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Research Article

MARKER ASSISTED SELECTION FOR BACTERIAL LEAF BLIGHT RESISTANCE IN SEGREGATING POPULATIONS OF COTTONDORA SANNALU

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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 B95-1. A popular high yielding and fine grain rice variety, MTU1010 (Cottondora Sannalu), susceptible to BLB and B95-1 carrying resistant genes for BLB (*xa13* & *Xa21* genes) was selected as the parent for crossing. B95-1 was verified for the presence of target genes by using gene linked primers viz., *xa13 promotor* and *pTA 248*. These primers were also used to study polymorphism between resistant (B95-1) and susceptible (MTU1010) parents. The cross viz., MTU1010 x B95-1 was affected during Rabi, 2010 and F_1 progenies were confirmed during Kharif, 2011. The F_1 plants confirmed as true hybrids for both the genes were advanced to F_2 generation and foreground selection was done using gene linked markers. Genetic analysis in F_2 populations 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 (0-2 scale), while the gene combinations viz., *Xa13Xa13Xa21Xa21*, *Xa13Xa13Xa21xa21*, and *Xa13xa13Xa21Xa21*, *Xa13xa13Xa21xa21* showed BLB resistance (0.5-3.0).

Key words: Rice; F_2 population; Bacterial Leaf Blight; Marker Assisted Selection.

Introduction

Rice is the important staple food for more than half of the world's population. But the 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 as the most economical and eco-friendly strategy for 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). Kalaichelvan (2009) used 78 SSRs 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 tight linkage with the target gene in order to have 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 stacking for biotic stress resistance can be accelerated through the process of marker assisted selection (MAS) (Joshi and Nayak, 2010).

BLB is caused by the *Xanthomonas oryzae pv. oryzae* and is one of the 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 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, 'ADT43' and 'ASD16' from isoline IRBB60 and F_2 populations 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* onto *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 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 B95-1 into the genetic background of MTU1010. The gene linked markers viz., *xa13 promotor* and *pTA 248* were validated in the resistant parents and the parental polymorphism was studied between susceptible and resistant parents. The genotypic and phenotypic segregation was analyzed to determine the inheritance pattern of these genes in the single hybrid derived F_2 population.

Methodology

Plant Material

MTU1010 (Cottondora Sannalu), a short duration, high yielding, fine grain, BPH resistant rice variety released from Andhra Pradesh Rice Research Institute (APRRI), Maruteru, Andhra Pradesh (A.P) in 1999, was used as susceptible parent for BLB. B95-1 (Improved Samba Mahsuri), used as resistant parent as it possesses BLB resistance genes viz., *xa13* and *Xa21*, is developed by Directorate of Rice Research, Rajendranagar, Hyderabad (Sundaram *et al.,* 2008).

Genomic DNA isolation and quantification

Genomic DNA was isolated from parents (MTU1010 and B95-1), F_1, F_2 and their check materials viz., SS1113 following the mini preparation procedure (modified method of Zheng *et al.,* 1991). Quantification of the 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 *et al.* (2001).

Polymerase chain reaction

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 $MgCl_2$ and 0.01 mg/ml gelatin) and 1U *Taq* DNA polymerase polymerase (Genei, Bangalore, India) on Applied Biosystems verity 96 well thermal cycler. The template DNA was amplified in PCR profile with initial denaturation at 94° C for 5 min, denaturation at 94° C for 45 sec, primer annealing at 55° C (*xa13* promotor) and at 58° C (*pTA 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 50bp DNA ladder (Biolabs) for an hour in 1X·Tris–Acetic acid–EDTA (TAE) buffer. The resolved PCR bands were documented using Bio-Rad Molecular Imager Gel Doc XR System.

Verification of resistance genes in donor parent and Parental Polymorphism study

Two gene specific primer pairs viz., *xa13 promoter* and *pTA 248* that are closely linked to the BLB resistance genes viz., *xa13* and *Xa21*, respectively were verified in B95-1 by comparing with the check material, SS 1113. Once the resistant parents were confirmed for the presence of resistance genes, the two gene specific primers viz., *xa13 promotor* and *pTA 248* were used to survey the parental polymorphism (MTU1010 vs B95-1) for resistance genes. The SSR markers showing clear polymorphism between the parental lines were used for F_1 confirmation and co-segregation analysis in F_2 population (foreground selection).

