

Mapping Genetic Locus Linked to Brown Planthopper Resistance in Rice *Oryza sativa* L.

^{1,2}S. Santhanalakshmi, ¹S. Saikumar, ¹Shubhada Rao,
¹A. SaiHarini, ¹Pawan Khera, ¹H.E. Shashidhar and ^{1,3}P. Kadirvel
¹Barwale Foundation Biotechnology Centre, #8-2-703, A.G. Heights, Road No. 12,
Banjara Hills, Hyderabad, Andhra Pradesh, India
²EI Du Pont, India Pvt. Ltd, Hyderabad, India
³Molecular Breeding/Entomology, AVRDC-The World Vegetable Centre,
P.O. Box 42, Shanuhua, Tainan, Taiwan 74199, Republic of China

Abstract: The aim of present study was to identify the genetic locus associated with brown planthopper resistance in rice. We studied the response of segregating population (F_3) from cross between the susceptible popular indica variety Swarna and the resistant variety PTB33 for its reaction against Indian biotype of BPH. A population comprising of 106 F_3 families along with their parents and the resistant (PTB33) and susceptible check Taichung Native1 (TN1) were evaluated for BPH resistance at seedling stage using the standard seed box method. The results indicated the presence of dominant gene controlling the resistance. Linkage analysis with SSR markers through bulked segregant analysis method was employed for the identification of DNA markers linked to the resistance genetic locus. Single-marker analysis through one way analysis of variance with crop stat showed that the markers RM3766, RM14687, RM251 and RM7 on chromosome 3 were linked to the resistance locus. Further study led to the identification of markers RM3766 and RM14687 linked to a major QTL associated with BPH resistance physically mapped on short arm of chromosome 3. This QTL showed an additive effect of 0.7 and dominance effect 0.3. The identified QTL on chromosome 3 is new source of resistance derived from the resistant parent PTB33.

Key words: ANOVA, BPH, biotype, QTL, rice, SSR markers

INTRODUCTION

Rice is a staple crop grown in the world providing food for more than quarter of the population. Rice is however, subjected to wide array of abiotic and biotic stress factors. Sucking pests cause serious damage to the crop directly by feeding on its tissue and by acting as vectors for plant pathogens. All the growth stages of the rice plant from the seedlings in the nursery to the mature plant stage are vulnerable. Brown plant hopper (*Nilaparvata lugens* Stal, BPH) is considered as the most serious sucking insect pest in rice growing regions in the world. Variations are known to exist between the BPH biotypes. A significant loss in annual rice yield is caused by insect infestation in which the brown planthopper (BPH) is the most destructive insect pest for rice crops in Asia (Park *et al.*,

Corresponding Author: Shubhada Rao, Barwale Foundation Biotechnology Centre, #8-2-703,
A.G. Heights, Road No. 12, Banjara Hills, Hyderabad,
Andhra Pradesh, India

2008). Chemical control is often incomplete for which the insects readily develop resistance and emerge as major pest. Understanding the mode of resistance to BPH is crucial for pest management. Vertical resistance is controlled by one (monogenic) or a few (oligogenic) major genes are short lived. Quantitative trait loci on the other hand, are known to impart durable resistance against the pest. Several major genes and Quantitative Trait Loci (QTL's) have been reported for BPH resistant varieties as well as wild species of rice (Park *et al.*, 2008). The native indica cultivars are known to possess higher levels of resistance compared to japonicas (Chen *et al.*, 2006).

Mapping and tagging of pest resistance genes have been greatly facilitated by a wide array of molecular markers in crop plants. Marker-assisted selection has been an integral part of plant breeding as it improves the efficiency of plant breeding by facilitating the transfer of genomic regions of interest followed by accelerated recovery of recurrent parent genome. About 22 major genes and several QTL's associated with BPH resistance have been identified and mapped on the rice chromosomes. The genes Bph14, Bph15, Bph19t and Bph1 3t along with QTL's qbph1, qbph3 etc., have been mapped on the short and long arms of chromosome 3 (Renganayaki *et al.*, 2002; Chen *et al.*, 2006). Resistant cultivars are the most economic and effective way to contend with BPH pests. Consequently, breeding for rice cultivars with insect resistance is a priority in crop improvement programs (Park *et al.*, 2008).

