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RESEARCH ARTICLE

Marker Assisted Selection of xa5, xa13 and Xa21 Gene in Breeding Populations Derived from Karma Mahsuri x IRBB 59

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ABSTRACT

Bacterial leaf blight, caused by the Gram negative bacterium *Xanthomonas oryzae* pv. *oryzae* (Xoo), is a serious disease throughout the rice growing world. Resistant cultivars are the primary and most effective means of control. Marker assisted selection (MAS) can help in screening more efficiently for the presence or absence of resistant genes. Molecular markers have made it possible to identify and pyramid valuable genes of agronomic importance in resistance rice breeding. In the present study, to incorporate durable resistance against bacterial blight three resistance genes, xa 5, xa13 and Xa21, from an indica donor IRBB 59 were introgressed into high yielding susceptible rice cultivar Karma Mahsuri. Karma Mahsuri is one of the most popular varieties of Chhattisgarh and mega varieties of India. These three genes were pyramided through marker-assisted breeding. For MAS of *xa5*:- RG556, RM122, RM390, RM13; *xa13*:-RG136 and RM 230 and *Xa21*: Xa21 and RM21 are the known linked markers. Markers xa5R and xa5S specific for xa5 resistant and susceptible genes respectively, xa13Pro for xa13 gene and PT248 for Xa21 gene obtained from Dr Sundaram (DRR, Hyderabad) were also used in the present study for MAS. High-resolution maps generated *in silico* around *xa5* and *xa13* will be useful for the precise placement of a gene of interest and the analysis of regional and sub-regional rates of recombination and appropriate combinations of markers for marker assisted selection in plant-breeding. In Karma Mahsuri X IRBB 59 cross we got Three lines (03) containing three gene (*xa5, xa13 and Xa21*), Twenty three (23) line contain a combination of *xa5* & *xa13*, only one (01) with *xa5* and *Xa21*. There were eight lines with *xa5* gene Seventeen (17) lines with *xa13* gene. We therefore report herein the development of nil, two and three gene pyramids of xa5, *xa13* and *Xa21* in the background of Karma Mahsuri.

Key words: Bacterial blight (BB), Broad-spectrum resistance, Gene pyramiding marker-assisted selection (MAS), Rice.

INTRODUCTION

Bacterial blight disease (BB) of rice, caused by Xanthomonas oryzae pv. oryzae (Xoo), is one of the most destructive diseases of rice affecting production in irrigated and rainfed lowland ecosys-tems throughout Asia, northern Australia, Africa, southern part of the United States and Latin America (Mew, 1987; Séré et al., 2005).It is a limiting factor to rice yield in all major rice-growing regions of the world. In some areas of Asia it can reduce crop yield by up to50% and also affects grain quality (Noh et al., 2007).Chemical control for BB is not effective. This may be because the pathogen population is highly variable in its sensitivity to the antibiotics used for control. Therefore, the utilization of rice varieties carrying resistance genes proves to be one of the most economical, effective and environment-friendly strategies for the management of rice BB (Zheng et al., 2009). To date, at least 38 BB resistance genes conferring host resistance against various strains of Xoo have been identified (Bhasin et al. 2012). All these resistance genes follow a Mendelian pattern of major gene inheritance and express resistance to a diverse group of Xoo pathogens (Cheema et al. 2008; Gu et al. 2005; Korinsak et al. 2009; Lee et al. 2003; Sun et al. 2004).

Gene pyramiding is a very useful approach to utilize existing genetic resources. It has been successfully applied in several crop breeding programs, leading to the development and/or release of many varieties and lines possessing multiple attributes (Huanget al., 1997; Jiang et al., 2004). Through gene interaction and complementation, lines with pyramided genes were found to increase resistance quantitatively and provide a wider spectrum of resistance over those conferred by single genes (Yoshimura et al., 1995;Singh et al., 2001). Pyramiding resistance genes can be very difficult using conventional methods of breeding due to epitasis or masking effects of other genes (Raj Purohit et al., 2011). However, selection of plants with homozygous resistance loci will be possible at any growth stages if DNA markers linked with the target genes are available. Marker assisted selection (MAS) for pyramiding important genes along with rapid background recovery of the recurrent parent, while maintaining the exquisite quality characteristics of rice, could be an effective approach for rice improvement (Shanti et al. 2010; Singh et al. 2001; Suh et al. 2009a; Suh et al. 2011; Sundaram et al. 2008;).