Development and Confirmation of F_1 plants

The F_1 crosses were affected during Rabi, 2010 viz., MTU1010 x B95-1. F_1 seeds were raised in the main field by planting single seedling per hill at a spacing of 20 x 20 cm during Kharif, 2011. DNA isolated from all F_1 plants were used for genotyping of target genes. The seeds harvested from single hybrid plant carrying both *xa13* and *Xa21* genes (MTU1010 x B95-1) were selfed and advanced to F_2 generation during Rabi, 2011. These segregating populations were screened by using gene linked SSR markers for the resistance genes viz., *xa13* and *Xa21*.

Genotyping for marker segregation in F_2 population

A total of 420 F_2 plants from MTU1010 x B95-1 along with parents were genotyped to determine the inheritance of target genes. The inheritance of BLB resistant genes viz., *xa13* and *Xa21* was 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% and 3% agarose gel and 50bp or 100bp 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 '1' and those with a banding pattern similar to the resistant parent alleles were scored as '2' and the plants with heterozygous allelic pattern were scored as '3'.

Disease Evaluation in F_2 Population

To determine segregation patterns of BLB resistance genes, F_2 seedlings were inoculated with hyper-virulent isolate

(DX-066) of *Xanthomonas oryzae* pv. *Oryzae* collected from DRR, Rajendranagar. F_2 population was inoculated with 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 Haywards agar media for 2 to 3 days at 28°C, in sterile distilled water at a final concentration of approximately10^8 cfu/ml. Inoculum density was adjusted to 10^7-10^8 (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 inoculums. Disease scoring was done 15 days after inoculation. Five leaves per plant were taken for scoring and the plant reaction was rated on a 1-4 scale according to lesion length scores as given in Table 1.

Table 1: Lesion length scoring for BLB disease

Score	Lesion length	Category
1	< 3cm	Resistant
2	3.1 to 5.0 cm	Moderately resistant
3	5.1 to 7.0 cm	Moderately susceptible
4	>7.1 cm	Susceptible

Statistical Analysis

For inheritance studies of BLB resistance in the segregating population, the goodness of fit of expected genetic ratios were tested by χ^2 –test (Singh *et al.* 1977). The chi-square analysis for genotypic and phenotypic ratio was calculated by using following formula: $\chi^2 = \sum(O\text{-}E)^2 / E$, where, O is observed value, E is expected value, and $\sum$- Summation.

