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Research Article

Introgression of Blast Resistant Genes (Pita, Pikh, Pitp(t)) of Tetep variety into Local Rice Blast susceptible cultivar Bg94/1 by incorporating PCR-based DNA Markers

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Abstract

Rice blast, caused by the fungal pathogen Magnaporthe grisea (anamorph-Pyricularia grisea) is the major rice disease among 85 countries across the world. This research study made progress in the introgression of blast-resistant genes of resistant variety Tetep (Pita, Pikh and Pitp(t)) into local susceptible cultivar Bg94/1 with the aim of developing a durable blast-resistant cultivar. Vietnamese rice variety Tetep and local cultivar Bg94/1 were selected as the donor and recurrent parents. The above rice varieties were grown in the field of RRDI, Batalagoda. During the flowering period, Tetep (donor) and mother parent Bg94/1 (recurrent) were first crossed in order to obtain the F1 population. Once the F1 population was developed, it was grown the same as above, and the F1 plants were crossed with recurrent parent (Bg94/1) to produce the first backcross population (BC1F1). Following the same above mentioned procedure the backcross two generations (BC2F1) and back three generations (BC3F1) were established by crossing BC1F1 plants and BC2F1 plants with recurrent parent plants (Bg94/1). According to the results, most of the plants of all three backcross generations were found to be highly blast-resistant in the phenotypic screening process. The molecular marker genotyping process confirmed the presence of blast-resistant genes Pita, Pikh, Pitp(t) of the parent Tetep variety in the backcross three generation (BC3F1). This concluded the above blast-resistant genes transferred to our backcross generations in an orderly manner from Tetep, hence making them resistant against the blast pathogen.

Keywords: *Backcrossing, blast pathogen, F1 population, rice blast, marker genotyping*

1. Introduction

The rice blast disease is a prominent serious infection in rice. It is due to the fungal pathogen *Magnaporthe grisea* that affects rice growing regions in Sri Lanka and in the world (Rossman *et al*., 1990). This disease is known to be reported more than 85 countries in the world causing US dollars 66 billion in production lost annually. Therefore, the production loss by rice blast up to 50% when conditions are favourable for growth of fungus (Skamnioti and Gurr, 2009). Blast is considered the most destructive rice disease among all of them generally as it causes lesions to form in the whole plant including leaves, stems, peduncles, panicles, seeds, and even roots (Mithrasena *et al*., 2012). Blast pathogen can infect the rice plant from the seedling stage through its maturity. *Magnaporthe* is mostly a diverse class of fungal family that has been studied which includes prominent species like (*Magnaporthe grisea, Magnaporthe poae, Magnaporthe rhizophila* and *Magnaporthe salvinii* etc.) These species cause identical diseases in their respective host system. Blast is commonly controlled by using fungicides. Fungicides usually cause high production cost and environmental pollution (Ghazanfar *et al*., 2009). So, breeders have employed conventional rice breeding technology such as phenotypic screening in order to identify resistant cultivars at a very early generation that can be used in breeding programs future (Mackill and Bonman, 1992). However, conventional breeding is cumbersome, needs a lot of field space and is also influenced by environmental conditions (Dissanayake, 1995). So, scientists direct the effort of developing new strategies that can develop durably resistant blast cultivars that possess broad genetic diversity for blast resistance.

Marker-assisted breeding (MAB) is a new technology that uses closely linked (map distance <2 C.M) DNA markers to localize resistant genes. And these DNA markers select highly resistant varieties at DNA level from early generation (Miah *et al*., 2013). These markers are highly accurate, and reliable though they are slightly expensive. Further, these markers identify superior plants with fewer efforts compared to other methods (Dissanayake, 1995). Hence, the recent achievements made in DNA marker-assisted breeding technology may impart new ways for developing durable blast-resistant cultivars with desirable traits in the rice populations.

This research summarizes the accomplishments made in the introgression of blast-resistant genes using marker-assisted backcrossing from blast-resistant plant Tetep into the blast-resistant backcross population (BC3F1) of local susceptible cultivar

Bg94/1 in Sri Lanka by incorporating simple sequence repeats markers (SSR) and a gene specific marker. In this research to introgression of resistant genes (*Pita, Pikh, Pitp(t)*) of variety Tetep into blast susceptible variety Bg94/1, the conventional backcrossing technique was used for three generations. The SSR DNA markers RM206, RM246 (for *Pikh and Pitp(t)* genes) and a gene-specific marker YL/155/87 (for *Pita* gene) were used in locating resistant genes in newly developed backcross populations of Bg94/1 (Jayawardana *et al*., 2014). The SSR markers; RM206 and RM246 are linked to the blast resistant genes *Pikh*, *Pitp(t)*.YL/155/87 is a gene-specific marker in *Pita* gene of blast-resistant varieties that had been identified by earlier studies. The main objective of the present study was to develop a blast resistant backcross population of blast-susceptible local variety; Bg94/1 through marker assisted backcrossing (MAB) technology. These research findings contribute immensely to the rice breeding programs in Sri Lanka. In future, this will enable a better overview for rice breeders to establish blast resistance in breeding programs by transferring more prospective genes from resistant cultivars to susceptible cultivars using new techniques such as DNA maker assisted backcrossing.