Three BB resistance genes (xa5, xa13 and Xa21) were pyramided in cultivar PR106 using MAS. With the availability of tightly linked PCR-based markers for target genes Xa4, xa5, xa13 and Xa21 (Ronald et al. 1992; Yoshimura et al. 1995; Zhang et al. 1996) and a number of markers for background selection, precise pyramiding of the target genes is feasible in minimum number of backcrosses with significant savings in time, labour, space and money. We have used marker-assisted backcross breeding to introgress the xa5, xa13 and Xa21 resistance genes into the Karma mahsuri breeding lines. The study also provides evidence for the accuracy and consistency with which the methodology can be applied on large scale in MAS.

MATERIALS AND METHODS

Plant materials

Experimental materials consisted of IRBB59 (a near isogenic line in the background of IR24) carrying three BLB resistant genes xa5, xa13 and Xa21 which served as the donor for these crosses. The recipient parents included Karma Mahsuri. F3 lines (79 Lines), derived from the cross between Karma mahsuri x IRBB59 and was kindly provided by Dr. Ravindra Verma, Assistant Professor, Department of Genetics and Plant Breeding (COA,IGKVV,Raipur) were used as segregating breeding population for marker assisted selection.

Bacterial Culture (*Xanthomonas oryzae* pv *oryzae* isolates)

The isolate of ***Xanthomonas oryzae*** pv. ***oryzae*** (Xoo) collected from Dhamtari (by Dr. A. S. Kotasthane, Professor and Head of the department of plant pathology, COA,IGKVV, Raipur) was used for evaluation and genetic analysis of F3 segregating breeding populations and three parents.

Bacterial blight screening

Individual plants of 79 line of Karma Mahsuri x IRBB 59 derived F3 breeding segregating population were grown in the field. These segregating population and two parent were evaluated for field infection to bacterial blight. The rice plants were inoculated with Xanthomonas oryzae pv. oryzae isolates at maximum tillering stage. Scissors clipping at 5 cm below the leaf tips inoculated the leaf blades. Observation on reaction to bacterial leaf blight was recorded by physical measurement of lesion length to that of the leaf length and percent leaf area was worked out. Disease score was evaluated 21 days after inoculation. The lesion length and total leaf length were recorded on 5 leaves and were further categorized based on 0-9 score (IRRI, 1998). The disease score were rated as HR, R, MR, S, HS

DNA isolation

The leaves were cut into pieces and transferred into 2.0 ml micro centrifuge tubes up to 0.5 ml marking. Two beads were placed in each tube. EBA (100mMTris-Hcl,PH-

8,20Mm EDTA, PH-8, 100Mm NACL,4% PVP) 200 µl, EBB(100mMTris-Hcl,PH-8,50Mm EDTA, PH-8, 100Mm NACL) 600 µl and 80 µl of 20% SDS was added to the samples and homogenized in tissue lyser. The samples were vortexed and incubated at 65oC for 10 min. Then 200 µl of chloroform: isoamyl alcohol (24:1) was added and the mixture was shaken gently by inversion for 5 min.The tubes were centrifuged at 13000 rpm for 10 min. to separate the phase. After centrifugation, the upper phase was transferred to new tubes.

The chloroform extraction step was repeated one more time. Then 2/3rd volume of pre-chilled isopropanol was added and incubated at -20ºC for 30 min or longer until DNA was precipitated. The tubes were then centrifuged for 10 min. at 13000 rpm. DNA pellet was saved and supernatant was discarded. The DNA pellet was washed with 70% EtOH and air dried for 10 min. Resuspended the DNA pellet in 50 µl of TE(10mMTris-Hcl,PH-8,1Mm EDTA PH-8) buffer and allowed the pellet to dissolve.Then added 2-5µl of RNase (10 mg/ml) and incubated at 370C for 60 min. Then added 1/10th volume of 3M sodium acetate and 2 volume of pre-chilled absolute ethanol. Mixed gently and incubated at -20ºC for 30 min .The DNA pellet was centrifuged for 10 min at 13000 rpm.The pellet was washed with 70% EtOH by centrifuging the tubes at 13000 rpm for 5 min.The supernatant was discarded and the pellet was air dried till the smell of ethanol vanishes.The pellet was then resuspended in 50 µl of TE buffer and incubated overnight; the volume of TE buffer may depend on size of DNA pellet. Stored the DNAs at -200C until used.

