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Research Article

Marker-assisted selection of bacterial blight broad spectrum resistance genes *Xa33* and *Xa38* into CO43 in ICF$_3$ generation

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Abstract
Bacterial Leaf Blight (BB) is one of the devastating diseases in Southern India. An elite rice variety, CO43 which is a medium duration variety (135 days) with average yield (6.3t/ha) and does not show resistance to Bacterial Leaf Blight (BB). Scientists had discovered several BB resistant genes which are both dominant and recessive and is denoted as *Xa* and *xa* respectively. The current study is on the two genes *Xa33* and *Xa38* which are pyramided and is located on chromosome number 7 and 4 respectively. The rice genotypes FBR1-15 and PR114 were used as donors of *Xa33, Xa38.* The markers RMWR7.5 linked to *Xa33* and Os04g53050-1 specific to *Xa38* were validated in parents (CO43/ FBR1-15 and CO43/ PR114) and ICF3 populations to carry out Marker Assisted Selection (MAS). The segregating population of ICF$_3$ were genotyped and the homozygous plants were subjected to artificial screening. The plants which showed resistance compared to CO43 are potential source of disease resistance which could be evaluated further and can be utilized for further breeding program.

Key words
Rice, Bacterial leaf Blight (BB), *Xanthomonas oryzae pv. oryzae* (*Xoo*), Foreground analysis, Artificial screening (Clipping method).

Introduction

Rice is a staple food for millions of people and is well adapted to different agro climatic conditions. The cultivation of rice is affected by several abiotic and biotic factors which affect the crop at physiological, biochemical and molecular levels. This results in severe yield loss. The causal organism of Bacterial Leaf Blight (BB) is *Xanthomonas oryzae pv. oryzae (Xoo)* is one of the grievous disease to rice cultivation of irrigated and rainfed lowland ecosystems which affects almost all parts of the world including Asia, Northern Australia, Africa, Southern part of United States and Latin America (Mew, 1987; Niono and Niono, 2005). In Asia, the disease is reported to reduce the yield upto 50% and also affects grain quality, in its severe form, causes yield losses of 74- 81% (Srinivasan and Gnanamanickam, 2005). An economical and ecofriendly strategy to achieve disease resistance and yield stability is to bring in host plant resistance. To accomplish this, steps to widen the genetic variation by exploring the genetic diversity can be carried out in future rice breeding (Sattari *et al.*, 2014). Promising rice cultivars which are resistant to BB causing pathogens can be developed by the discovery of new resistant genes, incorporating them in breeding programs (conventional or Marker Assisted Backcross Breeding) and pyramiding two or a few resistance genes (Kumar *et al.*, 2012). Linked markers are already being used in marker-assisted selection (MAS) programs for developing improved rice cultivars (Hittalmani *et al.*, 2000; Sanchez *et al.*, 2000). The basis of MAB breeding is to transfer a specific allele at the target locus from donor line to a recipient line while selecting against donor introgression across the rest of the genome (Collard and Mackill, 2008). CO43 is a semi-dwarf, lodging resistant, saline tolerant, high yielding (6.3t/ha) and a medium duration variety, grows well in Tamil Nadu and is released by TNAU (Tamil Nadu Agricultural University, Coimbatore). This local cultivar despite its advantages is susceptible to Bacterial Leaf Blight (BB) (Subramanian *et al.*, 1984). Utilizing the host plant resistance is an effective means to manage this pathogen in rice. As of now, 41 BB resistance genes have been reported among which 8 have been cloned and characterized (Ellur *et al.*, 2016; Hutin *et al.*, 2015; Kim *et al.*, 2015). Many of the wild species of *Oryza* such as *O. longistaminata*, *O. rufipogon*, *O. minuta*, and *O. nivara* have been reported to be resistant to BB (Brar and Khush, 1997). Five BLB resistance genes namely *Xa21*, *Xa23*, *Xa27*, *Xa29t*, and *Xa30t* have so far identified from *O. longistaminata*, *O. rufipogon*, *O. minuta*, *O. officinalis*, and *O. nivara*, respectively (Gu *et al.*, 2004; Tan *et al.*, 2004; Cheema *et al.*, 2008;Zhang *et al.*, 2009;). A dominant BB resistance gene present in chromosome 7 shows a broad spectrum of resistance to BB and is designated as *Xa33*. A putative gene encoding serine threonine kinase appears to be a candidate for the *Xa33* gene (Kumar *et al.*, 2012). A new SSR marker RMWR7.5 (located at 4.09Mb in the chromosome) is closely linked to the resistance gene *Xa38 (Xa30(t)*, a novel resistance gene was discovered from *Oryza nivara* acc. IRGC 81825 and is mapped to be located in the 38.4kb on chromosome 4L (long arm) (Cheema *et al.*, 2008). A co-dominant InDel marker, LOC_Os04gg53050-1 had been developed (Bhasin *et al.*, 2012) expects to amplify a region near *Xa38*. Therefore, the current research was undertaken to incorporate the resistant genes *Xa33* and *Xa38* into CO43, to widen the resistance spectrum of the crop variety.

