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Introgression of effective bacterial blight resistant genes into rice cultivars and hybrid rice parental lines

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ABSTRACT

Using marker-assisted selection in a backcross-breeding program, four bacterial blight resistant genes namely Xa4, xa5, xa13 and Xa21 have been introgressed into the popular hybrid rice parental lines KMR3, PRR78, IR58025B, Pusa 6B and a popular variety Mahsuri. Only foreground selection was done using markers. Conventional breeding strategy was adopted for background selection. The pyramids showed very high level of disease resistance to 10 highly virulent isolates of Xoo. Grain quality parameters of the pyramids were on par with that of the original genotype. This work demonstrates the successful application of marker-assisted breeding for pyramiding four BB resistant genes into multiple backgrounds, simultaneously. These lines can be used directly or can be used as donors of bacterial blight (BB) resistance breeding.

Key words: *bacterial blight, resistance, rice, gene pyramiding, marker assisted breeding*

Bacterial blight (BB) is one of the most devastating diseases of rice across the tropics and semi tropics. Damages range from 6-60% in India, Japan and Indonesia. While seed treatment and phytosanitation practices are recommended, breeding for varietal resistance has been and will continue to be, the most eco-friendly and economical method of control of BB (Devadath 1989).

Long-term cultivation of varieties carrying single resistance gene has resulted in a significant shift in pathogen race frequency and consequent breakdown of resistance (Mew *et al*., 1992). One tangible solution to resistance breakdown is pyramiding of multiple resistance genes in the background of modern high yielding varieties. The probability of simultaneous pathogen mutations for virulence to defeat two or more effective genes is much lower than a single gene (Mundt 1990).

More than 30 resistance genes have been identified and designated in a series from *Xa1* to *xa32* till date (Lin *et al*., 1996 , Khush and Angeles 1999; Lee *et al*., 2003; Xiang *et al*., 2005). DNA fingerprinting studies and pathotype analysis have indicated a significant diversity in the *Xanthomonas oryzae* pv. *oryzae* populations in India and other rice growing countries (Adhikari *et al*., 1995; Shanti *et al*., 2001 and 2008; Gupta *et al*., 2001.

Gene pyramiding is difficult to achieve using conventional breeding alone because of linkage with some undesirable traits that is very difficult to break even, after repeated backcrossings (Young and Tanksley, 1989). When two or more genes are introgressed, phenotypic evaluation is unable to distinguish the effect of individual gene precisely since each gene confers resistance to and combats multiple races of the pathogen. Moreover, in the presence of a dominant and a recessive allele, the effect of the recessive gene is masked. The advent and easy availability of molecular markers closely associated with each of the resistance genes makes identification of plants with multiple genes possible. Earlier efforts elsewhere to generate three-gene pyramids in the backgrounds of PR106 (Singh *et al*., 2001), Pusa Basmati (Singh *et al.*,2003) and Samba Mahsuri by the collaborative work of DRR and CCMB at Hyderabad, India (Sundaram *et al.*, 2008) have been successful.

Considering the importance of rice variety

Mahsuri in the Indian agriculture and the necessity to introduce BB resistance in it, we have adopted marker-assisted backcross breeding to introgress *Xa4, xa5, xa13* and *Xa21*. MAS is particularly useful in the present breeding program, since resistance of *xa5* and *xa13* is manifested in recessive condition.

Hybrid rice is a beneficial option to increase productivity of rice, but it is susceptible to a wide range of pests and diseases. The susceptibility period for bacterial blight is longer in hybrid rice than in high yielding rice varieties. KMR3 is one of the best restorers for hybrid rice breeding programs and is the female parent of KRH2 one of the most popular non-aromatic hybrids. PRR78 is the female parent of Pusa RH10 aromatic hybrid. This hybrid is very popular for its superfine grain and aroma. These two popular lines are highly susceptible to BB and recorded yield losses as high as 40%. IR58025A is one of the most popular CMS line and many hybrids have been developed both in public and private sector using this as the male line. KRH2 is one such example. Pusa 6A is another important CMS line in the aromatic types and is the male parent for Pusa RH10 hybrid.