Polymerase chain reaction

A series of optimization experiments using parents and isogenic samples was carried out in which concentrations of template DNA, primers, dNTPs and Taq polymerase were varied to determine which conditions gave the strongest patterns. The PCR reaction mixture of 20 Fl contained 40 ng template DNA,

10µM each primer 1 mM dNTPs, 10X PCR buffer (Readymade PCR buffer was used.) and 1 unit of Taq polymerase.((Axygen make)) The template DNA was initially denatured at 940C for 4 min followed by 35 cycles of PCR amplification under the following parameters: 1 min denaturation at 940C, 1 min primer annealing at 500C to 68 oc and 1 min primer extension at 720C. A final 5min incubation at 720C was allowed for completion of primer extension on Thermal cycler.

Following amplification, the samples were run on 5% SDS PAGE :- Twenty µl of PCR amplified products were mixed with 7 µl of 10X loading dye and loaded on 5% SDS PAGE (UREA PAGE) sequencing gel in 1X TBE buffer along with 100 bp ladder for 25 min at 800 volts. For the amplified products of RG136, 5 µl of PCR product was used for gel electrophoresis to determine the success of PCR. The remaining product was used for restriction digestion. The reaction mixture consisted of 0.5 µl (10 U/µl) of restriction enzyme (Hinf I), 2.0 µl of 10X restriction buffer, 2.5 µl of sterile distilled water and 15 µl of PCR product. Then incubated it for 4 h at 37ºC and the products of restriction digestion were separated by gel electrophoresis (5% PAGE) and visualized under UV after staining with ethidium bromide.

Data Scoring

The bands appeared in the primers were scored in the form as P1, P2 and H where stand for P1= susceptible parent P2= resistant parent H= Heterozygous respectively.

In-silico analysis

Sequences alignment of the molecular markers sequence with the BAC/PAC clone contig (pseudomolecules built 6) were used to land the molecular markers on the BAC/PAC map. Sequences of the BAC/PAC were then analyzed for SSR loci using Batch primer 3 (http://probes.pw.usda.gov/cgi-bin/batchprimer3/) which runs through the web interface and locates microsatellite patterns in FASTA formatted sequence files and reports the Seq ID, sequence coordinates for each SSR, SSR motifs (di to hexa), motif length and SSR length.

Primer designing was done by putting the individual BAC/PACs sequences in FASTA format with desired specifications for GC content, annealing temperature (Tm values), primer length and length of amplified fragments with other parameters as default setting.

RESULTS AND DISCUSSION

Reaction of germplasm to bacterial blight Pathogen

The F3 population derived from cross between IRBB59 and Karma Mahsuri classified as Susceptible (S) or Resistant (R) according to reaction. Out of the 79 plants are on the basis of phenotypic observation (lession length) we select 64 plant resistance and 15 plants susceptible for genotyping (Table 1).

Table 1: Reaction of germplasm to bacterial blight Pathogen

Line No	Score	Line No	Score	Line No	Score	Line No	Score
P1	HS	20	HR	41	HR	62	HR
P2	HR	21	HR	42	HR	63	HR
1	HR	22	HR	43	HR	64	HR
2	HR	23	HR	44	HR	65	HS
3	HR	24	HR	45	HR	66	HS
4	HR	25	HR	46	HR	67	HS
5	HR	26	HR	47	HR	68	HS
6	HR	27	HR	48	HR	69	HS
7	HR	28	HR	49	HR	70	HS
8	HR	29	HR	50	HR	71	HS
9	HR	30	HR	51	HR	72	HS
10	HR	31	HR	52	HR	73	HS
11	HR	32	HR	53	HR	74	HS
12	HR	33	HR	54	HR	75	HS
13	HR	34	HR	55	HR	76	HS
14	HR	35	HR	56	HR	77	HS
15	HR	36	HR	57	HR	78	HS
16	HR	37	HR	58	HR	79	HS
17	HR	38	HR	59	HR		
18	HR	39	HR	60	HR		
19	HR	40	HR	61	HR		

Selection of previously known molecular markers for validation on Karma Mahsuri X IRBB59 derived population

Microsatellite markers provide the high levels of polymorphism needed to follow genomic segments through the narrow crosses and closely related pedigrees of a rice breeding program (Panaud et al. 1996). Out of 36we use 00 markers for selective genotyping and co segregation analysis (Table 2).

