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VALIDATION OF REPORTED CANDIDATE GENES IN PLANTHOPPER RESISTANCE IN RICE LINE RP2068

Agricultural Science

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ABSTRACT

Planthoppers, such as brown planthopper (BPH) and white backed planthopper (WBPH) are the most important destructive pests of rice. Even though 40 resistance genes for BPH and 19 genes for WBPH have been identified from different rice genotypes and its wild relatives, most of these genes are ineffective in India. In our study a breeding line RP2068 was identified as resistant to both the planthopper. Based on the molecular allelism test with reported linked markers and RT-PCR validation for reported candidate genes suggested that the gene present in RP2068 are new genes/QTLs. No gene has been mapped yet on chromosome# 1 against the WBPH, RP2068 may have new gene(s) conferring resistance.

KEYWORDS

SSR markers, insilico, BSA, BPH, WBPH

INTRODUCTION

Rice (*Oryza sativa* L) is one of the most important staple food for two thirds of the populations. About 52% of the total global rice production is lost annually by biotic factors, of which nearly 21% is attributed to the attack of insect pests (Sogawa *et al.,* 2003). Brown planthopper (BPH) [*Nilaparvata lugens* (Stal)] and white backed planthopper (WBPH) [*Sogatella furcifera* (Horváth)] are most important among the insect pests as these not only inflict losses by direct sap sucking from rice hosts but also as vectors of diseases causing plant viruses (Zhou *et al.,* 2008). Till now, farmers controlled these pests by heavy spray of harmful insecticides, but breeding resistant rice varieties is an ecologically acceptable way to manage these pests.

Forty major genes and 72 QTLs and 19 major genes and 75 QTLs have been identified against BPH and WBPH from cultivated and wild species of rice (Du *et al.,* 2020). Several rice varieties possessing BPH resistance with single or combination of genes have been developed and released for cultivation since 1980s. However, resistance in several of these varieties has been overcome in less than three years by evolution of virulent insect populations. Moreover, many of these BPH resistant rice varieties were susceptible to WBPH.

Hence, it is important to identify resistance sources against both the planthoppers. And thus, the ideal goal of developing rice varieties with durable resistance against both BPH and WBPH is still eluding us. Some rice land races like Sinna sivappu, ADR52 and BM71 etc. have been reported to be resistant to both BPH WBPH in India. But it is unclear whether a single gene/QTL in these lines confers resistance to both the planthoppers. Generally, gene(s) governing resistance against WBPH is (are) different from those governing brown planthopper resistance (Ling and Weillin, 2016).

The resistance mechanism(s) in the rice line RP2068 a derivative from the cross Swarnadhan x Veluthchera, and reported as a new source of resistance against gall midge, BPH WBPH (Sama *et al.,* 2014). A new gene *Bph33* have been identified in RP2068 on chromosome 1 (Naik *et al.,* 2018). The present study aims to identify that, the genes involved in resistance against BPH are same for WBPH or different.

MATERIALS AND METHODS:

Insects And Plant Material

Colonies of both BPH and WBPH were maintained separately on TN1 variety in greenhouse at ABF, Hyderabad. Initially both BPH and WBPH were collected from field in Nalgonda district of Telangana state, India, during 2014-2015 and regularly insects were added to the colonies to maintain virulence. Care was taken to prevent population mixture. Nymphs or adults, arising from these populations were used for the experiments.

A mapping population developed from a cross between TN1 (susceptible parent) X RP2068 (resistant parent) was used in the current study (Sama *et al.,* 2014). All the 180 F_{14} RILs were subjected to standard seedbox screening test (SSST).

Standard Seedbox Screening Test (SSST)

In standard seedbox screening test (SSST), the RIL lines were infested with 2ndinstar BPH/WBPH nymphs, on an average of 8-10 nymphs per seedling at 10-12 days after sowing (Naik *et al.,* 2018). The test lines were arranged in a randomized complete block design (RCBD) and replicated three times. Susceptible TN1 was sown in two rows at the edge of box on both the sides while the resistant check (PTB33 for BPH and MO1 for WBPH) was sown in one row in the middle. These plants were observed for damage and scored as per the standard evaluation system (SES) for rice (IRRI, 2013) on a scale 0 to 9 when 90% of TN1 on both the rows were dead in about 8 to 12 days after insect release. Each test entry was scored by recording damage to each seedling; subsequently, the score for each replication was averaged and then the grand mean of the three replications was derived for the entry. Entries with damage score 1 to 3 against BPH and with score 1 to 4 against WBPH for rice were considered as resistant (as per SES) while those with score $\geq$ 8.0 were treated as susceptible.

