Medbiotech Journal

MBTJ, 2(3): 190-197, 2018

Research Paper

Marker-Analysis of Leaf Resistance Bacterial in Rice

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Received: 18 June 2018 Accepted: 27 July 2018 Published: 01 September 2018

Abstract

This study was carried out with the aim of cultivating different types of fine-grain and high-growing rice. Moreover, due to the transfer of bacterial leaf blight (BLB) resistant genes, such as *xa13,* viz., and *Xa21* from B95-1, they take advantage of Broad-spectrum resistance and durable resistance. MTU1010 (Cottondora Sannalu), as a commonly used fine-grain and high-growing rice, was chosen as the parent in the crossing procedure. In addition, this particular variety is quite vulnerable to BLB and B95-1 bearing resistant genes for BLB (*Xa21 & xa13* genes). Using gene-associated primers viz., *xa13 promotor* and *pTA 248,* an experiment was carried out which proved that the target genes were widely present in B95-1. Furthermore, in order to study polymorphism between rather vulnerable (MTU1010) and resistant (B95-1) parents, the aforementioned primers were utilized as well. During Rabi, 2010 and Kharif, 2011, the cross viz., MTU1010 x B95-1 was heavily influenced and F_1 progenies were firmly established. While the F_1 plants were considered as true hybrids for both of the genes which progressed to the F_2 generation, gene-associated markers were implemented in order to carry out the foreground selection. The genes (*xa13* & *Xa21*) that essentially control BLB resistance obeyed Mendelian inheritance, which was suggested in a Genetic analysis of F_2 populations. Finally, the plants bearing a combination of two resistance gene (*xa13xa13 Xa21Xa21, xa13xa13Xa21xa21*) demonstrated BLB resistance (0-2 scale), while the combinations of genes including viz., *Xa13Xa13Xa21Xa21, Xa13Xa13Xa21xa21,* and *Xa13xa13Xa21Xa21, Xa13xa13Xa21xa21* illustrated BLB resistance (0.5-3.0), which was proposed using the phenotypic data analysis.

Keywords: Rice; F_2 Population; Bacterial Leaf Blight; Marker-assisted Selection

How to cite the article:
G. Jamal, Sh. Rahman, Marker-Analysis of Leaf Resistance Bacterial in Rice, Medbiotech J. 2018; 2(3): 190-197, DOI: 10.22034/mbt.2018.76931

1. Introduction

Over half of the world's population consider rice as one of the major staple food in their life. However, numerous abiotic and biotic factors, bacterial leaf blight (BLB) as one of the prevalent diseases, restrain the production of rice. Moreover, various strategies including Host plant resistance (HPR) have been pursued in order to manage biotic stresses in an economical and eco-friendly manner (Hulbert *et al.,* 2001). Molecular markers are broadly employed in agriculture, and Mackill and McNally in 2004; Jordan *et al. in* 2004; Xu *et al. also in* 2004; Toojinda *et al. in* 2005; Liu *et al. in* 2006 and Mackill in 2007 have all assessed the use of molecular markers in rice cultivation. Furthermore, 78 SSRs were utilized by Kalaichelvan in 2009 so as to identify different varieties of rice. While taking advantage of morphological and molecular markers, Kalaichelvan also evaluated the genetic association between the elite rice cultivars. Yunbi in 2010 suggested that in order to have comparatively high selection efficiency, there should be a firm linkage between the utilized marker and the target gene.

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Furthermore, Sanchez *et al. in* 2000 incorporated MAS to transfer genes into a new plant type from pyramided lines. Singh *et al. in* 2001 were also able to transfer genes into the enhanced varieties of Indian rice. In addition, Joshi and Nayak in 2010 indicated that via marker-assisted selection (MAS) procedure, biotic stress resistance may be enhanced and using gene pyramiding or gene stacking may improve broad spectrum durable resistance.

