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Identification of True Rice Hybrids Carrying the Brown Planthopper Resistance Gene through Foreground Marker Analysis

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ABSTRACT: The brown planthopper is the most widespread and damaging pests of rice, causing yield losses of over 50 percent. This study targeted on the introgression of the brown planthopper resistant gene into a high-yielding variety using marker-assisted backcross breeding. In this context, the investigation aimed to identify F_1 individuals possessing the BPH resistant gene *Bph33(t)* through linked markers. The F_1 progeny was obtained by crossing two rice varieties BPT5204 (well known for slender grain and quality traits) and RP2068-18-3-5 (a BPH-resistant donor) having *Bph33(t) gene*. Among the three SSR markers validated, only heat shock protein gene marker exhibited polymorphism between the parents and was used further for foreground selection. Among the 18 tested F_1 progenies, 15 were found to carry the *Bph33(t)* gene. The identified F_1 progenies will be utilized in marker assisted backcross breeding programs aimed at enhancing BPH resistance in rice.

Keywords: Rice, hybrids, brown plant hopper, resistance, foreground selection, markers.

INTRODUCTION

Rice, belongs to the Poaceae family, which also includes major cereals like wheat and maize. In Asia and Africa, rice contributes between 50% to 80% of daily caloric intake, making it an essential source of energy. Nutritionally, rice provides complex carbohydrates that fuel the body and is a significant source of protein in regions where animal protein consumption is lower

Global rice production for 2023-24 is projected at approximately 520.4 million metric tons (USDA, 2024). In 2023-24, India produced approximately 135.5 million metric tons of rice, maintaining its position as the second-largest producer globally. The total rice cultivation area in India was around 47.6 million hectares, with an average productivity ranging from 2.9 to 3 tons per hectare (USDA, 2024). Andhra Pradesh alone produced about 7.79 million tons of rice in 2023-24, with a cultivation area of 2.1 to 2.2 million hectares and an average yield of 3.7 to 3.8 tons per hectare (USDA, 2024).

It is a crucial staple food for over 3.5 billion people worldwide. To satisfy the needs of a increasing population, rice production must double by 2030.However, rice cultivation is frequently hindered by diseases and insect pests, leading to significant yield losses across many rice-growing regions. BPH is the important destructive pests of rice, resulting in significant annual losses across all rice-producing countries (Kumar *et al*., 2018). Severe infestations can result in "hopperburn," where plants dry out completely and die. Conventional methods to control BPH using chemical insecticides are costly, pose serious environmental risks, including pollution and disruption of natural predators like mirid bugs (e.g., *Cyrtorhinus lividipennis* and *Tytthus parviceps*), which help regulate BPH populations (Lakshmi *et al*., 2010).

Bosque-Perez and Buddenhagen (1992) suggested combining resistance breeding with control measures can reduce the ecological fitness of BPH. Development of resistant varieties through host plant resistance (HPR) and using developed varieties is cost-effective and powerful methods to provide long-term and broad-spectrum protection against virulent BPH populations (Khush, 2001). However, most of the BPH-resistant genes do not offer broad-spectrum resistance to different BPH biotypes.

Earlier from 1960s, rice researchers have identified approximately 41 BPH resistance genes and over 70 quantitative trait loci (QTLs) distributed across 12 rice chromosomes (Akanksha *et al*., 2019; Balachiranjeevi

et al., 2019; Muduli *et al*., 2021; Tan *et al*., 2022). However, only a few genes, such as *Bph17, Bph3/32, Bph31*, and *Bph33(t),* have demonstrated wide-spectrum tolerance to the Indian biotype 4 (Liu *et al*., 2015; Ren *et al*., 2016; Prahalada *et al*., 2017; Naik *et al*., 2018)

Marker-assisted selection (MAS) has emerged as a pivotal tool in contemporary plant breeding, enabling the identification of desirable traits, such as insect resistance, in individuals or populations. After the crossing procedure, confirming the hybridity of F_1 plants is a crucial step in breeding, as it ensures the desired genetic purity and distinguishes between parents and progeny. Conventional methods for testing hybridity are often time-consuming and may fail to identify true genotypes (Sundaram *et al*., 2008) because of environmental factors on morphological traits. In contrast, Marker-Assisted Selection (MAS) plays a vital role in modern breeding by providing accurate results in a shorter time frame, without environmental interference (Pallavi *et al*., 2011). This investigation focuses on testing the hybridity of F_1s using MAS, which is further applied in a backcrossing method to develop an improved brown planthopper resistant rice variety.