Materials and Methods

The plant material selected in this study consists of ICF_3 lines from an intercross (CO43/FBR1-15) / (CO43/ PR114) where, CO43 is the recurrent parent, FBR1-15 and PR114 are donor parents of the genes *Xa33* and *Xa38* respectively (Fig.1).

The plants were tagged individually in the field (Wetlands, Tamil Nadu Agricultural University, Coimbatore). Leaf samples were collected from 14 days old seedlings for isolating plant genomic DNA and samples were stored in -80^{o}C freezer after blot drying it with filter paper and the rice genomic DNA was isolated using the modified CTAB method (Dellaporta *et al.*, 1983).

The SSR markers closely linked to *Xa33* and *Xa38* are RMWR7.5 (Gidamo *et al.*, 2015) and Os04g53050-1 (Bhasin *et al.*, 2012) respectively were used for validation of the parents and ICF_3 progenies. PCR amplification was conducted with the reaction mixture consisting of 2.0µl PCR buffer+$MgCl_2$, 0.5µl dNTPs, 1µl primer (0.5µl forward and reverse each) 0.2µl Taq polymerase, 2µl template DNA (50-100ng concentration) and made the reaction volume upto 15µl with sterile water. The PCR profile was setup in the order of initial denaturation at 94^{o}C (4mins) and final denaturation at 94^{o}C (1min) for a cycle, 35 cycles of PCR amplification which includes primer annealing at 55^{o}C (*Xa33*) for 30s and 56^{o}C (*Xa38*) for 1min, primer extension at 72^{o}C (1min), final extension at 72^{o}C (7min) and finally stored in 4^{o}C.

The amplified product obtained from PCR reaction was separated using agarose gel electrophoresis. The samples were resolved in 3.5% agarose gel. The bands in the gel are visualized by UV-Transilluminator.
The bands visualized in the agarose gel electrophoresis is scored as A (if the resistant alleles are absent), B (if the resistant alleles are present) and H (if the genes are said to be segregating under Mendalian ratio).

The experiment was carried out in the glass house at Department of Rice, *Xoo* isolates were cultured in peptone sucrose agar media and incubated at 30^{o}C for 2-3days. A broth of *Xoo* is prepared by placing it in shaker (120rpm at 30^{o}C) for a day and a half. The OD value measured at 600nm of the culture broth prepared should be at 0.5 (approximately $3x10^9$ CFU/ml).

The top leaves of the plants (80-90DAS) are cut with scissors dipped in bacterial suspension along with the recurrent parent as check. For accessing resistance, the length of the longest lesions of three damaged leaves of each individual leaves and was recorded twice at 8 and 14 days after inoculation. Based on their mean lesion length scores individual progenies were distinguished as 'R'- resistance (<3cm), 'MR'- moderately resistant (3-6cm), 'MS'- moderately susceptible (6-9), 'S'- susceptible (>9cm) (Cheema *et al.*, 2008).The observations were subjected to Analysis of Variance (ANOVA) and the disease severity as PDI was calculated using SPSS 16.0.

Results and Discussion

Molecular markers can be used as a flag to identify plant with resistance genes. A total of 70 plants (14 lines- 5plants from each line) from of ICF_3 segregating generation for the genes *Xa33* and *Xa38* were genotyped using SSR markers, RMWR7.5 (*Xa33*) and Os04gg53050-1 (*Xa38*) respectively along with their recurrent (CO43) and donor (FBR1-15 and PR144) parents. These plants were subjected to foreground analysis to check the status of introgression of the resistant genes. A total of 20 plants were confirmed with *Xa33* gene (Fig.2), 10 plants with *Xa38* (Fig.3) and 18 plants with *Xa33* and *Xa38* in homozygous condition. The homozygous plants with two gene combination showed broader host plant resistance than the plants with single gene.

Noda and Ohuchi (1989) reported pyramiding of two or more BB resistant genes *viz.*, *Xa4, xa5, xa13, Xa21* in the breeding line Tapaswini- with higher level of resistance against most of the BB isolates from various places and also several promising varieties have been released such as Angke (*Xa4* and *xa5*), Conde (*Xa4* and *Xa7*) (Toenniessen *et al.*, 2003), Tubigan 7 (*Xa4* and *Xa21*) (Verdier *et al.*, 2012), Lalat (*Xa4,xa5, xa13, Xa21*) (Dokku *et al.*, 2013) Tubigan- 11 (*Xa4* and *Xa21*) (Khan *et al.*, 2014), MTU1010- BB resistance (*xa13, Xa21*) and blast resistance (Pi54) (Kumari *et al.*, 2014).