In some cases, the major gene resistance was short lived due to the adaptation of BPH population to highly resistant varieties, harboring any one of these major genes (Ketipearachchi *et al.*, 1998). Quantitative resistance is considered to be more durable (Sounderrajan *et al.*, 2004). Quantitative Trait Loci (QTLs) that confer BPH resistance have also been found in certain indica cultivars, such as B5 (Ren *et al.*, 2004) and Col.5 Thailand (Sun *et al.*, 2007).

The advent of new molecular genetic tools has led to the rapid search for genes involved in complex traits. Identification of chromosomal location of BPH resistance gene forms the basis of gene transfer into susceptible varieties. In the last decade, a great progress has been made to identify the chromosomal regions that influence quantitative resistance to insects (Yencho *et al.*, 2000). The availability of IR64/Azucena Double Haploid (DH) mapping population and saturated linkage map led to the identification of several QTL's underlying various resistance mechanisms in BPH populations of Philippines (Alam and Cohen, 1998). Thus, several QTL's for BPH resistance have been successfully detected and validated. The present study was taken up with an aim to identify the resistance genes in PTB33 which has been considered to be highly resistant to the Indian biotype of BPH.

MATERIALS AND METHODS

Plant Materials

The present research was conducted during the year 2007-2008. The plant material included the segregating F_{2,3} populations derived from a cross between the popular indica variety Swarna with BPH resistant variety PTB33.

Rearing Insects

The BPH colony used for research was Biotype 4 or Indian biotype which has been recognized as a predominant biotype in India. It was collected from the rice fields at Barwale Foundation research farm at Maharajpet, Hyderabad, India. The insects were fed on TN1 plants for about 5-6 weeks to produce a sufficiently large population. The first to second

instar larvae were selected for infestation; the remaining insects were maintained on TN1 for further screening activities.

Screening for BPH Resistance

Plant materials were screened using the standard seedling box test (Heinrichs *et al.*, 1985). The seeds of test lines (106 F₃ families) along with their parents, resistant check (PTB33) and susceptible check (Taichung Native1) were germinated in wooden seed boxes placed in galvanized iron trays filled with water to maintain humidity. The temperature in the polyhouse was maintained at 26-30°C and the relative humidity between 60-70%. The insects of *N. lugens* were maintained on susceptible check TN1 plants in polyhouse. The gravid females were selected randomly and allowed to oviposit on TN1 plants for 2-3 days. The 1st to 2nd instar larvae were released on 10 day old seedlings at the rate of 10-12 nymphs per seedling. The plants were carefully monitored periodically for symptoms of damage. The plants were scored phenotypically when the susceptible check TN1 wilted completely. The plants were rated individually for insect damage on 0-9 scale according to the Standard Evaluation System (IRRI, 1996). The plants with score 0-3 were considered as resistant, 3.1- 6.9 as moderately resistant and 7-9 as susceptible.

Identification of Markers Linked to BPH Resistance Genes

Bulked segregant analysis method (Michelmore *et al.*, 1991) was used to identify the molecular markers linked to BPH resistance in F₂ progeny derived from the cross between Swarna (susceptible) and PTB33 (resistant) indica varieties. A set of 15 resistant plants and 15 susceptible plants constituted the Resistant Bulk (RB) and Susceptible Bulk (SB), respectively. The bulks were sorted based on damage rating obtained by BPH bioassay. Genomic DNA was extracted from young leaves of individual plants using CTAB method. The DNA samples were then pooled to form bulk samples. The parental and bulk DNA was screened using 200 SSR primers on chromosomes 3, 6 and 12 from the genomic regions where BPH resistance has been reported. All samples were PCR amplified and the amplified PCR products were resolved on 6% polyacrylamide gel electrophoresis for 1 h at 100 v and detected by silver staining. The polymorphic bands between bulks and parents were scored to identify the co-segregation of DNA fragments with the resistant phenotype.

Twelve markers from chromosome 6 (RM190, RM469, RM111, RM17, RM225, RM402, RM541, RM19429, RM584, RM19425, RM8072 and RM19291) were found to be polymorphic while five from chromosome 3 (RM14515, RM14687, RM3766, RM7 and RM 251) and two each from chromosome 4 (RM687 and RM8213) and chromosome 12 (RM3331 and RM28561) showed polymorphism between the parents as well as bulks. The polymorphic markers identified by BSA were validated in individual plants.

