

## IDENTIFICATION OF BACTERIAL LEAF BLIGHT RESISTANT GENE *Xa-21* IN FILIAL GENERATION OF IRBB 60 x Bg 358

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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. *Xa-21* is a dominant gene, which is responsible for the resistance of rice varieties to BLB. Bg 358 is a 3½-month-old rice variety, which was released in 1999 by the Department of Agriculture and at present is susceptible to BLB. To incorporate resistance, Bg 358 rice variety was crossed with IRBB 60 a pyramid line carrying resistant gene *Xa-21* for BLB and the breeding program was carried out until F<sub>5</sub> generation. Marker Assisted Selection was carried out with the specific primer pTA248 for *Xa-21* gene. Seven lines of IRBB 60 x Bg 358 of F<sub>5</sub> generation were carrying resistant gene *Xa-21* for BLB whereas parental line of Bg 358 was without resistant gene. Field screening of F<sub>5</sub> generation was carried out with most virulent isolate, which was used for selection of rice variety for the breeding program. Lines screened for BLB showed 90% resistance in field. Therefore, it can be concluded that by breeding program, resistant gene *Xa-21* has been incorporated into rice variety Bg 358 to withstand BLB.

**KEYWORDS:** Bacterial leaf blight, Marker assisted selection, Pyramid line, Specific primer.

### INTRODUCTION

Bacterial Leaf Blight (BLB) caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) is one of the most destructive diseases of rice in Sri Lanka. Efficacy of chemicals is not significant in this disease. Therefore, planting resistant varieties provides an effective control and up to present, most breeders concentrate on the development of highly resistant varieties. *Xa-21* gene was identified in the wild species *Oryza longistaminata* (Khush *et al.*, 1990), which was resistant to all Philippine and Indian races of *Xanthomonas oryzae*. This locus *Xa-21* has been introduced into the commercial cultivar IR24 that is susceptible to all known six *Xoo* races (Mew *et al.*, 1987). *Xa-21* is linked to three other BLB resistant genes (*Xa-3*, *Xa-4*, *Xa-10*) on chromosome 11 (Ikeda *et al.*, 1991). It has been reported that unlike the other genes identified, the dominant locus *Xa-21* confers resistance to all isolates of *Xoo* tested (Mew *et al.*, 1987).

Bg 358 is a 3½-month-old, BLB resistant rice variety, which was released in 1999 as a recommended rice variety by the Department of Agriculture (DOA). However, severity of this disease has increased gradually over the years and at present this variety is susceptible to BLB. Bg 358 is a cross between Bg 12-1 and Bg 1492. It is a popular variety grown in most parts of the island and there is yield losses due to susceptibility to BLB in the

field. Therefore a breeding program was initiated at RRDI, Bathalagoda to incorporate resistance into Bg 358. Since the selection of lines resistant to BLB in the field is both time and labour consuming, identification of resistant genes using molecular markers is more preferable. Therefore, a study was undertaken at the Plant Genetic Resources Centre (PGRC), Gannoruwa to identify the resistant genes in filial generation of IRBB 60 x Bg 358. Rice variety IRBB 60 was selected for the incorporation of resistance because this variety carries resistant genes *Xa-21*, *xa-13* and *xa-5* (Huang *et al.*, 1997). Incorporation of resistant gene *Xa-21* using molecular markers could be found in filial generation of IRBB 60 x Bg 358 whereas attempts to incorporate *xa-5* and *xa-13* have yielded negative results.

## MATERIALS AND METHODS

### Extraction of DNA

Extraction of DNA was carried out with the leaf samples obtained from the nursery of F<sub>5</sub> generation from RRDI, Bathalagoda using CTAB method (Kurt, 1995) with some modifications. Leaf sample (150mg) was ground using liquid nitrogen in a cool mortar and pestle until it formed a fine powder and then transferred to an Eppendorf tube. Seven hundred and fifty  $\mu$ l of pre-warmed (65°C) isolation buffer was added and incubated for 30 minutes at 65°C in a shaking water bath. The samples were centrifuged at 3000 rpm for 10 minutes and the supernatant carefully transferred to a new tube. Five  $\mu$ l of (10g/ml) RNase A was added to each sample and incubated for 30 minutes at 37°C. Seven hundred and fifty  $\mu$ l of chloroform: isoamyl alcohol (24:1) was added and mixed well by inverting for 15 minutes. This was centrifuged at 13000 rpm for 10 minutes and the supernatant carefully pipetted out to a new tube and 0.6 volume of chilled iso-propanol was added and inverted slightly until it formed a DNA cluster. Then it was centrifuged at 13,000rpm for 10 minutes and the supernatant removed. The pellet was washed with 70% ethanol for 60 minutes. The ethanol was removed and the pellet was air-dried. The pellet dissolved in 200 $\mu$ l of Tris-EDTA (pH 8.0) buffer [1 M Tris-Cl (pH 8.0), 0.5M EDTA (pH 8.0)]. Samples were stored at 4°C.