Pyramiding resistant genes in the restorer lines alone is not enough as the hybrid will have these genes in heterozygous condition and the level of resistance imparted will be reduced. Since, *xa5* and *xa13* are recessive genes and so they have to be introgressed in the female line also. Presently most of the BB resistance genes are available in partial restorer backgrounds, thus making it impossible to transfer them directly into the A lines. This necessitates first to transfer them to the maintainer background and after stabilization; they can be transferred with ease to the male sterile background. Once transferred to the maintainer and the CMS line base, transferring multiple resistance to any other CMS line will be easier without having to face the problem of fertility restoration. Hence, it is necessary to insulate both the parents to be able to get resistance in the hybrid. This necessitates the introgression of BB resistant genes in the backgrounds of maintainers as well as restorers.

Studies conducted to identify the best gene combinations conferring broad spectrum resistance showed that the four-gene combination was the most effective and did not show any sign of breakdown of resistance to various strains of the pathogen from different parts of the country (Shanti *et al*., 2005). Therefore, we attempted pyramiding of four BB resistance genes *Xa4*, *xa5*, *xa13* and *Xa21* through marker-assisted selection (MAS) in a BB susceptible high yielding rice cultivar Mahsuri and parental lines of hybrid rice, maintainers IR58025B and Pusa 6B and the restorer lines KMR3 and PRR78 simultaneously. The main objective of this work was to introgress the four BB resistant genes and insulate against the yield loss without sacrificing the grain characteristics of the parents.

MATERIALS AND METHODS

IRBB60, a near isogenic line in the background of IR24, carrying the four resistant genes *Xa4 ,xa5, xa13* and *Xa21* served as the donor for all the crosses attempted. The recipient parents were Mahsuri, KMR3, PRR78, IR58025B and Pusa 6B, respectively for each cross.

Miniscale DNA isolation for PCR analysis of the parents and backcross progenies was carried out following Dellaporta *et al*., (1983). Three STS markers Npb 181, RG 136 and pTA 248, tightly linked to *Xa4*, *xa13* and *Xa21* and one SSR marker RM 122 tightly linked to *xa5* were used to confirm the presence of each gene and the different combinations. The PCR mixture contained 50 ng of template DNA, 5 picomoles of each primer, 0.05mM dNTPs, 1X PCR buffer(10mM Tris, pH 8.4, 50mM KCl, 1.8mM $MgCl_2$ and 0.01mg/ml gelatin) and 1 U Taq DNA polymerase in a reaction volume of 25µl. Template DNA was initially denatured at 94ºC for 5min followed by 35 cycles of PCR amplification with the following parameters: a 30 sec denaturation at 94 ºC, a 30 sec annealing at 55ºC and a 1 min primer extension at 72ºC. A final extension was done at 72ºC for 5 min. the amplified product of pTA 248 and Npb 181 was electrophoretically resolved on 1.4% agarose gel and visualized under UV.

For the amplified products of RG136, 5 µl of PCR product was used for gel electrophoresis. The remaining product was used for restriction digestion. The reaction mixture consisted of 0.5 µl (10U/ µl) of restriction enzyme (*Hinf I*) , 2.0 µl of 10X PCR buffer, 2.5 µl of sterile distilled water and 15 µl of PCR product. The reaction mixture was incubated for 4 hours at 37ºC and the products of restriction digestion were separated

by gel electrophoresis (1.4 % agarose) and visualized under UV after staining with ethidium bromide.

For *xa5* PCR was carried out using 20ng DNA as template for amplification, 5 picomoles of each primer, 0.05mM dNTPs, 1X PCR buffer, and 1U of Taq polymerase in a total volume of 15 µl. Template DNA was initially denatured for 94ºC for 5min followed by 35 cycles of PCR amplification with the following parameters: a 30 sec denaturation at 94 ºC, a 30 sec primer annealing at 55ºC and a 1 min primer extension at 72ºC. A final extension was done at 72ºC for 5 min. The PCR products were resolved in 3% agarose gel.

