

Original Research Article

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Genotyping of Advanced Backcross Progenies Derived From Tellahamsa and MTU1010 Rice Varieties for BB and Blast Resistance Genes

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ABSTRACT

Bacterial blight (BB) and rice blast are the two most important diseases causing significant yield loss in rice, and they are endemic to several rice growing states of India. In Telangana and Andhra Pradesh states, the yield loss is very severe due to BB and blast. In order to address the above said issues, the efforts have been made by Institute of Biotechnology, Rajendranagar, Hyderabad, and developed MTU1010 and Tellahamsa introgression lines with bacterial blight (*xa13* and *Xa21*) and blast (*Pi54* and *Pi1*) resistance genes by backcrossing with donor parents. GPP2 was used as a donor for bacterial blight resistance genes (*xa13* and *Xa21*), while NLR145 (Swarnamukhi) was used as donor for blast resistance genes (*Pi54* and *Pi1*). Genotyping of these advanced backcross progenies of MTU1010 (BC₄F₅) and Tellahamsa (BC₃F₄) was carried out to check the uniformity of plants with in the progeny in homozygous condition for target genes. DNA bulking method was used to reduce the time, resources and number of samples from 3465 to 99 for DNA isolation without compromising the quality of DNA. Genotyping was carried out in 29 advanced backcross progenies (BC₃F₄, BC₄F₅) for the presence of four target genes viz., *xa13*, *Xa21*, *Pi54* and *Pi1* by using gene specific / linked PCR based molecular markers viz., *xa13-prom*, *pTA248*, *Pi54 MAS* and RM224, respectively. The bulk sample analysis of 29 progenies exhibited uniformity for the target genes.

Keywords

Rice, Bacterial blight, Blast, Marker assisted selection, BC₃F₄ & BC₄F₅ generations

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Introduction

Rice is a monocotyledonous angiosperm. It belongs to the genus *Oryza*, which contains 20 wild and two cultivated species. *Oryza sativa* cultivated in South Asia and Japan, while *Oryza glaberrima* cultivated in West Africa (Watanabe, 1976). The production of rice was 104.32 million tonnes from an area of 43.39

million hectares in India during 2015-16. Total production of rice by May 9, 2017 was 109.15 million tons in India (Ministry of Agriculture and Farmers Welfare, 2017). In India, 65 % of the population depends on rice as their staple food and the country is a huge contributor to the global rice production. However, biotic and abiotic stresses are causing huge yield losses.

MTU1010 (Cottondora Sannalu), a short duration mega rice variety released in 2000 derived from the cross Krishnaveni / IR64. This variety is extremely popular among farmers which are evident from 397.25 quintals of breeder seed indents for 2018 (<http://seednet.gov.in>). This variety possesses brown planthopper tolerance with long slender grains.

Tellahamsa (RNR 10754), a rice variety released from PJTSAU has become popular for rabi season because of its cold tolerance, high yielding ability and long slender grain quality. However, both the rice varieties MTU1010 and Tellahamsa are susceptible to BB and blast diseases, which limits their spread to areas where the two diseases are endemic.

Bacterial blight (BB), caused by *Xanthomonas oryzae pv. oryzae* (*Xoo*), is one of the serious diseases of rice across countries and can cause yield losses as high as 74 to 81 % (Srinivasan and Gnanamanickam, 2005). The fungus *Magnaporthe grisea* is the causal agent of rice blast disease and belongs to phylum, Ascomycota and family Magnaporthaceae. It is one of the most devastating diseases in at least 85 countries worldwide. The disease often results in a significant yield loss, as high as 70 to 80 % during an epidemic (Ou, 1985).

Nearly 40 genes conferring BB resistance have been identified (Sundaram *et al.*, 2014) and many of them have been fine-mapped and cloned (Natrajkumar *et al.*, 2012). 101 blast-resistance genes (Rajashekara *et al.*, 2014) and 350 quantitative trait loci (QTLs) have been identified (Sharma *et al.*, 2012) and many are fine-mapped and a few are cloned. Closely linked or functional markers are available for many BB and blast resistance genes (Sundaram *et al.*, 2014). As there is availability of several resistance genes to BB and blast, pyramiding multiple genes into

MTU1010 and Tellahamsa is considered as an ideal strategy to improve its resistance to these major diseases.