Table 2: List of Co-segregating Molecular markers identifying the presence of respective genes

S. No.	Marker	S. No.	Marker	S. No.	Marker
xa5		xa13		Xa21	
1	RM122	1	E6a	1	pTA 248(1)
2	RM13	2	SR 11		
3	RM602	3	SR 6		
4	RM607				
5	RM611				

Identification of map position of eight polymorphic markers For xa5

Identification of map position was accomplished by identifying BAC or PAC clones that simultaneously contained a hit from the microsatellite marker. Forward primer sequences of eight polymorphic marker(s) RM122, RM13, RM602, RM607, RM611, RM601, and RA603, were used for blast analysis to detect the physical position of the molecular markers and the BAC / PAC clones to which they were anchored. In the order of their occurrence on the BAC clones eight markers, RM122, RM601, RM602 and RM607, RM611, RM13, RA603 anchored to BAC clones OSJNBb0035J08 and OSJNBa0068N01 respectively and were used to map and select xa5 in Karma Mahsuri X IRBB59 derivd populations. The physical position of seven polymorphic molecular marker(s) (Start and end of forward primer) RM122, RM601, RM602 and RM607, RM611, RM13 and RA603 covering the xa5 region flanked by RM122 and RM13, on the terminal end of chromosome # 5 accounting for a physical distance of 16,99,979 bases (1699.9Kb) (Table3).

Identification of map position of eight polymorphic markers For xa13

Identification of map position was accomplished by identifying BAC or PAC clones that simultaneously contained a hit from the microsatellite marker. Forward primer sequences of nine polymorphic marker(s) RM23496, RM230, SR6, RM23478, RM23495, RM23503, RM6070, RM6765, ST 9, were used for blast analysis to detect the physical position of the molecular markers and the BAC / PAC clones to which they were anchored. The physical position of nine polymorphic molecular marker(s) (Start and end of forward primer) RM23496, RM230, SR6, RM23478, RM23495, RM23503, RM6070, RM6765, ST 9 covering the xa13 region flanked by RM230 and SR6, on chromosome # 8 accounting for a physical distance of 1152382 bp (605kb) (Table4).

Genetic map construction for xa5, xa13 and Xa21 in Karma Mahsuri x IRBB59 derived breeding population through selective genotyping approach

To determine chromosomal position of the target gene , at first, a total of 5 known SSR markers selected from tarated region of chromosomes 5 and were tested in the 79 selected resistant (64) and susceptible(15) individuals, through the selective genotyping approach. The results showed that the five markers(RM607, RM602, RM13, RM611 and RM122), located on targeted region of chromosome 5, showed positive polymorphisms for the resistance gene in all the 79 selected resistant (64) and susceptible(15) individuals. The same set of 79 selected resistant and susceptible individuals derived from Karma Mahsuri x IRBB59 populations was also subjected to linkage analysis with the 3 SSR markers SR6, E6a, RG136. The result showed that 24, 17, 7 distinct recombinants were identified at SR6, E6a, RG136 loci, respectively defining the xa13 with genetic distance of 15.19, 10.76, 4.43 cM respectively (Table4). Similarly the same set of 79 selected resistant and susceptible individuals derived from Karma Mahsuri x IRBB59 populations were subjected to linkage analysis with the PTA248(1). The result showed 4 distinct

recombinants were identified at PTA248(1) defining the Xa21 with a genetic distance of 2.53 cM (Table 5).

