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Research Article

Improvement of rice cultivar for bacterial blight disease through marker assisted breeding approach

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Abstract
The present investigation was undertaken with the objective to develop high yielding, fine grain rice varieties possessing broad spectrum durable resistance by transferring bacterial leaf blight (BLB) resistant genes viz., xa13 and Xa21 from Improved Pusa Basmati 1. A popular high yielding and fine grain rice variety, HUBR 2-1 (Malviya Dhan 2-1), susceptible to BLB and Improved Pusa Basmati 1 carrying resistant genes for BLB (xa13 & Xa21 genes) were selected as parents for crossing. Improved Pusa Basmati 1 was verified for the presence of target genes by using gene linked primers viz., xa13 promoter and pTA 248. The cross viz., HUBR 2-1 x Improved Pusa Basmati 1 was undertaken during Kharif 2011 and F1 progenies were confirmed during rabi 2011. The F1 plants confirmed as true hybrids for both the genes were advanced to the F2 generation and foreground selection was done using gene linked markers. Genetic analysis in the F2 population confirmed that the genes (xa13 & Xa21) governing BLB resistance followed Mendelian inheritance. The phenotypic data analysis revealed that the plants carrying two resistance gene combinations (xa13xa13 Xa21Xa21, xa13xa13Xa21xa21) showed BLB resistance (0-3 scale), while the gene combinations viz., Xa13Xa13Xa21Xa21, Xa13Xa13Xa21xa21 and Xa13xa13Xa21Xa21, Xa13xa13Xa21xa21 showed BLB resistance (5-30).

Key words
Bacterial leaf blight; F2 population; marker assisted selection; rice

INTRODUCTION
Rice is the important staple food for more than half of the world’s population, but rice production is limited by various biotic and abiotic factors; bacterial leaf blight (BLB) being one of the major diseases. Host plant resistance (HPR) has been considered the most economical and eco-friendly strategy for the management of biotic stresses (Hulbert et al., 2001). Molecular markers are widely applied in agriculture, and their application in rice improvement has been recently reviewed (Mackill and McNally 2004; Jordan et al., 2004; Xu et al., 2004; Toojinda et al., 2005; Liu et al., 2006; Mackill, 2007; Soumya and Sindhumole 2016, Vennisa et al., 2018). Kalaichelvan (2009) used 78 SSR’s for varietal identification and also assessed the genetic relationship among the elite rice cultivars using morphological and molecular markers. The marker used in the selection must have a tight linkage with the target gene in order to have a relatively high selection efficiency (Yunbi, 2010).

MAS has also been employed for moving genes from pyramided lines into new plant type (Sanchez et al., 2000) as well as into improved varieties grown in India (Singh et al., 2001). Development of broad spectrum durable resistance through gene pyramiding or gene
staining for biotic stress resistance can be accelerated through the process of marker assisted selection (Joshi and Nayak, 2010). BLB is caused by Xanthomonas oryzae pv. oryzae and is one of the most devastating diseases of rice causing yield losses ranging from 74 to 81% (Srinivasan and Gnanamanickam, 2005) in severe conditions.

Till date, 34 BLB genes (Chen et al., 2011) have been identified in rice and a number of them have been deployed into breeding lines but disease breakdown has resulted due to a significant shift in pathogen race frequency (Mew et al., 1992). Such breakdown can be delayed by marker assisted gene pyramiding. The xa13 gene is fully recessive, conferring resistance only in the homozygous status (Khush and Angeles, 1999). Perumalsamy et al. (2010) introgressed three BLB resistance genes xa5, xa13 and Xa21 into two high yielding BLB susceptible indica rice cultivars, ‘ADT 43’ and ‘ASD 16’ from isoline IRBB60 and F$_2$ populations that were screened for the presence of all the three resistance genes by using functional markers. These pyramided genotypes with two or three resistance genes exhibited high levels of resistance against two predominant Xanthomonas oryzae isolates of South India.