2. Materials and Methods

2.1 Development of Backcross population

Tetep and Bg94/1 were selected as the donor and recurrent parents. The seeds of the traditional Vietnamese rice cultivar Tetep and blast-susceptible local rice cultivar Bg94/1 were obtained from RRDI, Batalagoda. The selected rice seeds of both donor and recurrent parents were germinated first, then grown in the pots and maintained for 18-21 days period in the plant house at RRDI, Batalagoda. After 21 days the seedlings of both parents were transferred into a well-designed field in RRDI, Batalagoda. Fertilizer was added according to a recommended scheme stipulated by RRDI, Batalagoda for two months period for all plants. All plants of parents were grown till the flowering time ($2_{1/2}$ to 3 months), at the flowering time firstly, Tetep (donor) and mother parent Bg94/1 (recurrent) were taken into individual pots and then crossed by using hot water emasculation method before dehiscence of anthers. In this method, pollens of the recurrent parent (Bg94/1) were killed by submerging spikes in hot water carrying in thermos flask at 45 ^{o}C for 5 minutes. After hot water treatment, the pollen emasculated flowers of the recurrent parent (Bg94/1) were crossed with pollens of the donor parent (Tetep) in order to obtain the F1 seed population. Followed by cross fertilization the entire flower of the recurrent parent was wrapped with glass line paper sealer and maintained in the plant house, RRDI

Batalagoda till F1 seeds collection. After one month, the collected F1 seeds were germinated and then grown in pots in a nursery (RRDI, Batalagoda) for 21 days period and seedlings were transferred to the allocated field in RRDI, Batalagoda. F1 plants were well maintained with adding recommended fertilizer scheme for $2_{1/2}$ to 3 months period.

Once the F1 population was developed in the F1 field, at the flowering time the F1 plants were removed to new pots and emasculated their pollens with the same aforestated hot water treatment method before anther dehiscence as mentioned above. Then, these F1 plants were again crossed with pollens of the recurrent parent (Bg94/1) obtained from other fields in RRDI, Batalagoda to produce the first backcross population seeds (BC1F1) (Hasan *et al*., 2015). As mentioned earlier the crossed flowers of F1 plants were covered with glass line paper bags after the crossing and well maintained in nursery RRDI, Batalagoda till obtaining the first backcross population seeds (BCIF1). Following the same aforestated procedure the backcross one generation seeds were collected and germinated. Then transferred into new pots and were grown in the pots in the nursery for 21 days period of time. Later, BCIF1 seedlings were transferred to a well-designed field in RRDI, Batalagoda. The BC1F1 plants were maintained in the field for a $2_{1/2}$ to 3 months period with adding recommended fertilizer stipulated by RRDI, Batalagoda. At the time of flowering, BC1F1 plants were removed to new pots from the field and emasculated their pollens by the same hot water treatment method and were crossed with the pollens of recurrent parent Bg94/1.The flowers of crossed BCIF1 plants were covered with glass line paper bags till the collection of the second backcross generation seeds "BC2F1" in the nursery RRDI, Batalagoda (Siriphat *et al*., 2015). The BC2F1 backcross population seeds were collected, germinated and potted in a nursery and well maintained for 21 days of time. Then the BC2F1 seedlings were transferred to the allocated field in RRDI, Batalagoda (Figure 01). The BC2F1 plants were grown by adding recommended fertilizer for $2_{1/2}$ to 3 months and at the flowering time BC2F1 plants were removed to new pots from the field and emasculated by incorporating the same hot water treatment method in order to cross it with pollens of recurrent parent Bg94/1.The cross between BC2F1 plants and recurrent parent Bg94/1 was performed according to the same aforementioned procedure.

As same as above the flowers of crossed BC2F1 plants were covered with glass line paper bags and maintained in the nursery RRDI, Batalagoda nursery till the BC3F1seed collection. The BC3F1 seeds were collected at the end of 21 days. And

these seeds (BC3F1) were germinated again and were grown in new pots (Harlan and Pope, 1922). The BC3F1 seedlings were maintained in the new pots for 3 months period by adding the recommended fertilizer scheme stipulated by RRDI, Batalagoda. The leaves of BC3F1 plant were collected at the time of maturity and preserved at the germplasm conservation unit, RRDI, Batalagoda.

Other than the leaves of BC3F1 plants, the leaves of donor and recurrent parents (Tetep and Bg94/1), F1 plants, BC1F1 plants, and BC2F1 plants also were collected in previous seasons. Together with leaves the seeds of BC1F1, BC2F1, and BC3F1 generations were also collected and preserved at the germplasm conservation unit, RRDI, Batalagoda. Later, these seeds were used for phenotypic screening procedures and morphological study. The leaves of all above-mentioned generations were brought to Genetech molecular diagnostic laboratory, Colombo, and maintained in -80 ^{0}C low-temperature freezer till marker-assisted genotyping.

