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Mapping of a Major Resistance Gene to the Brown Planthopper in the Rice Cultivar Rathu Heenati

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A Sri Lankan *indica* rice (*Oryza sativa* L.) cultivar Rathu Heenati was found to be resistant to all the four biotypes of the brown planthopper (BPH) (*Nilaparvata lugens* Stål). In the present study, we constructed a linkage map to identify the locus (loci) for the BPH resistance genes, using an F_2 population from a cross between Rathu Heenati and a susceptible cultivar 02428. Insect resistance was evaluated using 156 $F_{2:3}$ lines and the genotype of each F_2 plant was inferred from the phenotype of the corresponding $F_{2:3}$ lines. Three loci detected by QTL (quantitative trait locus) analysis, were assigned to chromosomes 3, 4 and 10. The phenotypic variance of the three QTLs indicated that the QTL on chromosome 4 is a major BPH resistance gene in Rathu Heenati. Through linkage analysis, it was found that this BPH resistance gene was located between two SSR markers RM8213 and RM5953 on the short arm of chromosome 4, with map distances of 3.6 cM and 3.2 cM, respectively. This gene, tentatively designated as *Bph17*, should be useful for the breeding of varieties resistant to BPH in a marker-assisted selection (MAS) program.

Key Words: *Bph17*, brown planthopper resistance, SSR, QTL analysis, *Oryza sativa* L.

Introduction

The brown planthopper (BPH), *Nilaparvata lugens* Stål, which causes serious yield reduction by directly sucking the plant sap and acting as a vector of various diseases such as rice grassy stunt (Rivera *et al*. 1966) and ragged stunt (Ling *et al*. 1978), is one of the major insect pests of rice (*Oryza sativa* L.) throughout the Asian rice-growing countries. The utilization of host resistance has been recognized as one of the most economic and effective measures for BPH management. Many donors of BPH resistance have been identified and the genetic characteristics of the resistance have been investigated. So far, 16 major effective BPH resistance genes have been identified in *indica* cultivars and four wild relatives, *O. australiensis*, *O. eichingeri*, *O. latifolia* and *O. officinalis*. Of these genes, 12—*Bph1*, *bph2*, *Bph3*, *bph4*, *Bph9*, *Bph10*, *Bph11*, *Bph12*, *Bph13*, *Bph14*, *Bph15* and *Bph16*—have been assigned to rice chromosomes (Hirabayashi and Ogawa 1995, Murata *et al*. 1998, 2000, Ikeda 1985, Kawaguchi *et al*. 2001, Ishii *et al*. 1994, Hirabayashi and Ogawa 1999, Liu *et al*. 2001, Takita 1996, Renganayaki *et al*. 2002, Yang *et al*. 2004). Analysis of quantitative trait loci (QTLs), which contributed to BPH resistance in IR64, Kasalath, DV85, Teqing and wild rice *O. officinalis*, has also been carried out (Alam and Cohen 1998, Su *et al*. 2002, Su *et al*. 2005, Xu *et al*. 2002, Huang *et al*. 2001).

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Lakshminarayana and Khush (1977), Sidhu and Khush (1978, 1979), Ikeda and Kaneda (1981) reported that the Sri Lankan *indica* rice cultivar Rathu Heenati displayed a resistance to all the BPH biotypes, controlled by a new dominant gene different from *Bph1*, that the resistance of Babawee was controlled by a recessive gene *bph4* different from *bph2*, and that these two resistance genes may be allelic or closely linked. Trisomic analysis demonstrated that *Bph3* was located on chromosome 10, and linked to a round kernel gene, *rk2*, with a recombination value of 30.3% (Ikeda 1985). The resistance gene *bph4* in Babawee was first reported to be located on chromosome 4 at a map distance of 40.4 cM and 36.2 cM from the RFLP markers C891 and C531 (Murata 1998, Nagato and Yoshimura 1998), while in another study, it was reported that *bph4* was located on chromosome 6 (Kawaguchi *et al*. 2001). However, molecular mapping of resistance genes in Rathu Heenati had not been reported. In the present study, we constructed the framework of a linkage map with an F_2 population and conducted at the molecular level a genetic analysis of BPH resistance genes in Rathu Heenati. The objective was to identify SSR (simple sequence repeats) markers closely linked to the BPH resistance genes in order to promote marker-assisted selection (MAS) in rice breeding and to map the major resistance genes on rice chromosomes for further gene cloning.