The broad spectrum BLB resistance gene Xa21 is expressed in dominant condition and was introgressed from a wild species O. longistaminata into O. sativa chromosome 11 through conventional breeding (Khush et al., 1989). Basavaraj et al. (2010) also used markers RG 136 and pTA 248 linked to BLB resistance genes xa13 and Xa21 respectively, for foreground selection to improve Pusa 6A by using improved Pusa 6B as a donor for xa13 and Xa21.

The present study was undertaken to develop a high yielding, fine grain, short duration rice variety resistant to BLB by introgression of two BLB resistance genes viz., xa13 and Xa21 from Improved Pusa Basmati 1 into the genetic background of HUBR 2-1. The gene linked markers viz., xa13 promotor and pTA 248 were validated in resistant parent and parental polymorphism was studied between susceptible and resistant parent. The genotypic and phenotypic segregation was analyzed to determine the inheritance pattern of these genes in the single hybrid derived F$_2$ population.

**Material and Methods**

The variety HUBR 2-1 (Malviya Dhan 2-1) is a short duration, high yielding, fine grain, blast resistant rice variety released from the Institute of Agricultural Sciences, Banaras Hindu University in 2004, was used in the present investigation as a susceptible parent for BLB. Improved Pusa Basmati 1 (PB 1460), was used as a resistant parent as it possesses BLB resistance genes viz., xa13 and Xa21 and released from the Indian Agricultural Research Institute, New Delhi.

Genomic DNA was isolated from parents (HUBR 2-1 and Improved Pusa Basmati 1), F$_1$, F$_2$ and their Backcross progenies (BC$_F$, BC$_C$, BC$_F$, and BC$_C$) following the mini preparation procedure (modified method of Zheng et al., 1991). Quantification of DNA samples was done by using 0.8% agarose gel electrophoresis with diluted uncut DNA ladder as standard and spectrophotometer (Thermo electronic corporation UV1) as per the procedure described by Sambrook and Russell et al. (2001).

The PCR amplification was performed in 10 µl volume containing 50 ng of template DNA, 5 picomoles of each primer, 2 mM dNTPs, 10X PCR buffer (10 mM Tris, pH 8.4, 50 mM KCl, 1.8 mM MgCl2 and 0.01 mg/ml gelatin) and 1U Taq DNA polymerase (Genei, Bangalore, India) on Applied Biosystems verity 96 well thermal cycler. The template DNA was amplified in PCR profile with an initial denaturation at 94°C for 5 min, denaturation at 94°C for 45 sec., primer annealing at 55°C (xa13 promoter) and at 58°C (p TA 248) for 45 sec., extension at 72°C for 1 min, final extension at 72°C for 10 min, and cooling at 4°C for ∞. These steps were repeated for 35 cycles for amplification of DNA. The amplified products were then mixed with bromophenol blue and resolved electrophoretically in 2% agarose gel along with the marker 50 bp DNA ladder (Biolabs) for an hour in 1X Tris Acetic acid EDTA (TAE) buffer. The resolved PCR bands were documented using BioRad Gel Doc XR System.

The F$_1$ crosses were affected during Kharif 2011 viz., HUBR 2-1 × Improved Pusa Basmati 1. F$_1$ seeds were raised in the main field by planting a single seedling per hill at a spacing of 20 × 20 cm during rabi 2011. DNA isolated from all the F$_1$ plants were used for genotyping of target genes. The seeds harvested from a single hybrid plant carrying both xa13 and Xa21 genes (HUBR 2-1 x Improved Pusa Basmati 1) were selfed and advanced to the F$_2$ generation during kharif 2012. These segregating populations were screened by using gene linked SSR markers for the resistance genes viz., xa13 and Xa21.

A total of 200 F$_2$ plants from HUBR 2-1 × Improved Pusa Basmati 1 along with parents were genotyped to determine the inheritance of target genes. The inheritance of BLB resistant genes viz., xa13 and Xa21 were studied with the help of gene linked SSR markers viz., xa13 promotor and pTA 248 respectively. Alleles at the SSR loci were detected on 2% or 3% agarose gel and 50 bp or 100 bp DNA ladder was added with the first load to confirm the allele sizes observed in the parental survey. Scoring of alleles was done to identify the plants carrying different genotypic combinations. The F$_2$ plants that showed a pattern similar to the susceptible parent alleles were scored as ‘9’ and those with a banding pattern similar to the resistant parent alleles were scored as ‘0’ and the plants with the heterozygous allelic pattern were scored as ‘3’.

