B7 were all resistant. However, out of 60 plants of B5 and B1, there were three and five susceptible plants, respectively.

Inheritance of imazapyr resistance

Under imazapyr stress, the reactions of the F_1, F_2, and F_3 progenies from the three crosses were shown in table 2.

The F_1 hybrids from all crosses showed a resistant reaction indicating that resistance to this herbicide in OMCF 9 (OM2395/B2); OMCF6 (IR64/B1; OMCF39 (OMCS2000 / B6) exhibited dominant gene action.

The F_2 progenies from these crosses segregated into 3:1 resistance to susceptible, suggesting that a dominant gene controls herbicide resistance. This was confirmed by reaction of F_3 progenies, which segregated into 1:2:1 homozygous resistant to segregating to homozygous susceptible. The data on the reaction to herbicide of F_1, F_2 populations and F_3 lines of crosses among the resistant varieties are presented in table 2. As expected the F_1 from all the crosses showed a resistant reaction. The F_2 populations from the crosses OM2395 / B2 and IR64 / B1, OMCS 2000 / B6.

Table 1. Reactions of rice cultivars to herbicide (imazapyr)

Cultivar	Reaction = 70 g ai	Reaction >210 g ai	Reaction < 210 g ai
OMCS2000	S	S	S
IR64	S	S	S
OM2395	S	S	S
AS996	S	S	S
OM4495	S	S	S
OM2717	S	S	S
OM1490	S	S	S
B1	R	R	R:S (5)
B2	R	R	R
B3	R	R	R
B4	R	R	R
B5	R	R:S	R:S(3)
B6	R	R	R
B7	R	R	R
PWC 16	R	R	R

Table 2. Reactions of F_1, F_2 and F_3 populations from the crosses to herbicide

Cross	F_1	F_2		χ^2	F_3			χ^2
		R	S	3:1	R	Segregating	S	1:2:1
OM2395/ B2	R	563	205	1.35	36	90	33	1.30
IR 64 / B1	R	530	200	2.24	44	82	30	2.70
OMCS2000 / B6	R	613	213	0.30	36	74	31	1.21

The F_2 populations from the crosses OMCS2000 / B7 segregated into 15:1 resistant to susceptible, while the F_3 lines approximated a segregation ratio of 7:8:1 resistant to segregating to susceptible (Table 3).

Table 3. Reactions of F_1, F_2 and F_3 populations from the cross of OMCS2000 / B7 to herbicide

Cross	F_1	F_2		χ^2	F3			χ^2
		R	S	15:1	R	Segregating	S	7:8:1
OMCS2000 / B7	R	751	50	0.03	69	72	10	0.72

The information obtained in this study is useful for rice breeders in the task of developing improved varieties with multiple resistance to imazapyr. Any one of newly identified genes for herbicide resistance can serve as a source of resistance to this herbicide. Because various genes for resistance to imazapyr are independent, they can be combined in cultivars of improved agronomic background.

In order to exploit the variation in source material as efficiently as possible, the breeder needs to have knowledge of the genetic architecture of the characters to be improved.

Means, ranges, genotypic coefficient of variation (GCV), phenotypic and genotypic variance, and broad-sense heritability estimates for yield and yield components are shown in table 4. Mean yield of population 2 (IR64 / B1) was higher than those of population 1, although population 1 (OM 2395/B2) had better spikelet number per panicle as compared to population 2. In contrast, population 2 had higher average yield than population 1.

Population 1 had slightly higher values for tillering than population 2, while population 2 offered higher plant height, growth duration than population 1.

Table 4. Means, ranges, genotypic coefficient of variation, genotypic variance (σ^2_G), phenotypic variance (σ^2_P), and broad-sense heritability (h^2_B) for breeding populations.

OM2395 / B2	Mean	Range	GCV (%)	σ^2_G	σ^2_P	h^2_{bs}
Plant height (cm)	101.9	100-101.9	7.2	10.37	9.35	0.54
Duration (days)	100.3	100-105	5.8	1.37	2.81	0.49
Panicle No./hill	10.0	9-12	5.9	0.17	0.21	0.83
Panicle length (cm)	25.0	23-27	4.4	2.80	3.20	0.34
Spikelets/ panicle	105.7	100.0-107.5	9.6	203.85	331.74	0.62
1000-grain weight (g)	26.4	25-27	11.8	5.24	6.82	0.77
Yield (ton/ha)	5.7	5.4-5.7	6.8	0.28	0.52	0.54
IR 64 / B1						
Plant height (cm)	101.9	99.8-105.6	10.3	1.49	2.05	0.73
Duration (day)	96.0	93.4-99.9	3.8	9.01	10.76	0.84
Panicle No./hill	13.0	12-13	5.5	9.29	10.75	0.86
Panicle length (cm)	23.7	22.0-25.5	7.0	56.04	71.05	0.47
Spikelets/ panicle	100.0	96.9-105.3	3.2	3.64	6.46	0.56
1000-grain weight (g)	25.3	24.1-26.5	6.2	2.05	2.62	0.78
Yield (ton/ha)	6.7	5.1-7.2	16.0	1.17	1.53	0.76

