Original Research Article

Gene validation for Brown Plant Hopper resistance in rice varieties using SSR markers

ABSTRACT

The Brown Plant Hopper (BPH), *Nilaparvatalugens* (Homoptera: Delphacidae) is one of the most destructive insect pests causing significant yield loss in most of the rice cultivars of Asia. Marker-assisted backcrossing is used to introgress the genes for BPH resistance in which the selection of donor parents that can ~~impart~~ to confer durable resistanceand validation of the presence of genes is a prerequisite. Jyothi the most popular, widely cultivated rice variety in Kerala is screened along with four parental lines viz., PTB33 (Variety released from RARS, Pattambi), IRRI introgressed lines-IR65482-7-216-1-2, IR7103-121-15-B, and a breeding line RP2068-13-5-8 for validating the presence of genes *Bph3*, *BPH18*,*Bph20*, and *Bph21* using SSR markers. Parental polymorphism was shown for markers specific to *Bph3*, *BPH18,* and *Bph20*. Besides this, cluster analysis using the UPGMA method produced 4 clusters. The variety 'PTB33' was obtained in a separate cluster which can be attributed to its resistant character against BPH and can be considered as a potential donor. The IRRI introgressed lines- IR65482-7-216-1-2, IR7103-121-15-B, and the breeding line RP2068-13-5-8 can also be well utilized as donors by proper standardized BPH screening tests for confirming the phenotypic expression of BPH resistance.

Keywords: *Brown Planthopper, Gene validation, Insect resistance, Polymorphism, Rice*

1. INTRODUCTION

Rice (*Oryzasativa*L.) is the primary source of food for more than half of the world's population and plays a significant role in the food security of Asia where more than 90 percent of the rice is produced [1]. Among the major cereal crops grown in India, rice alone contributes to 44.11 million hectares and produced 112.91 million tonnes during 2017-18 [2]. Rice encounters many biotic and abiotic constraints in production and productivity. Insects are the most important biotic stress causing significant yield loss. Among the insect pests, the brown plant hopper (*Nilaparvatalugens*Stal.) is a typical phloem sap-feeding pest of rice resulting in "hopper-burn"symptoms and its role as a vector of rice grassy stunt virus and ragged stunt virus have aggravated the posed by the insect. It accounts for 20% to 80% yield loss in rice with an overall annual economic loss of around $300 million in Asia [3].

Pest management strategies in managing BPH include chemical control, improving field practices, and developing resistant varieties. However excessive use of chemicals for BPH management is hazardous to human health, cost-ineffective, and unsafe for the environment and biodiversity. Alternatively, the most durable, precise, cost-effective, and environment-friendly strategy is to develop a sustainable host-plant resistance mechanism for BPH management. The quick evolution of BPH poses a primary concern. Hence, there is a need to attain durable and broad-spectrum resistance by theintrogression of novel genes to build resistance against BPH[4]. Four biotypes of BPH have been recognized, in which biotypes 1 and 2 are the most predominant in East and Southeast Asia[5], biotype 3 originated in the laboratory from rearing the insect on a resistant variety[6], and biotype 4 in South Asia especially Indian sun-continent[7].

The first ever BPH resistant gene, *bph1* was introgressed to the *indica* rice variety Mudgo, but the selection pressure on this gene leads to the involvement of new pathotypes in BPH which could tolerate the effect of *bph1* [8]. Accordingly, more than 38 major BPH resistance genes have been reported in several *Oryzasativa*sp. *indica*cultivars and wild relatives [9]. The genetics of BPH resistance revealed the presence of 32 major genes (*Bph1* to *Bph32*) in cultivated rice and seven wild relatives [10]. Whereas, cloning and characterization of nine potential genes (*Bph3*/*Bph17*, *Bph6*, *BPH9*, *Bph14*, *Bph15*, *BPH18*, *BPH26*, *BPH29*, and B*ph32*) were carried out to date [11].