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To determine the segregation patterns of BLB resistance genes, F₂ seedlings were inoculated with a hyper-virulent isolate (DX-066) of *Xanthomonas oryzae* pv. *oryzae* collected from DRR, Rajendranagar. The F₂ population was inoculated with the bacterial culture at maximum tillering stages by using the leaf clipping method described by Kauffman et al. (1973). The inoculum was prepared by suspending bacteria, grown on Hayward’s agar media for 2 to 3 days at 28°C, in sterile distilled water at a final concentration of approximately 108 cfu/ml. Inoculum density was adjusted to 107-108 (cfu/ml) and plant inoculation was carried out by clipping the tip (about 1 to 2 cm) of the fully expanded uppermost leaf with scissors that had been dipped into the inoculum. Disease scoring was done 15 days after inoculation. Five leaves per plant were taken for scoring and the plant reaction was rated on a 0-9 scale according to lesion length scores as given in Table 1.

Table 1. Infection per cent, score and host response

| INFECTION % | SCORE | HOST RESPONSE |
|-------------|-------|---------------|
| 0%          | 0     | Highly resistant (HR) |
| >1-10%      | 1     | Resistant (R) |
| >10-30%     | 3     | Moderately resistant(MR) |
| >30-50%     | 5     | Moderately Susceptible (MS) |
| >50-75%     | 7     | Susceptible (S) |
| >75-100%    | 9     | Highly susceptible (HS) |

For the inheritance studies of BLB resistance in the segregating population, the goodness of fit of expected genetic ratios was tested by χ² test (Singh and Chaudhary et al., 1977). The χ² analysis for genotypic and phenotypic ratios was calculated using the following formula: $\chi^2 = \Sigma (O-E)^2/E$, where, O is the observed value, E is the expected value and Σ stands for summation.

RESULTS AND DISCUSSION

Rice is one of the most important cereal crops for global food security. Resistance breeding with MAS has been employed to develop broad spectrum durable BLB resistance in rice. A clear marker trait association was established for BLB. Hence, it is possible to monitor the transmission of trait genes viz., *xa13* and *xa21* via closely linked markers, (*xa13 promotor* and *pTA 248*). The primer pairs viz., *pTA 248* (Huang et al., 1997) and *xa13 promotor* (Sundaram et al., 2008) were used as gene sequence based marker for BLB resistance genes viz., *Xa21* and *xa13* in marker assisted selection. Similarly, Mc Couch et al. (1997) and Olufowote et al. (1997) also used SSR’s to study the polymorphism in rice varieties.

The present investigation clearly indicated that two resistance genes viz., *xa13* and *Xa21* for BLB were present in Improved Pusa Basmati 1. The susceptible parent, HUBR 2-1 was carrying both corresponding susceptible alleles (Table 2). Since, the polymorphism was very clear among the parents for both the target genes, these markers were selected for foreground selection in the segregating generations. The F₂ crosses were made during *kharif* 2011 viz., HUBR 2-1 × Improved Pusa Basmati 1 and F₂ plants were raised in the field during *rabi* 2011. The primer pair *xa13 promotor* and *pTA 248* were used to confirm the hybridity of 100 F₂ plants from cross HUBR 2-1 × Improved Pusa Basmati 1 out of which 78 plants were confirmed as true hybrids (*Xa13xa13Xa21xa21*) for both BLB resistant genes viz., *xa13* and *Xa21* as shown in Fig. 1& 2.