Xanthomonas oryzae pv. oryzae is considered to be the cause of BLB which is one of the most destructive diseases of rice. In serious situations, yield losses ranging from 74% to 81% have been reported due to the presence of BLB (Srinivasan and Gnanamanickam 2005). Chen *et al.,* in 2011 recognized 34 BLB genes in rice where a number of them have been used in breeding lines, however, since there was a considerable shift in pathogen race frequency, numerous disease breakdowns have been reported (Mew *et al.,* 1992). Marker-assisted gene pyramiding may be able to avoid such breakdowns. According to Khush and Angeles (1999), the *xa13* gene is entirely recessive, and resistance is merely conferred in the status of homozygous. In 2010, Perumalsamy *et al.* introgressed three BLB resistance genes *xa5*, *xa13* and *Xa21* into two high-growing BLB vulnerable *indica* rice cultivars, 'ADT43' and 'ASD16' from isoline IRBB60. Moreover, functional markers were utilized in order to evaluate the existence of these three resistance genes in F_2 populations. Furthermore, satisfactory levels of resistance with respect to two main *Xanthomonas oryzae* isolates of South India were demonstrated by pyramided genotypes with two or three resistance genes. Based on the study of Khush *et al. in* 1989, the broad spectrum BLB resistance gene *Xa21 expression* was common in the dominant condition. This gene was also introgressed, via normal breeding, from a wild species *O. longistaminata* onto *O. Sativa* chromosome 11. While utilizing enhanced Pusa 6B as the donor for *xa13* and *Xa21,* markers *RG 136* and *pTA 248* associated with BLB resistance genes *xa13* and *Xa21*, respectively were implemented by Basavaraj *et al.* in 2010 for foreground selection in order to enhance Pusa 6A.

This research was conducted with the objective of cultivating different types of short-duration, fine-grain, and high-growing rice which was resistant to BLB. This goal may be achieved by the introgression of two BLB resistance genes viz., *xa13* and *Xa21* from B95-1 into the genetic background of MTU1010. In the resistant parents, validation experiments were carried out for the gene-associated markers viz., *xa13 promotor* and *pTA 248*. In addition, the parental polymorphism was investigated between vulnerable and resistant parents. Finally, in order to figure out the intrinsic pattern of these genes in the single hybrid derived F_2 population, an analysis was conducted with respect to the genotypic and phenotypic segregation.

2. Methodology

2.1 Plant Material

Andhra Pradesh Rice Research Institute (APRRI), Maruteru, Andhra Pradesh (A.P) developed a short-duration, fine-grain, and high-growing rice, MTU1010 (Cottondora Sannalu), in 1999 which is resistant to BPH. This variety of rice was utilized as the vulnerable parent for BLB. Moreover, Directorate of Rice Research, Rajendranagar, Hyderabad (Sundaram *et al.,* 2008) developed the B95-1 (enhanced Samba Mahsuri) and since it possesses BLB resistance genes viz., *xa13* and *Xa21*, it was utilized as the resistant parent.

2.2 Separation and quantification of genomic DNA

Based on the mini-preparation procedure or the modified method of Zheng *et al. (*1991), Genomic DNA isolation from parents (MTU1010 and B95-1), F_1, F_2, and their check materials viz., SS1113 was carried out. According to the procedure reported by Sambrook *et al.* in 2001, DNA samples were quantified by 0.8% agarose gel electrophoresis with diluted uncut DNA ladder as the standard and spectrophotometer (Thermo electronic corporation UV1).

2.3 Polymerase chain reaction

In the next stage, on the Applied Biosystems Veriti 96 well thermal cycler, 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 were used in order to perform PCR amplification (Genei, Bangalore, India). 5 minutes of initial denaturation at 94° C, 45 seconds of denaturation at 94° C, 45 seconds of primer annealing at 55° C (*xa13* promotor) and at 58° C (*pTA 248*), a total of 60 seconds extension at 72° C, 10 minutes of final extension at 72° C, and cooling at 4°C for an unlimited time were meticulously carried out in order to amplify the template DNA in the PCR profile. Then, for improved DNA amplification, these steps were exactly iterated for 35 cycles. Finally, for 60 minutes, these amplified products were carefully mixed with bromophenol blue and resolved in an electrophoretic fashion in 2% agarose gel alongside with the marker 50bp DNA ladder (Biolabs) in 1X·Tris–Acetic acid–EDTA (TAE) buffer. Bio-Rad Molecular Imager Gel Doc XR System was used in order to document the resolved PCR bands.