Genotyping

A set of 137 SSR markers were found to be polymorphic between the parents TN1 and RP2068 (Sama *et al.,* 2014; Naik *et al.,* 2018; Sahu *et al.,* Unpublished). The entire mapping population was genotyped with these 137 polymorphic SSR markers. Genotypic data were used for *in silico* bulked segregation analysis (BSA) (Naik *et al.,* 2018). RILs were filtered into R pool and S pool based on their phenotypic value within the range displayed by RP2068 and TN1 for each phenotype test. Marker tally was computed with total of RILs under R pool with R allele and of RILs under S pool with S allele and expressed as percentage of the total. Recombination between the marker and trait (%- tally) indicated linkage strength of the marker with the trait.

Genotyping For Reported Markers

Two reported gene linked markers to two BPH resistance genes Viz., Bph3/Bph17 and bph21 were used for parental polymorphism study between TN1 and RP2068 and also in the mapping population. Genomic DNA was isolated from TN1 and RP2068. Polymerase chain reaction (PCR) conditions were described as follows: 10 ul of PCR reactions contained, 10X PCR buffer 1 ul, (2.5 mM) dNTPs 0.5 ul, (5 pmole) primers forward and reverse 0.5 ul, 1 unit Taq polymerase 0.5 ul, (50 ng) genomic DNA template 2 ul and sterile distilled water 4 ul. The PCR was performed with a profile of 94°C for 5 min, followed by 30 cycles at 94°C for 30 s, at 55°C for 30 s, at 72°C for 1 min, and finally for 7 min at 72°C for the final extension.

Experiment for qRT-PCR

For expression studies using qRT-PCR, four RILs (TR3RR, TR139RS, TR60SR and TR7SS) were included based on the damage score against both the planthoppers. Seedlings were raised in 3L plastic bucket pots and 15 days after sowing they were infested with 1st-2nd instar nymphs of BPH or WBPH (5 nymphs for BPH and 10 nymphs

for WBPH/plant). Leaf sheath samples were collected from 6 replications of control and infested plants at one time points: 6 hours after infestation. RNA isolation and cDNA synthesis were carried out following manufacture protocols (Biorad, USA).

Quantitative Real Time PCR Real time

Real time PCR was performed using CFX96 Real Time PCR System with the SYBR green chemistry (Bio-Rad, USA) according to the manufacturer's instructions. Rice ubiquitin gene, OsUbq (GenBank accession no. AK059694), was used as the endogenous control. Real Time PCR reaction volume of 10 µl contained 5 µl SYBR Green PCR Master Mix (Bio-Rad, USA), 500 nM each of forward and reverse primers and 30 ng of the cDNA samples. To calculate mean relative expression levels, cDNAs from three independent biological samples in two technical replications each were used. PCR was initiated with denaturation at 95 °C for 5 min followed by 40 cycles of denaturation at 95 °C for 10s and annealing and extension at 60 °C for 30s. After 40 cycles, a melt curve analysis was carried out to determine the specificity of the reaction. After normalization, quantity of each mRNA was calculated from the threshold points (CT) located in the log-linear range. The data from different PCR runs or cDNA samples were compared by using the mean of CT values of the three biological replicates that was normalized to the mean of CT values of the endogenous gene. The relative standard curve method was used for the quantification of mRNA levels and displayed as Relative Expression Values (REV). Expression ratios were calculated using the 2–ΔΔCt method (Livak and Schmittgen, 2001). The data were analyzed using the Bio-Rad CFX Manager 3.1 Software (Bio-Rad, USA) with default baseline and threshold. Relative transcription levels are presented graphically.

