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Research Article

Marker-assisted selection for biotic stress (Bacterial leaf blight and gall midge) tolerance in Bc_4F_4 generation of rice (*Oryza sativa* L.)

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Abstract
Bacterial blight (BB) of rice caused by the pathogen *Xanthomonas oryzae* pv. oryzae and the insect gall midge (GM) (*Orseolia oryzae*) are two major limitations of rice production. The present study was carried out to improve CO 43 a popular variety of Tamil Nadu for BB and GM resistance through marker assisted backcross breeding. Two sets of genes *Xa21* and *xa13* for bacterial blight resistance and *Gm1* and *Gm4* for gall midge resistance were used for this study. A total of 95 plants in BC_4F_4 generation were genotyped with SSR markers RM1328, RM22550, xa13 prom and pTA248 for the genes *Gm1, Gm4, xa13 and Xa21* respectively. Artificial screening for bacterial blight screening was conducted in glass house condition and gall midge screening was conducted in the field condition. The lines selected for bacterial blight and gall midge resistance genes recorded a high level of resistance than the recurrent parent CO43.

Keywords
Bacterial blight, gall midge, foreground selection, marker assisted selection

Introduction

Rice (*Oryza sativa* L.) is the staple food for more than half of the world's population (Akhtar *et al*., 2010). China and India account for more than half of the world's rice areas and consume more than three – quarters of the global rice production (Hossain, 1997; Maclean *et al*., 2002). It is probably the most diverse crop as it is produced in a wide range of locations and under a wide variety of climatic conditions from the submerged areas in the world to the dried deserts.

A rising global population requires increased crop production. The rate of increase in crop yields is currently declining and traits related to yield, stability and sustainability should be the focus of plant breeding efforts. In the last five decades, adoption of green revolution technology had resulted in major increases in rice production. Though many high yielding varieties of rice have been released during the last few decades, yield potential is severely affected by various abiotic and biotic stresses. However, due to increasing population, it is estimated that we will have to produce 40 percent more rice in 2030. This increased demand will have to be met from less land, with less water, less labour and fewer chemicals. Among the biotic stresses, incidence of gall midge and diseases like blast, bacterial leaf blight are economically important as they cause significant yield reduction.

Use of chemical method plays a major role in reducing the losses in agricultural crops but it results in additional expenditure to the farmer and there has been a strong tendency for fungicides, like other crop protection products, to be overused (Bonman *et al*., 1992). This causes environmental problems like soil and water pollution and leads to imbalance in the ecosystem. Host plant resistance is the most economical and eco friendly way of disease control (Manandhar *et al*., 1992). Over the last three decades, rice breeders have extensively used resistance (R) genes which confer resistance to pest and diseases containing corresponding avirulence gene in rice breeding programs for developing cultivars with broad spectrum resistance.

Over recent decades, conventional breeding has played an important role in rice cultivar improvement. Molecular markers offer numerous advantages over conventional phenotype based alternatives as they are stable and detectable in all tissues regardless of growth, differentiation, development, or defense status of the cell and they are not confounded by the environment and epistatic effects (Agarwal *et al*., 2008). Hence, molecular markers can be exploited in plant breeding through an indirect selection of trait and having economic importance. It is called as marker assisted breeding (MAB) (Xu and Cruoch, 2008). DNA markers have enormous potential to improve the efficiency and precision of classical plant breeding *via* MAS by reducing the reliance on laborious and fallible screening procedures (Ragimekula *et al*., 2013).

Breeding for durable resistance involves the pyramiding strategy which includes successive to introduction of more than one gene to a single cultivar. Gene pyramiding approach poses difficulties due to dominance and the epistatic effect of genes that governs disease resistance in conventional breeding (Joseph *et al*., 2004). MAS is an effective approach for pyramiding important genes to a new cultivar by rapidly recovering the background quality characteristics of the recurrent parent (Shanti *et al*., 2010). The most widely used markers in major cereals are called simple sequences repeats (SSRs) or microsatellites (Gupta and Varshney, 2000). Hence the present study was conducted to improve the biotic stress resistance of the plants through marker-assisted selection approach.