Table 2. Polymorphism between resistant and susceptible alleles

| Trait          | Gene | Primer      | Resistant allele | Susceptible allele |
|----------------|------|-------------|------------------|--------------------|
| Bacterial Leaf Blight | *xa13* | *xa13 promotor* | 500bp | 250bp |
| Resistance     | *Xa21* | *pTA248*    | 925bp | 730bp |
**Xa 21**

Fig 1. Confirmation of F1 plants for Xa21 gene by using pTA248 promoter primer

**Xa 13**

Fig 2. Confirmation of F1 plants for x1a3 gene by using xa13 promoter primer
Fig. 3. Segregation of $F_2$ individuals derived from cross HUBR 2-1 × Improved Pusa Basmati 1 for $xa_{13}$ gene. $P_1$ is HUBR 2-1 is the susceptible parent and $P_2$ is Improved Pusa Basmati 1 is the resistant parent.

Fig. 4. Segregation of $F_2$ individuals derived from cross HUBR 2-1 × Improved Pusa Basmati 1 for $Xa_{21}$ gene. $P_1$ is HUBR 2-1 is the susceptible parent and $P_2$ is Improved Pusa Basmati 1 is the resistant parent.
Similarly, pTA 248 primer pair was used to study the co-segregation of the Xa21 gene. Out of 200 F₂ plants, pTA 248 primer pair amplified an allele of 730 bp in 50 F₂ plants identical to the susceptible parent, an allele of 925 bp in 54 F₂ plants identical to the resistant parent and 96 F₂ plants exhibited heterozygosity (Fig 4).

The χ² analysis indicated a good fit to the expected segregation ratio 1\(Xa21Xa21\): 2\(Xa21xa21\): 1\(xa21xa21\) for the single gene model. This result is in agreement with the results of Jiang et al. (2004) for the Xa21 gene in the F₂ population of 'Minghui 63'. The \(xa13\) gene confers resistance only when it is present in the homozygous recessive condition whereas, Xa21 is dominant in nature and can be expressed even in the heterozygous condition governing resistance to multiple races of Xoo.

The co-segregation analysis of the two BLB resistance genes viz., \(xa13\) and Xa21 together showed the goodness of fit to the expected ratio 1:2:4:1:2:1:2:1:2:1, for two genes with a high degree of significance (Table 3). This result indicated that the two BLB resistant genes viz., \(xa13\) and Xa21 followed Mendelian inheritance. Joseph et al. (2004) also reported that the two genes segregated into nine distinct classes as 1:2:2:4:1:2:1:2:1 out of which, the seven resistant genotypic classes viz., \(xa13xa13Xa21Xa21\), \(xa13xa13Xa21xa21\), \(xa13xa13xa21Xa21\), \(Xa13xa13Xa21Xa21\), \(Xa13xa13xa21xa21\), \(Xa13Xa13Xa21Xa21\) and \(Xa13Xa13Xa21xa21\) are expected to segregate in the ratio of 1:2:1:2:4:1:2 for the two gene combinations.

### Table 3. Co-segregation analysis of the two BLB resistance genes \(xa13\) and Xa21

| S. No. | Genotypes          | Observed value | Expected ratio | Expected value | \(\chi^2\) value |
|------|--------------------|----------------|----------------|----------------|-----------------|
| 1.   | \(Xa13\) \(Xa13\) \(Xa21\) \(Xa21\) | 12             | 1              | 12.5           | 0.02            |
| 2.   | \(Xa13\) \(Xa13\) \(Xa21\) \(xa21\) | 27             | 2              | 25             | 0.16            |
| 3.   | \(Xa13\) \(xa13\) \(Xa21\) \(Xa21\) | 24             | 2              | 25             | 0.04            |
| 4.   | \(Xa13\) \(xa13\) \(Xa21\) \(xa21\) | 49             | 4              | 50             | 0.02            |
| 5.   | \(xa13\) \(xa13\) \(Xa21\) \(Xa21\) | 14             | 1              | 12.5           | 0.18            |
| 6.   | \(xa13\) \(xa13\) \(Xa21\) \(xa21\) | 24             | 2              | 25             | 0.04            |
| 7.   | \(Xa13\) \(Xa13\) \(xa21\) \(xa21\) | 12             | 1              | 12.5           | 0.02            |
| 8.   | \(Xa13\) \(xa13\) \(xa21\) \(xa21\) | 26             | 2              | 25             | 0.04            |
| 9.   | \(xa13\) \(xa13\) \(xa21\) \(xa21\) | 12             | 1              | 12.5           | 0.02            |
| Total|                   | 200            | 16             | 200            | 0.54**          |