The genetic base of the breeding population have an impact on the effectiveness of introgression of genes due to gene to gene or gene to environment interactions (Liao *et al.*, 2001). A total of 15 homozygous plants were screened along with CO43 (recurrent parent) as a check. The scoring was recorded wherein the progenies showed high level of resistance (R) with lesion length ranging from 0.5 to 3cm (Fig.4) while CO43, which was used as check showed moderate resistance (S) with lesion length of 10cm.

The screened plants with resistant genes *Xa33* and *Xa38* can be phenotypically evaluated which indicated that the advantage of marker-assisted selection. The present study reveals that plants with two genes recorded an enhanced level of resistance and will be evaluated for further agronomic traits and for disease resistance.

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Table 1. Scoring data on Artificial screening for BB

S.No.	Plant Lines	Gene combination	Lesion length in cm (8th day)			Disease severity as PDI (8th day)	Lesion length in cm (14th day)			Disease severity as PDI (14th day)	Resistant/ Susceptible
1.	487	*Xa38*	1	1.5	0.5	1.00^{a}	1	1.5	0.5	1.00^{a}	**R**
2.	763	*Xa38*	3	2	0.5	1.83^{a}	3	3	0.5	2.16^{a}	**R**
3.	508	*Xa38*	3	3	0.5	2.16^{a}	3	3	0.5	2.16^{a}	**R**
4.	168-236	*Xa33*	2	0.5	0.5	1.00^{a}	2	0.5	0.5	1.00^{a}	**R**
5.	168-248	*Xa38*	2	1	0.5	1.16^{a}	2	2	1	1.66^{a}	**R**
6.	168-245	*Xa38*	0.5	0.5	0.5	0.50^{a}	1	1	0.5	0.83^{a}	**R**
7.	577	*Xa33*	0.5	0.5	3	1.33^{a}	0.5	0.5	3	1.33^{a}	**R**
8.	168-242	*Xa33*	0.5	5	1	2.16^{a}	0.5	5	1	2.16^{a}	**R**
9.	3-7-70	*Xa38*	0.5	0.5	0.5	0.50^{a}	1	0.5	0.5	0.66^{a}	**R**
10.	168-237	*Xa38*	1	0.5	1	0.83^{a}	1	0.5	1	0.83^{a}	**R**
11.	168-249	*Xa38*	2	2.5	1.5	2.00^{a}	2	3	4	3.00^{a}	**R**
12.	168-234	*Xa38*	1	0.5	0.5	0.66^{a}	1	0.5	0.5	0.66^{a}	**R**
13.	3-7-13	*Xa38*	3	1	0.5	1.50^{a}	3	1	0.5	1.50^{a}	**R**
14.	168-247	*Xa33*	2	2	0.5	1.50^{a}	2	4	0.5	2.16^{a}	**R**
15.	CO43	Check	10	9.2	8	9.06^{b}	10	9.5	8	9.16^{b}	**S**

Scale - <3cm = Resistant; 3-6 cm = Moderately resistant; 6-9cm = Moderately susceptible;
>9 = Susceptible
*PDI- Percent Disease Index

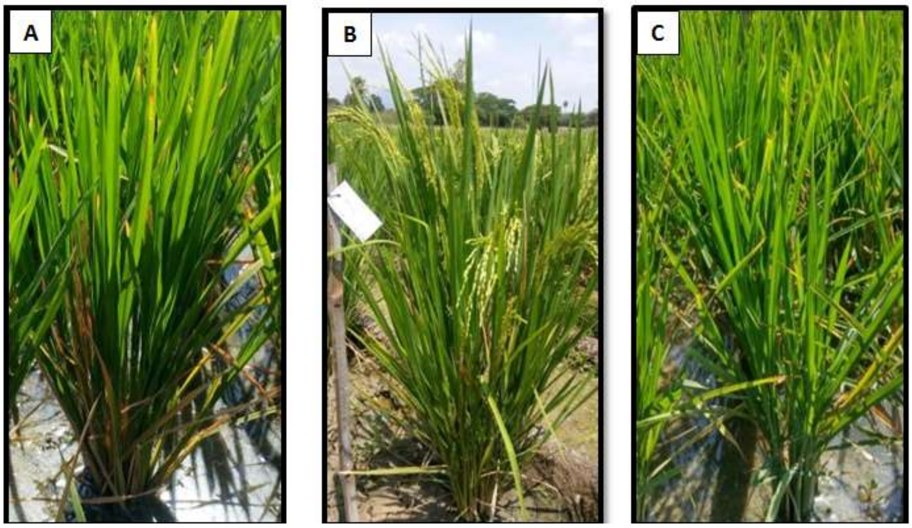

Fig. 1. A. CO43 (Recurrent parent); B. PR144 (*Xa38* donor parent); C. FBR1-15 (*Xa33* donor parent)

