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PYRAMIDING OF BACTERIAL LEAF BLIGHT RESISTANT GENES *Xa-21* AND *xa-5* IN RICE BY MOLECULAR MARKER ASSISTED BREEDING

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ABSTRACT

Bacterial Leaf Blight (BLB) caused by *Xanthomonas oryzae* pv. *Oryzae*, is one of the most destructive diseases of rice in Sri Lanka. Cultivation of varieties having multiple resistant genes will minimize the pesticide use while avoiding the risk of disease epidemics. Pyramiding of genes by molecular Marker Assisted Selection (MAS) has proven to be the best practical approach to incorporate more than one resistant gene. In 2003, a popular variety Bg358 was crossed into IRBB60 having *Xa-21, xa-13, Xa-4 and xa-5* resistant genes. Generations were advanced along with the field screening for BLB resistance using most virulent isolate and by selecting lines for desirable agronomical traits. Molecular Marker Assisted Selection (MAS) was carried out in F_5 generation onwards. Specific primers, pTA248 and RG556 (digested with *Dra*1) identified the presence of *Xa-21* and *xa-5* genes in line 1033 of F_7. The same line also showed a high level of field resistance for BLB in F_8 generation. Therefore, it can be concluded that the required level of BLB resistance was achieved in rice line 1033 derived from the cross IRBB60 x Bg358 by pyramiding *Xa-21* and *xa-5* genes.

KEYWORDS: Bacterial Leaf Blight, Gene pyramiding, Rice.

INTRODUCTION

Bacterial Leaf Blight (BLB), caused by *Xanthomonas oryzae* pv. *oryzae* (Xoo) is one of the most destructive diseases of rice throughout the world. The disease contributes to the loss of the entire crop at seedling stage and yield loss up to 50 % at a later stage. Pathogens from different locations having different degree of virulence have been found and there is clear eco-geographic variation among bacterial isolates at molecular (DNA) level (Ou, 1985). Furthermore, any effective agricultural practice or agro-chemical has not been used in the field to control the disease to date. Therefore, the most effective approach to combat BLB is the use of resistant varieties (Kush *et al.*, 1989). Scientists have found that there are genes in some rice varieties that could withstand the disease. These genes are not widely distributed among rice varieties (rice germplasm), instead are restricted to a few (Kush *et al.*, 1989).

The pyramid lines have shown a wider spectrum of resistance than of resistance given by lines having a single resistant gene. The BLB resistant genes *xa-5, xa-13* and *Xa-21* together have shown resistance to all

known pathogens. Pyramiding of these genes could be impossible using conventional methods due to the epistasis and/ or the masking effects (Huang *et al.*, 1997). Therefore, DNA Marker Assisted Selection (MAS) was used to pyramid four BLB resistance genes into rice varieties. A three-gene combination appeared to be the most effective, with *Xa-21* contributing the largest component of resistance. It has been shown that the incorporation of these resistant genes into commercial cultivars is successful and effective in controlling the disease (Kush *et al.*, 1989).

Bg 300, Bg 358 and Bg 352 are some of the popular local rice varieties released by the Department of Agriculture (DOA) which are susceptible to the disease at present (Fernando *et al.*, 2007). An IRRI pyramid line IRBB60, which carries the resistant genes *Xa-4*, *xa-5*, *xa-13* and *Xa-21* has been developed successfully (Huang *et al.*, 1997). The objective of this work was to pyramid BLB resistant genes in IRBB60 into popular local rice varieties by breeding and MAS.

MATERIALS AND METHODS

Breeding programme

A breeding programme was initiated to incorporate resistant genes into the local rice varieties at the Rice Research and Development Institute (RRDI), Batalagoda. IRBB60 was used as the donor parent and crosses were made with the BLB susceptible varieties Bg358, Bg300 and Bg352. Generations were advanced through the conventional breeding and selection. Field screening for BLB was carried out in F_3, F_5 and F_8 generations. Molecular marker screening was carried out for progeny lines in F_5 (Fernando *et al.*, 2007) and F_8 at the Plant Genetic Resources Centre (PGRC), Gannoruwa.