Gene pyramiding through conventional phenotype based breeding is considered to be difficult and often impossible, due to the dominance and epistasis effects of genes governing disease resistance and also due to limitations related to screening against the two diseases across the year (Sundaram *et al.*, 2009). The availability of molecular markers, closely linked to or located within the resistance genes (i.e., functional markers), makes the task of gene pyramiding easier (Singh *et al.*, 2001; Sundaram *et al.*, 2008; Shanti *et al.*, 2010; Zhao *et al.*, 2014). Functional markers are developed from polymorphic sites within genes that affect target trait variation i.e. based on functional characterization of polymorphism. Hence, they are more reliable to be used in marker assisted backcross breeding, circumventing the recombination issue there by getting rid of false positives. Among the BB resistance genes identified so far, the dominant gene, *Xa21*, originally discovered from an accession of the wild rice, *Oryza longistaminata* confers broad spectrum resistance to many *Xoo* isolates in India and elsewhere. The gene has been cloned and fine-mapped on the long arm of rice chromosome 11. A PCR based STS marker, pTA248 (Ronald *et al.*, 1992), was developed for marker assisted breeding. BB resistance gene *xa13* was first discovered in the rice variety BJ1, mapped on the long arm of rice chromosome 8 (Ogawa *et al.*, 1987; Zhang *et al.*, 1996; Sanchez *et al.*, 1999) and very tightly linked markers were identified for this gene (Sundaram *et al.*, 2014). *xa13-prom* a gene specific functional PCR based marker was used for *xa13* gene by several rice groups. The BB resistance gene combination, *Xa21* + *xa13* is known to be very effective across India (Joseph *et al.*, 2004; Gopalakrishnan *et al.*, 2008).

Pi-54, is one of the major blast resistance genes and has been observed to show resistance against many isolates of the blast pathogen in India (Ramkumar *et al.*, 2011), which was considered as the widest spectrum resistance resource favored by the rice breeders. *Pi54* gene was originally identified from Tetep, a Vietnam indica source and mapped on chromosome 11L with two tightly linked simple sequence repeat (SSR) markers TRS26 and TRS33 has been cloned (Sharma *et al.*, 2005). Ramkumar *et al.*, 2011 developed PCR based functional marker *Pi54 MAS* and it was observed to perfectly co-segregate with no recombinants. RM224 marker was used for *Pil* gene. The rice cultivar “Tetep” has been found to be resistant to most of the pathogenic races occurring in India (Padmanabhan *et al.*, 1979). Tetep was one of the parents for NLR 145 (Swarnamukhi) variety, released from Agricultural Research Station (ARS), Nellore. The resistance of rice varieties to rice blast was mostly controlled by a pair or several pairs of main effective dominant genes.

The advanced backcross progenies of MTU1010 (BC₄F₅) and Tellahamsa (BC₃F₄) pyramided with both Bacterial blight (*xa13* and *Xa21* from GPP2) and blast (*Pi54* and *Pil* from NLR145) resistance genes were developed by Institute of Biotechnology, Rajendranagar, Hyderabad. In continuation to above research project the present research study entitled “Genotyping of advanced backcross progenies derived from Tellahamsa and MTU1010 rice varieties for BB and Blast resistance” was carried out to check the uniformity of plants with in progenies.

Materials and Methods

GPP2 and NLR145 were used as original donor parents in the recurrent backcross breeding where MTU1010 and Tellahamsa were used as recurrent parents. Subsequently

advanced backcross progenies having BB and blast resistance genes were developed at Institute of Biotechnology, Rajendranagar, Hyderabad. A total of 29 advanced backcross lines of Tellahamsa (BC₃F₄) and MTU1010 (BC₄F₅) were evaluated in our study along with parental lines viz., NLR145, GPP2, MTU1010 and Tellahamsa (Table 1).

Genotyping of advanced backcross progenies

Genotyping studies were carried out to assess the homozygosity of individual plants with in each backcross progeny for all the target genes. Gene specific and closely linked gene markers were used to identify the plants carrying the target genes in homozygous condition (Table 2). This study is also known foreground selection. Genotyping was executed to identify plants possessing the target R genes in homozygous condition.

Molecular analysis (Genotyping)

Molecular analysis was carried out in 29 advanced backcross progenies and four parents to determine the presence of target genes in homozygous condition.

Collection and preservation of leaf material

Leaf samples were collected from advanced backcross lines of Tellahamsa (19 BC₃F₄ lines) and MTU1010 (10 BC₄F₅ lines) along with two recipient and two donor parents at 15 days after transplanting. Leaf samples were collected in the morning and immediately stored at -80°C for DNA isolation.

Bulking of leaf samples for DNA isolation

A total of 33 (29 backcross progenies and four parents) entries were evaluated in three replications. Thirty five plants were planted per row with a spacing of 15x20 cm. For DNA

isolation the sample size from 3465 (33x3x35=3465) was reduced to 99 by following DNA bulking method (Nas *et al.*, 2002, Arunakumari, 2009 and Sravanthi, 2010). During the bulking of leaf material, precaution was taken to have uniform representation of all 35 plants present in a particular row. Leaf samples were collected from all 35 plants present in a row and bulked, thus a total of 99 bulks from a total of 3465 plants were prepared.