Figure 1: Fields of back population at RRDI, Batalagoda

2.2 Phenotypic screening

The selected seeds of all three backcross generations (BC1F1, BC2F1, BC3F1) together with parental seeds of Tetep (Donor) and Bg94/1 (recurrent)and their F1 plants seeds obtained from germplasm conservation unit, Batalagoda and were sown in the screening field RRDI, Batalagoda in order to identify the blast resistance of new three backcross populations. All these plants of the above generations were grown in the upland blast nursery field for 14 days of period. For three backcross populations different replicates were maintained in order to get average blast score to enhance the accuracy of blast scoring. The field was set up according to a complete randomized design. Once all plants were grown to proper young seedling stage they were subjected to infection by fungal dry leaves spore suspension. This was carried out for another 14-day period by covering the field at night with moisture chambers in order to increase the level of infection (Jayawardana *et al*., 2013). Later, the blast reactions were scored by using the International Rice Research Institute (IRRI) stipulated standard blast scoring system (IRRI, 1996) at the end of the infection period.

2.3 DNA extraction

The method used for DNA extraction was C-TAB by Doyle and Doyle, 1987. The leaves of all three backcross populations, F1 plants and their parental plants (Tetep and Bg94/1) were pre-stored in -80 ^{o}C refrigerator and were taken for DNA extractions. In DNA extractions leaves of 10 plants of each above generation were used. Approximately 0.6g - 0.8 g of the tender healthy fresh leaf tissues of each sample without veins was finely ground with liquid nitrogen using a motor and pestle and then 100 mg of a homogenous mixture of each sample was transferred to a 1.5 ml eppendorf tube. A volume of 900 µl of CTAB extraction buffer (preheated at 65 ^{o}C) was added to each eppendorf tube containing the samples. The eppendorf tubes containing the samples of leaves were kept in 65 ^{o}C dry bath for 1 hour while shaking the tubes. Eppendorf tubes were centrifuged at 8000 r.p.m for 5 minutes after one hour. Consecutively, supernatants of all samples of leaves were transferred into new 1.5 eppendorf tubes. Added 5 µl RNAse solution to each eppendorf tube and incubated at 56 ^{o}C dry bath for 30 minutes. Then volume of 570 µl of chloroform: Isoamyl alcohol (24:1) solution was added to each eppendorf tube and gently inverted up and down for three times to mix well. The tubes were centrifuged at 12000 r.p.m for 5 minutes (this step was performed two times for better extraction).

After the centrifugation, aqueous phase of each eppendorf tube was decanted into new eppendorf tubes of 1.5 µl and was added 500 µl of isopropanol solution (1 volume) to each tube, then incubated at -20 ^{o}C for 30 minutes. After low-temperature incubation, all new tubes containing the aqueous phase of each leaf sample were centrifuged at 10000 r.p.m for 5 minutes. Decanted the isopropanol and washed the pellet of each tube with 70 % alcohol 500 µl (performed twice) then drain dried the tubes keeping them inverted on a tissue paper. The DNA pellets of all samples of leaves were air-dried until all alcohol evaporated. Finally, the DNA pellet of each tube was dissolved in 100 µl -150 µl of nuclease-free sterilized water and kept the tubes in 4 ^{o}C freezer to dissolve DNA pellets fully for 24 hours period (Doyle and Doyle, 1987). On the following day, the dissolved DNA pellet tubes were kept for 15 minutes at 65 ^{o}C dry bath. DNA pellets were mixed well with a micropipette by pipetting in and out for further dissolving of DNA properly in nuclease-free sterilized water before transferring them to -20 ^{o}C freezer. All extracted DNA of all above populations was stored in -20 ^{o}C Genetech molecular diagnostic laboratory till the marker genotyping process.

2.4 PCR amplification

The PCR process was performed in 25 µl reaction mixture volumes by incorporating Biorad T100^{TM} thermal cycler, California, USA. This PCR mixture consisted of 2.5 µl of 10X buffer containing $MgCl_2$ (10X Dream Taq Buffer), 0.75 µl of 10 mMdNTP, 1.5 µl from 10 µM of primer mixture (forward and reverse primers), 0.3 µl of 5U/µl Dream Taq DNA polymerase (low fidelity) enzyme and 16.95 µl of sterile PCR water (Table 01). The PCR mixture was prepared in the PCR hood. Master mixtures were prepared by using multiples of the above-stated volumes of reagents according to the number of tested DNA samples in the PCR process (Table 02). The 22 µl of the PCR master mixture was added to eppendorf tube contained 3 µl of DNA in the pre-PCR DNA extraction bench when testing for one DNA sample in PCR. This PCR reaction consisted of 35 maximum cycles (Jayawardana *et al*., 2014).