Data Analysis for Localization of BPH Resistance Genes and Assigning them to Linkage Groups

Mean, Standard error, LSD and frequency distribution of phenotypic values were obtained using (IRRI, 2007). The SSR marker data of F₂ individuals and their corresponding phenotypic values were used for single locus analysis through one-way Analysis of variance using (IRRI, 2007) to establish marker-phenotype association. The probability of less than 0.01 was used as empirical threshold value to claim the association of SSR marker with putative locus for BPH resistance. A framework of linkage map for QTL analysis was reconstructed using genotypic data of the selected markers with Joinmap version 4.0 (Van Ooigen, 2006). The map locations of putative QTL's involving Simple Interval Mapping

(SIM) was done with QTL Cartographer v1.13 model (Basten *et al.*, 2002) at a threshold of LOD = 2.0 for declaring the presence of QTL (Yamasaki *et al.*, 2003). Average distance between pairs of markers was measured in cM derived using Kosambi function. The phenotypic effects, such as additive effects (a), dominance effects (d) were also estimated using QTL Cartographer.

RESULTS

Phenotypic Screening for BPH Resistance

The 106 F₃ families along with their parents Swarna and PTB33 were evaluated for resistance to Brown plant hopper of Indian biotype using the standard seed box technique of IRRI. The donor parent, PTB33 was found to be highly resistant to the Indian biotype while the popular variety Swarna was BPH susceptible. The response of F₃ progeny was assayed directly based on the phenotypic score. Among the 106 F₃ families screened for resistance, the percentage of resistant progeny was between 60-70% of total progeny. The Chi-square goodness of fit showed that the pattern of resistance in the segregating F₃ family was observed in the ratio of 1R:2MR:1S indicating the presence of dominant gene controlling resistance to BPH (Table 1, Fig. 1). The distribution of trait for BPH resistance was observed to be quantitative in nature with median value of 5.78.

Bulked Segregant Analysis and Linkage Analysis Using Microsatellite Markers

A total of 200 SSR markers from chromosomes 3, 6 and 12 were used to study parental polymorphism, of which 21 markers were found to be polymorphic between Swarna and PTB33. The genotypes of F₂ individuals for BPH resistance were inferred from their phenotypic results. Bulk Segregant Analysis (BSA) was carried out according to the method of Michelmore *et al.* (1991). The band profiles generated by a combination of SSR markers were compared among the parental DNA, resistant bulk and the susceptible bulk. Five markers on chromosome 3 (RM 14515, RM 14687, RM 3766, RM 7 and RM 251) showed

Table 1: Segregation for BPH resistance in 106 F₃ progenies of Swarna/PTB33 cross

Phenotypic class*	Frequency		$\chi^2 = ((\sum(O-E)^2)/E)$
	Observed	Expected	
Resistant (0-3)	28	27	0.037
Recombinants/moderately resistant (3.1-6.9)	56	54	0.055
Susceptible (7-9)	22	26	0.920