2.4 A Parental Polymorphism study and resistance genes presence verification in donor parent

The presence of two gene-specific primer pairs viz., *xa13 promoter* and *pTA 248 were verified* in B95-1 compared to the check material, SS 1113. They are closely associated with the BLB resistance genes viz., *xa13* and *Xa21*, respectively. In the next step, due to this verification, the two gene-specific primers viz., *xa13 promotor* and *pTA 248* were employed to assess the parental polymorphism (MTU1010 vs B95-1) of the resistance genes. Distinct polymorphism between the parental lines was indicated by the SSR markers which were utilized for the co-segregation analysis and F_1 confirmation in F_2 population (foreground selection).

2.5 F_1 plants Cultivation and Confirmation

The F_1 crosses were influenced during Rabi, 2010 viz., MTU1010 x B95-1. Moreover, during Kharif, 2011, single seedling per hill at a spacing of 20 x 20 cm was planted which caused F_1 seeds to grow in the primary field. In order to genotype the target genes, DNA was completely isolated from all F_1 plants. During Rabi, 2011, the harvested seeds of single hybrid plant bearing both *xa13* and *Xa21* genes (MTU1010 x B95-1) were selfed which made them progress to F_2 generation. Furthermore, gene-associated SSR markers were employed in order to evaluate these separating populations for the resistance genes viz., *xa13* and *Xa21.*

2.6 Marker separation Genotyping in F_2 population

In order to determine the target genes inheritance, a total of 420 F_2 plants from MTU1010 x B95-1 along with parents were genotyped. In addition, using gene-associated SSR markers viz., *xa13 promotor* and *pTA 248,* respectively, the inheritance of BLB resistant genes viz., *xa13* and *Xa21* was investigated. Alleles at the SSR loci were identified on 2% and 3% agarose gel. In order to verify the sizes of the detected allele in the parental evaluation, 50bp or 100bp DNA ladder was mixed with the first load. Identification of the plants bearing various genotypic combinations was achieved by the scoring of alleles. In other words, The F_2 plants indicating a pattern which was comparable to the vulnerable parent alleles were scored as '1', the plants possessing a banding pattern comparable to the resistant parent alleles were scored as '2' and finally, the ones with the heterozygous allelic pattern were scored as '3'.

2.7 Evaluating Diseases in F_2 Population

F_2 seedlings were vaccinated with hyper-virulent isolate (DX-066) of *Xanthomonas oryzae* pv. *Oryzae, which was* collected from DRR, Rajendranagar, in order to carefully determine the separation patterns of BLB resistance genes. Furthermore, the leaf clipping method developed by Kauffman *et al.* in 1973 was applied to vaccinate F_2 population with the bacterial culture at the highest tailoring levels. The process of vaccine preparation was as follow: the bacteria, grown on Haywards agar media for 2 to 3 days at 28°C, is suspended in sterile distilled water at a final

concentration of about 10^8 cfu/ml. the vaccine density was modified to around 10^7-10^8 (cfu/ml) and plant vaccination was conducted by cutting the tip (about 1 to 2 cm) of the entirely expanded uppermost leaf with a cutting tool which had been dipped into the vaccine. Moreover, 15 days after vaccination the disease scoring was carried out. In this process, five leaves of each plant were removed for the scoring procedure and according to lesion length scores, as shown in Table 1, the reaction of the plant was rated on a 1-4 scale.

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 vulnerable
4	>7.1 cm	Vulnerable

2.8 Statistical Analysis

χ^2 –test (Singh *et al.* 1977) was carried out in order to examine the quality of the expected genetic ratios fit, as part of the inheritance studies of BLB resistance in the separating population. The chi-square analysis of genotypic and phenotypic ratio was conducted by taking into account the following formula: $\chi 2 = \sum(O-E)^2/E$, where, O is the observed value, E is the expected value, and Σis the Summation.