MATERIALS AND METHODS

Plant Material: Rice varieties, BPT 5204 and RP 2068-18-3-5, were chosen to develop F_1 progeny. BPT 5204 is renowned for its medium slender type of grain and excellent quality attributes, while RP 2068-18-3-5, developed at the Indian Institute of Rice Research, Rajendranagar, Hyderabad from the cross Swarnadhan (an elite variety) × Velluthacheera (a landrace from Tamil Nadu) serves as a donor parent for the *Bph33(t)* gene. The *Bph33(t)* gene was identified on first chromosome linked with different markers (Naik *et al*., 2018).

The parental lines, BPT5204 and RP2068-18-3-5, were planted during the *rabi* season of 2021-22 with a spacing of 25 × 10 cm. Leaf samples from 30-day-old seedlings were collected to for isolation of DNA and to validate the markers in the parental lines. Three SSR markers reported by Naik *et al*. (2018) namely RM488, RM11522, and the heat shock protein gene (LOC_Os01g42190.1) were used for validation in the parents. RM488 and RM11522 served as flanking markers for *Bph33(t)* QTL, while the *hsp* (heat shock protein) gene was recognized as a candidate for BPH resistance. The details of the markers were presented in Table 1.

Upon confirmation, the F_1 progeny was generated through the crossing of these two parent lines. For hybridization, emasculation of anthers from the female parent (BPT5204) was carried out. Panicles with more number of spikelets (on the 3rd or 4th day of anthesis) were chosen for emasculation in the afternoon or evening, one day before pollination. The top and bottom one-third spikelets of the selected panicle were removed, leaving the middle spikelets intact. The top one-third of each spikelet was clipped at an angle, and the six anthers inside were extracted using forceps, ensuring the stigma remained undamaged. To avoid contamination, emasculated spikelets were enclosed with a glassine paper bag and labeled. The next morning, mature anthers from the donor parent (RP2068-18-3-5) were collected and dusted onto the emasculated spikelets stigmas of female parent. After pollination, the panicles were covered again for 5-7 days until the stigma became non-receptive and F_1 seeds were collected 25-30 days post-pollination. The F_1 seeds along with parents (BPT 5204 and RP 2068-18-3-5) were then sown during *kharif,* 2022, following a spacing of 25 × 10 cm for confirmation of true hybridity. Samples of leaf were collected from 30 days old seedlings for the isolation of DNA.

DNA isolation and PCR amplification: Isolation of Genomic DNA was done from the seedlings of the parental lines (BPT5204 and RP 2068-18-3-5) and 18 F1 plants using the modified CTAB method described by Doyle and Doyle (1990). The extracted DNA was quantified, and parental polymorphism studies were conducted using linked primers.

The PCR reaction was commenced by pipetting 2 µl of 100 ng DNA template into PCR tubes and then placed in the PCR plate at 4°C. Preparation of master mix was carried out, containing 1 µl each of 10 pmol forward and reverse markers, 0.5 µl of 2.5 mM deoxyribonucleotides (dNTPs), 2 µl of 10X Hi-buffer with 17.5 mM MgCl2, 0.5 µl of 50 mM $MgCl_2$, and 0.1 µl (0.5 U) of 5U/µl Taq DNA polymerase (HIMEDIA), with 3.9 µl of molecular-grade water was added to achieve a total volume of 10 µl. The master mix was briefly centrifuged for approximately 10 seconds to ensure thorough mixing. The amount of master mix prepared depended on the number of PCR samples. Subsequently, 8 µl of master mix was added to PCR tube containing 2 µl of template DNA, bringing the final volume to 10 µl. The PCR plate was covered, and PCR amplification was carried out using the Prima-96TM-HiMedia-Thermocycler, following the temperature profiles outlined in (Table 1). The resulting PCR amplicons underwent electrophoresis on a 4% agarose gel stained with ethidium bromide. (10 mg/ml) using the Labnet Enduro 20.20TM Large Horizontal Gel System, running at 100 V for 1 hour and then at 120 V for 30 minutes in 1X TAE buffer.