DNA isolation

DNA was isolated from the leaf material using a CTAB method of Zheng *et al.*, (1996) with some modifications Quantification of isolated genomic DNA was performed by running the genomic DNA on 0.8% agarose gel (Sambrook and Russell, 2001) with diluted uncut DNA as standard.

Based on the intensity and thickness of genomic DNA bands in comparison to (λ) DNA, the concentration and quality of DNA in individual samples was determined. Normalization of DNA samples was done to equalize the concentration of all the samples to be used for PCR reaction. The purpose of normalization was to avoid erroneous analyses due to differences in the brightness of the bands obtained after electrophoresing the PCR products.

Polymerase Chain Reaction (PCR)

PCR reaction was carried out in small PCR tubes containing 10 μ l PCR reaction mixture. The details of PCR components are furnished in the following table 3.

Thermo cycling was carried out on a 96- well PCR system (Eppendorf) which holds 0.2 ml thin walled PCR tubes. All tubes were placed into the thermo cycler and DNA amplification program mentioned in table was followed. The

annealing temperatures of the primers were standardized by using gradient PCR. The PCR program was set up with three steps per cycle and details are furnished in the following table 4.

The amplified products were resolved on 3% agarose (Seakem L Agarose) gel. The gel was observed under UV trans-illuminator and gel documentation system and amplified products were visualized. The size of the amplified fragments was calculated using 50 bp ladder (Biolabs, India) as size reference standards.

Results and Discussion

Genotyping of advanced backcross progenies were conducted to assess the uniformity of plants within each progeny for the target genes. The DNA samples which showed amplification pattern similar to donor parent allele were considered as the uniform progeny rows, while the samples different from that of donor parent allele will be considered as segregant rows.

Molecular markers enable breeders to exercise precise selection on genotypic differences between the phenotypic expressions. The markers have the potential efficiency to increase the selection and shorten the breeding cycle (Yencho *et al.*, 2000).

The use of molecular markers linked to resistance genes can accelerate gene pyramiding efforts and make the process more precise (Mohan *et al.*, 1997).

In our study, 29 pooled DNA samples of advanced backcross progenies along with recurrent parents (MTU1010 and Tellahamsa) and donor parents (GPP2 for BB and NLR145 for blast resistance) were amplified for the four target genes using the following markers. The details of resistance and susceptible alleles are furnished in following table 5.

Foreground analysis of BC₃F₄ lines derived from Tellahamsa and BC₄F₅ lines derived from MTU1010 for BB resistance genes

Foreground analysis or foreground selection in which the breeder selects those plants having the marker allele of the donor parent at the target locus. Selection by genetic markers helps the breeder to ascertain the presence of an introgressed gene when direct phenotypic evaluation is not feasible. The objective is to maintain the target locus in a homozygous state. Foreground analysis in BC₃F₄ lines of Tellahamsa and BC₄F₅ lines of MTU1010 was done for two BB resistance genes i.e., *Xa21* and *xa13*.

Foreground selection for BB resistance genes was carried out in plants using PCR based markers, viz., pTA248 (Ronald *et al.*, 1992) for *Xa21* gene and *xa13 prom* (Sundaram *et al.*, 2011) for *xa13* gene which were successfully used by Magar *et al.*, (2014), Arunakumari *et al.*, (2016), Hajira *et al.*, (2016) for tracking the target genes in various generations. 15 advanced backcross progenies of Tellahamsa and 10 progenies of MTU1010 carrying single or both the genes were screened using gene specific / linked markers.

Xa21 confers broad spectrum resistance (Khush *et al.*, 1990; Ikeda *et al.*, 1991) to many Indian isolates of *Xoo*. This approach of convergence breeding however can be very difficult or impossible when there is epistasis or masking effect of genes, particularly when a breeding line already harbors a gene like *xa13* which shows resistance to all known *Xoo* races. With the conventional approach, breeding lines with *Xa21* alone cannot be distinguished from breeding lines with *Xa21* plus other BB genes. However, with the availability of DNA markers for each resistance gene, the identification of plants with multiple resistance genes would become easy (Xu *et al.*, 1996).