3. Results and Discussion

As previously mentioned, rice may be regarded as one of the most vital cereal products in terms of food security around the globe. Thus, in order to cultivate rice with a broad spectrum durable BLB resistance, MAS has been utilized in conjunction with resistance breeding. A distinct marker-trait relation was developed for BLB, which makes it quite possible to track the transfer of trait genes viz., *xa13* and *Xa21* through the use of closely-associated markers, (*xa13 promotor* and *pTA 248*). Moreover, while comparing to their check material SS113, the marker verification was carried out in the resistant parent B95-1 for the BLB resistant genes viz., *xa13* and *Xa21* with gene-associated markers, (*xa13 promotor* and *pTA 248*). The results, as illustrated in Figure 1.1, suggested that an allele

of 500bp was amplified with *xa13 promoter* in the resistant parent. In addition, they also revealed that the marker *pTA 248* amplified an allele of 925bp in B95-1, as shown in Figure 1.2, which is exactly similar to the amplified band in the check material, SS1113. This observation leads to that fact that the resistant parent was bearing both *xa13* and *Xa21* genes. This result is in good agreement with the results of Sundaram *et al.* (2008). Initiating marker-assisted selection requires a Study of parental polymorphism and provided that the parents are polymorphic with respect to the traits of interest, further selection of the plants bearing traits of interest may be feasible in the progenies. Okoshi *et al.* in 2004, suggested that SSR Markers can, in fact, recognize a larger amount of polymorphism in rice. In this research, as shown in Figure 2.1, the primer pair, *xa13 promotor* amplified a distinct band of 250bp in MTU1010 while at the same time, another band of 500bp was amplified in the resistant parent, B95-1. In the same manner, as shown in Figure 2.2, when *pTA 248* primer pair was employed for *Xa21* gene amplification, polymorphism was clearly noticed between B95-1 (925bp) and MTU1010 (730bp). That is to say, at the time of amplification with *xa13 promoter* and *pTA 248* primer pairs, a distinct polymorphism was present between the parents, MTU1010 and B95-1 for *xa13* and *Xa21* genes, respectively. Moreover, in marker-assisted selection, the primer pairs viz., *pTA 248* (Huang *et al.,* 1997) and *xa13 promotor* (Sundaram *et al.,* 2008) were utilized as gene sequence-based marker for BLB resistance genes viz., *Xa21* and *xa13*. In the same fashion, in order to investigate the polymorphism in different types of rice, McCouch *et al.* (1997) and Olufowote *et al. (*1997) used SSRs in their studies. Our study suggested that two resistance genes viz., *xa13* and *Xa21* for BLB existed in B95-1, and as presented in Table 2, the vulnerable parent (MTU1010) was bearing both of the corresponding vulnerable alleles. Finally, these markers were chosen in the process of foreground selection as part of the separating generations since the polymorphism was highly distinct for the target genes of parents.

During Rabi, 2010 viz., MTU1010 x B95-1 and Kharif, 2011, F_1 crosses were created and F_1 plants were cultivated in the field, respectively. Moreover, in order to establish the hybridity of 25 F_1 plants from cross MTU1010 x B95-1, the primer pair *xa13 promotor* and *pTA 248* were utilized, where 15 plants were established as true hybrids (*Xa13xa13Xa21xa21*) for both BLB resistant genes viz., *xa13* and *Xa21* as depicted in Figure 3.1 and 3.2.

Table 2. Polymorphism between resistant and vulnerable alleles

Trait	Gene	Primer	Resistant allele	Vulnerable allele
Bactrial Leaf Blight Resistance	*xa13*	*Xa13 promotor*	500bp	250bp
	xa21	*pTA 248*	925bp	730bp