Materials and Methods

Location of work

The study on molecular marker analysis with regard to parents and progenies was conducted in the Department of Plant Biotechnology and Department of Plant Molecular Biology and Bioinformatics, Centre for Plant Molecular Biology and Biotechnology, Tamil Nadu Agricultural University, Coimbatore. The study on artificial screening for bacterial blight was done at Paddy Breeding Station, Tamil Nadu Agricultural University, Coimbatore. The gall midge screening was conducted in Agricultural College & Research Institute Madurai in the field condition.

Source of plant materials

The material for this study includes F_4 generation of the cross {[CO43*4 //// (B_{95-1} X Abhaya)]} / [CO43*4 //// (B_{95-1} X Tetep)] / [CO43*4 //// (B_{95-1} X Kavya)]} involving CO43 as the recurrent parent. B_{95-1} X Kavya was used as donor for *Gm1*, B_{95-1} X Abhaya was used as donor for *Gm4* and *xa13*, B_{95-1} X Tetep was used as donor for *Xa21*. These entries were genotyped for Gall midge (*Gm1* and *Gm4*) and bacterial blight (*Xa21* and *xa13*) resistance genes using molecular markers.

Foreground selection

Gene specific markers were used for the foreground selection of disease resistance genes. For selection of *Xa21* gene, STS primer pTA248 was used (Ronald *et al.,* 1992).
For foreground selection of *xa13* gene, functional primer xa13prom (Chu *et al.,* 2004) was used. The SSR marker used for *Gm1* was RM1328 (Himabindu *et al.,* 2007) and *Gm4* was RM22550 (Nanda *et al.,* 2010).

Methods

Total genomic DNA extraction from rice leaves

Genomic DNA was isolated using the modified CTAB method (Sambrook *et al.,* 1989). Prior to extraction, 100 to 300mg of young leaves were cut into pieces and transferred to pestle and mortar. One ml of extraction buffer was added immediately. About 500µl of the cell extract was transferred in to a 2ml eppendrof tube and then 250 µl of choloroform : isoamyl alcohol (24:1) was added into the tube and mixed well. The tube was kept at 65°C for 10-12 mins and then the tube was centrifuged for 10min at 12,000 rpm and 450 µl of the supernatant was transferred immediately to a new eppendrof tube. 450 µl of ice cold isopropanol was added and after mixing, the tubes were kept in ice for 20 min. The tubes were centrifuged for 15 min at 12,000 rpm and supernatant was discarded without disturbing the pellet. The pellet was washed with 500 µl of 70% ice cold ethanol and centrifuged for 10 min at 12,000 rpm. The supernatant was discarded and the pellet was air dried for 5 min and suspended in 40 µl of TE buffer or sterile water. DNA was stored at -20°C. The isolated DNA was checked for its purity using nanodrop (Thermo Scientific) for quantification and DNA quality check by agarose gel electrophoresis.

PCR analysis using gene specific marker

Gene specific markers were amplified by the PCR using forward and reverse primers.STS Primer pTA248 was used for foreground selection of *Xa21* gene. For the foreground selection of *xa13* gene, functional primer xa13 prom was used. PCR amplification of genomic DNA was done using forward and reverse primers, dNTPs, assay buffer, DMSO, *Taq* DNA Polymerase. The different PCR components used for PCR amplification are described in table below.The reaction mixture was given a momentary spin for thorough mixing of the cocktail components. Then 0.2 ml PCR tubes containing the PCR mix along with 2 µl DNA per sample were loaded in the thermal cycler. The reaction was programmed in thermal cycler (Eppendrof, Germany) for different primers.

Components	Concentration	Quantity
PCR Buffer with $MgCl_2$	10X	2.0µl
dNTPs	2.5 mM	0.5 µl
Forward primer	10 µM	1.0 µl
Reverse primer	10 µM	1. 0 µl
Taq DNA Polymerase	3U	0.2 µl
Template DNA	40µg/µl	2.0 µl
Sterile water		8.3 µl
Total		**15 µl**

Profile	Activity	Temperature(°C)	Duration (min)	Cycles
1	Initial denaturation	95	5	1
2	Denaturation	94	1	35
3	Annealing	55	1	
4	Extension	72	1	
5	Final extension	72	5	1
6	Storage	4	-	1

Artificial screening for bacterial blight resistance

Preparation of *Xanthomonas oryzae* pv. *Oryzae* (Xoo) culture

Preparation of media

For preparation of 1000ml medium, 20g sucrose, 5g peptone, 0.5g calcium nitrate, 0.82g disodium hydrogen phosphate, 0.05g iron sulphate and 20g agar was weighed and dissolved in 800ml of distilled water and pH was adjusted to 6.8-7.0 and final volume was made up to 1000ml. After preparation, medium was autoclaved and poured into petri plates in laminar air flow.