Screening of advanced backcross progenies for *Xa21* gene

Fourteen progenies of Tellahamsa (TPL-53, TPL-54, TPL-55, TPL-56, TPL-57, TPL-58, TPL-59, TPL-60, TPL-65, TPL-66, TPL-68, TPL-69, TPL-70 and TPL-71) and 9 (MPL-1, MPL-3, MPL-4, MPL-5, MPL-6, MPL-7, MPL-8, MPL-9 and MPL-10) progenies of MTU1010 were evaluated in three replications. Twenty three progenies were advanced from BC₃F₃ and BC₄F₄ generations (Rabi, 2016-17) to the present investigation to determine the uniformity of all the plants in each progeny for *Xa21* gene. Replication wise 23 bulked DNA samples were screened using pTA248 marker along with recurrent (Tellahamsa and MTU1010) and donor (GPP2) parents. pTA248 marker used in our study is as functional marker as per the earlier report from Ronald *et al.*, 1992. The *Xa21* gene was mapped and tagged on chromosome 8 by RAPD and RFLP markers (Ronald *et al.*, 1992) and further a PCR based STS marker pTA248 was developed which was very closely linked to the gene (0.1 cM). According to Ronald *et al.*, 1992, pTA248 amplified a 900 bp fragment in case of resistant genotypes while 600-800 bp fragments were observed in case of susceptible genotypes in a co-dominant fashion. Hence it is highly useful for marker assisted introgression of *Xa21*.

Twenty three bulked DNA samples along with four parents (MTU1010, Tellahamsa, NLR145 and GPP2) were amplified and loaded on 3% agarose gel. The gel documentation of PCR results showed 650 bp susceptible alleles in MTU1010, Tellahamsa and NLR145, while 900 bp resistance allele was observed in GPP2 (the donor parent for *Xa21* gene) and all 23 advanced back cross progenies (Plate 1 pertaining to replication 1). This result clearly indicated the presence of BB resistance gene, *Xa21* in all 35 plants in homozygous condition. Similar result was observed in other

two replications too. In a marker assisted breeding study, it is desirable to verify the progenies for uniformity of target genes till the entries are promoted to advanced yield trials. DNA bulking method saved us a lot of time and resources as we could reduce the sample number from 2835 to 81. The segregants or impurities or admixtures can be easily identified by the presence of susceptible allele or alleles in heterozygous nature. Arunakumari *et al.*, 2009 and Sravanthi *et al.*, 2010 used this methodology to determine the uniformity of various samples.

Screening of advanced backcross progenies for *xa13* gene

Thirteen progenies of Tellahamsa (TPL-53, TPL-54, TPL-55, TPL-56, TPL-57, TPL58, TPL-59, TPL-60, TPL-65, TPL-66, TPL-67, TPL-70 and TPL-71) and 7 progenies of MTU1010 (MPL-1, MPL-2, MPL-3, MPL-5, MPL-6, MPL-9 and MPL-10) were screened for *xa13* gene using *xa13 prom* marker (Sundaram *et al.*, 2011) along with recurrent (Tellahamsa and MTU1010) and donor parents (GPP2). Divya *et al.*, (2015) also carried out foreground selection for the presence of *xa13* gene using PCR based gene-specific marker, *xa13 prom*. *xa13 prom* is a functional marker designed from promoter

region of *xa13* gene (Sundaram *et al.*, 2011) and it was more accurate than earlier RG136, CAPS marker which is ~1.5 cM away from *xa13* gene. This marker was used for accurate genotyping by ArunaKumari and Durga Rani, 2015 as it is a perfect marker would elicit no false positive results.

Replication wise 20 bulked DNA samples were amplified with primer pair, *xa13 prom* and loaded on 3% agarose gel along with four parents (MTU1010, Tellahamsa, NLR145 and GPP2). The gel documentation of PCR results showed 250 bp susceptible allele in MTU1010, Tellahamsa, NLR145, while 500bp resistance allele was observed in all the backcross progenies. Sundaram *et al.*, (2011), Magar *et al.*, (2014), Padmaja *et al.*, (2017) and many others also used this marker and observed 250 bp and 500 bp susceptible and resistance alleles, respectively. The results clearly revealed that 500 bp resistance allele was observed in all 20 progenies (Plate 2 pertaining to replication 1) planted in three replications, indicating that all 35 plants present with in a progeny exhibited uniformity for *xa13* gene. Similar strategy was used by Arunakumari, 2009 and Sravanthi, 2010 to observe admixtures or impurities in breeder seed samples of promising rice varieties.

Table.2 Details of molecular markers used for genotyping

S. No	Marker	Chromosome	Sequence	Reference
1	<i>xa13 prom</i>	8	F-GGCCATGGCTCAGTGTTTAT R- GAGCTCCAGCTCTCCAAATG	Sundaram <i>et al.</i> , (2011)
2	<i>pTA248</i>	11	F-AGACGCGGGAAGGGTGGTTCCCGGA R-AGACGCGGGTAATCGAAAGATGAAA	Ronald <i>et al.</i> , (1992)
3	<i>Pi54-MAS</i>	11	F-CAATCTCCAAAGTTTTTCAGG R-GCTTCAATCACTGCTAGACC	Ramkumar <i>et al.</i> , (2011)
4	RM 224	11	F-ATCGATCGATCTTCACGAGG R-TGCTATAAAAGGCATTTCGGG	Hittalmani <i>et al.</i> , (2000)

