INTROGRESSION OF BACTERIAL BLIGHT RESISTANCE GENES; Xa33 AND Xa38 INTO RICE GENOTYPE ADT 47 THROUGH MARKER ASSISTED SELECTION

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ASTRACT

Bacterial blight is one of the serious diseases of rice. Utilization of resistant varieties is considered to be the most effective method of control. ADT 47 rice genotype has nearly been improved with the blight resistance genes xa5, xa13 and Xa21, through marker assisted selection (MAS). As the objective of this study, marker assisted backcrossing breeding (MABB) was adopted for targeted introgression of the broad spectrum resistance genes, Xa*33* and Xa38 to the Improved ADT 47 rice variety. The rice genotypes FBRI-15, PR114 were used as donors of Xa33 and Xa38 genes, respectively. The marker RMWR7.1 linked to Xa33 and the sequenced-tagged site marker Os04g53050-1 specific to Xa38 were validated in parents who were used for foreground selection. True F_1 hybrids were selected using the polymorphic markers. Out of one hundred and thirty seven SSR markers, forty-two were polymorphic for Improved ADT 47 x FBRI-16 which was used for background selection. Foreground selection revealed that a single F_1 plant was heterozygote in Improved ADT 47 x FBRI-15 and it was used to develop BC_1 generation. At BC_1F_1, one plant was found to be heterozygous for Improved ADT 47 x FBRI-15. At BC_2F_1, a single plant was found heterozygous as well from the cross. In the final BC_3F_1 population, one heterozygous plant was validated from the cross, the plant was subjected to background selection for selecting plants having the genetic background similar to that of the recurrent parent (i.e., confirmation of recurrent parent genome recovery) using the forty-two dominant SSR markers. In BC_3F_1, there was a 92.80% parental genome recovery in the Improved ADT 47 x FBRI-15. This high parental genome line was selfed to produce BC_2F_2 for selection of homozygous lines for xa5, xa13, Xa21 and Xa33/Xa38. The study recommends that the BC_2F_2 plants will be selfed using pedigree method and then advance to preliminary yield trial (PYT), then Advanced yield trial (AYT) and finally to on farm assessment for release.

Keywords: Bacterial Blight, genotype, Rice, ADT47, Xa33, Xa38.

INTRODUCTION

Rice is an important staple food crop and major source of livelihood for more than a half of the world population. A major concern in rice production is yield losses due to diseases and insect pests attack. Fungal and bacterial rice diseases such as blast, brown spot and bacterial blight are the major diseases resulting in large yield losses and crop failure causing threat to global food security.

Bacterial blight (BB) is one of the major destructive diseases of irrigated and rain fed lowland rice ecologies of the world. It is caused by rod shaped, gram negative bacteria, *Xanthomonas oryzae* pav. *oryzae*. The rice crop can be attacked at all stages which may results

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in 20-30% yield losses. It may even cause higher yield losses of up to 80 percent in susceptible plants when favourable environmental conditions such as drastic winds, frequent monsoon rains, warm temperature present (Singh *et al*., 1997; Noh *et al*., 2007).

Different strategies have been used to control bacterial blight such as antibiotics spray, use of biological control and good agronomic practices (Khan *et al*., 2014). These strategies were not effective because of variability of the pathogen which prevented development of suitable chemical and biological control agents. Host-plant resistance is considered to be an effective and environmentally friendly management strategy to reduce yield losses (Sundaram *et al*., 2008). With the availability of BB resistance genes, deploying resistant varieties is certainly achievable. Also, with the advent of low cost sequencing and molecular marker technologies: identification, cloning and functional analysis of resistance genes can be performed rapidly. Up to date, more than 38 BB resistance genes have been identified in cultivated and wild relatives of rice and some of them were deployed in different rice varieties (Kottapalli *et al.,* 2007; Sundaram *et al.*, 2008; Bhasin *et al*., 2012; Dokku *et al*., 2013a). Of which, Xa21 and Xa27 are the two dominant bacterial blight resistance genes that confer broad spectrum and race specific resistance to multiple Xoo strains, which are widely exploited in rice breeding programs. With the aid of molecular markers, a new broad spectrum BB resistance gene Xa33 (Kumar *et al.,* 2012) and Xa38 (Cheema *et al*., 2008) were also identified from *Oryza nivara*.