Genomic DNAs from two populations

The discovery of polymerase chain reaction (PCR) opened a new area of biological research. The impact can be observed not only by the great progress in the field of molecular biology, but also in many achievements in other related fields of science. Molecular biology techniques have been implemented successfully in biology, biotechnology, medical science, diagnostics, and many more. The microsatellite RM267, RM262, RM285, RM256, RM271, RM44, RM272 and RM261 were tested to detect parent and segregation of two crosses (IR64 / B1 and OM2395 / B2). The presence or absence of the associated molecular marker indicates at an early stage, the presence or absence of the desired target gene (Lang *et al.* 2005). Segregating populations were used to confirm co- segregation between SSR markers and use for marker-assisted selection for herbicide resistance gene. Polymorphic marker RM267, RM262, RM285, RM256, RM271, RM44, RM261 detected target gene in population OM2395/B2 (Figure 1.A). Then polymorphic markers RM262, RM285, RM271, RM44, RM261 did in IR64/B1 (Figure 1.B). Lane 1 presented B1 and lane 2 as OM 2395. This study suggested that electromorph size polymorphism is an adequate measure of genetic difference in studies involving closely related individuals. When phylogenetic or evolutionary inferences are being made over longer time scales, evaluation of SSR variation at the sequence level is essential. DNA markers can increase screening efficiency in plant breeding programs because of their ability to screen at the seedling stage for all traits.

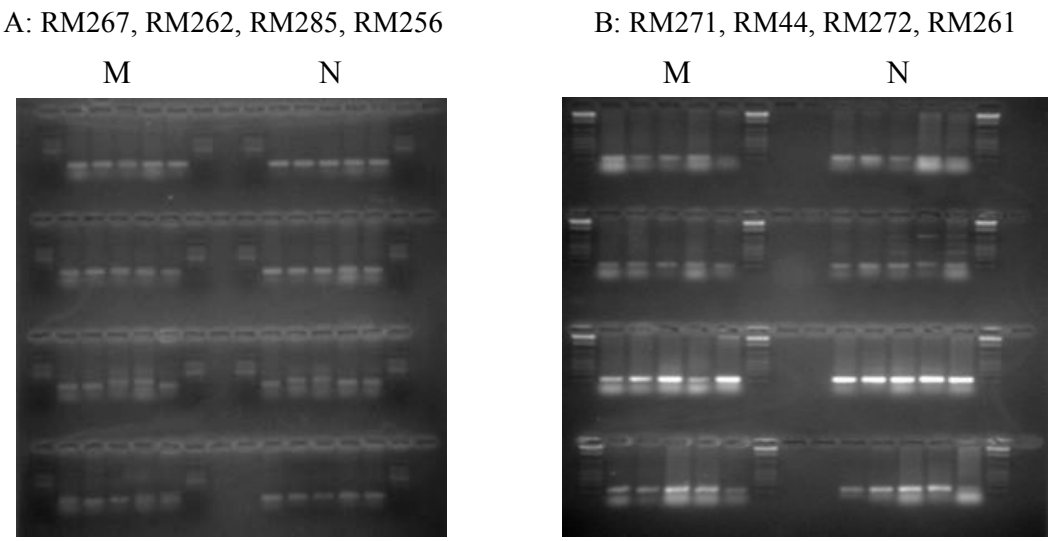

Figure 1. Polymorphism analysis for parents, F_1, and F_2 populations of two crosses OM 2395/ B2 and IR64 / B1.

Reactions of elite rice lines / varieties in field condition

The parents as controls and progeny populations were evaluated on herbicide resistance in the field. The progenies of five crosses were tolerant and there were considerable variation among the lines in terms of tolerance.