Extraction of DNA

DNA was extracted from leaf samples of fifteen lines collected from F_7 generation in RRDI, Batalagoda using CTAB "miniprep" protocol (ICGEB, 2003) and the pellets were dissolved in 100 µl of Tris-EDTA (pH 8.0) buffer [1 m Tris-HCL (pH 8.0), 0.5M EDTA (pH 8.0)]. DNA samples were quantified by using both UV Spectrophotometer (BIOMATE 3) and 0.8 % agarose gels. Agarose gels were stained by ethidium bromide and bands were visualized under UV light in gel documentation apparatus (BIO-RAD). Based on the spectrophotometric readings and intensity of DNA bands, DNA samples were diluted to 25 ng/µl for PCR.

Polymerase Chain Reaction (PCR)

DNA of advanced progeny lines and susceptible check were subjected to PCR analysis using primers, pTA248 for *Xa21*, RG136 for *xa13* and RG556 for *xa5* resistant genes (Table 1). Amplification was performed in 15 µl volume reaction mixture containing 0.05 µM each of two opposing primers, 10 ng of genomic DNA, 200 µM of each four dNTPs, 0.05 units/µl Taq DNA polymerase in a reaction buffer [10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$ and 0.001% gelatin]. A PCR programme (denaturation-1 min. at 94°C, primer annealing-1 min. at 56°C and primer extension-3 min. at 72°C) of 40 cycles was performed to amplify the DNA. Amplified products were separated in 1.4 % agarose gel in a voltage gradient of 5 V/cm and products of primers RG136 and RG556 were digested using *DraI* and *HinfI*, respectively. Digestion was carried out in a total reaction volume of 10 µl. The reaction mixture consists of, 1 µl restriction buffer (10X), 0.1 µl bovine serum albumin (10 µg/µl), 0.3 µl restriction enzyme (10 units/µl) and 4 µl of PCR products and 4.6 µl sterile distilled water. The mixture was incubated for 1½ hours at 37°C and products were separated in the agarose gels (1.4 %) and bands were visualized under UV light in gel documentation apparatus (BIO-RAD) after staining with ethidium bromide for 20 min. and destaining with water for 15 min.

Table 1. Details of three BLB resistant markers used in MAS.

Marker	*Primer (5'-3')*	*Linked gene*	*Size of amplified proucts*
pTA248	F-AGACGCGGAAGGGTGGTTCCCGGA R-AGACGCGGTAATCGAAAGATGAAA	*Xa-21*	R-1000 bp S-750 bp
RG136	F-TCCCAGAAAGCTACTACAGC R-GCAGACTCCAGTTTGACTTC	*xa-13*	1000 bp
RG556	F-TAGCTGCTGCCGTGCTGTGC R-AATATTTCAGTGTGCATCTC	*xa-5*	1500 bp

R-Resistant, S- Susceptible

Field screening by bacterial inoculation

A single colony of *Xanthomonas oryzae pv. Oryzae* was streaked on modified Wakimoto's medium (20 g/l sucrose, 5 g/l peptone, 0.5 g/l $Ca(NO_3)_2.4H_2O$, 1.8 g/l $Na_2HPO_4.7H_2O$, 0.05 g/l $FeSO_4.7H_2O$, 20 g/l agar). Three to four, 72 hours old cultures were dissolved in sterile distilled water and concentration of the cell suspension was adjusted to 10^8 CFU/ml (Ou, 1985). The plants were inoculated by clipping tips of 5–10 fully opened leaves at the maximum tillering stage (approximately 45 days after transplanting) as described in Kauffman *et al.* (1973). To evaluate the disease reaction, the standard method, based on the lesion length (> 5 cm – resistant, <

5 cm - susceptible) was used after 14 days of inoculation (Kaur *et al.*, 2005). As the lesion lengths were not different after 14 days, percentage of infected plants in a progeny line was taken as the measurement for disease resistance. Field screening of F_8 generation was carried out at RRDI, Batalagoda with the most virulent isolate, which is one of the seventy isolates collected from Sri Lanka (Fernando *et al.*, 2005). Before inoculating the field, 5-10 leaves of rice varieties; TN-1 (susceptible check), IRBB60 (resistant check), Bg358, Bg 300 and Bg352 at maximum tillering stage (approx. 45 days of transplanting) were inoculated at the PGRC plant house to check the virulence of pathogen.