The calculated \(\chi^2\) value, 0.54 less than tabulated value, 15.5 at df = 8 and P = 0.05 and 20.090 at P = 0.01

### Table 4. Co-segregation of two genes in F₂ population from cross MTu 1010 × B95-1 against Xoo isolate DX-066

| S. No. | Gene combinations          | Disease Reaction/Scale | Observed value | Expected ratio | Expected value | \(\chi^2\) value |
|------|---------------------------|------------------------|----------------|----------------|----------------|-----------------|
| 1.   | \(Xa13\) \(Xa13\) \(Xa21\) \(Xa21\) | Resistant (only due to \(Xa21\)) 0 to 2.0 | 111 | 9 | 112.5 | 0.02 |
| 2.   | \(Xa13\) \(Xa13\) \(Xa21\) \(xa21\) |             | | | | |
| 3.   | \(Xa13\) \(xa13\) \(Xa21\) \(Xa21\) |             | | | | |
| 4.   | \(Xa13\) \(xa13\) \(Xa21\) \(xa21\) |             | | | | |
| 5.   | \(xa13\) \(xa13\) \(Xa21\) \(Xa21\) | Resistant (due to both \(xa13\) & \(Xa21\)) 0.5 to 3.0 | 39 | 3 | 37.5 | 0.06 |
| 6.   | \(xa13\) \(xa13\) \(Xa21\) \(xa21\) |             | | | | |
| 7.   | \(Xa13\) \(Xa13\) \(xa21\) \(xa21\) | Susceptible (both genes in susceptible combination) | 40 | 3 | 37.5 | 0.16 |
| 8.   | \(Xa13\) \(xa13\) \(xa21\) \(xa21\) |             | | | | |
| 9.   | \(xa13\) \(xa13\) \(xa21\) \(xa21\) | Moderately Resistant (only due to \(xa13\)) | 10 | 1 | 12.5 | 0.5 |
| Total|                     | | | | | 0.74** |
The F$_2$ population from the cross HUBR 2-1 × Improved Pusa Basmati 1, showed segregation for resistance and susceptibility reactions for BLB with isolate, DX-066 (Table 4). The $\chi^2$ analysis of the result exhibited a good fit for the Mendelian segregation ratio. This indicated that resistance to BLB is governed by both single genes independently. Higher levels of resistance in gene pyramid lines containing multiple BLB resistance genes as compared to lines having single (or fewer) resistance genes have been reported earlier (Yoshimura et al., 1996).

In this study, the co-segregation analysis for the two gene combinations demonstrated a good fit to the phenotypic ratio of 9:3:3:1 indicating that the two genes segregated independently and revealed a simple dominant recessive relationship. The plants possessing $xa13$ gene in homozygous condition along with $Xa21$ gene in homozygous or homozygous condition showed BLB resistance (score ranged between 0 and 2), $Xa21$ gene alone also showed resistance (0.5-3.0), $xa13$ gene alone showed moderate resistance (2.4-5) and the plants with $Xa13$ and $xa21$ genes showed susceptible (>5) reaction.

Pandey et al. (2013) also improved traditional BB susceptible Basmati varieties (Taraori Basmati and Basmati 386) by introgressing two major BLB resistance genes, $Xa21$ and $xa13$, coupled with phenotype based selection. They reported improved lines possessing a single resistance gene (i.e., either $Xa21$ or $xa13$) both in homozygous condition ($Xa21Xa21$ or $xa13xa13$) displayed moderate resistance to BLB while, lines possessing both $Xa21$ and $xa13$ in homozygous condition ($Xa21xa21xa13xa13$) showed significantly higher levels of resistance.

The present investigation indicated that the use of molecular markers that are closely linked to traits of interest in combination with the phenotype based selection resulted in effective selection of the desired combination of genotypes. Identification of desired genotypes possessing more than one gene is efficiently carried out when compared to the conventional breeding method (Dwivedi et al., 2007). The results also further indicated that the selection based on genotypic data is reflecting at the phenotypic level.

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