Inoculation of *Xanthomonas oryzae* pv. *oryzae*

The plants showing typical bacterial blight symptom was brought to lab and the leaf bits were cut taking care that the three fourth of bits were healthy and one fourth with symptoms. These leaf bits were surface sterilized in 0.1percent sodium hypochloride and then the leaf bits were serially washed thrice in sterile water to remove the excess surface sterilant. Then the leaf bits were placed on modified Nutrient Agar (NA) medium containing in petri plates and the plates were incubated at room temperature. After 48 h the ooze from the cut ends were picked up with bacteriological loops and streaked in to modified NA medium serially and kept for 24h incubation. The single colonies were observed on the 3rd day and were then transferred to the modified nutrient agar slant.

Mass multiplication of the pathogen

The pathogen was multiplied on modified nutrient agar and after 48h, old pure culture of the pathogen was brought in to suspension by adding 10ml of water per slant to give a concentration of bacterial cells of about 10^8 to 10^9/ml. The lines were inoculated in between maximum tillering and booting stages.

Artificial screening for bacterial blight resistance by leaf clipping method

Leaf clip method as suggested by Kauffman *et al.* (1973) was used for the inoculation of the rice plants with *Xanthomonos oryzae* pv. *oryzae*. These tests were conducted at flag leaf stage (80-90DAS). The gene pyramided lines of BC_4F_4 generation were screened artificially for bacterial blight resistance at PBS, TNAU, Coimbatore. The gene pyramided lines along with parents namely, CO43, $B_{95\text{-}1}X$ Tetep, $B_{95\text{-}1}X$ Abhaya were grown. Inoculation was done at active vegetative phase at 80 to 90 DAS. For disease scoring, the length of the longest lesions of five damaged leaves of each individual plant was measured 21 days after inoculation.

Evaluation for the bacterial blight resistance (Chen *et al.*, 2000)

Lesion length	Description
<3	Resistant
3-6	Moderately Resistant
6-9	Moderately Susceptible
>9	Susceptible

Screening of gall midge in the field condition

The gall midge affects the plants by gall formation in the tillers. The larval gall midge bores the tillers in the ground portion and stays inside the plant. The adult one comes out of the gall producing a hole in the tiller. The whole tiller becomes a hallow tube like structure and the productive gets reduced. Onion leaf like symptom is shown in the infected plants.

Results and Discussion

Foreground analysis of BC_4F_4 segregating generation

The present study aims to genotype and select the gene pyramided plants in the background of CO43 as rice variety for bacterial blight (*Xa21* and *xa13*) and gall midge (*Gm1* and *Gm4*) resistance genes using marker–assisted selection. CO43 is a popular rice variety in Tamil Nadu and it is tolerant to salinity and alkalinity (Subramanian *et al.*, 1984). Multiple gene pyramided plants possessing bacterial blight and gall midge resistance genes in BC_4F_4 generation were selected using tightly linked molecular markers. The selected plants were artificially screened for bacterial blight resistance. Gall midge screening in the field condition was also performed. Agronomic performance was carried out to identify lines which are similar to CO43 and having resistance to bacterial blight and gall midge.

A total of 95 plants in BC_4F_4 generation were genotyped with SSR markers RM1328, RM22550, *xa*13 prom and pTA248 for the genes *Gm1, Gm4, xa13 and Xa21* respectively.

Three plants were identified with four gene combinations namely *Xa21, xa13, Gm1, Gm4*; Twenty five plants were identified with three gene combinations namely *Xa21, Gm1, Gm4*; One plant was identified with two gene combinations namely *Xa21, xa13*; Twenty five plants were identified with two gene combinations namely *Gm1, Gm4*; Four plants were identified with two gene combinations namely *Xa21, Gm1*; Twelve plants were identified with two gene combinations namely *Xa21, Gm4*; Two plants were identified with *Gm4*. One plant each was identified with the following genes namely *Xa21, xa13* and *Gm1* (Fig. 1-4).