### Polymerase chain reaction (PCR) analysis for marker assisted selection

PCR was carried out using the amplification reaction conditions as described by Huang *et al.* (1997) with some modifications as follows. Fifty ng/ $\mu$ l template DNA, 2.5mM of each primer, 10X Ex Taq buffer (10mM Tris-Cl, pH 8.8, 50mM KCl, 1.5mM MgCl<sub>2</sub>, 0.01% gelatin), 2.5mM dNTPs and 5 units/ $\mu$ l Taq DNA polymerase in a volume of 10 $\mu$ l for the primer pTA248 and 20 $\mu$ l for primers RG 556 and RG 136. PCR conditions were as follows: denaturation at 94°C for 1 min, 2 min of primer annealing at 56°C and 3 min

of primer extension at 72°C for 35 cycles. The amplified products of *Xa-21* using pTA248 primer (Table 1) (Huang *et al.*, 1997) were electrophoretically resolved on a 1.4% agarose gel in 0.5 x TBE buffer. Agarose gel was stained with ethidium bromide and observed under UV illumination. Ten µl of amplified products of primers for *xa-5* using RG 556 primer and *xa-13* using RG 136 primer (Table 1) (Huang *et al.*, 1997) were initially tested on a 1.4% agarose gel in 0.5 x TBE. The confirmed products were then used to carry out restriction digestion with the remaining PCR product. The amplified product of *xa-5* was digested with *DraI* and the amplified product of *xa-13* was digested with *HinfI*. Digestion was carried out in a total reaction volume of 10µl. The reaction mixture consisted of 4.6µl sterile distilled water, 1µl restriction buffer (10X), 0.1µl Bovine Serum Albumin (10µg/µl), 0.3µl restriction enzyme (10units/µl) and 4µl of PCR product. The reaction mixture was incubated for 1h and 30 min. at 37°C. The DNA fragments produced by restriction digestion were separated through gel electrophoresis (1.4% agarose) and visualized under UV light after staining with ethidium bromide.

**Table 1. Distribution of alleles of three PCR marker loci linked to resistance genes.**

Marker	Primer (5'-3')	Linked gene	Size of amplified product
pTA248	F-AGACGCGGAAGGGTGGTTCCTCCGGA	<i>Xa-21</i>	Resistant 1000bp
	R-AGACGCGGTAATCGAAAGATGAAA		Susceptible 750bp-500bp
RG 136	F-TCCCAGAAAGCTACTACAGC	<i>xa-13</i>	1000bp
	R-GCAGACTCCAGTTTGAATTC		
RG 556	F-TAGCTGCTGCCGTGCTGTGC	<i>xa-5</i>	1500bp
	R-AATATTTTCAGTGTGCATCTC		

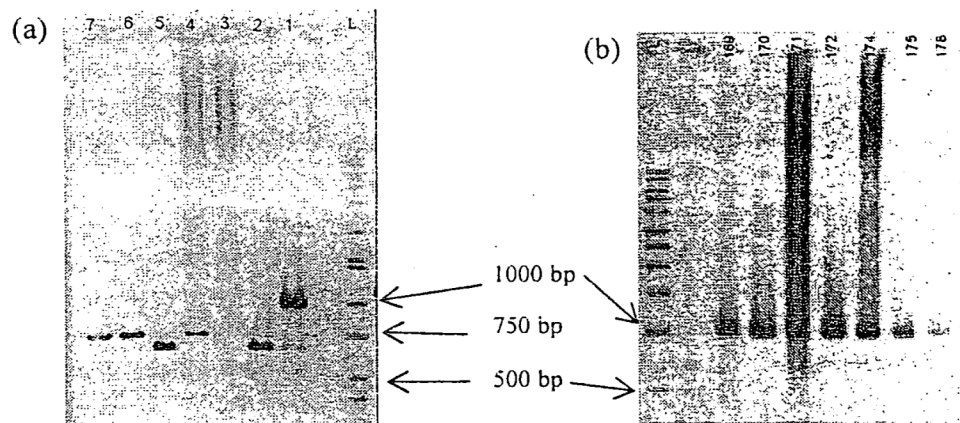
### Field screening by bacterial inoculation