Table.1 Advanced backcross progenies of Tellahamsa and MTU1010

S. No	Code No. of advanced backcross progenies	Generation	Recurrent parent	Target genes
1	TPL-53	BC ₃ F ₄	Tellahamsa	<i>Xa21, xa13, Pi54, Pi1</i>
2	TPL-54	BC ₃ F ₄	Tellahamsa	<i>Xa21, xa13, Pi54, Pi1</i>
3	TPL-55	BC ₃ F ₄	Tellahamsa	<i>Xa21, xa13, Pi54, Pi1</i>
4	TPL-56	BC ₃ F ₄	Tellahamsa	<i>Xa21, xa13, Pi54, Pi1</i>
5	TPL-57	BC ₃ F ₄	Tellahamsa	<i>Xa21, xa13, Pi54</i>
6	TPL-58	BC ₃ F ₄	Tellahamsa	<i>Xa21, xa13, Pi54</i>
7	TPL-59	BC ₃ F ₄	Tellahamsa	<i>Xa21, xa13, Pi54</i>
8	TPL-60	BC ₃ F ₄	Tellahamsa	<i>Xa21, xa13, Pi54</i>
9	TPL-61	BC ₃ F ₄	Tellahamsa	<i>Pi54, Pi1</i>
10	TPL-62	BC ₃ F ₄	Tellahamsa	<i>Pi54, Pi1</i>
11	TPL-63	BC ₃ F ₄	Tellahamsa	<i>Pi54, Pi1</i>
12	TPL-64	BC ₃ F ₄	Tellahamsa	<i>Pi54, Pi1</i>
13	TPL-65	BC ₃ F ₄	Tellahamsa	<i>Xa21, xa13, Pi1</i>
14	TPL-66	BC ₃ F ₄	Tellahamsa	<i>Xa21, xa13, Pi1</i>
15	TPL-67	BC ₃ F ₄	Tellahamsa	<i>xa13, Pi54, Pi1</i>
16	TPL-68	BC ₃ F ₄	Tellahamsa	<i>Xa21, Pi54, Pi1</i>
17	TPL-69	BC ₃ F ₄	Tellahamsa	<i>Xa21, Pi54, Pi1</i>
18	TPL-70	BC ₃ F ₄	Tellahamsa	<i>Xa21, xa13</i>
19	TPL-71	BC ₃ F ₄	Tellahamsa	<i>Xa21, xa13</i>
20	MPL-1	BC ₄ F ₅	MTU1010	<i>Xa21, xa13, Pi54, Pi1</i>
21	MPL-2	BC ₄ F ₅	MTU1010	<i>xa13, Pi54, Pi1</i>
22	MPL-3	BC ₄ F ₅	MTU1010	<i>Xa21, xa13, Pi54</i>
23	MPL-4	BC ₄ F ₅	MTU1010	<i>Xa21, Pi54, Pi1</i>
24	MPL-5	BC ₄ F ₅	MTU1010	<i>Xa21, xa13, Pi1</i>
25	MPL-6	BC ₄ F ₅	MTU1010	<i>Xa21, xa13</i>
26	MPL-7	BC ₄ F ₅	MTU1010	<i>Xa21, Pi54</i>
27	MPL-8	BC ₄ F ₅	MTU1010	<i>Xa21, Pi1</i>
28	MPL-9	BC ₄ F ₅	MTU1010	<i>Xa21, xa13, Pi54, Pi1</i>
29	MPL-10	BC ₄ F ₅	MTU1010	<i>Xa21, xa13</i>

Table.3 Components of PCR reaction

Components	Stock concentration	Quantity required for one reaction (µl)
Template DNA	50 ng/µl	2.0
PCR buffer	10X	1.0
dNTPs	2.5 Mm	1.0
Forward Primer	2.5 pmole	0.3
Reverse Primer	2.5 pmole	0.3
Taq polymerase	1 U/µl	1.0
Sterile distilled water		4.4

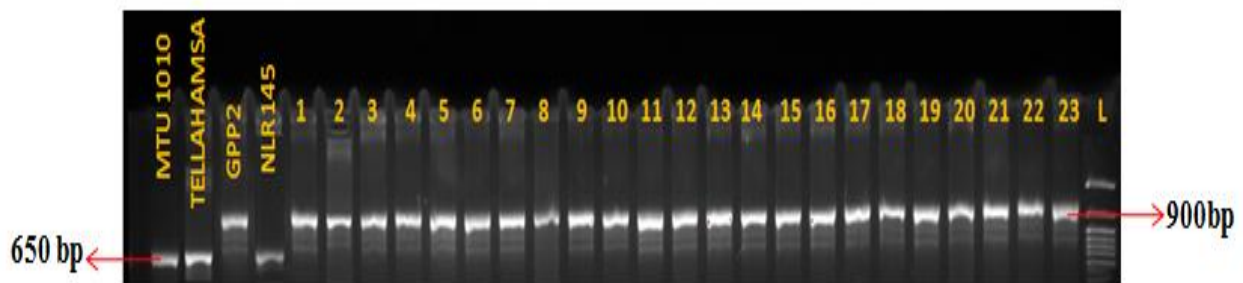
Table.4 Thermal profile followed for PCR reaction