The pyramided lines in the backgrounds of Mahsuri, KMR3 and PRR78 were evaluated for their reaction to BB under glass house conditions using 10 very virulent strains of *Xoo* from Maruteru, West Godavari, Andhra Pradesh, India. The cultures were maintained on modified Wakimoto's semi-synthetic medium (Karaganilla *et al.,* 1973) (per liter: 20 g sucrose, 5 g peptone, 0.5 g calcium nitrate, 1.82 g disodium hydrogen phosphate, 0.05 g ferrous sulphate, 18 g agar, pH 6.8-7).

For long-term storage, the cultures were maintained as glycerol stocks at -70 °C. The stored cultures were revived and grown on modified Wakimoto's medium for inoculation and DNA experiments. The strains used for inoculation were passed through the susceptible cultivar Karuna and re-isolated in the lab before use in inoculation experiments.

Individual plants were grown in plastic pots under flooded conditions with a mixture of soil and farmyard manure (3:1 ratio). The pots were fertilized with N:P @100: 50 kg/ha with P applied as basal dose and top dressing at 25 days after sowing. Approximately forty-day-old plants were clip inoculated (Kauffman *et al.,* 1973). Top three or four leaves of plants at maximum tillering stage were clip-inoculated with a cell suspension of 10^8 cfu ml^{-1} prepared from 48 h old cultures. For each culture-strain combination, five leaves of a plant were inoculated per replication. Each test was replicated thrice. Observations were recorded 15 days after inoculation and lesion lengths were measured to the nearest centimeter for classification of disease response. Each plant was classified as resistant (0-4cm) and susceptible (>4 cm). Grain quality parameters were tested adopting standard methods at the Grain Testing Laboratory at Central Rice Research Institute, Cuttack, India.

RESULTS AND DISCUSSION

Nine F_1 plants from each of the crosses between Mahsuri/IRBB 60 (donor for the four BB resistant genes), KMR3/IRBB60, PRR78/IRBB60, IR58025B/IRBB60, Pusa 6B/IRBB60 were tested for their heterozygosity for the R gene linked markers and were backcrossed using the female parent. The resulting BC_1F_1 lines were first checked for presence of the *Xa21* resistance allele. All plants carrying the resistant allele were checked for the presence of *xa5* allele in heterozygous condition. Plants containing resistant alleles for both the genes were further screened for *Xa4* gene using Npb 181. Finally, the triple positives were screened for the presence of *xa13* allele using the CAPS marker and subjecting them to digestion as described in Materials and Methods (Fig.1). Phenotyping of these target plants was done at the field level and only those showing the maximum similarity to the recurrent parent and showing high yield were selected. This was continued up to BC_3F_1 generation. BC_3F_2 that were screened using the R gene linked markers to identify plants that were homozygous for different R genes or their combinations.

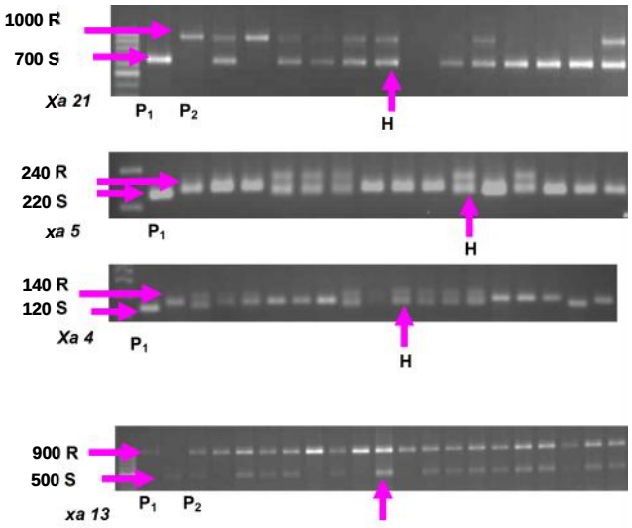

Fig.1. Foreground selection using R gene linked PCR based markers for the four BB genes at BC_1F_1 stage. (Gel photographs showing banding pattern of the markers used in the introgression in Mahsuri. P_1 – Mahsuri, P_2- IRBB60. R indicates Resistant and S indicates Susceptible)