Five lines were selected for further observation. For the performance test, the agronomic characteristics such as plant height, panicle length, number of tillers per hill, spikelets per panicle and grain yield were investigated and compared with those of the original plant. Analysis of variance and mean comparisons were carried out. The t-test values at 5% and 1% level of significance determined the superiority of the tested lines over varieties control. Almost varieties had early flowering, early growth duration (90-100 days), short plant stature. They yielded from 5 to 6 tons/ha in dry seasons. Five lines OM5712, OM5727, OM5728, OM5749, OM5755 exhibited survival ability when herbicide applied. An herbicide-resistant rice variety would be a strong selective advantage for these hybrid families' resistances. Generally, in this study, increased panicle number/hill and filled grains were recognized. Forty-three lines have reduced to survive with stress for imazapyr (40-95% died)

Yield testing on rice varieties

This aims to select the rice varieties with high yield potential, insect pest resistance, adaptation to the condition in the Mekong Delta and to find out the rice varieties with desirable traits for hybridization in rice improvement programs.

Experiments included 13 rice varieties with PWC 16 as check, grown in dry season 2006. Yield testing experiments were laid out in randomized complete block design with three replications in CLRRI's experimental plots. Statistical analysis was cited (Gomez and Gomez 1982). Data recorded on agronomic characters, yield and yield components were also guided as SES (IRRI 1996). Insect and disease reactions were informed by Plant Protection Department's screening. Evaluation of insect and disease reaction was done at seedling stage (IRRI 1996). Agronomic characters were presented in table 6.

Table 5. Agronomic characters, insect and disease reactions (dry season 2006)

Designation	Duration (days)	Height (cm)	Panicle No. /hill	1000-grain weight (g)	Yield (t/ha)	Reaction* (1-9)	
						BPH	Blast
OMCF9	100	105.3c	7.67ab	25.24g	6.6	3	3
OMCF6	95	98.3e	7.67ab	26.30de	6.7	3	3
OMCF46	100	110.2b	8.33a	25.90f	6.7	3	3
OMCF39	100	105.0c	6.33ab	27.32a	7.1	5	5
AS996	100	104.7c	7.00ab	26.60cd	6.2	3	3
OMCF47	100	104.4c	6.00ab	25.31g	6.7	5	5
OMCF48	95	109.5b	8.33a	26.76bc	7.0	5	5
OMCF24	100	101.40d	7.33ab	26.14ef	6.2	5	5
OMCF17	100	104.80c	6.67ab	26.60cd	6.6	5	5
PWC16	105	104.73c	5.67b	27.31a	6.7	7	5
OM2395	95	114.27a	6.67ab	27.01ab	7.1	3	3
OMCS2000	95	110.40b	7.33ab	26.81bc	6.5	5	5
OM1490	90	90.27f	6.67ab	26.22ef	6.4	5	7
CV (%)		0.84	18.73	0.68			

* Standard Evaluation System for rice. 1996

Table 6. Agronomic characters, insect and disease reaction (Wet season 2006)

No	Designation	Origin	Duration (days)	Plant height (cm)	Reaction (1-9)	
					BPH	Blast
4	OMCF9	OM2395 / B2	105.8	102.3	3	5
6	OMCF6	IR64 /B1	98.5	98.7	3	3
3	OMCF46	OMCS2000 / B7	100.0	101.4	3	3
1	OMCF39	OMCS2000 / B6	106.4	100.2	3	3
2	AS996 (check)	IR64 / *O. rufipogon*	107.2	98.7	3	3
7	OMCF47	AS996 / B7	105.7	99.5	7	5
8	OMCF48	OM2717 / B7	103.4	94.3	7	5
5	OMCF24	OM1490 / B4	105.2	102.4	7	3
10	OMCF17	OM1490 / B3	103.6	104.3	7	5
9	PW26	ClearField	105.4	98.6	5	5

It indicated that growth duration of tested varieties were less than 100 days. Plant height ranged from 104.3 cm for OMCF17 to 94.3cm for OMCF48.

OMCF47, OMCF48, OMCF24, OMCF17 were susceptible to brown plant hopper (BPH) (score 7), other rice varieties were as moderately resistance to BPH and blast (Bl) as AS996 (check). It indicated that these rice varieties would be planted by farmers if they have high yielding and good grain quality.