RESULTS AND DISCUSSION

Molecular analysis

Out of 15 advanced progeny lines screened, 10 lines amplified the markers for resistant genes *Xa-21, xa-13 and xa-5* and the rest did not amplify any markers (Table 2) despite pure DNA being present in all lines. This was confirmed by repeating the analysis once. The particular genes may have escaped during the selection for desirable morphological characters in the conventional breeding programme.

Table 2. Molecular screening of F_7 and Field screening of F_8.

Cross	*F_7 generation*				*F_8 generation*					
		Molecular screening			*Field screening (Susceptible %)*					
	Lines	*Xa-21*	*Xa-13*	*Xa-5*	*Lines*	*%*	*Lines*	*%*	*Lines*	*%*
IRBB60 xBg300	1006	-	-	-	2837	10%	2838	10%	2839	10%
	1008	-	-	-	2222	50%	2223	50%	2224	50%
	1009	R	-	-	2840	10%	2841	15%	2842	15%
	1014	R	-	-	2843	15%	2844	15%	2845	15%
IRBB60 xBg352	1015	S	-	-	2225	25%	2226	25%	2227	25%
	1018	-	-	-	2228	75%	2229	25%	2230	50%
	1019	-	S	-	2231	25%	2232	25%	2233	50%
	1020	-	-	-	2846	20%	2847	10%	2848	10%
	1026	S	S	-	2234	25%	2235	25%	2236	15%
	1027	S	S	S	2849	0%	2850	0%	2851	0%
	1028	-	-	-	2237	15%	2238	15%	2239	15%
	1030	S	S	S	2240	15%	2241	10%	2242	25%
IRBB60 xBg358	1033	R	S	R	2243	15%	2244	15%	2245	15%
	1039	R	S	S	2852	0%	2853	0%	2854	0%
	1041	R	S	S	2246	15%	2247	15%	2248	15%

R - Resistant, S – Susceptible, (-) – not amplified

Field screening for BLB in F_8 generations showed a level of resistance such as S (Susceptible), MS (Moderately Susceptible), MR

(Moderately Resistant) and R (Resistant) in the progeny lines advanced from F_7 (Table 2). In some of the lines, although the particular genes such as *Xa-21, xa-13 and xa-5* were not observed at molecular analysis, a higher level of field resistance was recorded (Table 2). This may be either due to the experimental errors in field screening or presence of some other resistant genes which could not be detected by the currently available molecular markers (Huang *et al.*, 1997).

The primer pTA248 (for the marker for *Xa-21*) amplified resistant fragments of 1000 bp and a susceptible fragment in between 750-500 bp (Chuwongse *et al.*, 1993) in parents, IRBB60 and Bg 358, respectively (Fig. 1). In molecular screening of 15 lines of F_7 generation, the marker for gene *Xa-21* was observed only in 1009, 1014 lines of IRBB60 X Bg 300 and 1030, 1033, 1039 and 1041 lines of IRBB60 X Bg 358 crosses (Fig. 1, Table 2). All these lines, which carry *Xa-21*, showed 85 – 90 % of field resistance that is desirable for commercial varieties to achieve durable resistance (Rajapakse, 2007). This also supports the fact that the *Xa-21* gene expresses a major contribution for the resistance in the donor IRBB60 (Kush *et al.*, 1989). None of the progeny lines amplified the marker for resistant gene *xa-13* as detected in the donor parent IRBB60, but 7 lines amplified the band for susceptible allele as appeared in the susceptible cultivar TN-1 [Fig. 2(b)]. Molecular screening for resistant genes *xa-5* detected the resistant allele in the line 1033 and detected susceptible allele in 4 lines which was identical to susceptible check TN-1 [Fig. 2 (a)].