The gene pyramids in the backgrounds of Mahsuri, KMR3 and PRR78 showed a very high degree of resistance as compared to their parents to all the 10 isolates of *Xoo* inoculated upon (Table1). There were varying degrees of resistance to each of the isolates, but no isolate could break down the resistance of any of the four-gene pyramids. In Mahsuri background, isolates 3 and 5 showed a higher level of virulence and isolate 7 was the least virulent (Figure 2). In KMR3 background isolates 4, 6 and 10 showed almost same levels of virulence and the least virulent was isolate 3 (Figure 3). In case of PRR, isolates 6, 7 and 10 showed a high degree of virulence as compared to isolate 9, which was the least virulent (Figure 4). This is indicative that the isolates 6 and 10 showed a similar pattern of virulence in the restorers. Figure 5 shows the comparison between the three pyramids and IRBB60 the donor of all the four BB genes. The isolates showed a varying degree of disease spectrum to all the genotypes. Our earlier studies with these isolates proved that they were highly virulent and even broke down the three gene combination in IR24 near isogenic lines (Shanti *et al*.,2005). These four gene pyramids have been inoculated with 20 isolates collected from different parts of the country but have not shown any sign of susceptibility to any one of the isolates indicating that this is the most effective gene combination to combat the ever changing pathogen population.

Grain quality parameters between the parents and pyramid were comparable and there were no significant differences between the parents and pyramids (Table 2).

The complete recovery of yield and grain quality characters is a very significant achievement since these are multigenic traits encoded by loci distributed across the rice genome (Sundaram *et al*., 2008). The maintainer lines are in BC_4F_1 stage. The lines containing all the four genes showed a very high degree of resistance to isolates from different parts of the country. The yield levels of the four-gene pyramid lines were not significantly different from those of the parents, indicating that there is no yield penalty associated with presence of resistance genes. Under BB infection there was a 20% reduction in yield in the original varieties. This proves that cultivation of the four gene pyramids in BB endemic areas would be of a great advantage to reduce the yield loss. These lines are also being used as donors for transfer of these resistant genes to other backgrounds. This work is the first successful example of the use of molecular markers in foreground selection in conjunction with conventional breeding for simultaneous introgression of genes of interest into multiple backgrounds.This work demonstrates that marker assisted backcrossing can be successfully utilized for simultaneous multiple gene introgression.

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Table 1. Reaction of the 10 isolates from Maruteru to the parents and pyramids

BB resistant genes	Isolates from Maruteru				Lesion length in cm					
	Xoo 1	***Xoo 2***	***Xoo 3***	***Xoo 4***	***Xoo 5***	***Xoo 6***	***Xoo 7***	***Xoo 8***	***Xoo 9***	***Xoo 10***
Mahsuri parent	21.3	20.0	22.3	20.0	18.3	11.0	10.0	17.8	21.0	20.3
Mahsuri pyramid	1.5	2.5	3.0	3.2	3.3	2.0	1.3	2.5	2.3	2.5
KMR3 parent	18.0	20.0	20.0	25.0	21.0	15.0	14.0	14.0	15.0	21.0
KMR3 pyramid	2.0	2.0	1.0	3.0	2.5	3.0	1.5	2.0	2.0	3.0
PRR 78 parent	22.0	24.0	22.0	23.0	22.0	25.0	22.0	21.0	22.0	22.0
PRR78 pyramid	1.8	2.0	1.5	2.0	2.0	2.5	3.0	1.5	1.0	2.5
IRBB60	3.2	2.5	2.6	2.2	2.5	2.3	2.0	3.0	2.0	2.0
Malagkit Sung Song (resistant check)	2.2	2.0	2.8	3.0	1.2	2.0	2.2	1.3	2.0	3.3
TN1 (susceptible check)	20.6	18.0	20.0	16.3	20.0	18.0	23.0	17.5	18.2	25.0