Table 1: Primer sequences of markers; *Pikh, Pitp(t),* and *Pita*

Gene	Linked marker	Forward primer	Reverse primer
Pikh	RM206	CCCATGCGTTTAACTATTCT	CGTTCCATCGATCCGTATGG
Pit(p)	RM246	GAGCTCCATCAGCCATTCAG	CTGAGTGCTGCTGCGACT
Pita	YL155/87	AGCAGGTTATAAGCTAGGCC	CTACCAACAAGTTCATCAAA

Table 2: The PCR protocol for RM206, RM246, and YL155/87 markers

PCR process	Temperature	Time
Initial denaturation	94 ^{o}C	4 min
Denaturation	94 ^{o}C	1 min
Annealing	52 ^{o}C	1 min
Extension	72 ^{o}C	1 min
Final extension	72 ^{o}C	10 min
Number of cycles = 35		

The agarose gel electrophoresis and polyacrylamide gel electrophoresis were performed as has been shown in (Geneprint, 2010).

2.5 Overall procedure for marker-assisted genotyping of backcross populations, their F1 plants, and parental plants with selected DNA markers

The extracted DNA of three backcross populations, their parents and F1 generation were tested with simple sequence repeats (SSR) markers; RM206 and RM246 and a gene-specific marker; YL155/87 linked to three blast-resistant genes; *Pikh, Pitp(t),* and *Pita* of Tetep the blast resistant Vietnamese variety. This is performed in order to confirm the presence and absence of these three blast-resistant genes in each stage of backcross development. For the above mentioned process, the PCR products were given by each population with the three DNA markers; RM206 (*Pikh),* RM246 (*Pitp(t),* andYL155/87 (*Pita*) after PCR reactions were injected into the wells of already prepared 2 % agarose gel set in an electrophoretic apparatus and were subjected to the electrophoresis. Once the electrophoresis process was completed the band sizes of each population for the above three markers were determined using

respective ladder sizes (50 bp, 100 bp or 1000 bp DNA ladders). In the case of *Pita* gene marker; YL155/87 only the presence and absence of a specific PCR product (1042 bp amplicon) was considered for each population as YL155/87 was a gene-specific marker.

For the rest of the two DNA markers; RM206 (*Pikh)* and RM246 (*Pitp(t)* as they were closely linked SSR markers the band lengths were determined by using both agarose gel electrophoresis (Lee *et al*., 2012) and polyacrylamide gel electrophoresis with respective ladders. To confirm the blast resistance of the final backcross three population (BC3F1) and to validate whether the newly developed blast-resistant backcross population of Bg94/1 (BC3F1) withstand the infection of blast fungal pathogen, both phenotypic screening process and molecular techniques such as marker-assisted genotyping were incorporated simultaneously to the testing procedure (Joshi *et al*., 2009).

2.6 Morphological analysis of BC3F1 plants in order to confirm their morphological similarity to local variety Bg94/1

A morphology study was carried out in the greenhouse, Department of Plant Sciences, University of Colombo in order to study the similarity in morphology between BC3F1 plants and local recurrent parent Bg94/1 used for the backcross process at the end of the backcrossing protocol by using the following characters such as plant height, nature of rice plant, plant maturity time, leaves colour, seeds morphology, and a number of tillers.

For this purpose, the BC3F1 seeds brought from RRDI, Bathalagoda were used. These seeds were sown in pots. The growing BC3F1 seedlings were maintained in the greenhouse till the morphological study. Ultimately, by the morphological analysis, the similarity in morphology between BC3F1 plants and local recurrent parent Bg94/1 used for the backcross process was determined.

3. Results and Discussion

3.1 Evaluation of blast resistance phenotypically

Most of the plants of all three backcross generations of the Bg94/1 variety, parent Tetep, and their F1 population were found to be highly resistant in the phenotypic screening process which was performed and assessed based on the blast scoring system stipulated by the international rice research institute (IRRI, 1996). Average

blast scores by considering different replicates were taken for three backcross generations. The blast scores for these plants of above generations relied on between the score of "1" and "0 which indicated that all three backcross populations, parent Tetep, and the F1 population were highly resistant to rice blast disease. The plants of three backcross generations (BC1F1, BC2F1, and BC3F1) were highly blast resistant most likely due to gaining blast-resistant genes from the Tetep resistant donor parent during the backcrossing process (Figure 02 a and b). So, in the phenotypic screening, it was confirmed the backcross generations were highly blast-resistant. On the other hand, the local blast-susceptible parent Bg94/1 remained susceptible to rice blast disease during phenotypic screening.

