RESEARCH PAPER

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Validation and fine-mapping of genetic locus associated with resistance to brown plant hopper [*Nilaparvata lugens* (Stal.)] in rice (*Oryza sativa* L.)

A. SAI HARINI[1], S. SANTHANA LAKSHMI[2], S. SAI KUMAR[1], S. SIVARAMAKRISHNAN[2] AND P. KADIRVEL[1]

[1]Barwale Foundation, Banjara hills, HYDERABAD (A.P.) INDIA

[2]Department of Agricultural Biotechnology, Acharya N.G. Ranga Agricultural University, HYDERABAD (A.P.) INDIA

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In the present study, an attempt was made to validate and fine-map the genetic locus associated with brown planthopper (BPH) resistance locus from IR71033-62-24, a derivative of *O.sativa / O.minuta* cross, using simple sequence repeats (SSR markers). A F_6 segregating population 300 individuals were developed by crossing IR71033-62-24 with a highly susceptible variety, Mahsuri scoring 4.5 and 9.0 (on the scale 1-9) to BPH in greenhouse screening experiments. A subset of 84 F_6 individuals was phenotyped for BPH resistance using a standard seedbox screening test (SSST) under greenhouse conditions. The mean phenotypic score of the subset population was 6.7 and the population was skewed towards the susceptible parent by -0.32. A subset of 61 individuals with extreme phenotypic score was selected from the population for marker trait association analysis. Through single marker analysis,it was found that the BPH resistant locus Bph 22(t) was located at a distance of 6.7cM from the SSR markers RM 585,RM 225,RM 584, RM 19429 and 12.7cM from RM 204 on the long arm of chromosome 6 with a probability value of 0.00.A subset of 75 F_2 lines was also genotyped with the identified markers RM 225, RM 584,RM 19429 and single marker analysis showed the probability value 0.05 in F_2 lines.

Key words : Rice, Brown planthopper, Validation, Fine-mapping, Genetic, Resistance

INTRODUCTION

Rice (*Oryza sativa* L.) is the staple food for about three billion people of the world providing more than 20 to 80 per cent of daily calorie intake. It is also becoming an ideal model plant among cereals for molecular genetic studies due to its relatively small genome size 430 Mb, significant levels of polymorphism, comparatively easy transformability, and large amount of well-conserved genetically diverse materials. Rice genetic resources, comprising land races, modern and obsolete varieties, genetic stocks, breeding lines and wild races are as the basis for the world food security.

During green revolution of the 1960's many high yielding, short duration, semi-dwarf rice varieties were introduced to the farmers. These varieties coupled with greater use of agricultural inputs brought increased yields of rice and lighted the possibility of stable food production in developing countries.

The brown planthopper (BPH) is one such insect pest of rice, which was a minor pest before and emerged as a major pest during green revolution (Dyck and Thomas, 1979). Chemical control using insecticides is harmful, high residual effect and, expensive for poor farmers and it was found neither economical nor safe as it also pose health and environmental risks. There are many resistant varieties in rice germplasm collection, which can be potential donors for BPH resistance genes. At least 19 such genes have been discovered so far (Khush and Brar, 1991; Jena *et al.*, 2005; Chen *et al.*, 2006; Rahman *et al.*, 2009). Many varieties *viz.*, IR26,IR36,carrying single resistance gene in homozygous condition (*Bph1* or *Bph2* or *Bph3*) were also developed and released for cultivation worldwide since 1970. However, such varieties became susceptible, due to adaptation of BPH, and outbreaks continue to occur. Therefore, non-availability of rice cultivars with durable resistance to BPH is a major concern for farmers. Recently, the advent of molecular marker technology has facilitated the identification, mapping and development of gene specific markers for selecting plants carrying specific genes, for the trait of interest, in breeding programmes, the process widely referred as marker-assisted selection (MAS).