Fig. 2. Foreground selection of ICF$_3$ plants using RMWR7.5 marker (*Xa33* gene)

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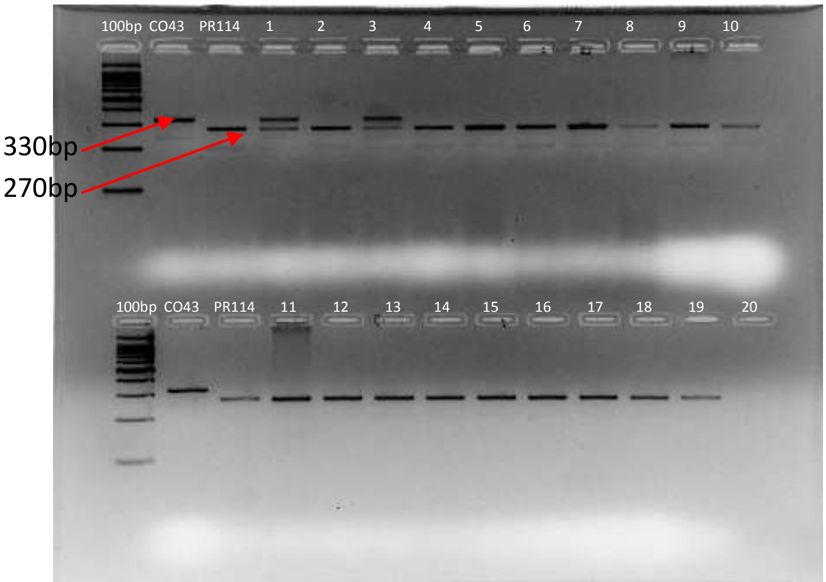

Fig. 3. Foreground selection of ICF$_3$ plants using Os04gg53050-1 marker (*Xa38* gene)
*1- ladder, lane 2- recurrent parent, lane 3- donor parent, lane 4 to 13- ICF$_3$ progenies

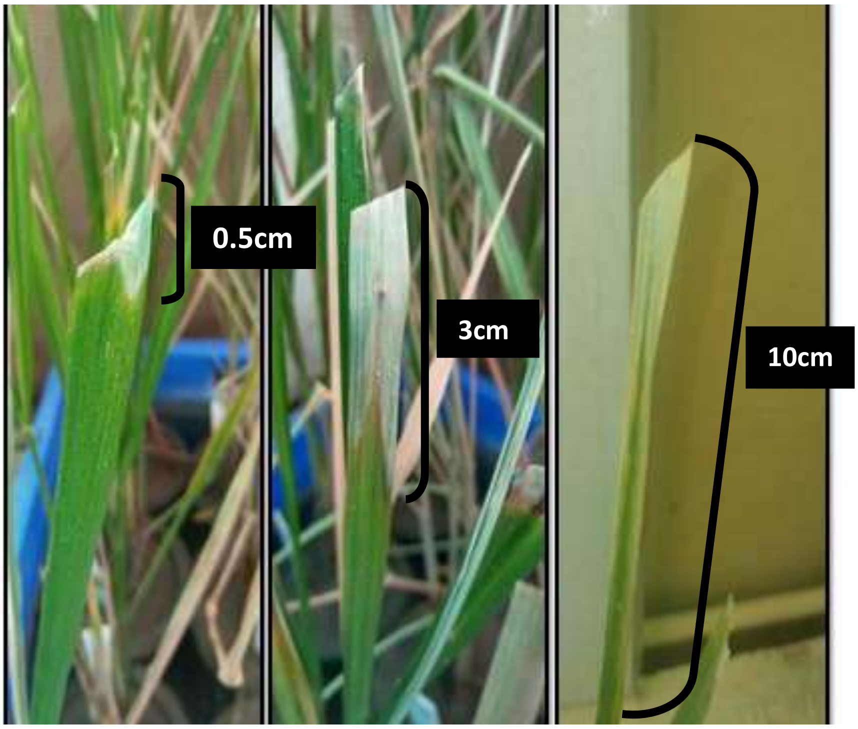

Fig. 4. Bacterial leaf blight screening by clipping method- Lesion length in different plants a) line 168-234 b) line763 c) CO43 (recurrent parent)