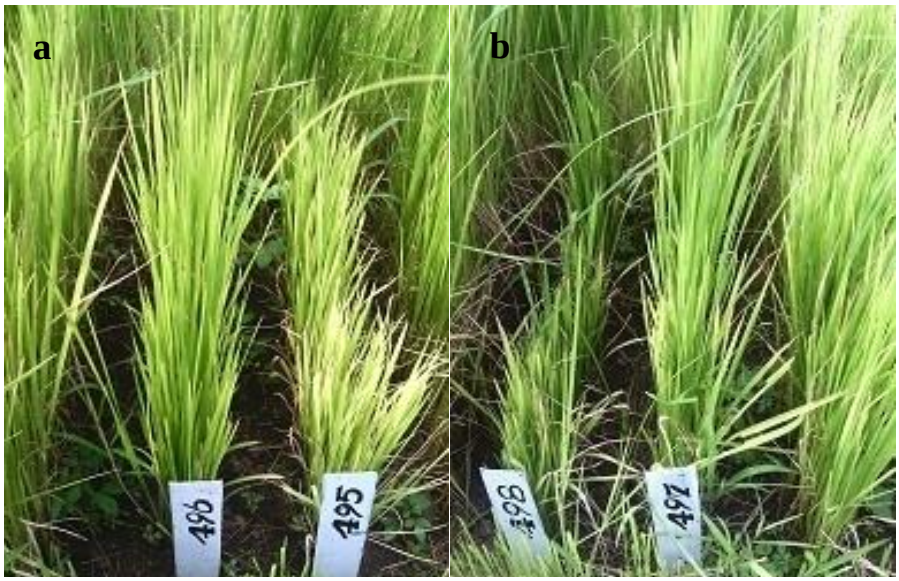

Figure 2: The label numbers 496 and 497 show backcross two plants in different replicates in the phenotypic screening process (a and b).

3.2 Analysis of DNA marker profiles of RM206, RM246, and YL155/87 in BC1F1, BC2F1, and BC3F1 backcross populations

The marker genotyping process was performed according to the protocol mentioned above and the following results were obtained for each DNA marker separately.

3.2.1 Analysis of marker profile of RM206 linked to blast resistant gene; *Pikh* in BC1F1, BC2F1, and BC3F1 populations

Firstly, in the marker genotyping process, DNA of Tetep and Bg94/1 parents were PCR amplified by primers specific to the RM206 marker and the PCR products were separated by agarose gel electrophoresis and observed after staining revealed a 141 bp fragment for Tetep (blast resistant parent) and a 171 bp fragment for Bg94/1 (blast susceptible parent).This observation indicates that Tetep and Bg94/1 parental varieties by amplifying 141 bp and 171 bp fragments demonstrate very clear polymorphism for RM206. Furthermore, the two amplified fragments are alleles of the RM206 SSR marker which is linked to blast resistant *Pikh* gene. The presence of the 141 bp allele of RM206 identifies blast-resistant rice plants and the 171 bp allele identifies blast-susceptible rice plants that originate from the Tetep×Bg94/1 cross. Phenotypic evaluation of Tetep and Bg94/1 for blast disease and outcome of genotyping of the parents are taken together it points to the fact that the 141 bp allele of RM206 marker can be taken as a reliable marker to identify blast resistance in rice plants which have originated from Tetep×Bg94/1 cross. Similarly, the 171 bp allele of the RM206 marker can be used to identify blast susceptibility in rice plants which have originated from Tetep×Bg94/1. DNA extracted from rice plants of F1 generation and rice plants of BC1F1, BC2F1, and BC3F1 backcross populations were PCR amplified by primers specific to RM206 marker, subjected to agarose gel electrophoresis, stained and observed. Moreover, when DNA extracted from rice plants belonging to of F1 generation, BC1F1, BC2F1, and BC3F1 were amplified using RM206 specific primers and subsequently PCR products were analyzed by agarose gel electrophoresis, the RM206 marker profile depicted all samples tested demonstrated the presence of 141bp allele as in Tetep, the blast resistant parent (as shown in Figure 03).

Secondly, to further confirm these results the PCR products of all these populations were subjected to polyacrylamide gel electrophoresis (Table 03) in order to determine accurately whether the backcross populations' band lengths for the RM206 marker of *Pikh* blast-resistant gene were similar to the band length of resistant parent (Tetep). In the polyacrylamide gel electrophoresis, BC2F1 and BC3F1 backcross populations were found to be heterozygous (141 bp and 171 bp alleles) for the RM206 marker of *Pikh* blast resistant gene as shown in Table 03. The backcross population BC1F1 produced a homozygous resistant 141 bp allele for the RM206 marker of *Pikh* blast-resistant gene as shown in Table 03. The F1 population

heterozygosity was not quite clear in the polyacrylamide gel electrophoresis. But it produced the 141 bp allele same as the blast-resistant parent Tetep. This was assumed to be most likely due to the phenomenon called microsatellite slippage (Oliveira *et al*., 2006). The resistant parent Tetep produced the 141 bp allele of RM206 while the susceptible parent Bg94/1 produced the 171 bp allele associated with blast susceptibility when tested for RM206 marker in the polyacrylamide gel electrophoresis.

