Journal of Entomology and Zoology Studies

Available online at www.entomoljournal.com

E-ISSN: 2320-7078
P-ISSN: 2349-6800
www.entomoljournal.com
JEZS 2020; 8(3): 2048-2051

Received: 21-03-2020
Accepted: 23-04-2020

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Molecular screening of *Nilaparvata lugens* (Brown plant hopper) resistance genes in Hasanta rice variety using SSR markers

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Abstract
The menace of Brown Plant Hopper has been a problem mainly in the rice growing regions of India. Hasanta variety, reported to be resistant to *Nilaparvata lugen*, at the phenotypic level in the field has been chosen in this study to confirm the presence of resistance genes against BPH in it. This paper focuses on the molecular validation part of the experiment. Six flanking SSR markers were selected for one major resistant gene (*BPH31*) and three QTLs (*qBPH4.3*, *qBPH4.4* and *qBPH9*). The outcomes were compared with a resistant check; CRCPT 2 and a susceptible check; TN1. The banding pattern of gel electrophoresis studies for the genomic DNA has confirmed the presence of one major resistance gene *BPH31* and minor QTLs, *qBPH4.3*, *qBPH4.4 and BPH9*. It was validated that the resistance displayed by Hasanta variety is due to the presence of these genes and further expression studies can be carried out to study their functional proteins.

Keywords: Hasanta, BPH31, *Nilaparvata lugens*, qBPH4.3, qBPH4.4, qBPH9

Introduction
Rice (*Oryza sativa* L.) is one of the oldest domesticated food crops in the world and provides two-third of calorie intake of more than 3 billion people in Asia and one-third of calorie intake of nearly 1.5 billion people in Africa and Latin America [1]. In India, the area under rice cultivation is 43.79 million hectares with an annual production of 112.91 million metric tonnes and an average yield of 3.87 metric tonnes per hectare (USDA, 207-18). It is cultivated globally under various agro-climatic conditions and hence faces major abiotic and biotic stresses due to adverse climatic changes, susceptible varieties and breakdown of resistance genes in elite cultivars.

About 52% of the global production of rice is lost annually by biotic factors, out of which 21% damage is due to insect pests [2]. As the crop is genetically very diverse it is attacked by more than 100 number of pests, 20 are considered as major ones [3]. Particulary, *Nilaparvata lugens* Stål. (Brown plant hopper), a typical phloem sap feeder, an erstwhile minor pest, has grown to be a major pest widely distributed in South, South East and East Asia, the South Pacific Island and Australia. It causes yield loss up to 60%. Both nymphs and adults suck sap from the leaves and leaf sheaths, which results in yellowing of leaves, reduced tillering, reduced plant height and increase in number of unfilled grains. Brown Plant Hopper (BPH) also causes the reduction in chlorophyll, protein content of leaves and photosynthetic rate, whereas severe attack of BPH causes 'hopper burn' symptoms [4-6]. It also transmits virus diseases like grassy stunt, ragged stunt and wilted stunt [7]. The losses to rice production caused by BPH in Asia have been estimated as more than US$300 million annually [8]. The control of this pest has predominantly relied on chemicals, over which it has already acquired resistance. Field population collected in 2016 in China reflected extremely high resistance to imidacloprid, thiamethoxam and buprofezin [9]. Four different biotypes of the insect have been identified in this regard that has developed virulence e.g., IR26 possesing *BhpI* resistant gene became susceptible in 1976-77 due to biotype 2. The resistance conferred by *bph2* gene was considered durable as BPH resistance of IR36 lasted for 14 years until 1991 [10]. Several major genes and QTLs conferring resistance to BPH have been located on various chromosomes of rice [11, 12].

Varieties with various levels of resistance are deployed for insect control in combination with other components of pest management.

It also helps in conservation of natural enemies and minimizing the number of pesticide applications. So availability of BPH resistant rice varieties with desired agronomic traits should be ensured to the farmers for reducing the BPH menace in rice. One such promising variety, Hasanta (OR2328-5), released from OUAT, Odisha, India, has shown potential against BPH attack. During *kharif* 2017, there was a large scale BPH infestation in Odisha and caused major losses. Under field condition, while all the other varieties were completely damaged, Hasanta withstood the infestation with minimal crop loss. Observing the field level tolerance of Hasanta to BPH, it was thought imperative to take up the validation and confirmation studies about the presence of BPH resistance genes at the molecular level.

Materials and Methods

An experiment was carried out in OUAT, Bhubaneswar to study and confirm the presence of BPH resistance genes. Three varieties *viz.*, TN1 (15120 days duration) as susceptible check, CRCPT 2 (135-140 days duration) as resistant check and Hasanta (143145 days duration) as test variety were taken. All the varieties were collected from National Rice Research Institute, Cuttack, Odisha and OUAT Research Farm for various experiments.