Plant resistant mechanism towards insect attack includes antibiosis, antixenosis and tolerance, in which antibiosis that affects insect behaviour such as survival, feeding or reproduction upon infestation is the most resistant mechanism in rice on BPH attack [11][12]. Reduction in pest colonization and oviposition by repelling and disturbing the pest on attack becomes antixenosis mechanism while, tolerant plants produce little or zero damage by insect attack with good quality and fitness[13].

Rice variety 'IR62' was observed with consistently high resistance to plant hoppers from screening in South and South East Asia [14] and was due to the genes for BPH resistance derived from PTB33, which is a popular source of resistance in modern rice breeding programs [15]. [16] Introgressed BPH-resistantgenes *Bph20* and *Bph21* into an elite variety CO43*Sub1* using IR71033-121-15 as a donor and two introgressed lines 32-4-34 and 32-4-35 showed resistance to BPH population.

Identification of genes for BPH resistance from different rice cultivars, the study of resistant mechanisms, and the introgression of resistant genes to elite well-adapted but susceptible rice varieties become needful for sustainable economic yield under the BPH outbreak. Advancement in molecular genetics plays an important role in the search and confirmation of resistant genes from various genotypes and helps in efficient and fast insect-resistant breeding methods. Molecular markers are employed for the selection of plants with desired genomic regions that control the expression of the trait of interest. Marker-assisted selection in plant breeding enables the selection of individual plants based on their genotype. Selection of parents in backcross breeding programmes and other breeding techniques is more efficiently done with aid of molecular markers reducing the cost, time, and resources. Screening of rice varieties and validating the presence or absence of genes of interest is essential for the selection of donor and recipient parents in breeding programmes for the development of resistant varieties.

So that the present study was undertaken to validate the presence or absence of four genes that impart BPH resistance (*Bph3*, *BPH18*,*Bph20*, and *Bph21*) in five genotypes of rice varieties to identify the best variety with multiple resistant genes, that could be used as a donor for marker-assisted gene pyramiding breeding programs and confirmation of the absence of the genes in Jyothi variety which is high yielding but susceptible to be used as recipient parent.

2. MATERIALS AND METHODS

2.1. Plant materials

The high-yielding rice variety in Kerala namely Jyothi, PTB33 (Variety released from RARS, Pattambi), IRRI introgressed lines- IR65482-7-216-1-2, IR7103-121-15-B, and a breeding line RP2068-13-5-8 were screened for validating the presence of genesBph3, BPH18,Bph20, and Bph21. The details of the markers used in the study are listed in table 1.

Gene	Marker	Sequence (5'-3')	Annealing temp. (□ c)	References
Bph 3	RM589	F-GAGGTTGTTTGGATGGATAGATGG R- AATCCCGTCCTAGAGTTCTTCTACC	55.3	[22]
Bph 18	RM3331	F-CCTCCTCCATGAGCTAATGC R-AGGAGGAGCGGATTTCTCTC	55.3	[26]
Bph 20	RM8213	F-TGTTGGGTGGGTAAAGTAGATGCTG R-CCCAGTGATACAAAGATGAGTTGG	55.3	[16]
Bph 21	RM28561	F-CTTCAAGACTGGCCCAATATTACTGC R-TGACTGAAGCCTTCTTCACTTGC	55.3	[26]

2.2. Genomic DNA extraction and quantification

Genomic DNA was isolated using theCTAB method [17]. About 100 mg of each leaf sample was ground using a pestle and mortar with liquid nitrogen (-196℃), and 400 µL CTAB extraction buffer (2% CTAB, 100 mMTris-HCL, 20 mM EDTA, 1.4 M NaCl, 1% PVP) was added, and the solution was transferred into a 2 ml microcentrifuge tube. The tubes were kept in the water bath at 60℃ for 1 hr with manual shaking in every 10 minutes. After that, the plant debris was removed by centrifuge (14,000 rpm, 10 minutes), and the supernatant was collected ina fresh centrifuge tube and 400 µl mixture, the ratio of phenol: chloroform: isoamyl alcohol (25:24:1%), was added. The mixtures were then well mixed by inverting and centrifuge (14,000 rpm, 5 minutes) at 4℃. The supernatant was then transferred into a freshmicrocentrifuge tube and an equal volume of isopropanol was added. The mixtures were gently mixed by inverting the microcentrifuge tube and then incubated at -20℃overnight. Following, the next day the mixture was centrifuged again (14,000 rpm, 5 minutes) at 4℃. The supernatant was then discarded and thepellets were washed with 70% alcohol two times. Subsequently, pellets were air-dried and re-suspended in 80 µL of TE buffer, and stored at-20°C. Agarose gel electrophoresis was carried out to resolve the genomic DNA in a horizontal gel electrophoresis Unit of BIO-SYS. An amount of 0.8% agarose was used for quantifying the genomic DNA in 0.5X TBE buffer. The gel was visualized under UV light using the SYNGENE gel documentation system in order to check the intactness and shearing of DNA and RNA contamination.