*Data significant at 5% level of significance

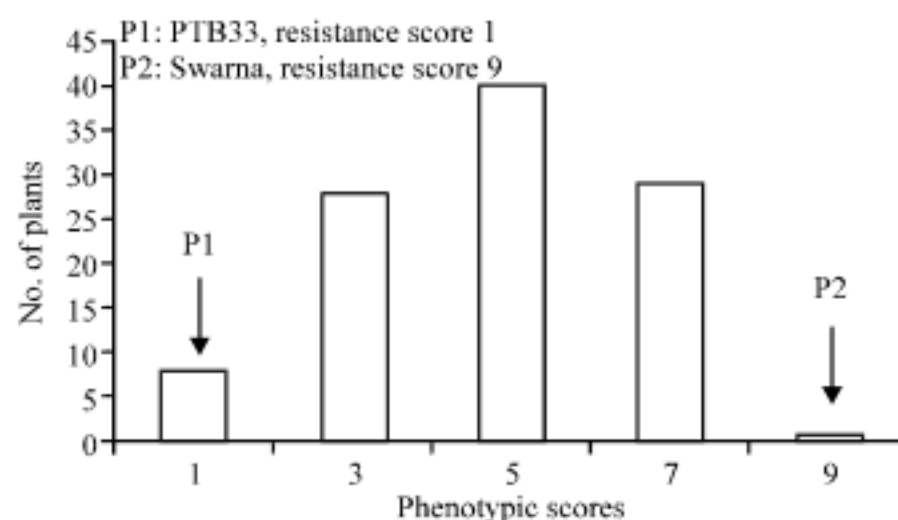


Fig. 1: Frequency distribution for BPH resistance in F₃ families derived from cross Swarna/PTB33. Phenotypic screening was based upon Standard Seedling Box Test

Table 2: Chi-square test of F₂ population, segregation of polymorphic markers on chromosome 3

Markers	Heterozygote P1	Heterozygote h	Heterozygote P2	$\chi^2 = ((\sum(O-E)^2)/E)$
RM 14687	27	55	24	4.10
RM 7	28	54	24	0.34
RM 251	24	59	23	1.18
RM 3766	26	56	24	0.42

*Data significant at 5% level of significance

Table 3: Putative SSR markers identified for BPH resistance on chromosome 3 using One-way ANOVA for F_{2;3} population

Markers	P1 Homozygote*	P2 Homozygote*	Heterozygote*	LSD (5%)	p-value**
RM 3766	6.072	5.782	4.327	0.747	0.000
RM 14687	6.417	4.830	5.451	0.773	0.006
RM 251	6.067	5.085	5.471	0.796	0.001
RM 7	6.869	4.333	5.593	0.678	0.000

*Mean phenotypic values of F₃ families carrying marker alleles where, P1= PTB33; P2= Swarna and Heterozygote of both parents. **Markers showing p<0.01

polymorphism between the parents as well as bulks identified by BSA. These markers were further used to validate the individual F₂ plants. The genotyping of corresponding 106 F₂ individuals showed segregation of the marker for the trait in the ratio of 1: 2: 1. Chi square test of goodness of fit was performed to analyze the segregation of polymorphic markers. Data was found to be significant at 5% level of significance (Table 2).

Single Marker Analysis

The single marker analysis with QTL cartographer showed that the mean trait value for BPH resistance was 5.50, while the variance for the trait was 2.88 and standard deviation 1.69. The coefficient of variation was 0.30. The average deviation being 1.42, respectively. The data analyzed was fit into simple regression model to assess the likelihood of marker linked to a QTL. The F-statistic ratio with smaller probability for RM3766, p = 0.000 significant at 0.01% level is indicative of the marker being associated with QTL for resistance in PTB33 (Table 3).

Interval Mapping/QTL Analysis

A genetic linkage map was constructed with LOD scores ≥ 2.2 . Map distances were estimated by Kosambi function. To tag the QTL for BPH resistance, we tried to analyze the co-segregation of SSR markers associated with BPH resistance using QTL cartographer. The QTL analysis using Simple Interval Mapping (SIM) was carried out with LOD score ≥ 2.2 and one QTL peak with major effects was detected between the markers RM3766 and RM14687 at (LOD = 10), another QTL with minor effects detected between RM14687 and RM7 showed a peak at (LOD = 4) (Fig. 2). To identify the mode of inheritance, the phenotypic effects such as additive gene effects and dominance gene effects were determined. Results were indicative of the role of additive genetic variance (0.703) in addition to the dominance gene effects (0.292) in contributing to the phenotype (Table 4). The QTL with major effect at marker interval (RM3766-RM14687) contributed more to the phenotype. The genetic distances of the markers on physical map were correlated well with the standard linkage map. (Fig. 3). The reported markers linked to resistant genes *Bph19t* and *Bph13t* mapped on short arm of chromosome 3 were found to be close to the QTL identified in the present study. Chromosomal regions around these markers indicate their involvement in conferring resistance to BPH is suggestive of gene clustering in this chromosomal region. The resistance gene/QTL identified in the present study is found to be new resource of BPH resistance on chromosome 3 in PTB33.