A single colony of *Xanthomonas oryzae* pv. *Oryzae* was streaked on modified Wakimoto's medium (20g/l sucrose, 5g/l peptone, 0.5g/l Ca(NO<sub>3</sub>)<sub>2</sub>.4H<sub>2</sub>O, 1.8g/l Na<sub>2</sub>HPO<sub>4</sub>.7H<sub>2</sub>O, 0.05g/l FeSO<sub>4</sub>.7H<sub>2</sub>O, 20g/l agar). Three to four 72 h-old cultures were dissolved in sterilized distilled water and concentration of the cell suspension was adjusted to 10<sup>8</sup> 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). Disease reaction [lesion length in cm] was scored 14 days after inoculation. Plants having lesion lengths of more than 5cm were considered as susceptible and the ones with lesion lengths of less than 5cm were considered as resistant (Kaur *et al.*, 2005). Field screening of F<sub>5</sub> generation was carried out at RRDI, Bathalagoda with the most virulent isolate, which is one of the seventy isolates collected from different locations of Sri Lanka (Fernando *et al.*, 2005).

## RESULTS AND DISCUSSION

**Molecular analysis**

Parental sample IRBB 60 (resistant variety) amplified a resistant fragment of 1000bp. Bg 358 which is a susceptible variety amplified a susceptible fragment in between 750bp-500bp (Chunwongse *et al.*, 1993) which is similar to the amplified fragment of the susceptible variety TN-1 confirming the absence of *Xa-21* resistant gene in rice variety Bg 358 [Fig. 1(a)]. Lines of F<sub>5</sub> generation showed a banding pattern identical to that of its resistant parent IRBB 60 [Fig. 1(b)]. Therefore it can be assumed that these lines carry *Xa-21* resistant gene. The results of molecular analysis showed presence of *xa-5* resistant gene and *xa-13* resistant gene in the parental sample IRBB 60 (resistant variety) and absence of *xa-5* and *xa-13* resistant genes in TN-1 (susceptible variety), Bg 358 and lines of F<sub>5</sub> generation (Fig. 2 and 3) In order to incorporate the two genes which confer a broad spectrum of resistance, breeding has to be continued.



**Figure 1.** (a) DNA amplification of resistant and susceptible varieties using specific primer pTA 248 (L- 10 Kb Wide range ladder, 1-IRBB 60, 2-TN-1, 3- Bg 360, 4- Bg 358, 5- Bg 357, 6- Bg 352, 7- Bg 300) (b) DNA amplification of lines of the cross IRBB 60 x Bg 358 in F<sub>5</sub> generation using specific primer pTA 248 (L-1 Kb ladder, 169 to 178 - Samples from the progeny lines of the cross IRBB 60 x Bg 358 in F<sub>5</sub> generation).