A total of 64 selected resistant individuals derived from Karma mahsuri x IRBB59 populations were subjected selective genotyping with SSR markers to determine the presence of xa5, xa13 and Xa21 in Karma Mahsuri X IRBB59 derived selected population. On combining the results of selective genotyping with the segregating molecular markers determining the presence of different gene(s) combinations of xa5, xa13 and Xa21 in Karma Mahsuri X IRBB59 derived selected lines it was observed that xa5, xa13, and Xa21 genes were present in 3 lines, xa5 and xa13 gene combination in 28 lines, xa5 and Xa 21 gene combination was present only in one line. Line #9, 10, 11, 13, 15, 16, 56, 64contained only xa5 and line #1, 3, 6, 21, 22, 26, 27, 32, 33, 35, 36, 41, 43, 45, 52, 61,63contained only xa13 resistant gene (Table 9).

CONCLUSION

The goal of our result is to develop rice variety with multiple-resistance. With increasing number of cloned resistance genes and mapping of useful genes for rice genetic transformation, multiple gene pyramiding is a wise and efficient strategy in rice resistance breeding. Pyramiding multiple genes by means of MAS represents the predominant and effective method in multiple-resistance rice breeding.

RM610 covering the xa5 region accounts for a physical distance of 24369bases was identified useful for MAS.The present study reports a successful transfer of three bacterial blight resistance genes xa5, xa13 and Xa21 into Karma Mahsuri which is most popular varieties of Chhattisgarh.Three lines (03) containing three gene (xa5, xa13 and Xa21), Twenty three (23) line contain a combination of xa5 & xa13, only one (01) with xa5 and Xa21. There were eight lines with xa5 gene Seventeen (17) lines with xa13 gene. We therefore report herein the development of nil, two and three gene pyramids of xa5, xa13 and Xa21 in the background of Karma Mahsuri.

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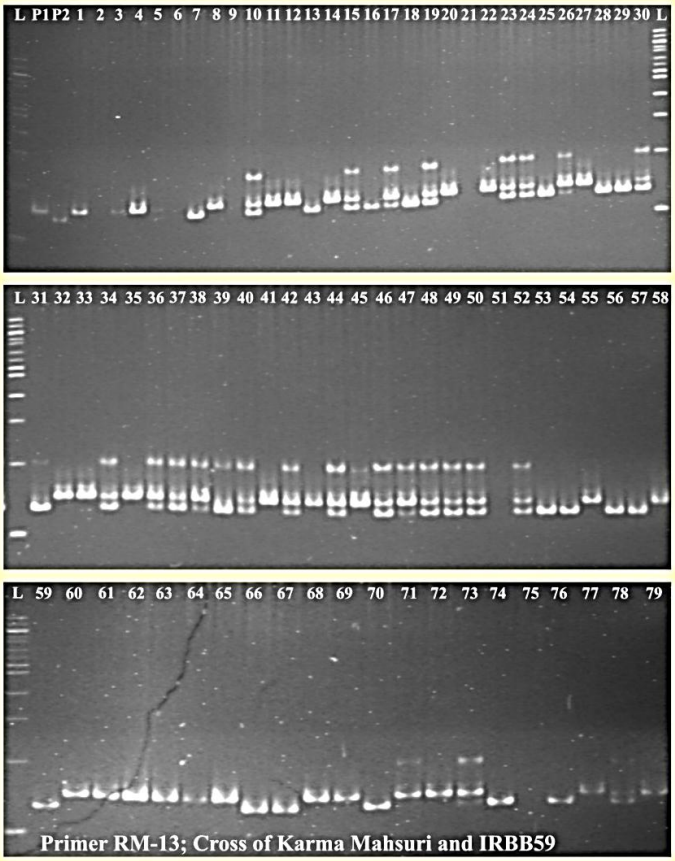

Plate 4.2 PCR amplification of population derived from cross of Karma Mahsuri and IRBB59 with primer RM-13 (*xa5* gene specific primer)