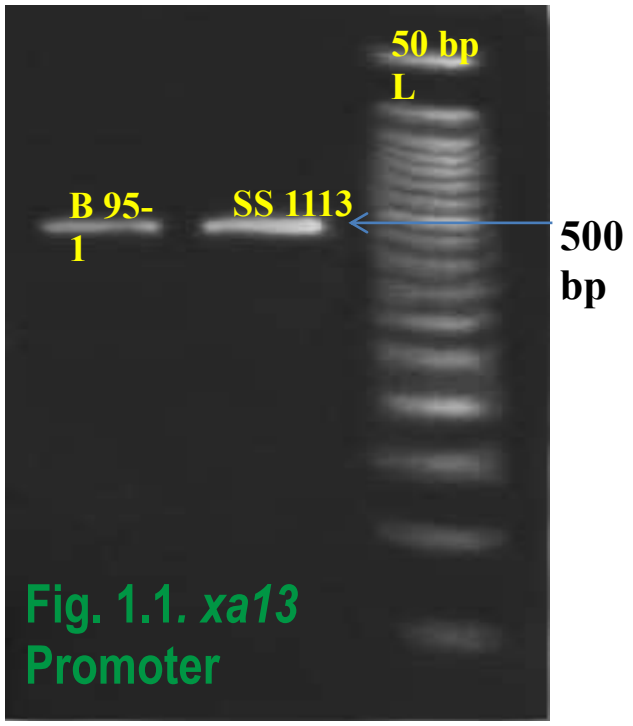
Results and Discussion

Rice is one of the most important cereal crops for global food security. Therefore 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 makers, (*xa13 promotor* and *pTA 248*). The marker validation was done in the resistant parent B95-1 for the BLB resistant genes viz., *xa13* and *Xa21* with gene linked markers, (*xa13 promotor* and *pTA 248*) by comparing with their check material SS113. The results (Fig.1.1) revealed that an allele of 500bp was amplified with *xa13 promoter* in the resistant parent and the marker *pTA 248* amplified an allele of 925bp (Fig.1.2), in B95-1 which is exactly identical to the band that was amplified in the check material, SS1113, confirming that the resistant parent was carrying both *xa13* and *Xa21* genes.This result is in confirmation with the results of Sundaram *et al.* (2008). Study of parental polymorphism is a prerequisite to begin marker assisted selection. Unless the parents are polymorphic for the traits of interest, the further selection of plants carrying the traits of interest is not possible in the progenies. SSR Markers can detect a significantly higher degree of polymorphism in rice (Okoshi *et al.* 2004). In the present study, the primer pair, *xa13 promotor* amplified a clear band of 250bp in MTU1010 while another band of 500bp was amplified in the resistant parent, B95-1 (Fig.2.1). Similarly, polymorphism was observed between B95-1 (925bp) and MTU1010 (730bp) when *pTA 248* primer pair was used for amplification of *Xa21* gene (Fig.2.2). The clear polymorphism was existed between the parents, MTU1010 and B95-1 for *xa13* and *Xa21* genes when amplified with *xa13 promoter* and *pTA 248* primer pairs, respectively. 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, McCouch *et al.* (1997) and Olufowote *et al. (*1997) also used SSRs to study the polymorphism in rice varieties. The present investigation clearly stated that two resistance genes viz., *xa13* and *Xa21* for BLB were present in B95-1. The susceptible parent, MTU1010 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.

F_1 crosses were made during Rabi, 2010 viz., MTU1010 x B95-1 and F_1 plants were raised in the field during Kharif, 2011. The primer pair *xa13 promotor* and *pTA 248* were used to confirm the hybridity of 25 F_1 plants from cross MTU1010 x B95-1 out of which 15 plants confirmed as true hybrids (*Xa13xa13Xa21xa21*) for both BLB resistant genes viz., *xa13* and *Xa21* as shown in Fig.3.1 and 3.2.

Table 2: Polymorphism between resistant and susceptible alleles.

Trait	Gene	Primer	Resistant allele	Susceptible allele
Bacterial Leaf Blight Resistance	*xa13*	*xa13 promotor*	500bp	250bp
	Xa21	*pTA 248*	925bp	730bp



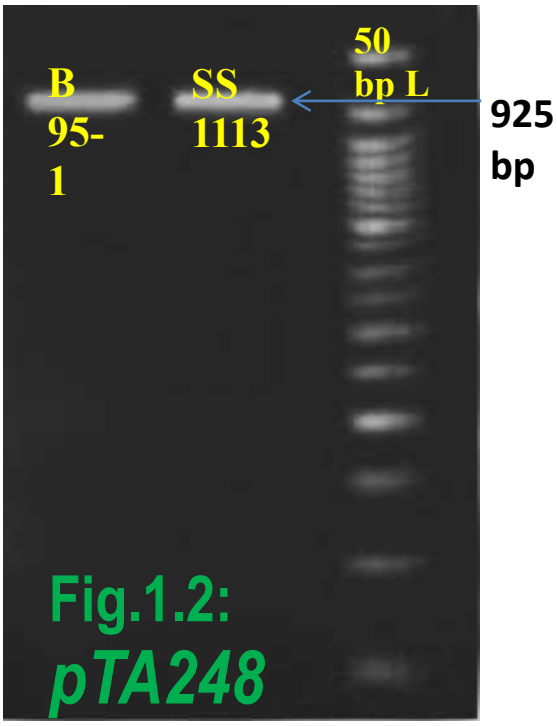
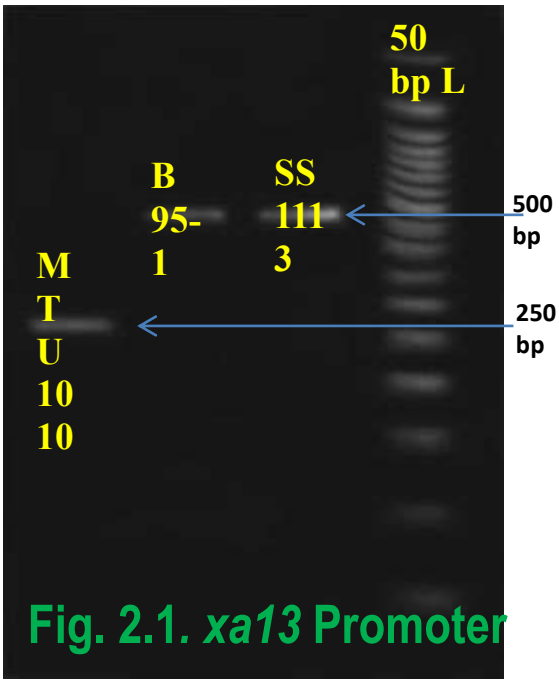


Fig. 1: Validation of SSR Markers linked to the bacterial leaf blight resistance genes viz., *xa13* (Fig.1.1) and *Xa21* (Fig.1.2) in resistant parents viz., B95-1.