RESULT

Phenotypic Screening

All the 180 RILs were subjected to SSST test against BPH and WBPH, separately. Under standard seedbox screening test (SSST) performance RILs were grouped into RR with resistance to both the planthoppers; RS with resistance to BPH only; SR with resistance to WBPH only and SS with no resistance against both the planthoppers. SSST results suggest that nine RILs as RR reaction; 16 were RS; 28 were SR and the remaining were SS. While resistance to BPH among RILs segregated in 25R:155S suggesting possible involvement of three genes (χ^2= 0.343; P=0.558), resistance to WBPH among RILs segregated in 37R:143S suggesting two gene involvement (χ^2=1.896; P=0.168). Four RIL lines; TR3RR, TR139RS, TR60SR, TR7SS were selected for the further studies (Table 1)**.**

Table 1 Damage Score And Rection Pattern For Parents And Four RIL Lines Against BPH And WBPH

S.No	Lines	BPH DS	Reaction against BPH	WBPH DS	Reaction against WBPH
1	TR3	1.00	R	3.31	R
2	TR139	3.33	R	8.76	S
3	TR60	9.00	S	3.95	R
4	TR7	9.00	S	9.00	S
5	TN1	8.86	S	8.80	S
6	RP2068	3.68	R	3.70	R
7	Ptb33	6.00	R	NT	R
8	MO1	NT		3.37	R

Note: NT-not tested

Bulk Segregant Analysis:

In-silico bulked segregant analysis (BSA) was performed in RILs lines with extreme phenotypic values as bulks suggested that at least four markers HSP, RM11342, RM488, RM11522 on chromosome 1, likely to be linked against both the planthoppers; three markers on chromosome 4 RM8213, RM7279 and RM16757 are likely to be linked against BPH and three markers on chromosome 10 RM216 and RM25176 against WBPH (Table 2).

Table 2 Details Of Reported Bph Genes Tested In TN1, RP2068 And RIL Lines.

S. No	Resistant Gene	Chromosome	SSR marker	Status	Status in RILs	Donar
1	*Bph33*	1	RM488	Polymorphic	Segregated	RP2068
2	*Bph33*	1	RM11522	Polymorphic	Segregated	RP2068
3	*Bph3/17*	4	RM8213	Polymorphic	Segregated	Rathu Heenathi
4	*Bph3/17*	4	LRK1	Monomorphic		Rathu Heenathi
5	*Bph3/17*	4	LRK2	Monomorphic		Rathu Heenathi
6	*Bph3/17*	4	LRK3	Polymorphic	No segregation	Rathu Heenathi
7	*Bph3/17*	4	LRK4	Monomorphic		Rathu Heenathi
8	*bph21(t)/ Bph30*	10	RM222	Monomorphic		RBPH54
9	*bph21(t)/ Bph30*	10	RM244	Monomorphic		RBPH54

Molecular Allelism

To know whether the resistance in RP2068 against BPH and WBPH is a new gene or already reported genes molecular allelism test was performed. We have also looked for the genes/QTLs reported in these regions for further confirmation. Three BPH genes (Bph33, *Bph3/Bph17* and *bph21(t)/Bph30)* on chromosomes 1,4 and 10 were detected in the same regions as detected through insilico BSA. Some of the available gene-linked SSR markers were tested for polymorphism between the parents (Table 3). In our earlier study we have already identified Bph33 in RP2068 against BPH on chromosome 1. In present study, these SSR markers showed linkage to WBPH resistance. Two SSR markers, RM8213 and RM5953 and four candidate gene-based markers LRK1, LRK2, LRK3 and LRK4 linked to Bph17 on chromosome 4 were tested between parents. Out of six markers tested, only two markers RM8213 and LRK3 showed polymorphism but only RM8213 showed segregation in R and S pool suggested that this is not a same gene. However, two markers RM222 and RM244 linked to *bph21(t)/Bph30* on chromosome 10 did not show polymorphism.