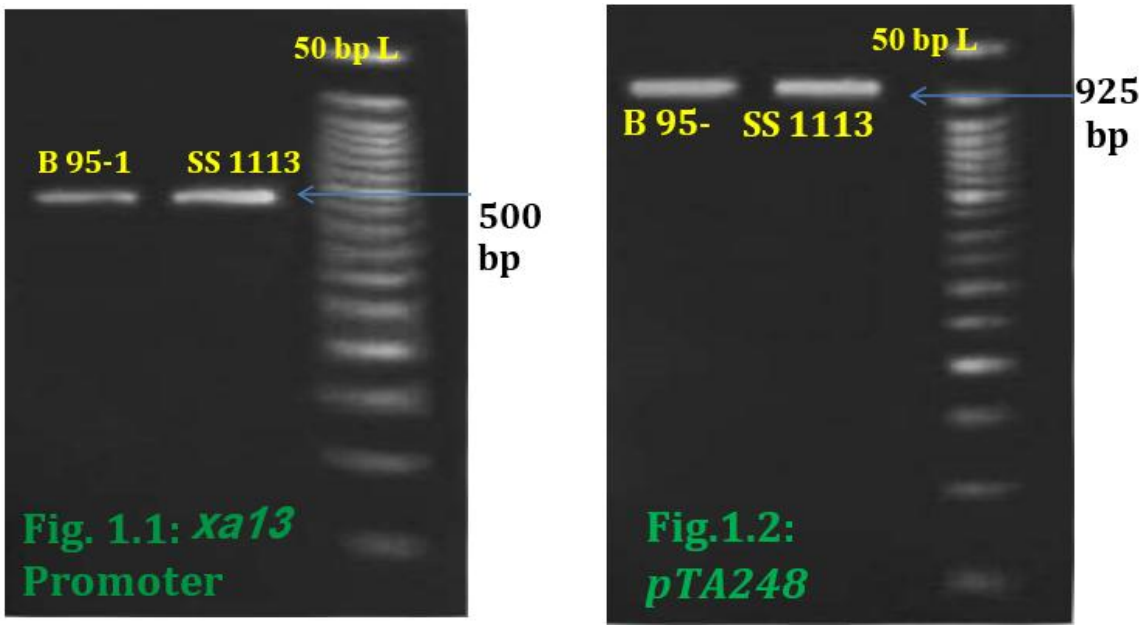

Figure 1. Validation of SSR Markers associated with the bacterial leaf blight resistance genes viz., *xa13* (Figure 1.1) and *Xa21* (Figure 1.2) in resistant parents viz., B95-1**.**

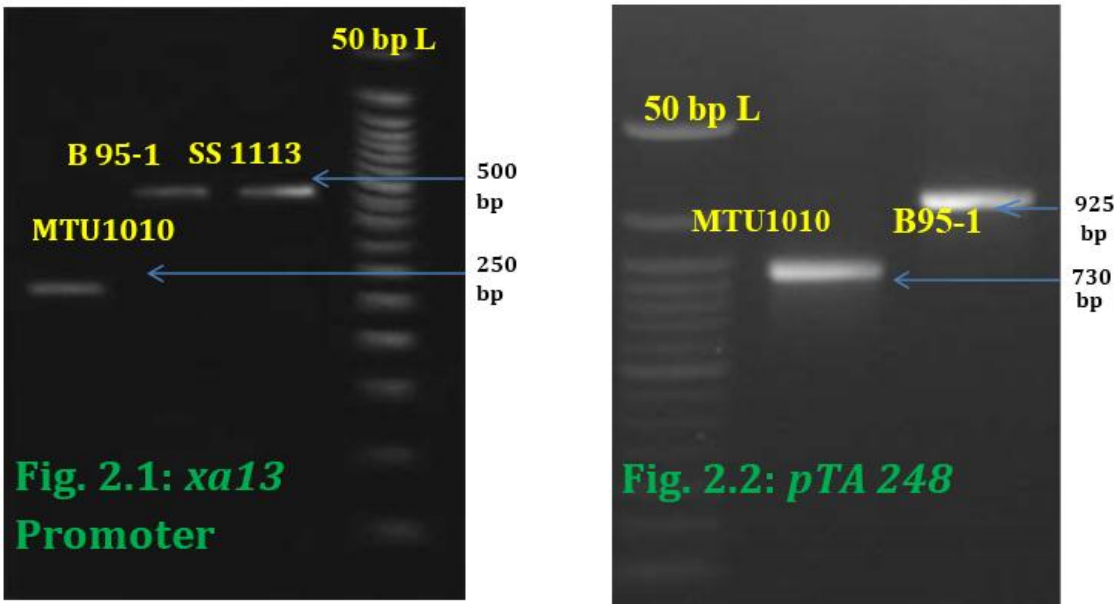

Figure 2. Polymorphism between parental lines for four target genes viz., *xa13* (Figure 2.1) and *Xa21* (Figure 2.2) using gene-specific primer pairs.