MATERIALS AND METHODS

Plant material :

Mahsuri (BF-selection) is highly susceptible to BPH but is of good grain quality type variety cultivated in south

India and IR71033-62-24 is a derivative of *Oryza sativa* and *O. minuta* cross. A F_6 population was generated by crossing Mahsuri (BF-selection) and IR71033-62-24 and a total of 84 F_6 families were used for screening against BPH. A subset of 75 F_2 individuals of the population was used for marker-phenotype association analysis.

Phenotypic screening for BPH resistance :

Insects :

Taichung Native1 (TN) a susceptible rice variety on which BPH was mass reared following the method of Heinrichs *et al* (1985). Initial BPH population was collected from the rice fields at Maharajpet Farm, Barwale Foundation, Hyderabad. The adults were confined on 35 day-old potted plants of TN1 placed in oviposition cages (45 x 45 x 60cm) having wooden frames, glass top, door and wire mesh sidewalls. The ovipositing insects were removed three days later and plants with eggs were taken out of cages, placed in separate cages for the nymphs to emerge. The emerged nymphs were then transferred to the 15- day – old TN 1 seedlings raised in the germination trays, which in turn were placed in galvanized iron trays (62 x 47 x 15cm) containing 5cm depth of water to increase humidity and to avoid watering daily. The seedling trays were changed as and when necessary. Using this technique, a continuous culture of the BPH was maintained during the period of study.

Phenotyping of mapping populations :

Screening of parents and segregating progenies for resistance to BPH were done using seed box screening test (SSST) in greenhouse conditions. The experiment was conducted at a temperature of 28 to 30^{0}C and relative humidity of 70 to 80 per cent in controlled greenhouse at Maharajpet Farm of Barwale Foundation, Hyderabad. A brief description of the procedure is provided below. The seeds were presoaked and sown in rows (3 cm interval) in 60 x 45 x 10 cm seed boxes along with parents, Mahsuri (BF-selection), IR71033-62-24, and checks, PTB33 (resistant) and TN1 (susceptible). A row of 30 seedlings were maintained per F_6 family. Ten day-old seedlings were infested with first instar nymphs at the rate of 5-8 per seedling. Approximately one week after infestation 'hopperburn' symptom was observed. When more than 90 per cent of TN1 plants started wilting, the plants were rated individually. The damage rating based on scoring system proposed by the International Rice Research Institute (IRRI, 1996) was used to rate each seedling *viz*., 0 = No visible damage; 1 = partial yellowing of first leaf; 3 = first and second leaves partially yellowing; 5 = pronounced yellowing or some stunting; 7 = mostly wilting, the plant was still alive; 9 = the plant completely wilted or dead. The families with a mean rating of 0 to 3.9, 4 to 6.9 and 7 to 9 were designated as resistant (homozygous), segregating (heterozygous) and susceptible (homozygous), respectively. Phenotypic extremes *i.e.* F_6 families showing high level of resistance and high level of susceptibility were identified based on the damage rating for molecular analysis.

Molecular marker analysis :

DNA extraction :The seeds of Mashuri, IR71033-62-24, F_2 individuals and F_6 individuals were kept for germination in Petri plates at 37^{0}C in incubator for about 48 hours. From these germinated seeds the first leaf was taken for DNA extraction. Total cellular DNA was extracted following protocol described by Dellaporta *et al*. (1983).

Agarose gel electrophoresis :

The PCR products were run on 3 per cent gels to verify amplification. The gel was electrophoresed in 1x TAE at 100V for 50min. The gel was then stained in ethidium bromide solution (1μg /ml) for 15 min. and de-stained with water and observed on a UV transilluminator (SYNGENE G Box). This exercise was made just to confirm whether the amplification is satisfactory or not before the PCR products are separated on 5% poly acrylamide gel for better resolution.

Polyacrylamidegelelectrophoresis(PAGE) :

SSR analysis was carried out following the protocol described by Paunaud *et al*. (1996) with some modifications.