The evolution of molecular marker technology facilitated introgression and pyramiding of resistance genes/QTL to a desired genetic background by contributing for rapid and precise selection of the target gene. As a result of advancement in genome research, large number of markers such as RFLP, RAPD and SSR markers tightly linked to the BB, blast genes were developed. The SSR markers are the most extensively used marker to identify resistance genes in rice. However, functional markers are superior in predicting the phenotype by eliminating false selection (Andersen and Lubberstedt, 2003). Functional markers for *xa5*, Xa21 and Xa38 from coding sequence *(*(Iyer and McCouch, 2007; Perumalsamy *et al*., 2010; Bhasin *et al*., 2012*)* and xa13 from promoter variation (Chu *et al.*, 2006) were developed which help in direct selection of genes involved in BB resistance, thus enhance reliability of marker-assisted selection. Introgression of resistance genes to an established variety is an important approach to overcome crop losses due to diseases and insect pests in rice breeding programs.

Pyramiding of resistance genes increases effectiveness and durability, thus preventing or delaying the evolution of new strains or biotypes. The probability of simultaneous pathogen mutations for virulence to defeat two or more effective genes is much lower than for a single gene (Mundt, 1990). Several reports confirmed the higher resistance for pyramided lines. Dokku *et al.* (2013a) reported pyramiding of four genes Xa4, xa5, xa13 and Xa21 in the elite variety Tapaswini background using marker-assisted selection. The present study was thus designed to introgress broad spectrum BB resistance genes Xa33 and Xa38 into Improved ADT 47 through markers assisted back cross breeding.

MATERIALS AND METHODS
The Study Area
The aim of this work is to develop bacterial blight resistance rice genotype through gene pyramiding. The field study was carried out at the Paddy Breeding Station, Department of Rice at Tamil Nadu Agricultural University, Coimbatore. The laboratory experiments were

conducted at the Centre for Plant Molecular Biology and Biotechnology (CPMB&B), Tamil Nadu Agricultural University (TNAU), and Coimbatore, India during 2014-2016.

Materials of the Study

Marker assisted backcross breeding method was attempted to introgress *Xa33* and Xa38 into Improved ADT 47 rice genotype. The donor line for Xa33, FBR1-15 (Samba Mahsuri x *O. nivara)* was received from the Indian Institute of Rice Research (IIRR, Hyderabad). The donor for Xa38, PR114 (O. *nivara* (Accession IRGC81825) x PR114) was received from Punjab Agricultural University, India. The recurrent parent, Improved ADT 47 which harbours xa5, xa13 and xa21 was developed at Tamil Nadu Agricultural University through marker assisted breeding (Perumalsamy *et al.,* 2010). The F_1 and BC_1F_1 generations were raised along with the parents and confirmed the presence of bacterial blight resistance alleles using linked markers. Identified heterozygote for Xa33 and Xa38 was backcrossed with the recurrent parent, Improved ADT 47 to produce BC_2F_1 and subsequently BC_3F_1.

Methods of the Study

A crossing was made between the recurrent parent (Improved ADT 47) and the donor parents FBR1-15 to pyramid bacterial blight resistance genes through molecular markers in rice. The F_1, BC_1F_1, BC_2F_1 and BC_3F_1 were screened and the heterozygote identified in each generation was moved to the next generation accordingly by marker assisted selection. The bacterial blight resistant donor lines were raised in staggered sowing at two weeks interval in order to achieve pollination. All the cultural operations were carried out similar to the normal rice crop. Hand emasculation of panicles at 50-60% emergence was carried out in early morning before 8:00 AM by using modified clipping method which was developed at IRRI. All the spickelets from the top portion of the panicle which have already completed anthesis and all the young immature spickelets in the lower portion of the panicle was removed with scissors.

Warm air on a cloth covered panicle was blown from mouth to facilitate opening of spickelets. All the six anthers were removed carefully without causing injury to stigma. The emasculated panicle was bagged using a wax paper cover to prevent entry of foreign pollen. To pollinate the emasculated panicle, the whole panicle from donor plant was carefully lifted and warm air was blown to open the spickelets. Then, the top portion of paper bag was opened to expose emasculated panicle and pollen from donor panicles was tapped over the emasculated panicle.

After pollination, each pollinated panicle was covered again with waxy paper and parentage and pollination dates were carefully labelled. Twenty five to twenty eight days after pollination, the crossed seeds were harvested by hand picking, air dried and safely stored for next sowing.

Development of BC_1F_1, BC_2F_1 and BC_3F_1 generations

A minimum of thirty crossed seeds was sown in raised beds along with parents. The BC_1F_1s were backcrossed with their respective recurrent parents to produce BC_2F_1 and the final backcross produced BC_3F_1.