Genomic DNA extraction and quantification

The three rice genotypes were collected and germinated in tray under controlled condition of RGA-cum-Phytotron facility. Big trays were made ready for putting sterilized soil mixture and placing seeds for germination. Seeds were placed in rows for germination. The seedlings were harvested (15 days) for Deoxyribonucleic Acid (DNA) extraction. Healthy leaf sample of 2-4gms was collected from 15days old plant in microfuge tubes and stored in ice. The leaf tissue were cut into small pieces and crushed by liquid nitrogen to make fine powder form. 300µl CTAB buffer was added to the sample and tissue was homogenized. The sample was transferred to 2ml tubes and volume as adjusted to 600µl using CTAB buffer. It was then kept in water bath at 65 °C for 1 hour. To this 600µl of Chloroform : Iso amyl alcohol (24:1) was added mixed properly and then centrifuged at 1700 °C for 1hr. The top aqeous phase was then transferred to 2ml microfuge tube. 1ml of pre-chilled iso propanol was added and kept for 1hr in -20 °C and centrifuged at 4 °C at 10000rpm for 10mins. Then the supernatant was decanted. The pellet was washed with 70% of ethanol and air dried. The DNA was resuspended in 100µl of 1X TE buffer and stored in -20 °C. DNA was checked for its purity and intactness and then quantified. The crude genomic DNA was run on 0.8% agarose gel stained with ethidium bromide following a standard method [13] and was visualized in a gel documentation system.

Validation of Hasanta variety through SSR markers

DNA templates from all the three varieties were amplified using a set of 6 flanking Simple Sequence Repeat (SSR) markers mentioned in Table 1. The PCR cycle regime was carried out with following specifications for 35 cycles: Denaturation at 94 °C for 1 minute, Annealing at 55 °C for 1 minute, extension at 72 °C for 1 minute and Final extension at 72 °C for 10 minutes. For quantification of genomic DNA 2µl sample along with 2µl of loading dye was run in 0.8% of agarose gel, whereas aliquots of 10µl of DNA products from PCR amplification were loaded in 2.5-3% agarose gel containing 0.8µg/ml Ethidium Bromide for electrophoresis in 1X TBE (pH 8.0). At least one lane was loaded with 50bp DNA ladder. The gel was run at 25V/cm for 4hrs and photographed using a Gel Documentation System (Syngene).

Results and Discussion

The molecular validation was taken up to confirm the presence of resistant genes in the variety. One resistant gene (*BPH31*) and three QTLs (*qBPH4.3*, *qBPH4.4 and qBPH9*) conferring resistance to the Indian biotypes of the insect are targeted in this validation work. For *qBPH4.3* resistance gene, SSR markers RM551 and RM335 were deployed to know the presence of the gene in the variety. DNA banding pattern obtained using these two markers are shown in Fig 1 and Fig 2. The banding analysis using these two primers revealed the expected resistant band in Hasanta variety and the resistant check. Thus the variety is positive for *qBPH4.3*. The analysis for *qBPH4.4* and *qBPH9* also showed the presence expected resistance band size by amplifying the markers RM5633 and RM242, respectively. Hence, the variety also shows the presence of *qBPH4.4 qBPH9* also. Genotyping for major gene *BPH31* was performed using markers RM251 and RM2334. Banding results confirmed the presence of *BPH31*. Thus the variety Hasanta was found to contain all the three genes/QTLs tested for their presence.

Conclusion

Genotyping results revealed the presence of one major resistance gene *BPH31* and minor QTLs, *qBPH4.3*, *qBPH4.4 and qBPH9*. Apart from the genes (that has been used in this study) it is also possible that many other gene locus for BPH resistance might be present in the candidate variety. Earlier mapping results of [14] at ICAR-NRRI is validated in this experiment for presence of major resistance gene *BPH31*. Also two QTLs (*qBPH4.3* and *qBPH4.4*) reported by [15], also *qBPH9* are validated in this experiment to be present in Hasanta. Therefore with a major gene and QTLs in its background Hasanta variety can be expected to tolerate well the biotic stress posed by BPH with minimal economical damage to the crop. Further the crop variety can be subjected to expression studies using the mRNA and protein product to confirm the expression of the already present genes.