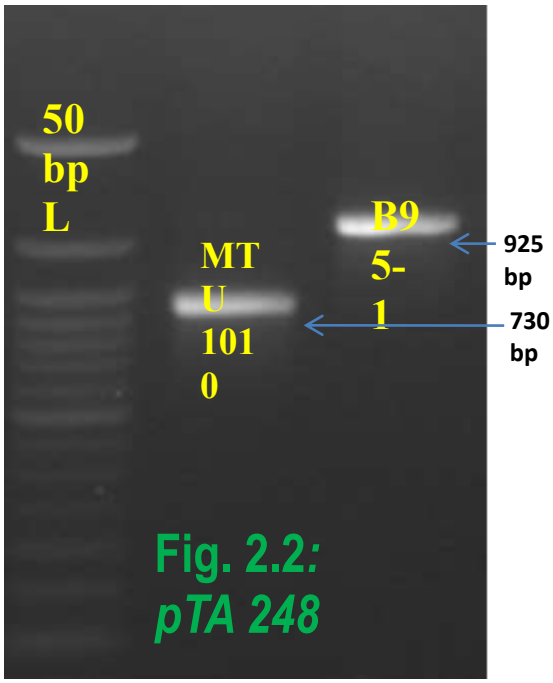


Fig. 2: Polymorphism between parental lines for four target genes viz., *xa13* (Fig.2.1) and *Xa21* (Fig.2.2) using gene specific primer pairs.