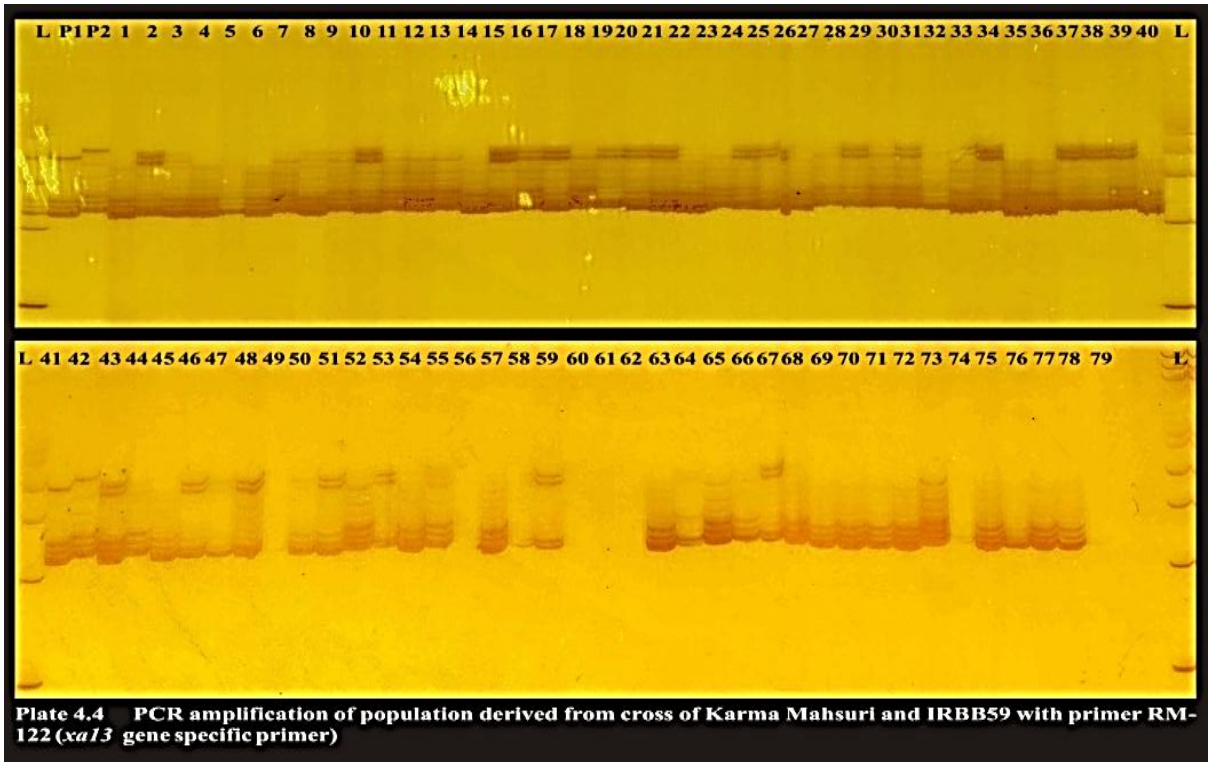

Plate 4.4 PCR amplification of population derived from cross of Karma Mahsuri and IRBB59 with primer RM-122 (*xa13* gene specific primer)

Table 3 : Anchoring molecular markers to a physical position on BAC/ PAC clones on chromosome #5 following blast analysis against pseudo molecules from Nipponbare a japonica genome sequence of rice

	Marker						
Physical Position of markers in (bp)	RM122	RM601	RM602	RA603	RM607	RM611	RM-13
BAC / PAC Clone	OSJNBb0035J08			OSJNBa0068N01			
Start (F Primer)	311189	357437	359145	432938	444773	488061	2011170
End (F Primer)	311209	357455	359167	433078	444792	488080	2011186
Size of primer	20	18	22	20	19	19	16
Distance between F Primer sequences		46246	1708	73793	11835	43288	1523109
Total region		16,99,981 bases (1699.9Kb)					

Table 4 : Identification of physical map position of eight polymorphic markers

	Molecular Marker								
1	RM230	RM23478	RM6070	RM6765	RM23496	RM23495	RM23503	ST9	SR6
2	P0689E12	OJ1770_H02	OJ1770_H02	OJ1770_H02	P0702E04	P0702E04	P0702E04	P0702C09	OSJNBa0033D24
3	25837022	26310189	26322440	26380990	26504554	26507347	26576709	26714889	26989404
4	25837039	26310209	26322463	26381016	26504576	26507370	26576731	26714908	26989423
5	17	20	23	26	22	23	22	19	19
6		473167	12251	58550	123564	2793	69362	138180	274515
7	1152382 bp (605kb)								

1= Physical Position of markers in (bp); 2= BAC / PAC Clone; 3= Start (F Primer); 4= End (F Primer); 5= Size of primer; 6= Distance between F Primer sequences; 7= Total region