Table 1: List of microsatellite or SSR markers used in the study

Marker	Chromosome And QTL	Expected Product size (bp)	Forward Primer	Reverse Primer	Repeat motif
RM251	3, Bph31	147	GAATGGCAATGGCGCTAG	ATGCGGTTCAAGATTCGATC	(CT)29
RM2334	3, Bph31	155	CATGCSTCTGATCTGATTAT	TGTGAAGAGTACAAGTAGGG	(AT)25
RM551	4, Bph4.3	215	AGCCAGACTAGCATGATTG	GAAGGCGAGAAGGATCACAG	(AG)18
RM335	4, Bph4.3	115	GTACACACCCACATCGAGAAG	GCTCTATGCGAGTATCCATGG	(CTT)25
RM5633	4, Bph4.4	250	GTGTAGCTGCTAGGCCGAAC	TTCCTTTCGCTACGTTGGAC	(AAT)9
RM242	9, qBph9	225	GGCCAACGTGTGATGTCTC	TATATGCCAAGACGGATGGG	(CT)26

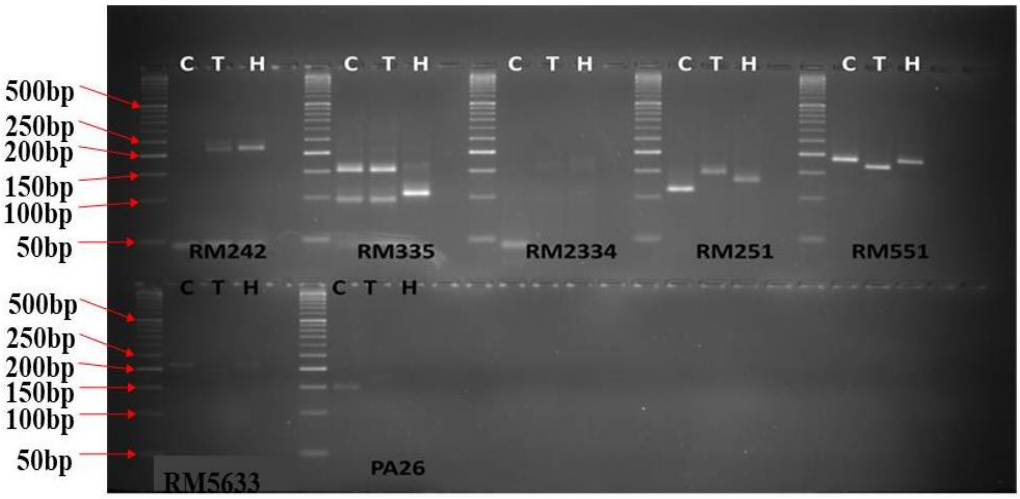

Fig 1: Electrophoregram in 3 rice genotypes using SSR market RM242, RM335, RM2334, RM251, RM551 and RM5663. In each Lane1: Molecular ladder (50bp); Lane2: Resistance check; Lane3:TN1 & Lane4: Hasanta

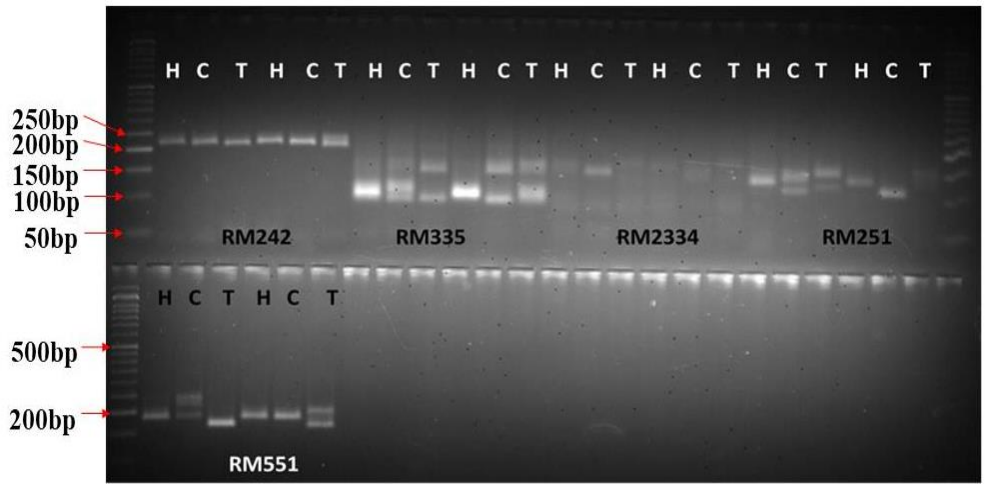

Fig 2: Electrophoregram in 3 rice genotypes using SSR market RM242, RM335, RM2334, RM251 & RM551. In each Lane1: Molecular ladder (50bp); Lane2: Hasanta; Lane3: Resistant check & Lane4:TN1

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