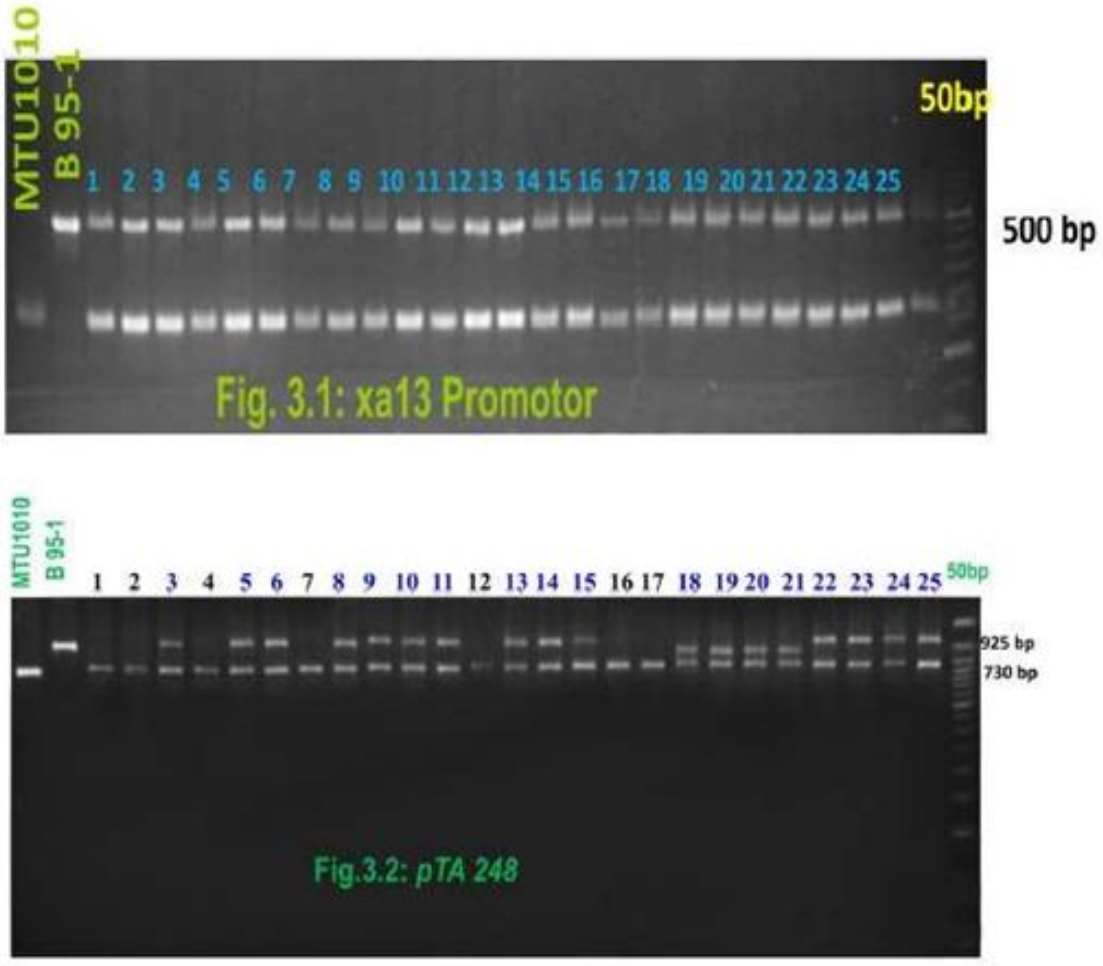

Figure 3.1 and Figure 3.2. Confirmation of F1 Plant for xa13 gene and Xa21 gene by using xa13 promotor and pTA primers, respectively.