Table 5 : Linkage analysis with the five selected SSR markers in 79 selected resistant and susceptible individuals derived from Karma Mahsuri x IRBB59 populations

xa5	RM607	RM602	RM13	RM611	RM122
Co segregating with resistance line	38	42	39	43	36
Co segregating with susceptible line	12	10	9	11	10
Total	**50**	**52**	**48**	**54**	**46**
Recombinant with resistance lines	24	22	25	19	22
Recombinant with susceptible lines	3	4	6	3	2
Total	**27**	**26**	**31**	**22**	**24**
Not Amplification	2	1	0	3	9
Total	**79**	**79**	**79**	**79**	**79**
cM	17.09	16.46	19.62	13.92	15.19

Table 6 : Linkage analysis with the three selected SSR markers in 79 selected resistant and susceptible individuals derived from Karma Mahsuri x IRBB59 populations

xa13	SR6	E6a	RG136
Co segregating with resistance line	45	54	38
Co segregating with susceptible line	9	8	8
Total	**54**	**62**	**46**
Recombinant with resistance lines	18	10	6
Recombinant with susceptible lines	6	7	1
Total	**24**	**17**	**7**
Not Amplification	1	0	26
Total	**79**	**79**	**79**
cM	15.19	10.76	4.43

Table 7: Linkage analysis with the selected molecular markers in 79 selected resistant and Susceptible individuals derived from Karma Mahsuri x IRBB59 populations

Xa21	PTA248(1)
Co segregating with resistance line	4
Co segregating with susceptible line	2
Total	6
Recombinant with resistance lines	3
Recombinant with susceptible lines	1
Total	4
Not Amplification	69
Total	79
cM	2.53

Table 9: Co-segregating Molecular Markers determining the presence of different gene(s) combinations of xa5, xa13 and Xa21 in Karma Mahsuri X IRBB59 derived selected lines

Gene combination	Line number	Freq.
xa5, xa13 and Xa 21	#47,51,54	3
xa5 and xa13	#2, 7, 12, 14, 18, 19, 23, 24, 25, 28, 29, 30, 31, 34, 37, 38, 39, 40, 42, 44, 46, 48, 49, 50, 53, 57, 60, 62	28
xa5 and Xa 21	#17	1
xa5	#9,10,11,13,15,16,56,64	8
xa13	#1,3,6,21,22,26,27,32,33,35,36,41,43,45,52,61,63	17

Table 8 : Co-segregating Molecular Markers determining the presence of xa5, xa13 and Xa21 in Karma Mahsuri X IRBB59 derived selected population

Co segregating Molecular Markers		Line number	Freq.
xa5			
RM 607, RM 602, RM611, RM 122 and RM 13	5	# 2, 7, 9, 10, 13, 16, 17, 18, 19, 23, 24, 25, 28, 29, 30, 31, 34, 37, 38, 40, 46, 47, 51, 53, 54, 56, 57, 62, 64,	29
RM 607, RM 602, RM 611and RM 122	4	#11, 12	2
RM 607, RM 602, RM 611 and RM 13	4	#15, 39, 49, 60	4
RM 602, RM 611, RM 122 and RM 13	4	#42	1
RM 607, RM 602, RM 122 and RM 13	4	#50	1
RM 607, RM 611 and RM 13	3	#48	1
RM 602, RM 611 and RM 13	3	#44	1
RM 602 , RM 611and RM 122	3	#14	1
RM 611 and RM 122	2	#35	1
RM 122 and RM 13	2	#36	
RM 607 and RM 602	2	#63	1
xa13			
SR6, E6a and RG136	3	# 1 2, 3, 6, 7, 12, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 39, 40, 45, 46, 50, 52, 54, 57, 60, 63	29
SR 6 and E6a	2	# 14, 21, 22, 36, 37, 38, 41, 42, 43, 44, 47, 48, 51, 61, 62	15
E6a and RG 136	2	# 15, 16, 17, 18, 19, 20, 53, 58	8
SR6	1	# 56	1
E6a	1	# 8,55	1
RG 136	1	# 64	1
Xa21			
PTA 248	1	# 17,47,51,54	4