Two or more major disease resistance genes having wider resistance spectra can be pyramided into elite cultivars using marker assisted selection strategy combined with stringent phenotyping. Through this approach resistant cultivars can be developed. Joseph *et al.,* (2004) reported the pyramiding of bacterial blight resistance genes *Xa21* and *xa13* into Pusa Basmati 1 rice cultivar which led to the development of improved Pusa Basmati 1. It was released for commercial cultivation. The improved cultivar was resistant to bacterial blight and retained the agronomic features of Pusa Basmati 1. Taraori Basmati and Basmati 386 were also introgressed with bacterial blight resistant genes through marker assisted selection combined with phenotype based selection and the pyramided line was found to be highly resistant to bacterial blight (Pandey *et al.,* 2013). Bacterial blight and gall midge resistance genes (*Xa21, xa13, Gm1 and Gm4*) were pyramided into an elite variety Samba Mahsuri by marker assisted selection (Divya *et al.,* 2015).

Artificial screening for bacterial blight resistance in BC_4F_4 generation

A total number of seven BC_4F_4 lines along with parents CO43 and B95-1xTetep and ADT38 (Susceptible check), were screened artificially for bacterial leaf blight resistance are presented (Table 1).The screening was conducted in the nursery bed and all the lines were sown row wise manner. After the 30th day from the date of sowing, the plants were in two leaf stage. The leaves of the plants were made to cut at the top portion and the *Xanthomonas* culture was sprayed. After the 14th day, the infestation was noticed and the reading is taken.

From the observations taken, the progeny lines recorded a high level of resistance (lesion length of 2 cm) when compared to the recurrent parent (CO43) (lesion length of 9 cm) and the susceptible check (ADT38) (lesion of 9 cm). The donor parent (B95-1xTetep) was showing high level of resistance (lesion length of 1 cm) (Fig. 5).

4.3 Screening of gall midge in field condition

Gall midge screening was conducted at Agricultural college & Research Institute, Madurai. A set of nine lines were screened for gall midge in the field condition. Along with these lines, a set of parents (CO43, B95-1xKavya, and B95-1xAbhaya) were also analyzed. The population selected for the screening was with *Gm1* and *Gm4* separately as well as in combination (Table 2).

Out of 9 lines, three lines were with both the genes (*Gm1* and *Gm4*). Three lines were with the *Gm1* gene and three plants were with *Gm4* gene. From the population screened, the plants containing both the *Gm1* and *Gm4* genes recorded complete resistance and the other lines with single gene were also recorded complete resistance.

The study reveals that the paramount importance of marker–assisted selection for the development of resistant lines for biotic stresses (bacterial blight and gall midge). The potential of marker assisted selection is to reduce the cost and increase the precise and efficient selection in breeding programmes. The lines selected through the present study will be further multiplied and evaluated in multi location trials. The selected lines can also be used for future breeding programmes.

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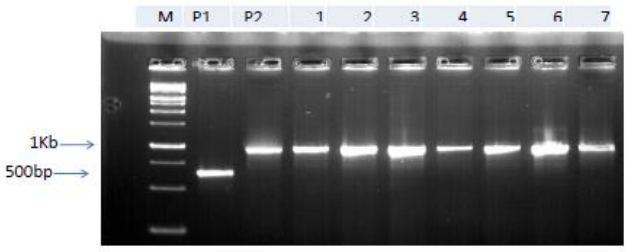
Table 1. Artificial screening of BC_4F_4 plants for bacterial blight disease

S.NO	Line number	Lesion length(cm)	Resistance/Susceptible
1	CO43	9	Susceptible
2	B95-1xTetep	1	Resistant
3	ADT 38	9	Susceptible
4	10-1	2	Resistant
5	10-2	1	Resistant
6	10-3	2	Resistant
7	10-4	2	Resistant
8	10-5	2	Resistant
9	10-6	2	Resistant
10	10-7	9	Susceptible

Scale - <3cm = Resistant; 3-6 cm = Moderately resistant;
6-9cm = Moderately susceptible; >9 = Susceptible

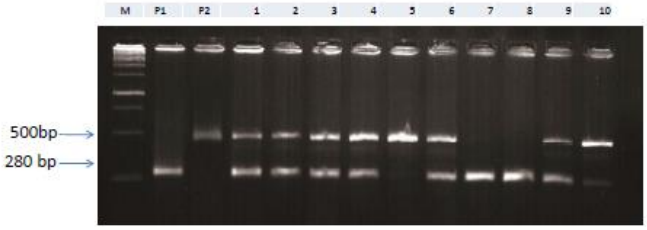
Table2. Screening of gall midge in field condition at AC&RI, Madurai.