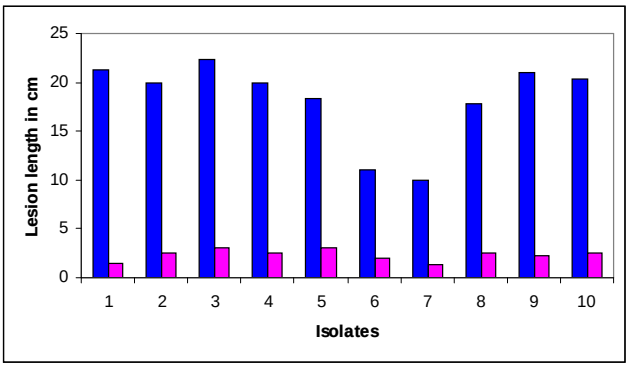

Fig. 2. Level of resistance imparted in Mahsuri pyramid (containing the four BB resistant genes) as against the parent

Fig. 4. Level of resistance imparted in PRR78 pyramid (containing the four BB resistant genes) as against the parent

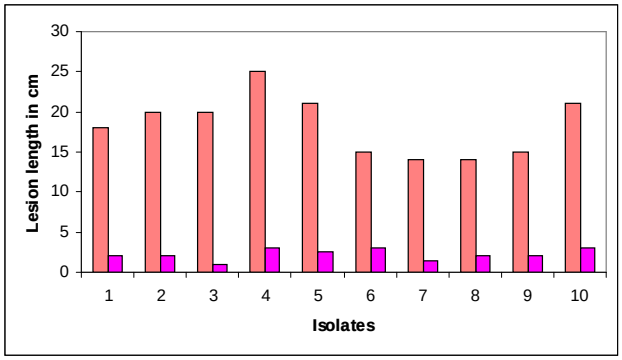

Fig. 3. Level of resistance imparted in KMR3 pyramid (containing the four BB resistant genes) as against the parent

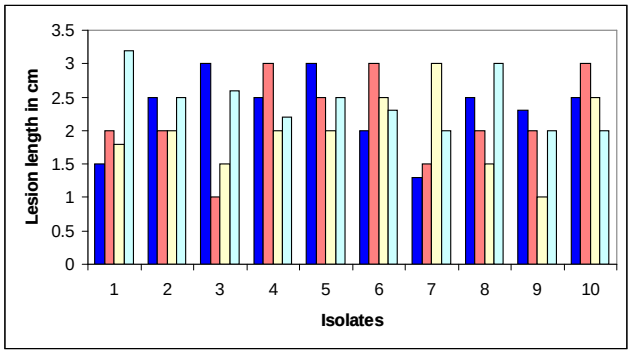

Fig. 5. Comparision of the disease spectrum between the three pyramids and the donor IRBB60.

Table 2. Grain quality parameters of the parents and the pyramids containing the four BB resistant genes

Sample	Hulling %	Milling%	Head rice recovery%	Length (mm)	Breadth (mm)	Length/Breadth ratio	Class
Mahsuri parent	77.50	69.75	65.00	4.64	1.93	2.40	MS
Mahsuri pyramid	78.00	70.50	67.50	4.98	2.08	2.39	MS
KMR3 parent	79.00	69.00	45.00	5.10	2.45	2.08	SB
KMR3 pyramid	79.00	69.00	45.00	5.12	2.40	2.13	SB
PRR78 parent	78.50	67.50	48.00	7.62	1.90	4.01	LS
PRR78 pyramid	79.00	67.00	48.00	7.57	1.86	4.07	LS

Sample	Alkali Spreading Value	Water Uptake	Volume Expansion Ratio	Kernel Length After Cooking (mm)	Elongation Ratio	Amylose %
Mahsuri parent	4.00	160	4.00	8.10	1.75	26.32
Mahsuri pyramid	5.00	130	4.25	9.10	1.83	25.12
KMR3 parent	3.50	200	4.25	9.60	1.88	27.30
KMR3 pyramid	4.00	155	4.25	9.50	1.72	26.50
PRR78 parent	7.00	290	4.00	13.50	1.77	23.78
PRR78 pyramid	7.00	335	4.00	12.75	1.72	24.00