Table 7. Yield and yield components of OMCF varieties (wet season 2006)

No	Designation	Panicle No./hill	Filled grains /panicle	Unfilled grains (%)	1000-grain weight (g)	Yield (t/ha)
1	OMCF9	12.3	110.4	10.3	26.1	5.80
2	OMCF6	12.3	112.9	9.6	25.6	5.80
3	OMCF46	10.2	113.6	9.7	25.7	5.70
4	OMCF39	10.5	108.8	11.3	26.3	5.60
5	AS996 (check)	11.1	112.2	10.4	26.0	5.60
6	OMCF47	11.6	100.4	9.8	25.0	5.60
7	OMCF48	10.5	112.4	12.5	25.9	5.20
8	OMCF24	9.5	109.4	11.4	26.7	5.20
9	OMCF17	10.8	114.7	8.7	25.4	4.20
10	PWC16	6.1	109.6	12.6	25.6	2.60
	CV%	10.3	10.5	9.7	-	7.20
	LSD5%	3.4	20.4	2.6	-	0.73

Grain yield and yield components of 10 varieties were presented in table 7. It was indicated that OMCF9 and OMCF 6 obtained the highest yield in experiment (5.8t/ha); higher than AS996 check but not-significant difference. Panicle/hill of OMCF 9 and OMCF 6 were the highest (12.3). Filled grains /panicle were 114.7 for OMCF17. These rice varieties as OMCF 17 and PWC 16 were significantly lower yield than check (Table 7).

Figure 3. Some susceptible and herbicide-resistant rice lines

CONCLUSION AND SUGGESTION

Results of analysis of variance for the 12 traits indicated that significant amounts of genetic variability exist among the progenies for almost all traits in both populations. This variability could be exploited for future improvement of the essential traits in the populations. The wide range of phenotypic variability existing for yield in both populations might be due to the fact that this trait was easily influenced by environment.

From the genotypic coefficient of variation (GCV) analysis, low GCV values were recorded for yield, yield components, in population 1. This could be due to the effect of continuous selection on the parental. That emphasized on these traits in the past, resulting in the reduction of genetic variability in these traits. To broaden the genetic variability for these traits in populations 2, introgression of new genes from germplasm collected in the future. On the other hand, moderate GCV values were exhibited for spikelet and panicle/ plants in both populations. This suggests that a lower progress from selection is expected in succeeding generations. Selection should be practiced using these traits.

These results showed that most of the yield and yield components in these populations are strongly controlled by genetic factors. Moderate to high broad-sense heritability estimates revealed for plant height and yield components, indicate that the traits offered highly genetic influence, and selection based on them could produce good response if their variability could be improved in the populations.

DNA markers can facilitate screening efficiency in plant breeding programs at seedling stage for all traits. Some promising rice varieties will be continuously tested in next season.

OMCF6, OMCF9, OMCF46, OMCF39 were the highest yield genotypes, resistant to herbicide, BPH and Bl. It should be recommended to be tested and evaluated at many sites to stability performance. This is one of the most desirable properties of a genotype to be released as a variety in large-scale cultivation

LOOKING TO THE FUTURE

- To backcross between selected OMCF to recurrent parents.
- To continue screening to pests and diseases.
- To apply MAS for herbicide tolerance in selected lines.

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Nghiên cứu cơ sở di truyền và chọn tạo giống lúa kháng thuốc cỏ imidazolinone

Sử dụng đột biến bằng tác nhân hóa học ethyl methane sulfonate thuộc nhóm "acetolactate synthase" để tạo ra nguồn vật liệu có khả năng kháng thuốc cỏ imidazolinone trên giống lúa 'CL 121' and 'CL 141' do Clear Field, Hoa Kỳ sản xuất. Sáu giống lúa cao sản của CLRRI là OM2717, AS996, OM2395, OMCS2000, IR64, OM1490 được lai với các dòng đột biến Clear Field (BASF company) (B1, B2, B3, B4, B5, B6, B7 mang gen kháng thuốc cỏ). Tỉ lệ phân ly tính trạng kháng và nhiễm là 3:1 ở F_2 cho thấy một gen trội điều khiển tính trạng mục tiêu. Xác định lại quần thể phân ly F_3, tỉ lệ 1:2:1 được kiểm chứng. Những microsatellite marker đa hình RM267, RM262, RM285, RM256, RM271, RM44, RM261 liên kết với gen mục tiêu trong quần thể con lai cho phép thực hiện MAS. Có 5 giống OM5712, OM5727, OM5728, OM5749, OM5755 biểu thị mức độ sống sót cao khi xử lý thuốc cỏ. 43 dòng con lai suy giảm mức độ sống sót khi xử lý imazapyr (40-95% chết). Bằng phương pháp hồi giao cải tiến, Viện Lúa ĐBSCL đã chọn được OMCF9 (OM 2395 / B2) và OMCF6 (IR64 / B1) cho năng suất cao nhất và biểu hiện tính kháng với stress do imazapyr.