Line	Gene combinations	Percentage of plants affected (%)	Resistance/Susceptible
CO 43	-	83.33	Susceptible
B95-1xAbhaya	*Gm4*	0	Resistant
B95-1xKavya	*Gm1*	0	Resistant
Line 2	*Gm1*	0	Resistant
Line 9	*Gm1*	0	Resistant
Line 13	*Gm1*	0	Resistant
Line 11	*Gm4*	0	Resistant
Line 60	*Gm4*	2.5	Resistant
Line 65	*Gm4*	0	Resistant
Line 22	*Gm1+Gm4*	0	Resistant
Line 23	*Gm1+Gm4*	0	Resistant
Line 24	*Gm1+Gm4*	0	Resistant



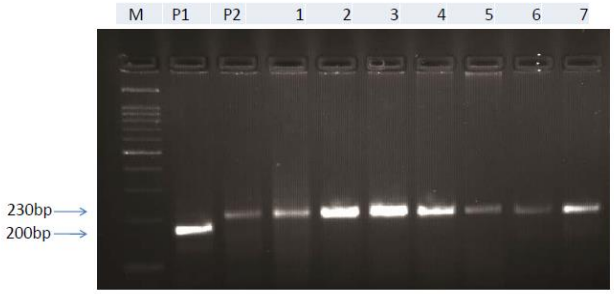
M – 1Kb , P1 – CO43, P2 – B95-1x Tetep

Fig. 1. Genotyping pattern in BC_4F_4 generation for pTA248 marker (*Xa21*)



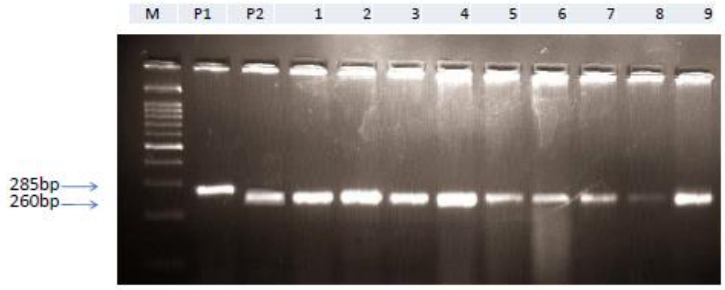
M – 1Kb , P1 – CO43, P2 – B95-1x Abhaya

Fig. 2. Segregation pattern in BC_4F_4 generation for *xa13* –prom marker (*xa13*)



M – 100bp, P1 – CO43, P2 – B95-1x Kavya

Fig. 3. Genotyping pattern in BC_4F_4 generation for RM 1328 marker (*Gm1*)



M – 100bp, P1 – CO43, P2 – B95-1x Abhaya

Genotyping pattern in BC_4F_4 generation for RM22550 marker *(Gm4)*

Fig. 4. Genotyping pattern in BC_4F_4 generation for RM22550 marker *(Gm4)*

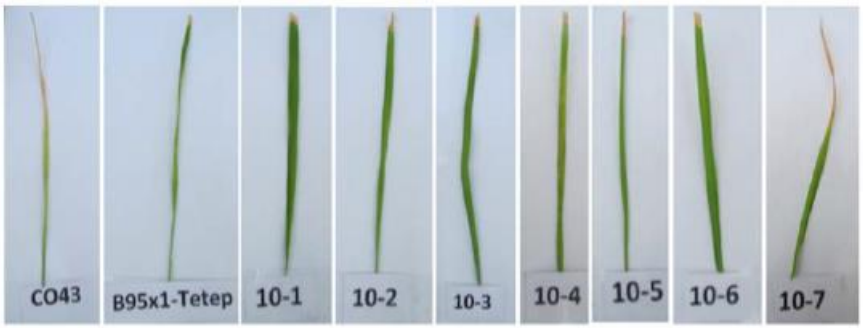


Fig. 5. Bacterial blight disease screening for BC_4F_4 generation