RESULTS AND DISCUSSION

The validation of three molecular markers reported by Naik *et al*. (2018) was conducted during the *rabi* season of 2021-22 across both the parent lines BPT 5204 and RP 2068-18-3-5. The details of the markers were presented in Table 2. Among the SSR markers tested *viz.,* RM 488, RM 11522 and heat shock protein gene (LOC_Os01g42190.1) only heat shock protein gene exhibited polymorphism and was selected for use as a marker for foreground selection in subsequent studies. In the parents, the heat shock protein gene (LOC_Os01g42190.1) produced allelic bands of 200 bp in BPT5204 (the recurrent parent) and 190 bp in RP2068-18-3 (the donor parent). Similar results were reported by Naik *et al*. (2018), where the heat shock protein gene produced a 190 bp allelic band in a

population with RP2068 as one of the parents (Fig. 1). After validation, the parents were crossed during *rabi,* 2021-22 and subsequently, 18 F_1 plants were generated. F_1 plants were planted during the kharif season of 2022 and evaluated for the presence of the *Bph33(t)* gene. (BPH), using the heat shock protein gene marker (LOC_Os01g42190.1) for foreground selection. The marker produced allelic bands of size 200 bp and 190 bp (Fig 2.) in 15 out of the 18 F_1 plants, indicating the presence of the *Bph33(t)* gene in a heterozygous condition. Therefore, 15 of the 18 F_1 plants tested positive for *Bph33(t).* Similar results, where the validated marker successfully distinguishing the donorparent allele from the recipient parents allele in the hybrids were reported by many researchers. Similar findings were reported by Anil Kumar (2012) during the validation of four markers in the parents BPT5204 and B0162 for the *Bph18* gene, where only one marker exhibited polymorphism. Likewise, Mohanapriya *et al.* (2019) found that among 17 validated markers for the *Bph20* gene in the parents CO43Sub1 and IR71033-121-15-B, only one was polymorphic. Similarly, Thulasinathan *et al.* (2020) reported that while validating 13 SSR-linked markers for the *Bph20* resistance gene in the parents, only one showed polymorphism between them.

Table 1: Thermal profile for PCR.

Sr. No.	Steps	Temperature in ºC for one cycle	Time for one cycle	No. of cycles
1.	Initial denaturation	94ºC	5 min	1
2.	Denaturation	94ºC	45sec	35
3.	Annealing	56ºC	45sec	
4.	Extension	72ºC	1 min.	
5.	Final Extension	72ºC	7 min.	1

Table 2: Details of the markers validated in the parents.

QTL	Chromosome	Position (bp)	Primer	Forward	Reverse	Reported by
Bph33 (t)	1	190	RM488	CAGCTAGGGTTTTGAGGCTG	TAGCAACAACCAGCGTATGC	Naik *et al*., 2018
		390	RM 11522	TAACTGCAGTGCTCAACAAAGG	CTAGGTACCGGATTAAGATTCACC	
		190	Heat shock protein gene (LOC_Os01g42190.1)	GCAACATCATCACCTGCATA	ATCCCATCGAAACAAGACAA	