Table 4: Characteristics of QTL's identified for BPH resistance on chromosome 3 in PTB33

Locus	Peak LOD	Additive effect	Dominance effect
RM 3766-RM 14687	10	0.703	0.292
RM 14687-RM 7	4	0.423	0.071

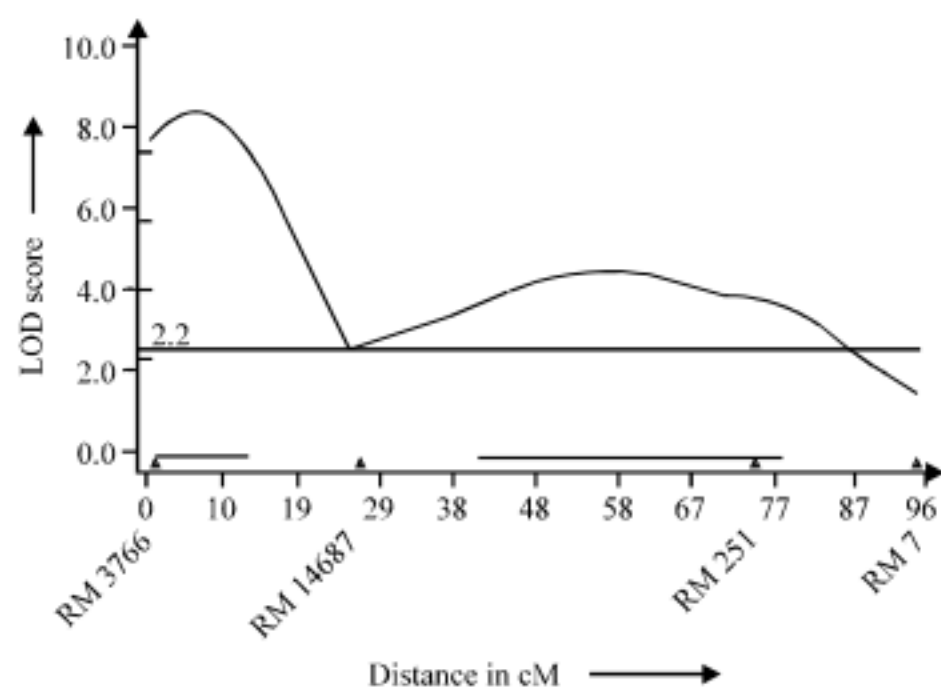


Fig. 2: QTL likelihood curves for BPH resistance on chromosome 3

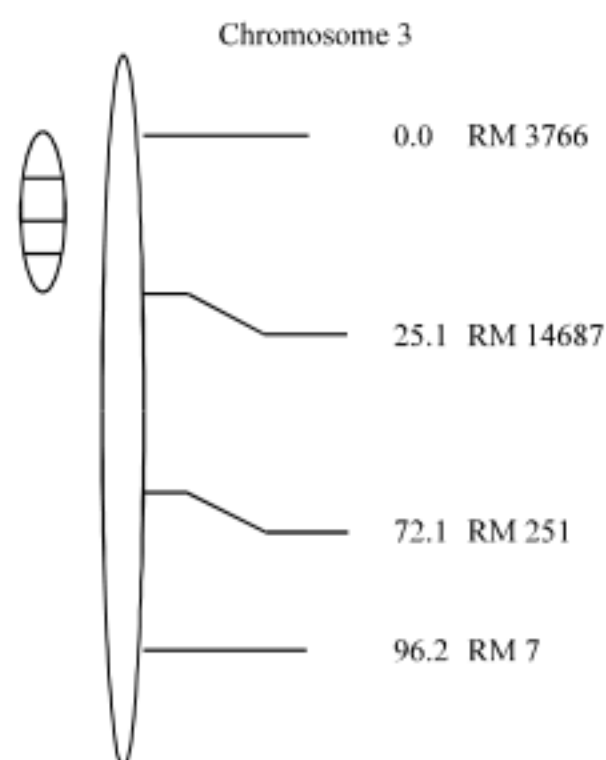


Fig. 3: Physical map of marker positions linked to BPH resistance chromosome 3

DISCUSSION

PTB 33 has been reported to be highly resistant to the various biotypes of BPH. Several improved breeding lines derived from PTB33 were found to be promising in all the regions of Asia. It has been reported that genes carrying resistance to *N. lugens* in PTB33 appeared to be different in South Asia from those in the rest of Asia, as evident from the differential reactions of the semi-dwarf selections derived from that variety (Seshu, 1992). A number of BPH resistance genes and QTL's have been mapped on different chromosomes. The identification of tightly linked SSR markers facilitate the marker-assisted selection and also provide an insight into map based cloning of the gene.