Scoring of the gel :

The amplified fragments were scored as '1' for the presence, '0' for the absence of the alleles from higher to lower molecular weight products. Genotypic data of the F_2 and F_6 population were generated by scoring the SSR alleles segregating in the population. The score 1 was used to indicate the homozygous SSR alleles derived from IR71033-62-24 and the score 3 was used to indicate the homozygous SSR alleles derived from Mahsuri (BF-selection). The score of 2 was used to indicate heterozygous alleles found in the F_2 population.

Data analysis :

Mean, SE, LSD and frequency distribution of phenotypic values were obtained using CROPSTAT (IRRI, 2005). SSR marker data of F_2 and F_6 individuals and their corresponding phenotypic values of F_2 and F_6

families were used for single locus analysis through One-way ANOVA using CROPSTAT (IRRI, 2005) to establish marker-phenotype association. The probability of less than 0.01 was used as empirical threshold value to establish association of SSR marker with putative locus for BPH resistance.

RESULTS AND DISCUSSION

The results obtained from the present investigation as well as relevant discussion have been summarised under following heads :

Phenotypic trait means, frequency distribution and chi-square test of F_6 population :

It was observed that the overall mean phenotypic score of 84 F_6 parents was 6.7, *i.e.* the population mean falls under moderately resistant (score 4 - 6.9) category. The frequency distribution graph was plotted for the number of individuals falling against different phenotypic means (Fig. 1). The phenotypic analysis had shown that the population has a skewness of -0.32 towards the susceptible parent. Further, one-way analysis was performed and it was found that the individuals in the moderately resistant class have a mean phenotypic score of 5.68 which was not significantly different from the individuals in the susceptible class with mean phenotypic score of 7.88. Further, Chi-square analysis was performed for the fitness of good for 1:1 ratio for the individuals in moderately resistant and susceptible classes. The Chi-square value of 0.0476 was found to be statistically not significant, for 1 degree of freedom against the tabulated value, thus, indicating that the trait under study exhibited monogenic segregation for moderately resistant and susceptible phenotype.

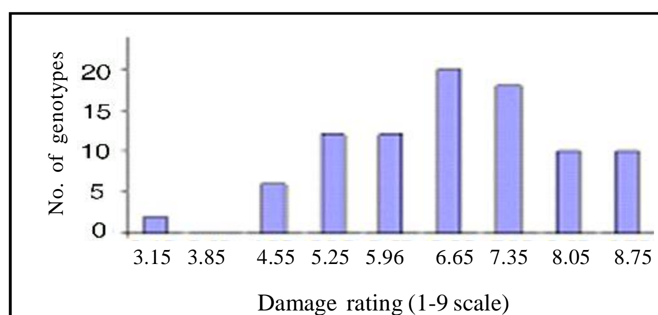

Fig. 1 : Frequency distribution of phenotypic values of Fe genotypes for resistance to BPH

Parental polymorphic survey :

A total of 50 SSR primer pairs were analysed for parental polymorphism between the parents IR71033-62-24 and Mahsuri. Five SSR primers *viz.,* RM585, RM 204, RM584, RM225 and RM19429 were found to be polymorphic.

Genotyping and identification of SSR markers associated with putative locus for BPH resistance in the F_2 population :

Based on the identified polymorphic SSR markers, RM225, RM19429, RM584 were used for genotyping a subset of 75 F_2 individuals and tested for goodness of fit in 1:2:1 ratio using chi-square test. The results showed that all the markers segregated in the expected ratio. Further one way-ANOVA was performed to find association of marker with BPH resistance. The analysis showed that SSR markers RM 225, RM 19429, and RM 584 on chromosome 6 manifested significant association with BPH resistance at 5% level and P-value of 0.05 for all the markers (Table 1).