RESULTS AND DISCUSSION

This study was conducted with the aim of introducing BB resistance genes Xa33, Xa38 into Improved ADT 47 with the aid of marker assisted selection. Backcross populations were developed using Improved ADT 47 as recipient parent and FBRI-15 as the donor parent (Plate 1).
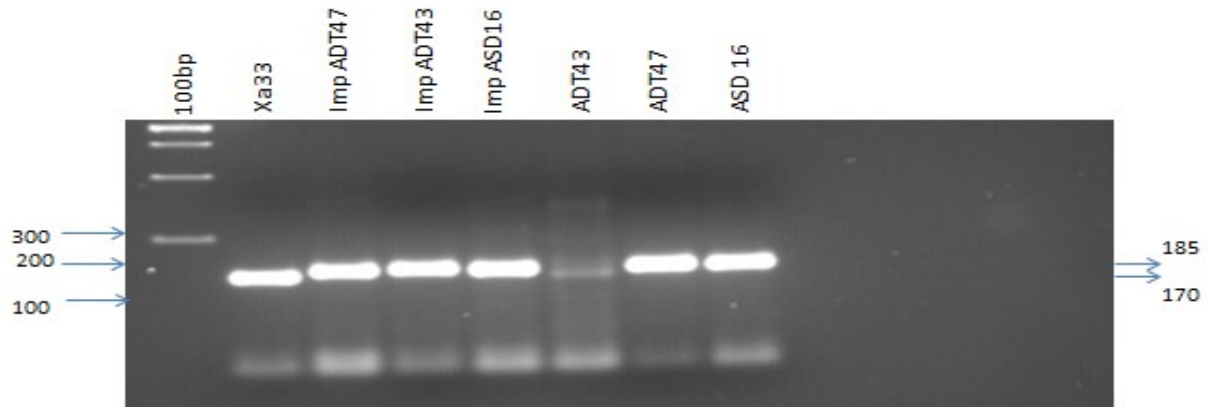
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Plate 1: Parental polymorphism between improved ADT 47 and FBRI-15 (*Xa33)* by
RMW7.1 marker

The backcross plants were used for background marker screening in order to know the recurrent parent genome recovery in introgressed plants. Plants with the heterozygous and homozygous combinations were identified. Attempt has been made to pyramid two bacterial blight resistance genes, Xa33 and Xa38 into Improved ADT 47 line that harbours xa5, xa13 and Xa21. Therefore, the study which aimed at incorporating resistance to BB through marker assisted selection into improved line is a timely initiative. (Plate 1).

Foreground Selection

The F_1s of the crosses between improved ADT 47 x FBRI-15 were raised in the field along with the parents. A total of 36 plants from improved ADT 47 × FBRI-15 were genotyped. A single progeny in Improved ADT 47 × FBRI-15 was confirmed as heterozygous for the respective alleles (Plate 2). In BC_1F_1, the verified heterozygous lines were surveyed for the presence of previous pyramided genes xa5, xa13 and Xa21 and were found to be homozygous for the parents' alleles. The single heterozygote plant for improved ADT 47 x FBRI-15 was backcrossed to develop BC_2F_1 (Table 1).
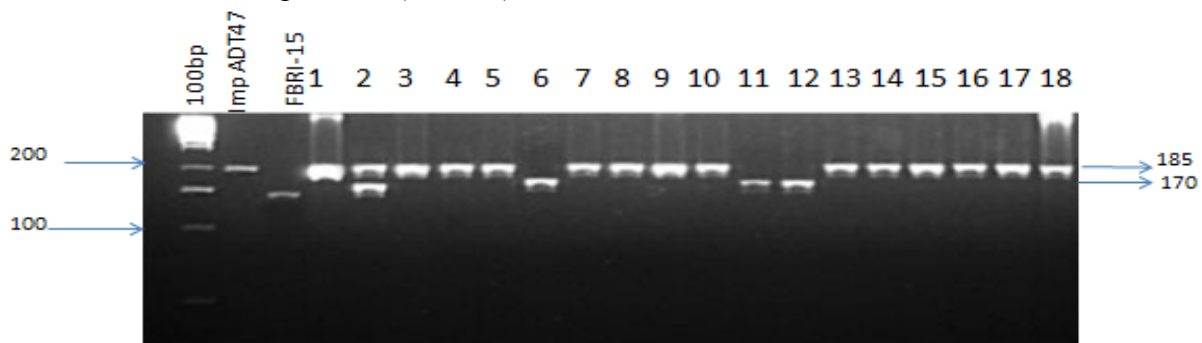
(a) PCR amplification of Improved ADT 47 x FBRI-15

Plate 2: *Showing heterozygosity for parent's alleles*

Plants with BB Resistance Gene

Subsequently, foreground screening was conducted in the BC_2F_1 generation in which 18 plants of Improved ADT 47/FBRI-15 were scored and one heterozygote plant (Table 1) in the crosses was identified and such plant was moved to BC_3F_1. In BC_3F_1, a total of twenty-one plants were scored for improved ADT 47 × FBRI-15, one heterozygote plant from the cross

carrying alleles of both parents was identified. (Table 1).The heterozygote BC_3F_1 plant was subjected to background analysis to identify the current genome contribution.