Table 3 SSR Markers Linked With Damage Score Identified Through In Silico BSA

S.No	SSR marker	Chromosome	Physical position	Recombination against Bph (%)	Recombination against Wbph (%)
1	RM11522	1	28.06	67.7	77.3
2	RM488	1	24.8	71	59.1
3	RM11342	1	24.5	64.5	56.8
4	HSP	1	23.91	67.7	65.9
5	RM8213	4	4.44	74.2	22.7
6	RM7279	4	13.82	61.3	20.5
7	RM16757	4	17.08	77.4	27.3
8	RM216	10	5.35	61.3	70.5
9	RM25176	10	8.08	58.1	72.7

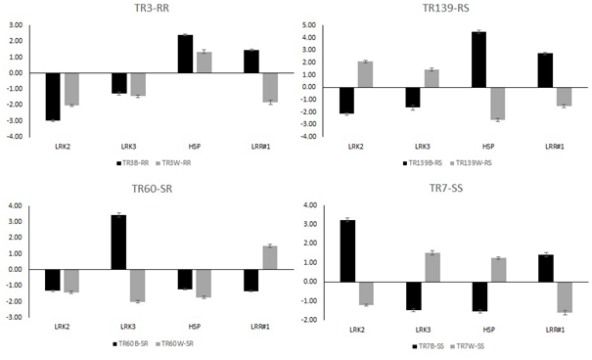

Fig 1 RT-PCR Analysis For Four Genes In TR3RR, TR139RS, TR60SR And TR7SS Lines Against BPH And WBPH

Real Time PCR

Based on our earlier report, two genes on chromosome 1 viz. HSP located at 23.0 Mb and a LRR gene located at 25.0 Mb were reported to be involved in BPH resistance in RP2068 (Naik *et al.,* 2018). Taking this information, in the present study the same candidate genes were

also tested against WBPH resistance. Based on the above BSA analysis, another region was also detected on chromosome 4 between the markers RM8213 and RM5953. In this region *Bph3/Bph17* was cloned and encoded by three lectin receptor kinases were also tested LRK1, LRK2, and LRK3 on chromosome 4 for their expression. Of the three genes tested, LRK1 did not amplify in any of the samples. LRK2 and LRK3-were found to be downregulated in most of the lines irrespective of compatible and incompatible interactions (Fig.1). HSP-the candidate gene for *Bph33* was upregulated in incompatible interactions of TR3(RR) with BPH or WBPH and downregulated in compatible interactions of TR60(SR) with BPH and TR139(RS) and TR60(SR) against WBPH. The expression of LRR gene on Chromosome#1 was inconsistence.

DISCUSSION

The brown planthopper (BPH) and white-backed planthopper (WBPH) are among the major pests that cause huge production loss of rice in Asia. Development and release of resistant varieties are the most accepted strategy for the pest management. Most of the genes for which markers have been developed, by and large, are not effective in India and effective genes for the country have not been intensively studied to tag and map the genes. Another dimension to the problem is that genes effective against BPH are, generally, not effective against WBPH. Hence, it is imperative that we need to identify new and effective resistance genes within India, tag and map these genes to identify closely linked markers for regional MAS breeding. We also need combined resistance against both BPH and WBPH.

Already there have been several land races of rice reported to have resistance against both BPH and WBPH like Sinna Sivappu, ADR52, Veluthacheera. But only few studies have been carried out to understand the genetics of resistance or tagging and mapping of the genes. A mapping population from the cross TN1 X RP2068 was developed to tag, map and clone gall midge resistance gene *gm3* (Sama *et al.,* 2014). This mapping population was also used for identifying a new resistance gene *Bph33* effective against BPH located on chromosome#1 (Naik *et al.,* 2018). Our current investigations extended these studies with major focus on WBPH resistance in RP2068 and investigating if the genes/loci responsible for BPH resistance are same that are responsible for WBPH resistance. Initially we started with segregation analysis of BPH/WBPH resistance in the RILs. The segregation ratio for both the planthoppers did not fit perfect into simple Mendelian ratio for damage score against BPH and WBPH. Hence the present study was focused on identification of loci/genes responsible for resistance against WBPH and/ or BPH in the rice line RP2068.

Majority of the earlier reports on genetics of planthopper resistance are based on segregation patterns of F_2 or F_3 progeny against SSST test values (Du *et al.,* 2020). Range of values recorded by the donor parent RP2068 for SSST was taken as cut off value for grouping RILs into 'R' group while rest of RILs were considered as 'S' group. These 'R' and 'S' RIL were considered for testing F_{14} RILs against different segregation ratios like 1:1 (single gene); 1:3 (two genes), 1:7 (three genes) and 1:15 (polygenic). Based on the phenotypic results, we observed that damage score data for the RILs against WBPH fit well into (1R:3S) and are likely to be controlled by two genes.