Validation, fine mapping and Identification of SSR markers for BPH resistance in F_6 population :

All the five SSR markers RM585, RM204, RM584, RM225 and RM19429 found polymorphic between the parents were genotyped on randomly selected 84 F_6 families.The SSR markers were analyzed using Chi-square test, to know whether they are segregate in the ratio of 1:1.Results of this analysis show that all the markers do not deviate from the expected ratio.Further, recombination frequency was calculated from the genotypic data to identify linkage among the markers.

Single marker analysis was used to identify putative SSR markers associated with BPH resistance in the mapping population through marker-trait association. For this analysis a subset of 61 individuals was selected from the population of 84 genotypes. The selection was made by taking genotypes with extreme phenotypic scores. Five SSR markers on chromosome 6 had significant association

Table 1 : Putative SSR markers identified for BPH resistance in one-way ANOVA for F_2 population

Marker	Chr.	P_1 Homozygote[1]	P_2 Homozygote[2]	P_3 Heterozygote	LSD (5%)	P value
RM225	6	3.49	5.16	5.15	1.395	0.05
RM584	6	3.49	5.16	5.15	1.395	0.05
RM19429	6	3.49	5.16	5.15	1.395	0.05

[1]P1 - IR71033-62-24; [2]P2 - Mahsuri (BF-Selection)

with BPH resistance at 5% level and P-value of 0.00 for all the five markers. The mean phenotypic values of F_6 families carrying marker alleles (homozygous) of IR71033-62-24 (P_1) was always lower than F_6 families carrying marker alleles (homozygous) of Mahsuri (P_2) for the putative markers associated with BPH resistance in one-way ANOVA (Table 2). The map location of SSR markers associated with putative BPH resistance loci in the F_6 mapping population studied was calculated to be 6.9 cM south of the SSR markers RM585, RM584, RM19429 and RM225 (Fig. 2).

The success of any molecular mapping study depends on the choice of appropriate parents for the trait of interest. In the earlier study by Anil (2006) two markers RM585 and RM204 located on the long arm of chromosome 6 were found to be putatively associated with BPH resistance in the F_2 population derived from the cross between IR71033-62-24 and Mahsuri (BF-selection). In this study, IR71033-62-24 was chosen as a resistant parent and Mahsuri as a susceptible parent. The IR71033-62-24 is a derivative from the cross, *O. sativa* x *O. minuta* developed at the International Rice Research Institute (IRRI). *Oryza minuta,* one of the wild species of rice, is known to be the potential donors for BPH resistance (Wu *et al*., 1986; Heinrichs *et al*., 1985; Brar and Khush, 1997). Mahsuri (BF-selection) is widely grown in India for its high yield and good grain quality but is a highly susceptible variety for many pest and diseases.

It was interesting to note that IR71033-62-24 did not show high level of resistance, when several accessions of *O. minuta* were reported to show high level of resistance to BPH (IRRI, 2002). However, the resistance level in IR71033-62-24 was relatively higher than the susceptible parent Mahsuri suggesting that IR71033-62-24 could still be the potential donor for genes conferring moderate resistance to BPH.

Based on the phenotypic score of the 84 F_6 population it was observed that the overall phenotypic score was 6.759, *i.e.* the population mean falls under moderately resistant (score 4 - 6.9) class with skewness of -0.32 towards the susceptible parent. This observation may be explained based on the fact that the resistant parent IR71033-62-24 does not have high degree of resistance. Further, it was found that the individuals in the moderately resistant class were significantly different from the individuals in the susceptible class and Chi-square analysis indicates that the trait under study exhibited a monogenic segregation for moderately resistant and susceptible phenotype. It is noteworthy that in the study of F_2 population from the same cross considerable transressive segregants were observed in the resistant class and the number of individuals in Resistant: Moderately Resistant: Susceptible class does not fit in ratio of 1:2:1 (Anil, 2006). Since the population has undergone many recombination's (from F_2 to F_6), it is obvious that the major QTL of BPH resistance in F2 must has segregated as minor QTLs in F_6.