Molecular markers tightly linked to the target gene are useful in breeding program. The SSR markers are abundant and widely distributed across the rice genome (McCouch *et al.*, 1997) they are simple and rapid to assay, thus they are most likely to be chosen by researchers for their investigations.

The donor parent PTB33 has been reported to carry two genes *bph2* and *Bph 3* (Rongbei *et al.*, 2001). The major finding in our study is the identification of markers linked to BPH resistant locus derived from PTB33. The BPH resistance is difficult trait to measure as the scores vary with conditions of plant growth, insect, environmental conditions. Genetic heterogeneity could be observed within each of F₃ families. Among the F₃ progeny tested 60-70% were found to be resistant indicative of dominant mode of inheritance against the biotype studied. Similar studies on mode of inheritance of resistance against the Pantnagar biotype of India was reported by Rongbei *et al.* (2001) in their introgression line 94-42-5-1 derived from *O. rufipogon*.

Based on the physical map data of rice chromosomes 3, 6 and 12 (<http://www.gramene.org/>), 200 SSR markers from regions flanking one or more reported genes for BPH resistance were selected to study parental polymorphism followed by bulked segregant analysis. Twelve markers from chromosome 6 (RM 190, RM 469, RM 111, RM 17, RM 225, RM 402, RM 541, RM 19429, RM 584, RM 19425, RM 8072 and RM 19291) were found to be polymorphic, while five from chromosome 3 (RM 14515, RM 14687, RM 3766, RM 7 and RM 251) and two each from chromosome 4 (RM 687 and RM 8213) and chromosome 12 (RM 3331 and RM 28561) showed polymorphism between the parents as well as bulks.

The results from this study showed that segregation of all linked markers followed the expected co-dominant ratio of 1:2:1 indicating the presence of major dominant gene conferring resistance (Table 2). Based on the bulked segregant analysis which detect loci with large effects followed by linkage analysis with microsatellite markers, the locus for BPH resistance was assigned on chromosome 3 (Fig. 3). The high degree of resistance shown by PTB33 is indicative of the presence of minor genes or QTL's contributing to the overall phenotypic variance.

Present results are indicative of presence of QTL on chromosome 3. Other genes for BPH resistance have been mapped on chromosomes 3, 9 and 12 (Murata *et al.*, 1998) and long arm of chromosome 3 (Kawaguchi *et al.*, 2001). A major effect QTL was identified between the markers RM 3766 and RM 14687 at a LOD score of 10.0. The presence of minor QTL between markers RM 14687 and RM 7 with a LOD score of 4.0 was also observed. The phenotypic variance explained by the QTL was due to the dominance (0.3) and additive (0.7) gene effects. Moderate and durable level of BPH resistance conferred by polygenes has been reported in IR64 by Sounderrajan *et al.* (2004). Six QTL's associated with BPH resistance have been detected on chromosomes 1, 2, 6 and 7 (Sounderrajan *et al.*, 2004). Cultivar IR36 which carried resistance gene *bph2* which was released in 1975 to replace IR26 with *Bph1* quickly lost resistance in response to new biotype. This led to the adoption of varieties carrying *Bph3* gene in background of IR56 and IR60. The gene was later mapped on to short arm of chromosome 6 (Jairin *et al.*, 2007).

These findings are indicative of the facts that insect populations can quickly overcome single resistance genes hence new sources of multiple resistance genes would prove to be useful for rice improvement against BPH. The QTL's for several traits were mapped on different chromosomes with LOD scores ranging from 0.9 to 3.3 and percentage of phenotypic variance explained by each QTL ranged from 3.4 to 12.6 (Yencho *et al.*, 2000; Alam and Cohen, 1998).

In a study by Rahman *et al.* (2009) one QTL was mapped to 193.4 kb region located on the short arm of chromosome 4 and the other QTL was mapped to a 194.0 kb region on the long arm of chromosome 12. The two QTLs additively increased the resistance to BPH. These major QTLs were reported to be new BPH resistance loci and were designated as *Bph20 (t)* on chromosome 4 and *Bph21 (t)* on chromosome 12.