The phenotypic results of damage score, noted against BPH did not fit well into 1:1, 1:3, 1:7 and 1:15 ratios tested and this suggested that it is likely to be controlled by more than three genes. Based on P value; damage score, against BPH showed much higher probability of 1:7 ratio than any other ratios tested. Hence, we propose resistance in RP2068 against BPH to be oligogenic rather than polygenic. It is likely that three major genes and additional factors may influence the inheritance of BPH resistance in RP2068.

Results of *in silico* BSA (Table 2) four markers on chromosome#1 (HSP, RM11342, RM488 and RM11522); were found to be associated with both BPH and WBPH resistance. In contrast, three markers on chr#4 (RM8213, RM7279, RM16757) were found associated against BPH. In other case, two of the markers (RM216 and RM25176) were found associated against WBPH. Though such *in silico* BSA suggest possible markers trait linkage (Naik *et al.,* 2018), this information needs to be further validated with more robust analysis, through QTL mapping.

Till now, for BPH two genes are reported on chromosome 1 viz. *BPH33*(t), which was reported by our group within the physical region of 24.52 Mb and 25.51 Mb in the rice line RP2068 (Naik *et al.,*2018). A HSP gene located at 23.0 Mb to be the candidate gene through expression profiling. Despite this information we decided to study expression of this gene in selected four RILs against both BPH and WBPH. This region was linked to damage score against WBPH and BPH. Besides Bph33(t), Yang *et al.* (2019) reported a novel QTL *BPH37* on chromosome 1 between RM302 and YM35 markers using IR64 as donor parent derived population. We have tested this marker RM302 and found it to be polymorphic between our parents TN1 and RP2068 but no segregation was observed in the mapping population.

Based on the *in silico* bulk segregation analysis, the marker RM8213 on chromosome 4 was found linked with BPH resistance. This marker is reported to be closely linked to *Bph3 (=Bph17*) on chromosome 4 identified from the rice land race Rathu Heenati (Sun *et al.,* 2005; Liu *et al.,* 2015). Later Liu *et al.* (2015) cloned this gene and found it to be a group of three Lectin Receptor Kinases (LRK1, LRK2 and LRK3) claimed that it confers resistance against both BPH and WBPH. We have also tested linked markers (RM8213 and RM5953) and also LRKs and found only RM8213 and LRK3 showed polymorphism between the parents but no segregation was observed for LRK3 marker in the mapping population. In the similar way we have also tested markers on chromosome 10 (RM222 and RM244) reported for *bph21(t)/Bph32* and found monomorphic between the parents. Hence our loci present on chromosome 4 and chromosome 10 could be new genes/QTL conferring resistance against BPH and WBPH.

So far, 15 BPH resistance genes have been cloned and their nature of action has been studied. *Bph14*, the first cloned gene codes for CC-NB-LRR protein expressed primary in vascular bundles and activate SA mediated defense leading to callose deposition in the phloem vessels (Du *et al.,* 2020). *Bph9* codes for CC-NB-NB-LRR protein localised to endomembrane and confer both antixenosis and antibiosis (Zhao *et al.,* 2016). *Bph6* encodes a typical LRR protein localised to exocycst facilitating exocytosis and cell wall strengthening and activates SA signalling pathway and is effective against both WBPH and BPH (Guo *et al.,* 2018). *Bph3*, earlier reported as *Bph17* on chromosome 4 identified from Rathu Heenati, is the locus with three LecRK genes (OsLecRK1, OsLecRK2 and OsLecRK3) which provide resistance to both BPH and WBPH (Liu *et al.,* 2015). Five genes selected for this study included four LRK genes on chr#4 representing *Bph3*, HSP and LRR#1 on Chromosome 1. Of the three LRK genes at Bph3 locus suggest that LRK2 and LRK3 were downregulated in most of the samples suggest the this gene is not involved in resistance in RP2068. Results suggested role of HSP in resistance against both BPH and WBPH, more studies are in progress to confirm these results. These studies have also brought out diversity in action of these R genes which may form the basis of selecting genes for pyramiding to develop durable planthopper resistant rice varieties and manage the pests.

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