Step 1	94°C - 3-5min	Initial Denaturation
Step 2	94°C - 1min	Denaturation
Step 3	50- 65°C - 30sec	Annealing- based on melting temp of primer
Step 4	70-72°C - variable	Extension- based on size of amplicons
Step 5	Repeat step 2-4 for 30-45 times	
Step 6	72°C - 7min	Final Extension
Step 7	Hold at 4°C till loading on Agarose gel electrophoresis	

Table.5 Resistance and susceptible allele sizes of target gene specific / linked markers

Gene	Markers	Resistance allele	Susceptible allele	Reference
<i>xa13</i>	<i>xa13-prom</i>	500 bp	250 bp	Sundaram <i>et al.</i> , 2011
<i>Xa21</i>	<i>PTA248</i>	900 bp	650 bp	Ronald <i>et al.</i> , 1992
<i>Pi54</i>	<i>Pi54 MAS</i>	200 bp	350 bp	Ramkumar <i>et al.</i> , 2011
<i>Pi1</i>	RM224	130 bp	150 bp	Hittalmani <i>et al.</i> , 2000

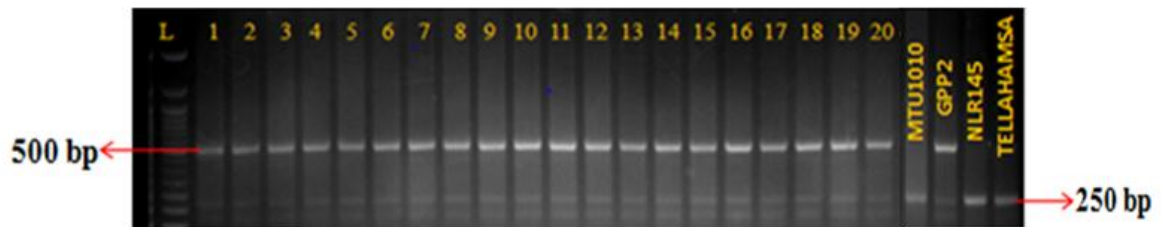
Plate.1 Genotyping of advanced backcross progenies for *Xa21* gene using pTA248 marker



L- 50 bp DNA ladder, resistance allele size- 900 bp, susceptible allele size- 650 bp
 MTU 1010 and Tellahamsa- Recipient parents
 GPP2- donor parent for BB resistance genes (*xa13* and *Xa21*)
 NLR 145- donor parent for blast resistance genes (*Pi54* and *Pi1*)
 1-23 indicate bulked DNA samples of advanced back cross progenies

S.No	Entries	S.No	Entries	S.No	Entries	S.No	Entries
1	TPL-53	7	TPL-59	13	TPL-70	19	MPL-6
2	TPL-54	8	TPL-60	14	TPL-71	20	MPL-7
3	TPL-55	9	TPL-65	15	MPL-1	21	MPL-8
4	TPL-56	10	TPL-66	16	MPL-3	22	MPL-9
5	TPL-57	11	TPL-68	17	MPL-4	23	MPL-10
6	TPL-58	12	TPL-69	18	MPL-5		

Plate.2 Genotyping of advanced backcross progenies for *xa13* gene using *xa13* *prom* marker



L- 50 bp DNA ladder, resistance allele size- 500 bp, susceptible allele size- 250 bp

MTU 1010 and Tellahamsa- Recipient parents

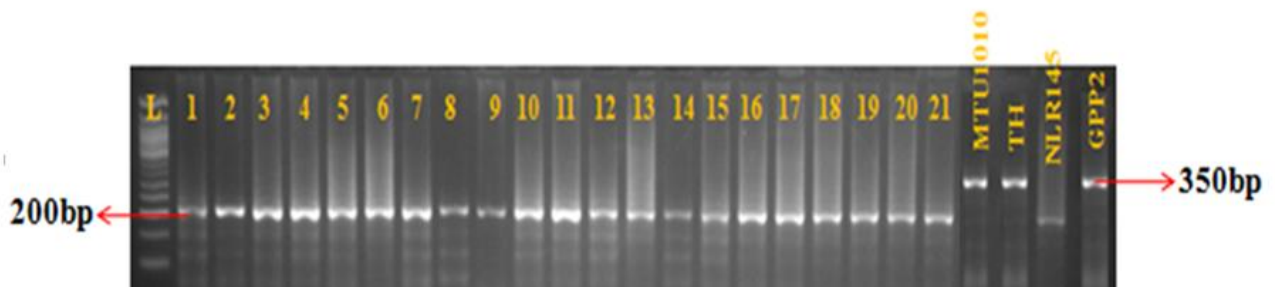
GPP2- donor parent for BB resistance genes (*xa13* and *Xa21*)