QTL mapping for ovicidal response to BPH and WBPH were mapped on an RFLP linkage map by Yamasaki *et al.* (2003) in a set of 98 backcrossed inbred lines derived from Nipponbare/Kalasath/Nipponbare was used to phenotype the rice ovicidal response to WBPH and BPH. Several QTL's were detected on chromosomes 3, 6, 7, 8 and 10. The RAPD markers linked to BPH resistance gene, *Bph13t* on short arm of chromosome 3 for biotype 4, prevalent in South Asia was reported by Renganayaki *et al.* (2002). The major gene *Bph13t* was found to be placed in the same region of chromosome 3 as the QTL for BPH resistance reported by Alam and Cohen (1998) which accounted for 5.6 and 13% variations in their population. The QTL identified by Huang *et al.* (2001) was mapped to the long arm of chromosome 3. The markers linked to single recessive gene *Bph19t* on short arm of chromosome 3, conferring resistance to BPH in ASD20-1 falls in the genomic region of QTL identified in PTB33 in the present study. The gene *Bph19t* was physically defined to an interval of 60 kb between RM 6308 and RM 3134. The genomic region pertaining to *Bph19t* was located in BAC clones OJ1071C11, OJ1081D05, respectively (Chen *et al.*, 2006) while the QTL identified in the present study between the markers RM 3766 and RM 14687 fall into BAC clones OJ1017C11 and OSJNBa0019J12.

Another BPH resistance gene *Bph13t* identified in introgression line IR54745-2-21-12-17-6, from *O. officinalis* conferring resistance to BPH biotype 4 is also located towards proximal end on linkage map of chromosome 3 (Renganayaki *et al.*, 2002). Dominant resistance gene *Bph14* previously named *qbp1* identified in B5 an introgression line from *O. officinalis* was mapped on long arm of chromosome 3, flanked by RFLP markers R1925 and G1318 (Huang *et al.*, 2001). The recessive gene *bph11t* in introgression lines of *O. officinalis* was mapped on long arm of chromosome 3. Chromosomal regions around these markers indicate their involvement in conferring resistance to BPH is suggestive of gene clustering in this chromosomal region.

The resistance gene/QTL identified in the present study has been found to be a new resource of BPH resistance in PTB33, which was earlier reported to carry only two resistance genes (*bph2* and *Bph3*). The *Bph3* was mapped on located on chromosome 6 (Jairin *et al.*, 2007). Phenotyping additional recombinant lines to fine-map particular QTL for BPH resistance would be difficult owing to the relatively smaller phenotypic variation contributed by it. An alternative strategy to facilitate cloning or marker assisted breeding with QTLs is the candidate gene approach in which the hypothesis are tested regarding the association of QTL's with known genes.

CONCLUSIONS

Brown planthopper (*Nilaparvata lugens* Stal, BPH) is considered as the most serious sucking insect pest in rice growing regions in the world. Breeding rice cultivars with monogenic resistance is often short lived because of the adaptation of BPH population to single gene resistance. Resistance derived from quantitative trait loci is therefore considered to be more durable.

Swarna is one of the popular rice varieties widely grown in India however, it is known to be susceptible to various biotic stresses like bacterial leaf blight, blast and brown plant

hopper. The rice accession PTB 33 has been reported to be highly resistant to the various biotypes of BPH. In the present study, PTB33 has been found to be highly resistant to the Indian biotype of BPH. Improved breeding lines derived from PTB33 would be promising sources for resistance to BPH. The cultivar PTB33 known to carry two single resistant genes *bph2* and *Bph3* mapped on chromosomes 12 and 6 could also be a source for several minor genes controlling resistance. This assumption led to the identification of new QTL on chromosome 3 associated with resistance derived from the resistant parent PTB33. The markers RM3766 and RM14687 linked to this new QTL on chromosome 3 were found to be in close proximity with the other resistance genes.

Fine-mapping of the resistance genetic locus identified could be the future strategy. This would further facilitate marker assisted breeding with QTLs. Another approach would be the candidate gene approach to test the association of QTL's with known genes.

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