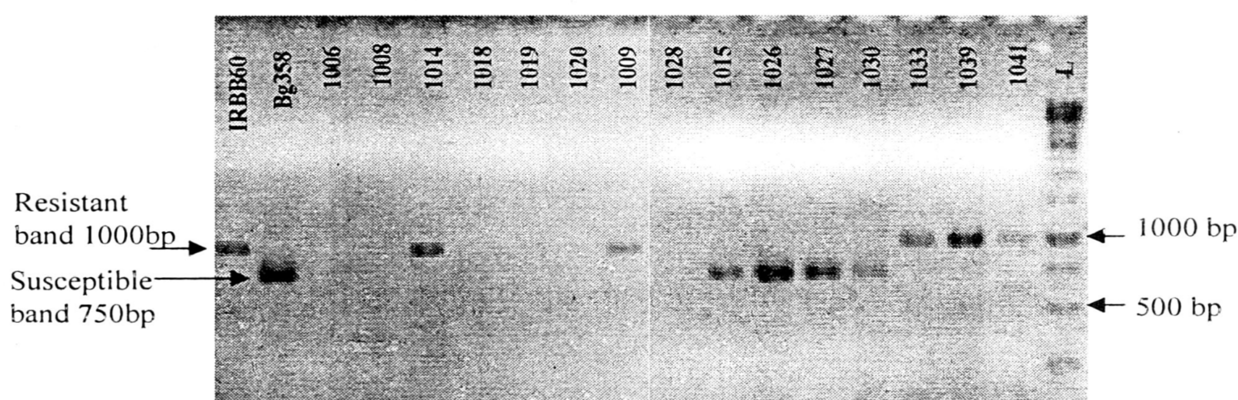

Figure 1. Amplified PCR products showing the presence of *Xa-21* resistant gene in lines of F_7 generation using specific primer pTA248. (IRBB60-resistant variety, Bg358-susceptible variety, 1006–1041 Lines of F_7 generation, L-1 kb Ladder).