2.3. Gene validation using SSR markers

The genomic DNA of five rice varieties was amplified using four SSR markers. The reaction was executed in a 25 µl reaction mixture consisting of 20ng template DNA, 2 µl of dNTP mix, 2.5 µl of PCR buffer, 2.5 µl of $MgCl_2$ and 0.3 µlTaq DNA polymerase, and 1 µl of both forward and reverse primers. Amplification compassed in an Eppendorf master cycler nexus PCR. Following PCR conditions were used: An initial denaturation at 94ºC for 5 minutes followed by 35 cycles of denaturation at 94 º C for 30 seconds, the annealing temperature of55.3 º C for 30 seconds, primer extension at 72 º for 1 minute, and final extension 5 at 72°C for 8 minutes.The amplified DNA was separated on 4% metaphor-agarose gels, visualized by staining with ethidium bromide and viewed under UV light using the SYNGENE gel documentation system and photographed.

2.4. Data analysis

The presence and absence of alleles were scored using the binary system ‘1’ and ‘0’ respectively. A genetic similarity matrix was constructed using Jaccard’s coefficient method [18]. The distance matrix was subjected to cluster analysis employing UPGMA (unweighted paired group method with arithmetic averages) using the SAHN (Sequential Agglomerative Hierarchical and nested Cluster) module of the software NTSYS PC [19].

3. RESULTS AND DISCUSSION

Table 1. List of SSR markers used for gene validation

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The rice varieties Jyothi, PTB33, IRRI introgression linesIR65482-7-216-1-2, IR7103-121-15-B, and a breeding line RP2068-13-5-8 were validated for the presence or absence of BPH-resistant genes *Bph3*, *BPH18*,*Bph20*, and *Bph21* using SSR markers RM589, RM3331, RM8213, and RM28561. All the markers were amplified at an annealing temperature of 55.3℃ (Fig. 1).

Jyothi (PTB 39) is the most widely accepted and popular high-yielding rice variety of Kerala released from RARS, Pattambi. At present, the variety is susceptible to brown plant hopper and was considered as a susceptible parent in the study. Parental polymorphic studies using the SSR marker ‘RM589’which is linked with the*Bph3* gene, produced five alleles. The obtained band size varied from 180bp in Jyothi (susceptible parent)to 220bp in the breeding line ‘RP2068-13-5-8’. The resistant donor variety PTB33 produced a band at 200bp (Table 2). Being resistant to all known biotypes of BPH, PTB33 can be used as a donor parentfor BPH resistance[20] [21]. [22] Mapped *Bph3* using markers RM589 and RM588 on the short arm of chromosome 6. A three-gene encoded plasma membrane-localized lectin receptor kinases were identified as a cluster in the *Bph3* gene conferring durable, broad-spectrum resistance against BPH. Also, enhanced resistance to BPH was obtained by the introgression of *Bph3* into susceptible varieties [23].

The SSR marker ‘RM3331’was used to detect the polymorphism between parental lines for the presence of the *Bph18* gene and produced five alleles with the band size of the screened varieties

ranging from 110bp to 150bp (Table 2). The IRRI introgression line 'IR65482-7-216-1-2' derived from wild species *Oryzaaustraliensis* showed a band size of 110bp and the susceptible parent 'Jyothi' produced a band of 130bp. The *Bph18* was mapped [4] on the sub-terminal region of the long arm of chromosome 12. A novel type of coiled-coil nucleotide-binding site and leucine-rich repeat motif (CC-NBS-NBS-LRR) in the introgression line 'IR65482-7-216-1-2' confers the resistance against BPH [24]. Being an indica donor line, it can be efficiently used to transfer the *Bph18* to other indica cultivars with reduced linkage drag.