Genetic variation and effective selection strategies to identify desirable alleles for target trait in breeding populations are the two key factors which determine successful breeding programme. Marker-assisted selection is an indirect selection which involves selection of plants based on the genotype of the linked marker. In this study, parental polymorphism survey showed that the SSR markers RMWR7.1, RMWR7.6, RMWR7.10 effectively identified the polymorphism between FBRI-15 and Improved ADT 47 for Xa33 (Plate 1).

Table 1: Number of Plants with BB Resistance Gene identified in F_1 and Backcross Generations of Improved ATD 47 × FBRI-15

| S/No.	Generation	Plants scored	No of heterozygous Plants
1	F_1	24	1
2	BC_1F_1	18	1
3	BC_2F_1	12	1
4	BC_3F_1	21	1

Background Selection

A total of 137 SSR markers were screened for polymorphism and 42 were found to be polymorphic in the Improved ADT 47 x FBRI-15 and were fairly distributed amongst the 12 rice chromosomes (Table 2). The experiment showed 137 SSR markers tested, of which 42 markers were polymorphic between Improved ADT 47 and FBRI-15 (Xa33*)* and as such were selected. Identification of these numbers of polymorphic markers indicates close relationships between the recipient and donor parents. In a related studies, use of SSR markers were reported to be useful in the recurrent parent genome analysis while incorporating xa5, xa13, Xa21 in Samba Mahsuri (Sundaram *et al.,* 2008), xa5, xa13, Xa21 in Lalat (Dokku *et al*., 2013b) and xa13, Xa21, Pi54, qSBR11-1 in improved *Pusa Basmati1* (Singh *et al*., 2012). Application of stringent phenotypic selection coupled with background analysis using molecular markers for recurrent parent features at BC_1F_1 and BC_2F_1 during marker-assisted selection reported to yield superior results (Joseph *et al.,* 2004). This scheme was also adopted in the development of Improved ADT 47 for BB resistance to hasten the recovery of recurrent parent genome.(Plate 3).

In a similar study, Chen *et al.* (2001) used 10 pairs of AFLP primers to select for the genetic background of the recurrent parent while incorporating Xa21 in "6078', an elite restorer line. They developed an improved version of '6078 (*Xa21*)' with about 98.8% of the recurrent parent genome after three generation of backcrossing. Gopalakrishnan *et al.* (2008), recovered recurrent genome up to the extent of 86.72% through AFLP assay and 76% through STMS markers in BC_1F_3 generation. Neeraja *et al.* (2007) has recorded a maximum of 94.2 % of recipient genome in just two backcross generation. It is achieved by increasing marker densities in each backcross generation. Maximum of 86.5 % of recipient genome was obtained by Gopalakrishnan *et al*. (2008) in Xa21 and xa13 pyramiding program in BC_1F_5. A few well-placed markers (two to four markers in a chromosome of 100cM) provide adequate coverage of genome in backcross program (Servin and Hospital, 2004).

Table 2: List of Microsatellite Markers Polymorphic between Improved ADT 47 and FBRI-15

Chromosome Locus	Total no. of Markers analysed	No. of Polymorphic markers
1	10	5(RM240, 371, 140, 443, 431)
2	7	3(RM305, 427,122)
3	16	4(RM3204, 6959, 1940, 3117)
4	12	4(RM241, 4405, 5686, 5633)
5	9	3(RM122, 153, 3575)
6	18	5(RM342, 1163, 402, 8226, 340, 2214)
7	13	3(RM427, 8037, 1268)
8	18	3(RM5808, 210, 447)
9	11	4(RM3919, 1896, 5799, 1099)
10	14	3(RM258, 3152, 171)
11	6	2(RM3717, 1124)
12	10	3(RM22, 3331, 110)
Total	137	42