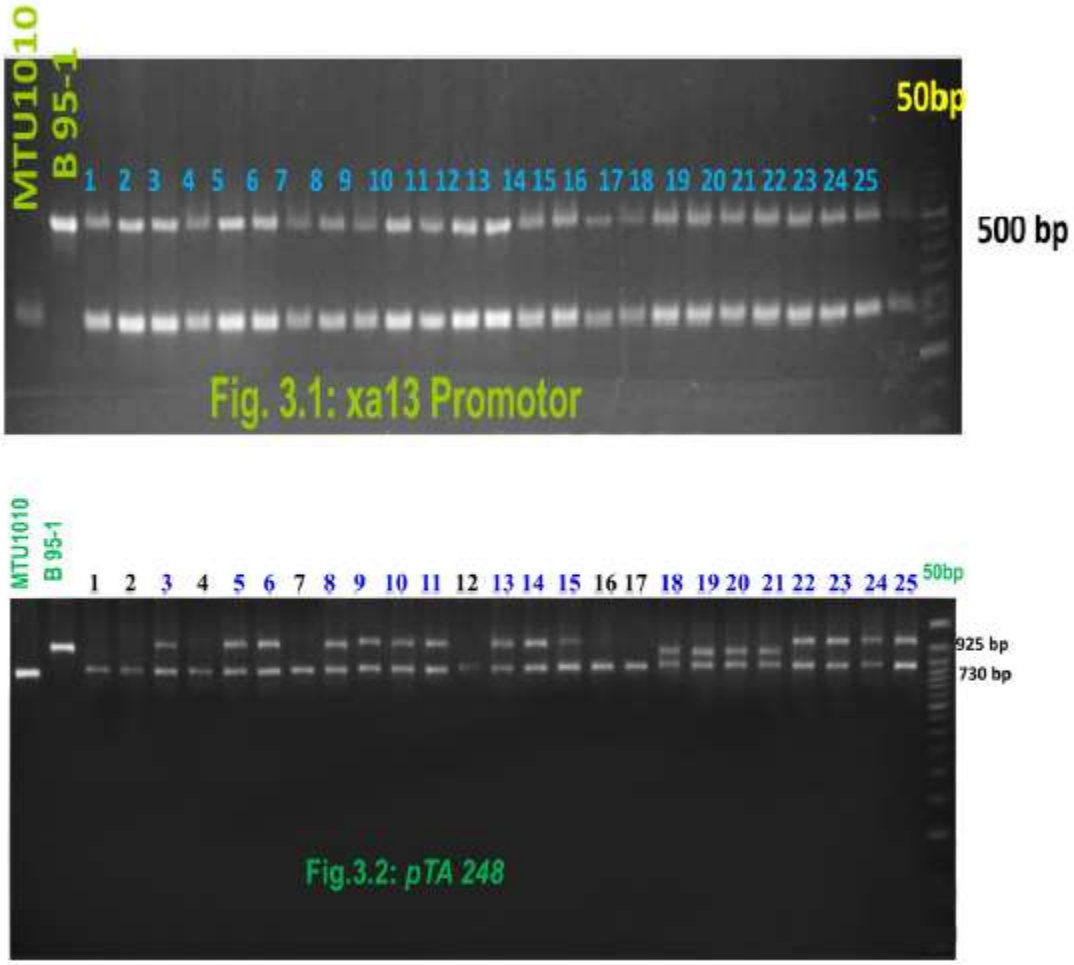


Fig. 3.1 & 3.2. Confirmation of F_1 plant for *xa13* gene and *Xa21* gene by using *xa13 promotor and pTA 248* primers, respectively.

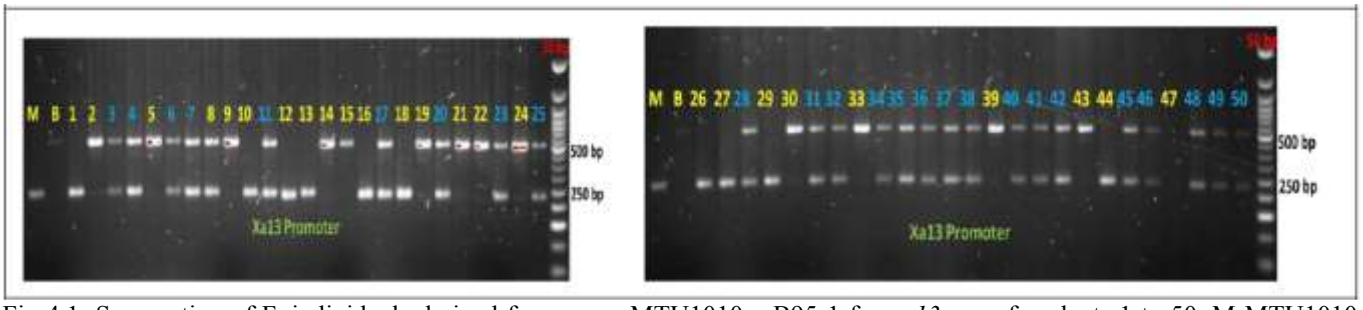


Fig 4.1: Segregation of F_2 individuals derived from cross MTU1010 x B95-1 for *xa13* gene for plants 1 to 50. M-MTU1010, susceptible parent and B-B95-1, resistant parent. (Note: The numbers in blue colour indicate heterozygous plants).

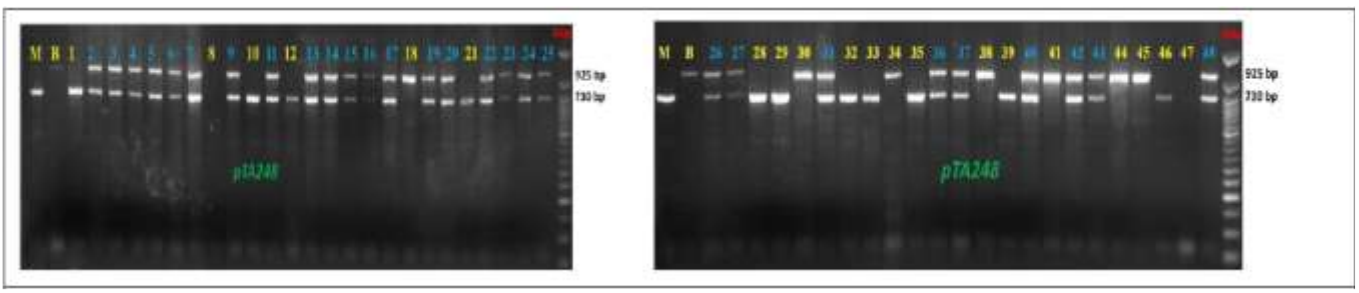


Fig 4.2: Segregation of F_2 individuals derived from cross MTU1010 x B95-1 for *Xa21* gene for plants 1 to 48, M-MTU1010, susceptible parent and B- B95-1, resistant parent. (Note: The numbers in blue colour indicate heterozygous plants).