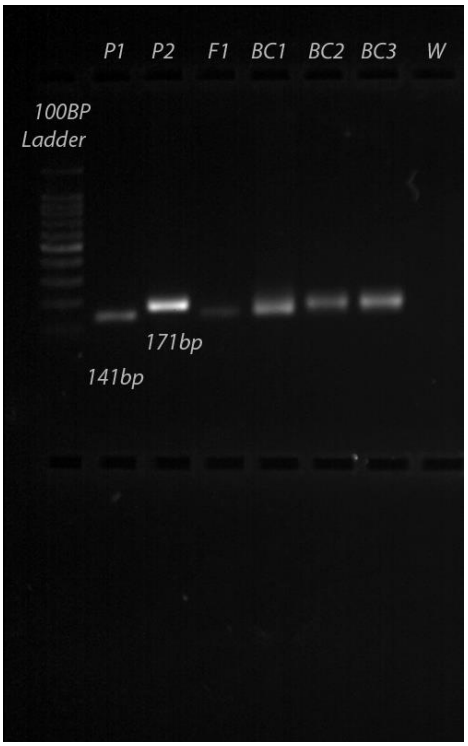

Figure 3: The image indicating the bands obtained for P1 (Tetep), P2 (Bg94/1), F1 (F1 plants), BC1 (BC1F1), BC2 (BC2F1), BC3 (BC3F1) generations with RM206 marker of *Pikh* blast resistant gene in the agarose gel electrophoresis. Ladder 10 bp.

It is evident that analysis of RM206 marker profiles of rice plants belonging to BC1F1, BC2F1, and BC3F1 populations contained the 141 bp allele of the RM206 marker and when phenotypic evaluation of rice plants of backcross populations is considered they exhibited blast resistance. This 141 bp allele of the RM206 marker linked to the *Pikh* blast gene probably suggests that resistance to blast as seen in rice plants containing the 141 bp allele, most likely due to the presence of *Pikh* gene of Tetep. The developed backcross population can be further selfing in order to produce dominant homozygous resistant plants for *Pikh* gene. These result outcomes suggest

that the *Pikh* blast-resistant gene most likely has been transferred to the three backcross populations from blast-resistant donor parent Tetep during the backcross development process.

Table 3: The bands obtained for Tetep, Bg94/1, F1 (F1 plants), BC1 (BC1F1), BC2 (BC2F1), and BC3 (BC3F1) generations with RM206 marker of *Pikh* blast resistant gene in the polyacrylamide gel electrophoresis. Ladder vWA range (139-167 bp).

Marker	Tetep	Bg94/1	F1	BC1	BC2	BC3
RM206	141bp	171bp	141bp	141bp	141/171bp	141/171bp

3.2.2 Analysis of marker profile of RM246 linked to blast resistant gene; *Pitp(t)* in BC1F1, BC2F1, and BC3F1 populations

Initially, in the marker genotyping process, DNA of Tetep and Bg94/1 parents were PCR amplified by primers specific to the RM246 marker and the PCR products were separated by agarose gel electrophoresis and observed after staining revealed a 111 bp fragment for Tetep (blast resistant parent) and a 98 bp fragment for Bg94/1 (blast susceptible parent). This observation indicates that Tetep and Bg94/1 parental varieties by amplifying 111 bp and 98 bp fragments demonstrate very clear polymorphism for RM246. Furthermore, the two amplified fragments are alleles of the RM246 SSR marker which is linked to blast resistant *Pitp(t)* gene. The presence of the 111 bp allele of RM246 identifies blast-resistant rice plants and the 98 bp allele identifies blast-susceptible rice plants that originate from the Tetep×Bg94/1 cross. Phenotypic evaluation of Tetep and Bg94/1 for blast disease and outcome of genotyping of the parents are taken together it points to the fact that the 111 bp allele of RM246 marker can be taken as a reliable marker to identify blast resistance in rice plants which have originated from Tetep×Bg94/1 cross. Similarly, the 98 bp allele of the RM246 marker can be used to identify blast susceptibility in rice plants which have originated from Tetep×Bg94/1.

DNA extracted from rice plants of F1 generation and rice plants of BC1F1, BC2F1, and BC3F1 backcross populations were PCR amplified by primers specific to RM246 marker, subjected to agarose gel electrophoresis, stained and observed. Moreover, when DNA extracted from rice plants belonging to of F1 generation, BC1F1, BC2F1, and BC3F1 were amplified using RM246 specific primers and

subsequently PCR products were analyzed by agarose gel electrophoresis, the RM246 marker profile depicted all samples tested demonstrated the presence of 111 bp allele as in Tetep, the blast resistant parent (as shown in Figure 04).

Secondly, to further confirm these results the PCR products of all these populations were subjected to polyacrylamide gel electrophoresis (Table 04) in order to determine accurately whether the backcross populations' band lengths for the RM246 marker of *Pitp(t)* blast-resistant gene were similar to the band length of resistant parent (Tetep). In the polyacrylamide gel electrophoresis, the BC1F1 backcross population was found to be heterozygous (111 bp and 98 bp alleles) for the RM246 marker of *Pitp(t)* blast resistant gene as shown in Table 04. BC2F1 and BC3F1 backcross populations did not produce bands in the polyacrylamide gel electrophoresis as the incorporated PCR products of the aforementioned backcross populations were insufficient in volume. This was due to the repeated use of PCR products of the above backcross populations previously for agarose gel electrophoresis. However, in agarose gel electrophoresis BC2F1 and BC3F1 backcross populations produced the same allele as the BC1F1 backcross population. Also, it has assumed the shaded bands produced in agarose gel electrophoresis are tended to be heterozygous in polyacrylamide gel electrophoresis when the same PCR products run in the polyacrylamide gel electrophoresis. So, based on the above fact BC2F1 and BC3F1 backcross populations were also found to be heterozygous (111 bp and 98 bp alleles) as BCIF1 population. The F1 population heterozygosity (111 bp and 98 bp alleles) was very clear in the polyacrylamide gel electrophoresis for this marker. The resistant parent Tetep produced the 111 bp allele of RM246 while the susceptible parent Bg94/1 produced the 98 bp allele associated with blast susceptibility when tested for RM246 marker in the polyacrylamide gel electrophoresis.