The single plant from the Improved ADT 47 (xa5, xa13, Xa21 and Xa33) crosses was identified and selfed to produce F_2 generation (Plate 3).
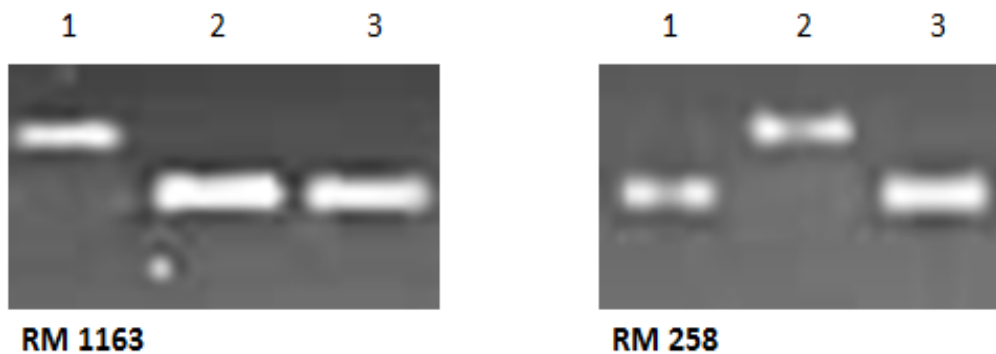
Line: 1- Improved ADT 47, 2- FBRI-15, 3- Progeny
Plate 3: *Screening of parents using microsatellite markers for background analysis*

The recurrent parent genome contribution of the cross between Improved ADT 47 x FBRI-15 was found to be 92.8% (Table 3).

Recurrent Genome in BC_3F_1 Generation of Improved ADT 47

Recurrent parent genome recovery of above 92% for Improved ADT 47 of the selected plants was observed at the last backcross (Table 3). Parent with the desirable features along with agronomic and grain quality traits were derived within three consecutive backcrosses. High background recovery of the recurrent parent genome (92%) was observed for selected BC_3F_1 plant of the cross `Improved ADT 47/FBRI-15`. This is due to the application of stringent selection for genotypic features of recurrent parent to selects BC_3F_1 plant for background analysis. This technique speeds up recovery of the recurrent parent genome within

single backcross. In a similar study by Joseph *et al.* (2004), 84% to 86.7% recovery of recurrent parent genome was reported by applying phenotypic screening for BB, agronomic traits and basmati quality characteristics in BC3 generation. Similar genetic similarity studies in introgression lines carrying BB resistance genes xa5, xa13 and Xa21 BB resistance genes in Lalat cultivar was found, where the generated dendrogram grouped the pyramided lines and recurrent into single cluster (Dokku *et al*., 2013b). Genetic background has influence on effectiveness of the introgressed gene due to gene to gene and/or gene to environment interactions (Liao *et al*., 2001). In addition, growth stage also has influence on the action of the gene.

Table 3: Estimation of Recurrent Genome in BC_3F_1 Generation of Improved ADT 47

Plant number	Number of polymorphic markers (n)	Number of homozygous markers (x)	Number of heterozygous markers (y)	Recurrent genome contribution (G) %
11	42	36	6	92.85

Rate of recovery of recurrent parent features depends on efficiency of the selection procedures used during backcross breeding. In this study, Xa33, Xa38 were pyramided into the genetic background of improved ADT 47. The pyramided lines carry xa5, xa13, Xa21 and Xa33/Xa38 in the background of ADT 47. This is the attempt to introduce four R genes into a single plant line. In this study, the objective of enhancing BB resistance while keeping the agronomic and grain quality traits of improved ADT 47 intact was successfully achieved (Table 3).

CONCLUSION AND RECOMMENDATIONS
As a final conclusion, in the current study, two BB resistance genes were introgressed into an improved line, ADT 47 through backcrossing with a maximum recurrent genome recovery of 92.8% (plant no. 45 from Improved ADT 47 x FBRI-15) and retaining the parental gene combinations of xa5, xa13 and Xa21. The above foreground and background selected plants were selfed in order to maintain their recurrent genome contribution thereby developing strong BB resistance. Its recommended that the BC_2F_2 plants will be selfed using pedigree method and then advance to preliminary yield trial (PYT), then Advanced yield trial (AYT) and finally to on farm assessment for release.

COMPETING INTEREST STATEMENT AND AUTHOR'S CONTRIBUTION
All the Authors declare that they have no competing interest. In terms of the authors' contributions, Dr. Ramalingam Jagdeep conceived and designed the study while Mr. Ahmed Auwal and Sheriff Hadiza Haladu conducted the experiment and performed the data analysis. Mr. Ahmed, A., also wrote the manuscript. Finally, all the authors read and approved the final manuscript for publication.

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