NLR 145- donor parent for blast resistance genes (*Pi54* and *Pi1*)

1-20 indicate bulked DNA samples of advanced back cross progenies

S.No	Entries	S.No	Entries	S.No	Entries
1	TPL-53	8	TPL-60	15	MPL-2
2	TPL-54	9	TPL-65	16	MPL-3
3	TPL-55	10	TPL-66	17	MPL-5
4	TPL-56	11	TPL-67	18	MPL-6
5	TPL-57	12	TPL-70	19	MPL-9
6	TPL-58	13	TPL-71	20	MPL-10
7	TPL-59	14	MPL-1		

Plate.3 Genotyping of advanced backcross progenies for *Pi54* gene using *Pi54* MAS marker



L- 50bp ladder, resistance allele size- 200 bp, susceptible allele size- 350 bp

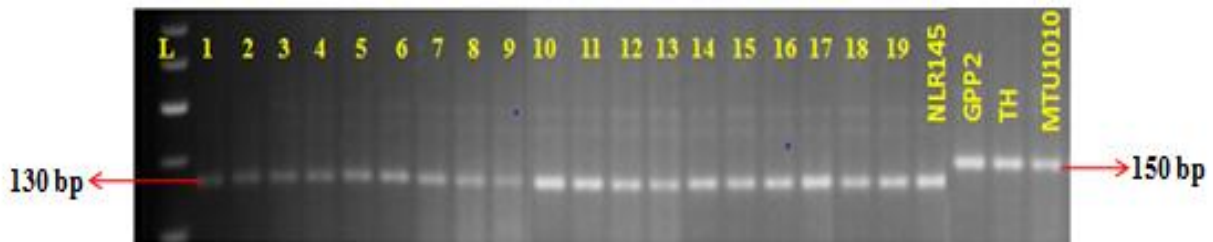
MTU 1010 and Tellahamsa- Recipient parents, GPP2- donor parent for BB resistance genes (*xa13* and *Xa21*)

NLR 145- donor parent for blast resistance genes (*Pi54* and *Pi1*)

1-21 indicate bulked DNA samples of advanced back cross progenies

S. No	Entries	S.No	Entries	S.No	Entries
1	TPL-53	8	TPL-60	15	TPL-69
2	TPL-54	9	TPL-61	16	MPL-1
3	TPL-55	10	TPL-62	17	MPL-2
4	TPL-56	11	TPL-63	18	MPL-3
5	TPL-57	12	TPL-64	19	MPL-4
6	TPL-58	13	TPL-67	20	MPL-7
7	TPL-59	14	TPL-68	21	MPL-9

Plate.4 Genotyping of advanced backcross progenies for *Pil* gene using RM224 marker



L- 50bp ladder, resistance allele size- 130 bp, susceptible allele size- 150 bp
 MTU 1010 and Tellahamsa- Recipient parents, GPP2- donor parent for BB resistance genes (*xa13* and *Xa21*)
 NLR 145- donor parent for blast resistance genes (*Pi54* and *Pi1*)
 1-19 indicate bulked DNA samples of advanced back cross progenies

S.No	Entries	S.No	Entries	S.No	Entries
1	TPL-53	8	TPL-64	15	MPL-2
2	TPL-54	9	TPL-65	16	MPL-4
3	TPL-55	10	TPL-66	17	MPL-5
4	TPL-56	11	TPL-67	18	MPL-8
5	TPL-61	12	TPL-68	19	MPL-9
6	TPL-62	13	TPL-69		
7	TPL-63	14	MPL-1		

Foreground analysis of BC₃F₄ lines derived from Tellahamsa and BC₄F₅ lines derived from MTU1010 for blast resistance genes

Foreground analysis was done in BC₃F₄ lines derived from Tellahamsa and BC₄F₅ lines derived from MTU1010 for observing uniformity of blast resistance genes i.e., *Pi54* and *Pi1*. In the current study, foreground selection for blast resistance genes was carried out using PCR based markers, viz., *Pi54 MAS* (Ramkumar *et al.*, 2011) for *Pi54* gene and RM224 (Hittalmani *et al.*, 2000) for *Pi1* gene. Seventeen advanced backcross progenies of Tellahamsa and 8 progenies of MTU1010 carrying single or both the genes were screened.