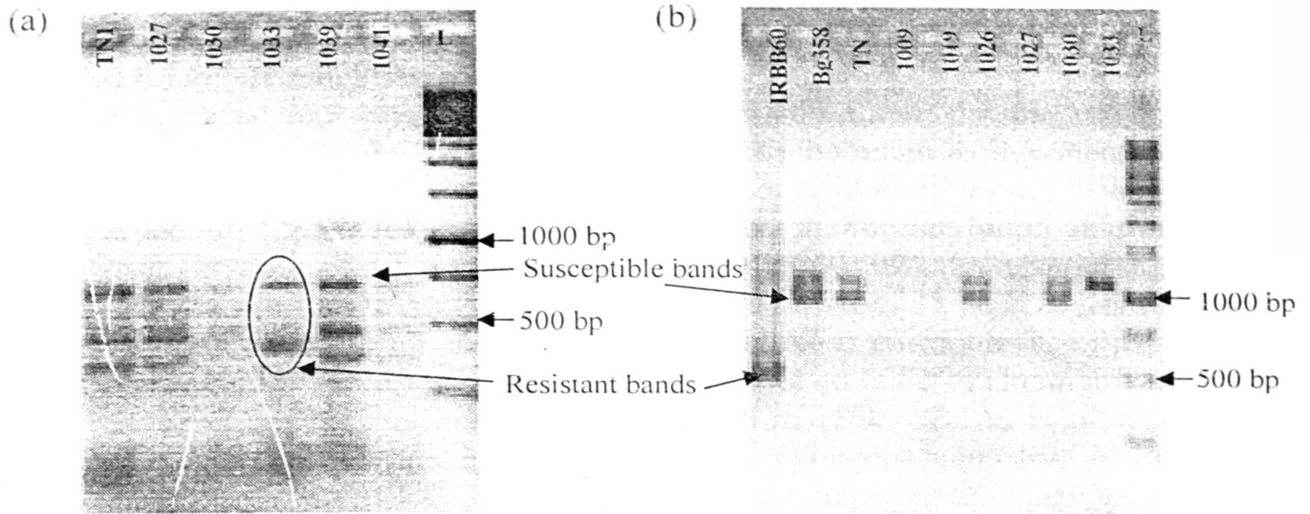

Figure 2. **(a) Agarose (1.4 %) gel showing the presence of *xa-5* resistant gene in line 1033 of F_7 generation, amplified by specific primer RG556 and digested with *Dra*I. (TN-1- susceptible variety, 1027–1041 Lines of F_7 generation, L-1 kb Ladder).**

(b) Agarose gel (1.4 %) showing the absence of *xa-13* resistant gene in lines of F_7 generation amplified by specific primer RG136 and digested using *Hinf*I. (IRBB60- resistant variety, Bg358/ TN-1- susceptible varieties, 1009 – 1033 Lines of F_7 of generation, L- 1 kb Ladder).

The presence of *Xa-21* and *xa-5* resistant genes in 1033 line in contrast to the banding patterns of IRBB60 while absence of *xa-13* in the same line was confirmed by repeating the analysis twice (Fig. 3).

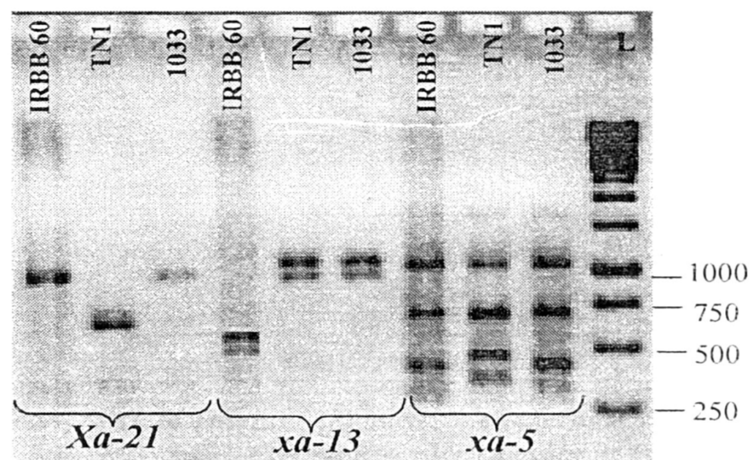

Figure 3. **Agarose gel (1.4 %) of PCR products showing the presence of *Xa-21* and *xa-5* (digested with *Dra* I) resistant genes and absence of *xa-13* (digested with *Hinf*I) resistant gene in line 1033 of F_7 generation using specific primers. (IRBB60- resistant variety, TN-1-susceptible variety, L-1 kb Ladder).**

Field screening

In F_5 generation, it showed a level of 90.48% resistance to the most virulent isolate of *Xanthomonas oryzae* pv. *oryzae* (Fernando *et al.*, 2007). Since inoculation of near generations are not effective with respect to the same isolate (I18), inoculation of progeny lines in F_8 generation was done to screen the field resistance and confirm the results of the molecular maker analysis as progenies have reached homozygosity for the resistance characters at the F_8 (Rajapakse, 2007). Comparison of results of molecular screening of F_7 and the results of field screening of F_8 are shown in Table 2.

It is evident that field screening complemented by molecular marker screening enables to identify gene pyramid line for BLB resistance with a high accuracy. This also gives the confidence for rice breeders for selecting genes for pyramid line early at the F_2 generation using molecular makers for *Xa-21*, *xa-13* and *xa-5* genes.

CONCLUSIONS

BLB resistant genes *Xa-21* and *xa-5* were pyramided to F_7 line No. 1033 of IRBB60 x Bg 358 cross which can be further advanced to develop varieties with durable resistance for bacterial leaf blight. This also gives the confidence for rice breeders for selecting genes pyramid line early at the F_2 generation using molecular makers for *Xa-21*, *xa-13* and *xa-5* genes.

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