The IRRI introgression line 'IR7103-121-15-B' derived from an interspecific cross between IR31917-45-3-2 and a wild species *Oryzaminuta*, mapped with two genes *Bph20* and *Bph21* imparts resistance to BPH biotypes [25]. The 'RM8213' marker was employed in screening the rice varieties for the gene *Bph20* producing four alleles and the band size ranged from 110-140bp (Table 2). A band size of 130bp and 140bp was produced by the susceptible parent 'Jyothi' produced and the introgression line 'IR7103-121-15-B' respectively. The *Bph21* gene was screened using the marker 'RM28561' which produced only two alleles among the varieties screened. The band size obtained was 290bp in PTB33 and 280bp in Jyothi, PTB33, IRRI introgression lines IR65482-7-216-1-2, IR7103-121-15-B, and a breeding line RP2068-13-5-8 (Table 2). Monomorphic bands were obtained in the susceptible varietyJyothi and the resistant variety IR7103-121-15-B using the marker 'RM28561'.

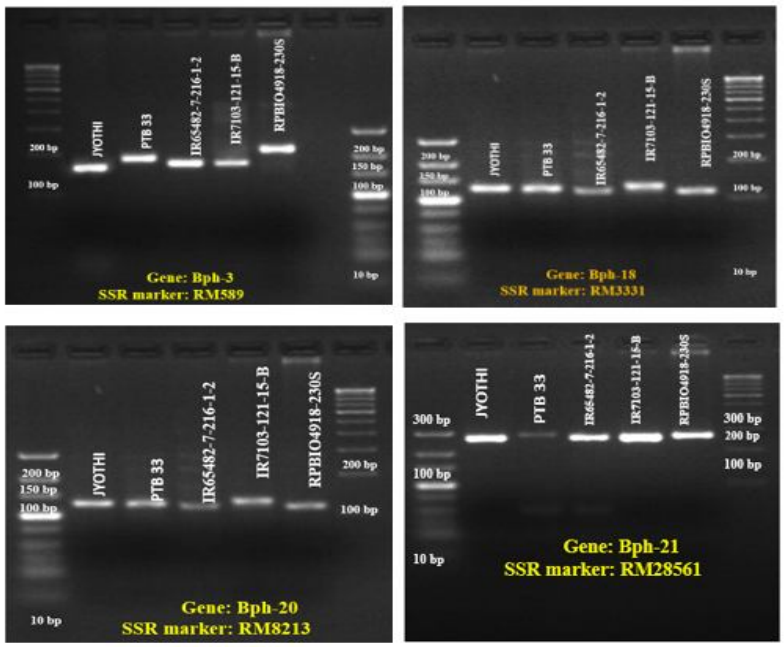

Fig 1. The banding pattern of SSR makers in rice varieties

Table 2. Details of marker amplification in rice varieties

Marker	Genotypes	bp	Number of alleles
RM589	Jyothi	180	5
	PTB33	200	
	IR65482-7-216-1-2	195	
	IR7103-121-15-B	190	
	RP2068-13-5-8	220	
RM3331	Jyothi	130	5
	PTB33	125	
	IR65482-7-216-1-2	110	
	IR7103-121-15-B	150	
	RP2068-13-5-8	120	

RM8213	Jyothi	130	4
	PTB33	120	
	IR65482-7-216-1-2	110	
	IR7103-121-15-B	140	
	RP2068-13-5-8	110	
RM28561	Jyothi	280	2
	PTB33	290	
	IR65482-7-216-1-2	280	
	IR7103-121-15-B	280	
	RP2068-13-5-8	280	