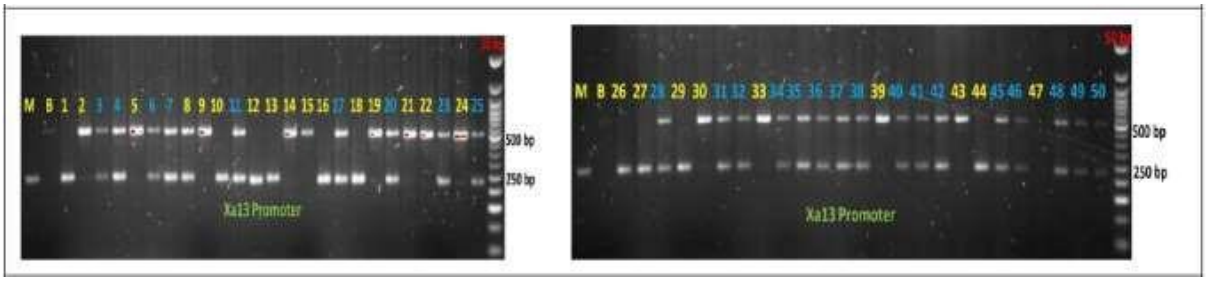

Figure 4.1. Separation of F_2 individuals derived from cross MTU1010 x B95-1 for *xa13* gene for plants 1 to 50. M-MTU1010, vulnerable parent and B-B95-1, resistant parent. (Note: The numbers in blue color indicate heterozygous plants).

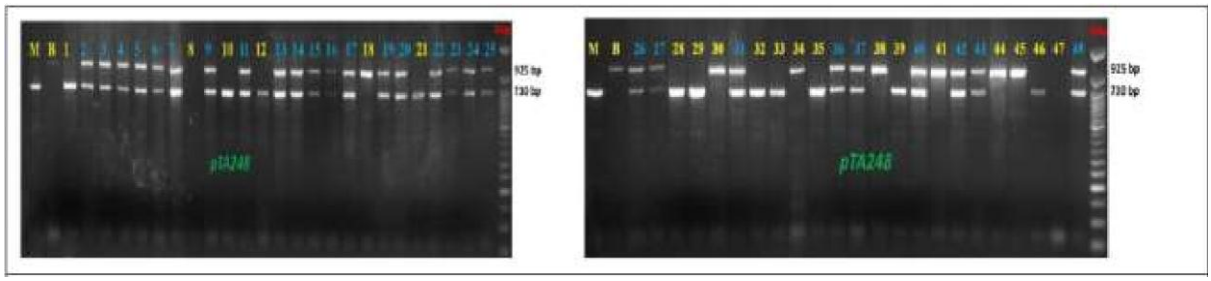

Figure 4.2. Separation of F_2 individuals derived from cross MTU1010 x B95-1 for *Xa21* gene for plants 1 to 48, M-MTU1010, vulnerable parent and B- B95-1, resistant parent. (Note: The numbers in blue color indicate heterozygous plants).

In order to investigate the co-segregation of the disease resistant genes viz., *xa13* and *Xa21,* the F_2 population was exposed to gene-associated markers. As depicted in Figure 4.1, 420 F_2 plants cultivated from cross MTU1010 x B95-1 and were evaluated with *xa13 promotor*. The result clearly suggested that 93 F_2 plants were exactly similar to the vulnerable parent (250bp), while 101 F_2 plants were identical to the resistant parent (500bp) and the rest (226) of F_2 plants displayed heterozygous property for both of the alleles.