In the F_6 generation, we expect the genetic structure of the population to be in near-homozygous condition. Chi-square test clearly showed that all the markers segregate in the ratio of 1:1, thus, supporting the state of almost complete homozygosity in the F_6 population. Further, for F_2 generation marker segregation was observed in 1:2:1 ratio as tested by Chi-square.

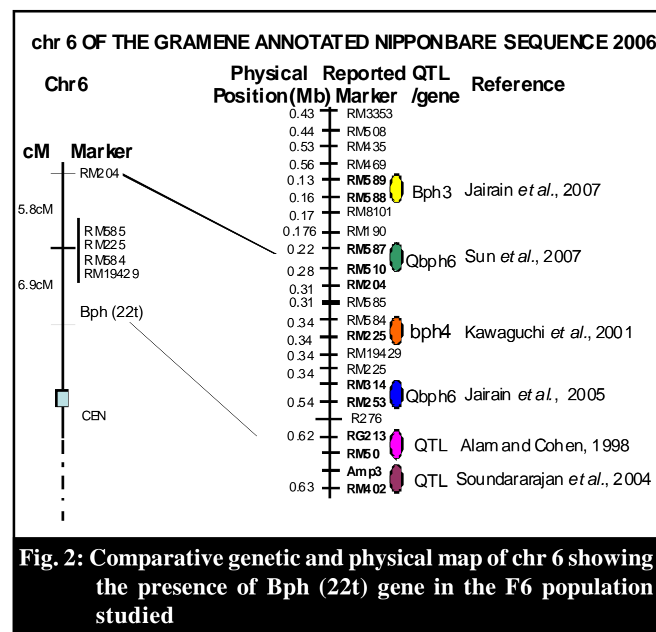

Fig. 2: Comparative genetic and physical map of chr 6 showing the presence of Bph (22t) gene in the F6 population studied

Table 2 : Putative SSR markers identified for BPH resistance in one-way ANOVA for F_6 population

Marker	Chr.	P_1 Homozygote[1]	P_2 Homozygote[2]	LSD (5%)	P Value
RM585	6	5.77	7.50	0.445	0.000
RM204	6	5.58	7.35	0.436	0.000
RM225	6	5.77	7.50	0.445	0.000
RM584	6	5.77	7.50	0.445	0.000
RM19429	6	5.77	7.50	0.445	0.000

[1]P1 - IR71033-62-24; [2]P2 - Mahsuri (BF-Selection)

It was interesting to note that the SSR markers RM585 and RM204 showed consistent association with trait in F_2 (Anil, 2006) and F_6 population currently studied than indicating the consistency of QTL across generation. Further, three new markers RM584, RM19429 and RM225 were identified in the present study to be associated with the BPH trait in F_2 and F_6 population. Linkage mapping analysis showed that the BPH gene is putatively located at a distance of 12.7 cM from RM204 and 6.9 cM from of the markers RM584, RM584, RM19429 and RM225 on chromosome 6 in the F_6 population (Fig. 2).

The same part of the genomic region was also reported to be associated with BPH resistance in different studies (Alam and Cohen, 1998; Kawaguchi *et al.,* 2001 Soundararajan *et al*., 2004; Jairain *et al*., 2005; Jairain *et al*., 2007; Sun *et al.,* 2007). These observations strongly suggest the possibility of a significant putative genetic locus associated with BPH resistance in chromosome 6 near the markers RM584, RM585, RM204, RM19429 and RM225.

The results of the present study help to validate the marker in the region surrounding RM204 and RM585 and further identifying three new markers, RM19429 RM584, and RM225 to be associated with BPH resistance. However, the location of moderately resistant BPH gene in IR71033-62-24 is located 6.9cM southwards of RM584, RM19429, RM584 and RM225 (Fig. 2). Hence, the present work gives the direction to work on more number of markers from the region of BPH resistance of chromosome 6, which will help in further fine mapping the gene for BPH resistance in IR71033-62-24.

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