The similarity coefficient as shown in the dendrogram varied from 0.53 to 0.68 (Fig. 2). Dendrogram reflects the similarity among the genotypes using seriation methods with most similar objects placed together [27]. All the genotypes used for the study were grouped into 4 clusters at 0.64 similarity coefficient. Group 1 consists of the genotypes Jyothi and RP 2068-13-5-8 and groups 2, 3, and 4 consist of single genotypes namely IR 65482-7-216-1, IR 7103-121-15-B, and PTB33 respectively. Clustering analysis reveals the similarity between the genotypes evaluated for a number of traits [28]. Each genotype showed dissimilarity with the variety Jyothi in terms of BPH resistance and carrying the resistant gene. The genotypes, IR 7103-121-15-B and IR 65482-7-216-1 were grouped into separate clusters at 0.56 and 0.63 similarity coefficients respectively. The rice variety 'PTB33' which is resistant to almost all biotypes of BPH was placed in a separate cluster from 0.53 similarity coefficient onwards. This revealed the distinct nature of the variety 'PTB33' in imparting resistance to BPH which can be screened using gene-specific markers making it suitable to be used as a potential donor.Cluster 1 consists of two genotypes, Jyothi(susceptible variety) and RP2068-13-5-8 (breeding line with BPH resistance) may be inferred as there exists some extent of genetic similarity between the two genotypes but the latter exhibited significant resistance in BPH screening tests [29] suggesting that it is phenotypically different. Varieties that show genotypic similarity may differ phenotypically depending on environmental and geographical conditions as reported [30] [31].

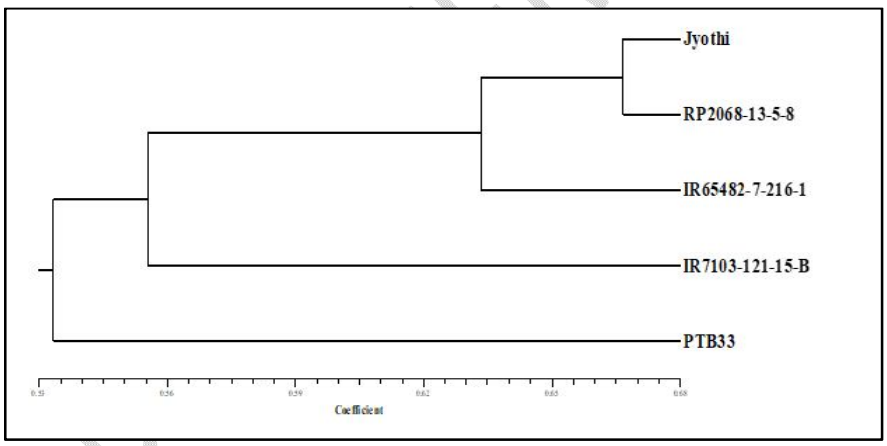

Fig.2 Cluster diagram using the UPGMA method showing the relationship among the rice varieties

4. CONCLUSION

India is home to the majority of BPH-resistantsources, especially landraces. Genetic studies in rice varieties had revealed valuable sources of BPH resistance. With the current improvements in plant molecular biology, it is now easier to identify the individuals with the gene of interest using molecular markers and incorporate it into the susceptible cultivars through marker-assisted backcrossing. It is important to detect parental polymorphism prior to the initiation of any breeding programme. Among the molecular markers, simple sequence repeats (SSRs) are widely employed in detecting polymorphism among parental lines for genes specific to BPH resistance. In this study, SSR markers were used for screening rice parental genotypes for validating the presence of genes in genotypes considered donor parents and the absence of genes in the susceptible parent 'Jyothi'. The varieties

PTB33, IRRI introgression lines IR65482-7-216-1-2, IR7103-121-15-B showed polymorphism for the markers specific to the genes *Bph3*, *BPH18*, and *Bph20*. These genotypes may be effectively used in marker-assisted backcrossing as donor parents to transfer the genes for resistance to the high-yielding but susceptible variety 'Jyothi'.

Comment [401C175B2]: In conclusion: 1. The varieties are NOT resistant or can't see resistant genes with these markers. 2. Jyothi are high-yielding but susceptible like the other studied varieties (at least with these markers)

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