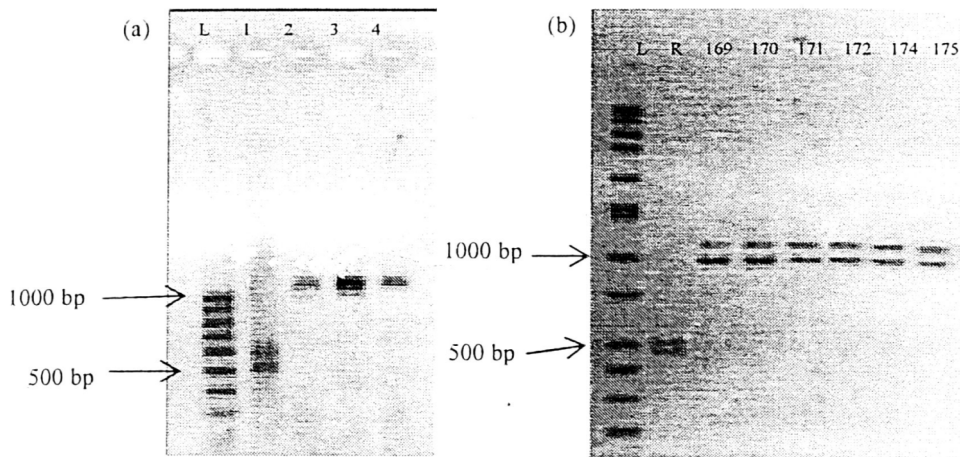


Figure 2. (a) DNA amplification of resistant and susceptible varieties using specific primer RG 136 (L - Low ladder, 1-IRBB 60, 2-TN-1, 3 - Bg 360, 4 - Sample from progeny line 178 of the cross Bg 358 x IRBB0 in F<sub>5</sub> generation) (b) DNA amplification of lines of the cross IRBB 60 x Bg 358 in F<sub>5</sub> generation using specific primer RG 136 (L - 10 Kb Wide range ladder, 169 to175 - Samples from the progeny lines of the cross IRBB 60 x Bg 358 in F<sub>5</sub> generation).

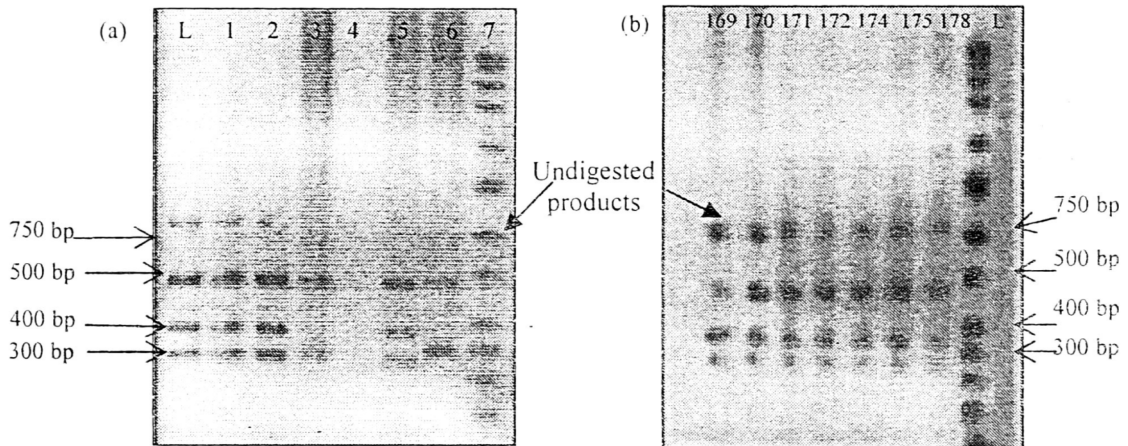


Figure 3. (a) DNA amplification of resistant and susceptible varieties using specific primer RG 556 (L - 10 Kb Wide range ladder, 1-IRBB 60, 2-TN-1, 3 - Bg 300, 4 - Bg 352, 5 - Bg 357, 6 - Bg 358, 7 - Bg 360) (b) DNA amplification of lines of the cross IRBB 60 x Bg 358 in F<sub>5</sub> generation using specific primer RG 556 (L- 10 Kb Wide range ladder, 169 to178 - Samples from the progeny lines of the cross IRBB 60 x Bg 358 in F<sub>5</sub> generation).

**Field screening**

Table 2 shows screening results of the lines in IRBB 60 x Bg 358 of F<sub>5</sub> generation.

**Table 2. Screening results of the lines in IRBB 60 x Bg 358 of F<sub>5</sub> generation.**

Number of the progeny line	169	170	171	172	174	175	178
Total number of plants inoculated	15	15	15	15	15	15	15
Number of resistant plants	13	15	11	13	15	13	15
Percentage resistance (%)	86.67	100	73.33	86.67	100	86.67	100
Average of percentage of resistance	633.34 / 7 = 90.48%.						

The field screening of cross IRBB 60 x Bg 358 of F<sub>5</sub> generation showed approximately 90% resistance in the field. Resistance in the field may be due to the presence of dominant gene *Xa-21*.

### CONCLUSIONS

Plants of F<sub>5</sub> generation of IRBB 60 x Bg 358 carry the recessive gene *Xa-21* and pTA 248 is a marker to identify the *Xa-21* resistant plants. Plants of filial generation of IRBB 60 x Bg 358 are resistant to most virulent isolate of BLB in the field.

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