The F_2 population was subjected to gene linked markers to study the co-segregation of the disease resistant genes viz., *xa13* and *Xa21*. Four hundred twenty F_2 plants developed from cross MTU1010 x B95-1 were analyzed with *xa13 promotor* (Fig.4.1). The result showed that 93 F_2 plants were identical to susceptible parent (250bp), while 101 F_2 plants were identical to resistant parent (500bp) and 226 F_2 plants exhibited heterozygous nature for both the alleles. The χ2-square analysis indicated that the *xa13* gene segregated in a genotypic ratio 1*Xa13Xa13*: 2*Xa13xa13*: 1*xa13xa13* and exhibited a good fit to the expected segregation ratio for single gene model with χ2-square value 2.73 at $p<0.05$. Genetic analysis of *xa13 promotor* clearly exhibited a goodness of fit to the expected segregation ratio of 1*Xa13Xa13*: 2*Xa13xa13*: 1*xa13xa13* for single gene model for *xa13* gene. Phenotypically it is not possible to differentiate all the genotypes. However, it is possible to make selection of plants carrying desirable gene combination i.e.*xa13xa13* by MAS. Similarly, *pTA 248* primer pair used to study the co-segregation of *Xa21* gene. Out of 420 F_2 plants, *pTA 248* primer pair amplified an allele of 730bp in 109 F_2 plants identical to susceptible parent, an allele of 925bp in 94 F_2 plants identical to resistant parent and 217 F_2 plants exhibited heterozygosity (Fig.4.2). The χ2-square analysis indicated a good fit to the expected segregation ratio 1*Xa21Xa21*: 2*Xa21xa21*: 1*xa21xa21* for single gene model. This result is in agreement with the results of Jiang *et al.* (2004), for *Xa21* gene in the F_2 population of 'Minghui 63'. The *xa13* gene confers resistance only when 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 two BLB resistance genes viz., *xa13* and *Xa21* together showed goodness of fit to the expected ratio 1:2:2:4:1:2:1:2:1, for two genes with 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 segregate into nine distinct classes to be 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 combination.

Table 3: Co-segregation analysis of two BLB resistance genes viz., *xa13* and *Xa21* in F_2 population of MTU1010 x B95-1

S.N.	Genotype	Observed value	Expected ratio	Expected value	χ^2 value
1	*Xa13 Xa13 Xa21 Xa21*	26	1	26.25	0.02
2	*Xa13 Xa13 Xa21 xa21*	48	2	52.5	0.38
3	*Xa13 xa13 Xa21 Xa21*	47	2	52.5	0.57
4	*Xa13 xa13 Xa21 xa21*	112	4	105	0.46
5	*xa13 xa13 Xa21 Xa21*	21	1	26.25	1.05
6	*xa13 xa13 Xa21 xa21*	59	2	52.5	0.80
7	*Xa13 Xa13 xa21 xa21*	19	1	26.25	2.00
8	*Xa13 xa13 xa21 xa21*	66	2	52.5	3.47
9	*xa13 xa13 xa21 xa21*	22	1	26.25	0.68
Total		420	16	420	9.43**

The calculated χ^2 value, 9.43 less then 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 F_2 population from cross MTU1010 x B95-1 against Xoo isolate DX-066.

S.N.	Gene Combination	Disease Reaction/Scale	Observed value	Expected ratio	Expected value	χ^2 value
1	*Xa13 Xa13 Xa21 Xa21*	Resistant (only due to *Xa21*) 0 to 2.0	233	9	236.25	0.04
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	80	3	78.75	0.01
6	*xa13 xa13 Xa21 xa21*					
7	*Xa13 Xa13 xa21 xa21*	Susceptible (both genes in susceptible combination) >5-7	85	3	78.75	0.49
8	*Xa13 xa13 xa21 xa21*					
9	*xa13 xa13 xa21 xa21*	Moderately Resistant (only due to *xa13*) 2.0 to 4.5	22	1	26.25	0.68
Total			420	16	420	1.22**

The calculated χ^2 value, 1.22 is less then tabulated value, 7.815 at df=3 and P=0.05 and 11.345 at P=0.01.

The F_2 population from the cross MTU1010 x B95-1, showed segregation for resistance and susceptibility reactions for BLB with isolate, DX-066 (Table.4). χ2-value analysis of the result shows good fit to Mendelian segregation ratio. This indicates 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 showed good fit to phenotypic ratio of 9:3:3:1 Indicating that the two genes segregated independently and show 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 was 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 (>5-7) 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. In which 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 equivalent to resistance with ISM and SS1113 possessing *Xa21*, *xa13* and *xa5*

Conclusion

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 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 phenotypic level.

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