In addition, the χ2-square analysis proposed that the *xa13* gene was separated in a genotypic ratio 1*Xa13Xa13*: 2*Xa13xa13*: 1*xa13xa13* and displayed an accurate fit with respect to the expected segregation ratio for single gene model with χ2-square value 2.73 at $p<0.05$. On the other hand, for single gene model of *xa13* gene, Genetic evaluation of *xa13 promotor* was in rather good agreement with the expected segregation ratio of 1*Xa13Xa13*: 2*Xa13xa13*: 1*xa13xa13*. However, it is not feasible to distinguish all the genotypes in a phenotypic manner. Nevertheless, it is quite feasible using MAS to exactly adjust the selection of plants which hold appropriate gene combination i.e. *xa13xa13*. In the same fashion, in order to investigate the co-segregation of *Xa21* gene, *pTA 248* primer pair was utilized. Out of the 420 F_2 plants, in 109 F_2, *pTA 248* primer pair was able to amplify an allele of 730bp plants which was exactly the same as the vulnerable parent. Furthermore, in 94 F_2 plants, an allele of 925bp was similar to the resistant parent, and 217 F_2 plants displayed heterozygosity property as depicted in Figure 4.2. The χ2-square analysis suggested that the result was in good agreement with respect to the expected segregation ratio 1*Xa21Xa21*: 2*Xa21xa21*: 1*xa21xa21* for single gene model. Moreover, For *Xa21* gene in the F_2 population of 'Minghui 63', this result was in perfect agreement with the results obtained by Jiang *et al.* in 2004. While *Xa21* is quite dominant intrinsically and may be expressed even in the heterozygous condition, the *xa13* gene faces resistance merely in the homozygous recessive condition. as depicted in Table 3, the combined co-segregation analysis of two BLB resistance genes viz., *xa13* and *Xa21* reveled rather highly overlapping fit with respect to the expected ratio 1:2:2:4:1:2:1:2:1, for the two genes. Moreover, this result may lead to the conclusion that the two BLB resistant genes viz., *xa13* and *Xa21*obyed Mendelian Inheritance. According to Joseph *et al.* (2004), the two genes separate into nine distinct classes so as to be 1:2:2:4:1:2:1:2:1, where seven resistant genotypic classes viz., *xa13xa13Xa21Xa21, xa13xa13Xa21xa21, xa13xa13xa21xa21, Xa13xa13Xa21Xa21, Xa13xa13Xa21xa21, Xa13Xa13Xa21Xa21* and *Xa13Xa13Xa21xa21* out of these nine distinct classes were expected to separate 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 the 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*	vulnerable (both genes in vulnerable 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.						

As illustrated in Table 4, the F_2 population from the cross MTU1010 x B95-1, displayed separation for resistance and vulnerability reactions for BLB with isolate, DX-066. The χ2-value analysis obtained from the result was in perfect agreement with respect to the Mendelian segregation ratio. This result, consequently, leads to the fact that resistance to BLB is dominated by both single genes independently. Yoshimura *et al.,* in 1996 suggested that in comparison to lines having single (or fewer) resistance genes, greater levels of resistance are present in gene pyramid lines consisting of multiple BLB resistance. In this research, for the two gene combinations, the co-segregation analysis proved to be in good agreement with respect to the phenotypic ratio of 9:3:3:1. Therefore, the two genes separated independently and displayed a simple dominant-recessive association. In addition, with a range of score between 0 and 2, the plants holding *xa13* gene along with *Xa21* gene, both in homozygous condition, displayed BLB resistance. Furthermore, *Xa21* gene alone also displayed resistance (0.5-3.0), *xa13* gene alone showed moderate resistance (2-4.5) and the plants containing *Xa13* and *xa21* genes proved to be vulnerable (>5-7). Pandey *et al.* in 2013 introgressed two major BLB resistance genes, *Xa21* and *xa13*, coupled with a phenotype-based selection which enhanced traditional BB susceptible Basmati varieties even further (Taraori Basmati and Basmati 386). While lines holding both *Xa21* and *xa13* in homozygous condition (*Xa21Xa21xa13xa13*) demonstrated considerably greater levels of resistance equal to resistance with ISM and SS1113 possessing *Xa21*, *xa13* and *xa5*, they, in fact, announced enhanced lines holding a single resistance gene (i.e. either *Xa21* or *xa13*), both in homozygous condition (*Xa21Xa21* or *xa13xa13*), which presented moderate resistance to BLB.

4. Conclusion

Our study proposed that an effective selection of the desired combination of genotypes can be obtained when incorporating molecular markers which are closely associated with traits of interest, in combination with the phenotype based-selection. Moreover, in comparison to the commonly used breeding method (Dwivedi *et al.,* 2007), efficient detection of desired genotypes holding more than one gene can be implemented. Finally, the results further suggested that the selection according to the genotypic data may reflect at the phenotypic level.

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