REFERENCES

Devadath S 1989. Chemical control of bacterial blight of rice. In: *IRRI (ed)* Bacterial blight of rice.1989. IRRI, Manila, Phillipines, pp 89-98

Mew TW, Vera Cruz CM, Medalla ES 1992. Changes in race frequency of *Xanthomonas oryzae* pv. *oryzae* in response to the planting of rice cultivars in the Philippines. Plant Dis., 76: 1029-1032

Mundt CC 1990. Probability of mutation to multiple virulence and durability of resistance gene pyramids. Phytopathol. 80: 221-223

Lin XH, Zhang DP, Xie YF, Gao HP, Zhang Q 1996. Identifying and mapping a new gene for bacterial blight resistance in rice based on RFLP markers. Phytopathol. 86: 1156-1159

Khush GS, Angeles ER 1999. A new gene for resistance to race 6 of bacterial blight in rice, *Oryza sativa* L. RGN ., 16: 92-93

Lee KS, Rasabandith S, Angeles ER, Khush GS 2003. Inheritance of resistance to bacterial blight in 21 cultivars of rice. Phytopathol., 93: 147–152

Adhikari TB, Vera Cruz CM., Zhang Q, Nelson RJ, Skinner DZ, Mew TW 1995. Genetic diversity of resistance of *Xanthomonas oryzae* pv. *oryzae* in Asia. Appl. Environ. Microbiol., 61: 966-971

Shanti ML, George MLC, Vera Cruz CM, Bernardo MA, Nelson RJ, Leung H, Reddy J N Sridhar R 2001. Identification of resistance genes effective against rice bacterial blight pathogen in eastern India. Plant Dis., 85: 506–512

Debabrata Nayak, Shanti ML, Bose LK, Singh UD, Nayak P 2008. Pathogenicity association in *Xanthomonas oryzae* pv. *oryzae*, the causal organism of rice bacterial blight disease. ARPN Journal of Ag and Biol Sci., 3: 12-27

Tanksley SD, Young ND, Paterson AH, BonierbaleMW 1989. RFLP Mapping in Plant Breeding:New Tools for an Old Science BioTechnol., 7: 257-264

Shanti ML, Shenoy VV 2005. Evaluation of resistance genes and their pyramids against rice bacterial leaf blight pathogen *Xanthomonas oryzae* pv. *oryzae*. *Oryza* 42, 3: 169-173

Singh S, Sidhu JS, Huang N, Vikal Y, Li Z, Brar DS, Dhaliwal HS, Khush GS 2001. Pyramiding three bacterial blight resistance genes (*xa5*, *xa13* and *Xa21*) using marker-assisted selection into *indica* rice cultivar PR106. Theor Appl Genet, 102: 1011-1015

Joseph M, Gopalakrishnan S, Sharma RK, Singh VP, Singh AK, Singh NK, Mohapatro T 2003. Combining bacterial blight resistance and Basmati quality characteristics by phenotypic and molecular marker-assisted selection in rice. Mol. Breeding, 1-11

Sundaram RM, Vishnupriya MR, Biradar SK, Laha GS, Reddy GA, Shoba Rani N, Sarma NP, Sonti RV 2008. Marker assisted introgression of bacterial blight resistance in Samba Mahsuri, an elite indica rice variety. Euphytica. 160: 411-422

Dellaporta SL, Woo J, Hicks JB 1983. A plant DNA mini preparation: version II. Plant Mol.Biol.Rep., 1: 19-21

Karaganilla A, Paris- Natural M, Ou SH 1973. A comparative study of culture media of *Xanthomonas oryzae* pv. *oryzae*. Phillippines Agriculture., 57:141-152

Kauffman HE, Reddy APK, Hsieh SPY, Merca SD 1973. An improved technique for evaluating resistance of rice varieties to *Xanthomonas oryzae*. Plant Dis Rep, 57: 537-541