Screening of advanced backcross progenies for *Pi54* gene

A total of 15 progenies of Tellahamsa (TPL-53, TPL-54, TPL-55, TPL-56, TPL-57, TPL-58, TPL-59, TPL-60, TPL-61, TPL-62, TPL-

63, TPL-64, TPL-67, TPL-68 and TPL69) and 6 progenies of MTU1010 (MPL-1, MPL-2, MPL-3, MPL-4, MPL-7 and MPL-9) in three replications were screened using *Pi54MAS* marker along with recurrent (Tellahamsa and MTU1010) and donor (NLR145) parents

The resistance allele size was noted as 200 bp whereas susceptible allele is of 350 bp. The gene *Pi-kh* from Tetep (an indica rice line) is highly effective against the pathogen population found in the Northwestern Himalayan region of India (Sharma *et al.*, 2002). Linkage analysis of the 208 individual plants in the mapping population with four rice markers and a CAPS marker revealed that (Sharma *et al.*, 2005a) S129700 and RM 206 were linked to the *Pi-kh* locus at distances of 4.5 cM distance and 0.7 cM distance cM, respectively (Sharma *et al.*, 2005b). Subsequently a PCR-based co-dominant molecular marker *Pi-54 MAS* was developed (Ramkumar *et al.*, 2011) which could effectively amplify *Pi54* gene compared to

the earlier reported markers. Twenty one bulked DNA samples along with four parents (MTU1010, Tellahmasa, GPP2 and NLR145) were amplified with primer pair, *Pi54 MAS* and loaded on 3% agarose gel. The gel documentation of PCR results exhibited 350bp susceptible allele in MTU1010, Tellahmasa and GPP2) and 200bp resistance allele was observed in NLR145, the donor parent for *Pi54* gene. The results further revealed that 200 bp resistance allele was observed in all 21 progenies (Plate 3 showing the progenies obtained from replication one) indicating that all 35 plants present in each progeny expressed resistance allele like NLR145 without any segregation. In a self-pollinated crop like rice as per the expectation, the gene will be fixed once we select the homozygous plant with *Pi54* gene from F₂ population. Madhavi *et al.*, (2012), Atulsingh *et al.*, (2012) and Hari *et al.*, (2013) also used *Pi54 MAS* for confirmation of *Pi54* gene in segregating populations and further generations.

Screening of advanced backcross progenies for *Pil* gene

A total of 13 progenies of Tellahmasa (TPL-53, TPL-54, TPL-55, TPL-56, TPL-61, TPL-62, TPL-63, TPL-64, TPL-65, TPL-66, TPL-67, TPL-68 and TPL-69) and 6 progenies of MTU1010 (MPL-1, MPL-2, MPL-4, MPL-5, MPL-8 and MPL-9) in three replications were screened for *Pil* gene using RM224 marker along with parents. According to Collard and Mackill (2008), the markers to be used should be close to gene of interest (<5 recombination unit or cM) in order to ensure that only minor fraction of the selected individuals will be recombinants. The SSR marker RM224 was chosen in our study because it is mapped in the same position (0.0 cM) as with the *Pil* gene. It is recognized as valuable marker for introgression and pyramiding of *Pil* gene into blast susceptible rice cultivars (Fuentes *et al.*,

2008). This was successfully used in several marker assisted breeding programmes for efficient selection. Hence RM224 marker for *Pil* gene screening was used in our study

Nineteen bulked DNA samples were amplified by using RM224 for *Pil* and loaded on 3% agarose gel along with four parents (MTU1010, Tellahmasa, GPP2 and NLR145). The gel documentation of PCR products shown 150 bp susceptible alleles in MTU1010, Tellahmasa and GPP2 and 130 bp resistance allele in all the 19 progenies and the donor parent, NLR145. This result indicated the presence of resistance gene in homozygous condition in them. The Plate 4 is showed the uniformity of 19 progenies in first replication for *Pil* gene. Similar results were observed in the works of Liu *et al.*, (2003), Prasad *et al.*, (2009), Jiang *et al.*, (2012) and Balakrishanan *et al.*, (2013).

DNA bulking method was used to reduce the time and number of samples for DNA isolation without compromising the quality of the test. Equal quantity of leaf sample was collected from all 35 single plants present in a row towards bulk DNA isolation.

Genotyping of 29 advanced backcross progenies for the presence of four target genes (*xa13*, *Xa21*, *Pi54* and *Pil*) by using gene specific / gene linked PCR based molecular markers *viz.*, *xa13-prom*, pTA248, *Pi54 MAS* and RM224, respectively was carried out.

The bulk sample analysis of 29 progenies exhibited uniformity of all the plants for the target genes.

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