It is evident that analysis of RM246 marker profiles of rice plants belonging to BC1F1, BC2F1, and BC3F1 populations contained the 111 bp allele of the RM246 marker and when phenotypic evaluation of rice plants of backcross populations is considered they exhibited blast resistance. This 111 bp allele of the RM246 marker linked to *Pitp(t)* blast gene probably suggests that resistance to blast as seen in rice plants containing 111 bp allele, most likely due to the presence of *Pitp(t)* gene of Tetep. The developed backcross population can be further selfing in order to produce dominant homozygous resistant plants for the gene *Pitp(t)*. These result outcomes suggest that the *Pitp(t)* blast-resistant gene most likely has been transferred to the

three backcross populations from blast-resistant donor parent Tetep during the backcross development process.

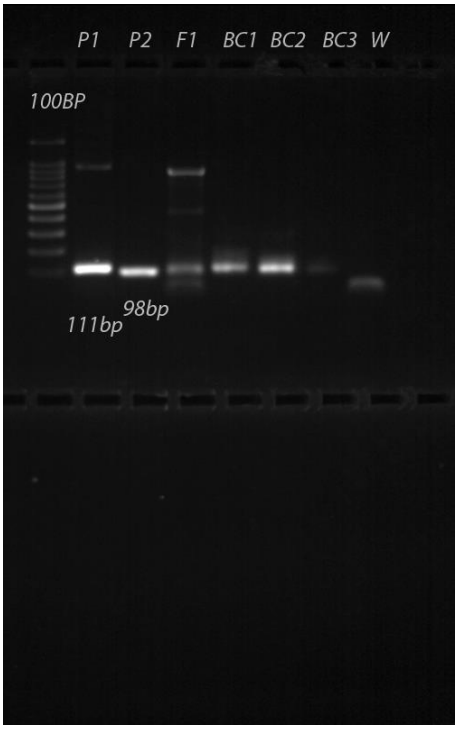

Figure 4: The image indicating the bands obtained for P1 (Tetep), P2 (Bg94/1), F1 (F1 plants), BC1 (BC1F1), BC2 (BC2F1), and BC3 (BC3F1) generations with RM246 marker of *Pitp(t)* blast resistant gene in the agarose gel electrophoresis. Ladder 100 bp.

Table 4: The bands obtained Tetep, Bg94/1, F1 (F1 plants), BC1 (BC1F1), BC2 (BC2F1), and BC3 (BC3F1) generations with RM246 marker of *Pitp(t)* blast resistant gene in the polyacrylamide gel electrophoresis. Ladder LPL range (105-133 bp).

Marker	Tetep	Bg94/1	F1	BC1	BC2	BC3
RM246	111bp	98bp	111/98bp	111/98bp	AB*	AB*

*****AB- Absence of bands

3.2.3 Analysis of marker profile of YL155/87 gene-specific marker of blast resistant gene; *Pita* in BC1F1, BC2F1, and BC3F1 populations

This gene-specific marker has been designed to amplify a specific region within the sequence of the dominant *Pita* gene. PCR amplification of a 1042 bp long amplicon using primers YL155/YL87 results in a 1042 bp long amplicon provides direct evidence for the presence of the *Pita* gene in the cultivars tested while no amplification is indicative of the absence of the *Pita* gene in the cultivar tested (Jia *et al*., 2002). The 1042 bp fragment amplified by YL155/87 as a gene-specific marker for the *Pita* gene exhibits polymorphism based on the presence or absence of the 1042 bp fragment. Analysis with gene-specific DNA marker for blast resistant gene *Pita* (as shown in Figure 05) revealed that Tetep the blast-resistant parent contains the *Pita* gene demonstrated by amplification of a 1042 bp fragment in the agarose gel electrophoresis. As Bg94/1 the blast susceptible parent didn't show the 1042 bp fragment in the agarose gel electrophoresis indicating the absence of the *Pita* gene in the susceptible parent (Figure 05). DNA extracted from rice plants of F1 generation and rice plants of BC1F1, BC2F1, and BC3F1 backcross populations were PCR amplified by primers specific to YL155/87 marker, subjected to agarose gel electrophoresis, stained and observed. Moreover, when DNA extracted from rice plants belonging to of F1 generation, BC1F1, BC2F1, and BC3F1 were amplified using YL155/87 specific primers and subsequently PCR products were analyzed by agarose gel electrophoresis, the YL155/87 marker profile depicted all samples tested demonstrated the presence of 1042 bp fragment as in Tetep, the blast resistant parent (as shown in Figure 05). So, in the current research study, the presence of PCR amplified 1042 bp fragment in BC1F1, BC2F1, and BC3F1 populations provided direct evidence for the presence of the *Pita* gene of Tetep in above backcross populations. These result outcomes suggest that the *Pita* blast-resistant gene has been transferred to the three backcross populations from blast-resistant donor parent Tetep during the backcross development process.