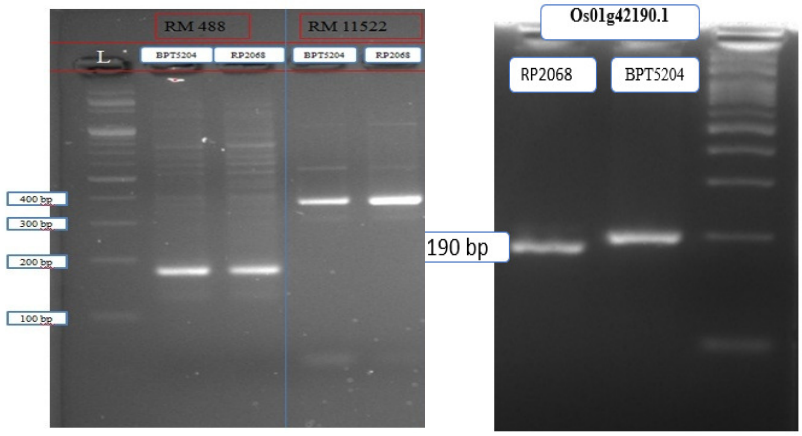

Fig. 1. Validation of linked markers in the parents.

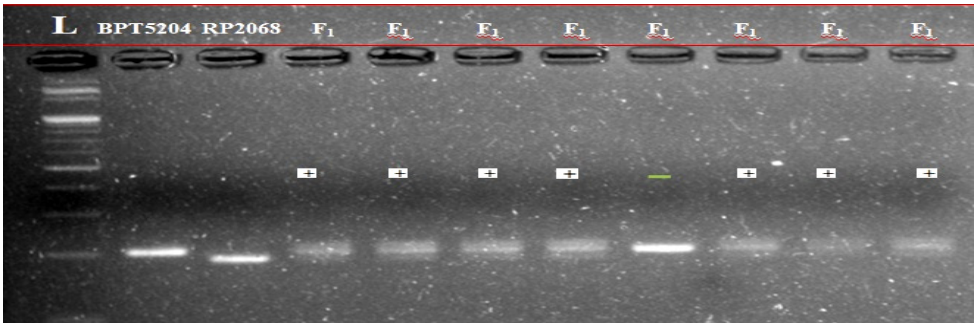

Fig. 2. Testing of hybridity in F_1s of BPT5204 × RP 2068-18-3-5 using foreground marker Os01g42190.1. '+' indicated the plants which were heterozygous and true hybrids, whereas '-' indicated the plant was false hybrid.

CONCLUSIONS

The study concludes that the heat shock protein gene(LOC_Os01g42190.1) effectively distinguished true F_1 plants from non-hybrids. SSRs are highly reliable codominant markers, ideal for identifying true hybrids. The identified polymorphic marker can be utilized as a foreground marker for identifying plants with *Bph33(t)* gene in Marker-Assisted Selection (MAS) programs. F_1 plants carrying the BPH resistance gene will be used in a Marker-Assisted Backcross Breeding (MABB) program to introgress the *Bph33(t)* gene from the donor parent, RP 2068 or selfed to produce an F_2 generation. MAS accelerate and enhances the efficiency of gene introgression compared to conventional breeding methods.

FUTURE SCOPE

The potential for F_1 plants derived from BPH-positive rice hybrids, especially those containing resistance genes like *Bph33(t),* is highly promising. These F_1 plants can be utilized to produce F_2 populations, which may further enhance the expression of BPH resistance traits through segregation. This process allows breeders to identify and select individuals exhibiting higher resistance levels or other desirable characteristics. Utilizing SSR markers for foreground selection can streamline the identification and selection of BPH-resistant plants in subsequent generations, thereby accelerating the development of stable lines with desired traits. Additionally, F_1 plants can be bred to simultaneously improve multiple traits, including nutritional quality, stress resistance (such as drought and salinity), and disease resistance, resulting in more robust rice varieties capable of addressing various agricultural challenges. Ultimately, the future of BPH-positive F_1 rice plants lies in their potential to enhance rice production systems through improved pest resistance, increased yields, and adaptability to evolving agricultural and environmental conditions. This progress is essential for food security and promoting sustainable farming practices in regions affected by BPH challenges.

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