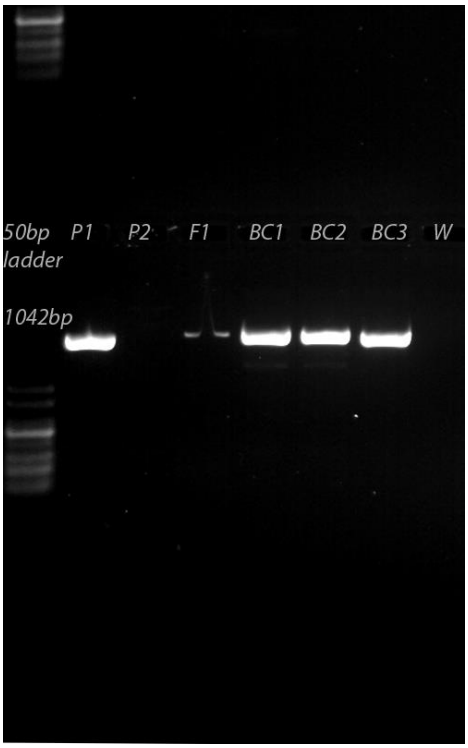

Figure 5: The agarose gel image showing the presence and absence of expected 1042 bp PCR amplified fragment in P1 (Tetep), P2 (Bg94/1), F1 (F1 plants), BC1 (BC1F1), BC2 (BC2F1), and BC3 (BC3F1) generations for gene-specific marker; YL155/87 of *Pita* blast resistant gene. Ladder 50 bp.

3.3 The investigation of morphological characteristics of BC3F1 rice plants

In order to confirm the similarity of general phenotype between backcross three generation plants (BC3F1) and the blast susceptible local recurrent parent plant Bg94/1, a morphological similarity study was carried out as mentioned in the methodology. Several characters were compared and the results revealed that the backcross three generation plants constituted of similar plant morphology as Bg94/1 blast susceptible local recurrent parent (Figure 06 a and b).

Figure 6: The BC3F1 plants grown in pots for morphological characteristics study; (a) The fully grown Bg94/1 plants in the field. (b) measuring morphological characteristics of BC3F1 plant.

Table 5: The observed characters in BC3F1 backcross plants

Compared characters	Observed characters in current BC3F1 plants
Plant height	83 cm
Nature of rice plant	Categorized under dwarf plants
Plant maturity time	105 days ($3^{1/2}$ months)
Leaves color	Dark green in color
The seeds' nature	*Nadu* rice variety type seeds observed
Number of tillers	3-4 tillers
Blast resistance	Resistant

According to the observations, it confirmed the general phenotype of the backcross three generation (BC3F1) plants developed in the current research study was similar to the general phenotype of recurrent parent Bg94/1 (Table 05). Therefore, the above results assured the backcross population development procedure adopted in the general methodology of the current research was accurate, as in a normal

backcrossing protocol the final backcross generation plants were morphologically similar to the recurrent parent plants though they gained the disease-resistant character from the donor parent.

All in all with above-mentioned outcomes in the current research study it confirmed the three backcross generations developed during the backcrossing process were highly blast resistant phenotypically and molecularly most probably due to transferring *Pikh*, *Pitp(t)*, and *Pita* genes from Tetep parent plant to backcross plants (Bertrand and David, 2008).

4. Conclusions

Overall in the phenotypic screening, analysis of DNA marker profiles of RM206, RM246 and YL155/87, and investigation of morphological characteristics, it suggested that the three backcross populations were highly resistant to blast pathogen (*Magnaporthe grisea*) probably due to the presence of three blast resistant genes; *Pikh*, *Pitp(t)*, and *Pita* in their genome.

With the above-mentioned outcomes in the current research study, it confirmed the three backcross generations developed during the backcrossing process were highly blast-resistant phenotypically and molecularly. In short, it successfully fulfilled the specific objective of this research to develop a blast-resistant backcross population (BC3F1 generation) of blast susceptible local variety Bg94/1 for rice blast disease through marker-assisted backcrossing (MAB) technology. The developed backcross population can be further selfing in order to produce dominant homozygous resistant plants for genes *Pikh* and *Pitp(t)*. So, this will enable to produce local rice varieties with durable blast resistance with the aim of reducing the cost of rice production and enhancing crop productivity in future breeding programs. Thus, the developed blast-resistant backcross population and the adopted procedure could be used towards sustainable blast resistance in local rice in the future.

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