Materials and Methods

Plant materials and insects

The genetic materials included an F_2 population

consisting of 156 plants and F_1 plants from a cross between Rathu Heenati and 02428. The *japonica* cultivar 02428 was susceptible to the BPH with a wide compatibility. Each F_2 plant was self-pollinated to obtain $F_{2:3}$ lines, which were used to investigate the reaction to BPH feeding. ASD7 harboring *bph2* (Athwal *et al*. 1971) and Taichung Native 1 without resistance genes were used as resistant and susceptible controls, respectively.

The BPH population with a mixture of biotypes 1 and 2, which was used for infestation, was firstly collected from rice fields in 2002 in Hangzhou, China. In the present experiment, the insects were maintained on Taichung Native 1 under natural conditions in the greenhouse of Nanjing Agricultural University, Nanjing, China.

Evaluation of BPH resistance

A seedling bulk test with modification was conducted to determine the phenotype for the reaction to BPH feeding. To ensure that all the seedlings were at the same growth stage for insect infestation, seeds were first germinated in petri dishes. Then, about 25 F_3 seeds harvested from each F_2 individual were sown in plastic pots 10 cm in-diameter with a hole at the bottom. The distance between the seedlings was 2.5 cm. Generally, twenty-eight pots, including each one pot for the parents and control varieties, were placed in a $68 \times 42 \times 16$ cm plastic seed-box. Water at a depth of about 2-cm was kept in the seed-box until the evaluation of the resistance was completed. A total of four pots for Rathu Heenati, three pots for 02428 and three pots for Taichung Native 1 were randomly arranged among the F_3 lines as controls. Seven days after sowing, the seedlings were thinned to 20 plants per pot. At the third-leaf stage, the seedlings were infested with 2nd to 3rd-instar nymphs of BPH at the rate of ten insects per seedling. When all the seedlings of Taichung Native 1 died, the plants of the $F_{2:3}$ lines were examined and each seedling was given a score of 0, 1, 3, 5, 7 or 9, according to the criteria listed in Table 1, which were based on the report of Athwal *et al*. (1971), IRRI (1988) and Huang *et al*. (2001). The significance among varieties was analyzed using the shortest significant ranges.

Table 1. Criteria for scoring brown planthopper resistance used in the present study

Resistance score	Plant status (investigated when most of the Taichung Native 1 plants died)	Resistance level
0	None of the leaves shrank and the plant was healthy	R
1	One leaf showed yellowing	R
3	One to two leaves showed yellowing or one leaf shrank	MR
5	One to two leaves shrank or one leaf shriveled	MR
7	Three to four leaves shrank or two to four leaves shriveled, the plant was still alive	S
9	The plant died	S

DNA preparation and SSR analysis

DNA samples were extracted from young leaves of each entry using the method described by Dellaporta *et al*. (1983). The extracted DNA samples were dissolved in TE buffer (10 mM Tris base, 0.1 mM EDTA) and tested for qualitative and quantitative characteristics using a MBA 2000 UV/VIS Spectrometer (Perkin Elmer Co.). The samples were then diluted into 20 ng/μl with autoclaved double distilled water (dd H_2O) for further analysis.

SSR analysis was performed according to the procedure of Chen *et al*. (1997) with minor modifications. The original sources and motifs for all the SSR markers used in this study could be found in the gramene database (http://www.gramene.org/) and the report of McCouch *et al*. (2002) (http://www.dna_res.kazusa.or.jp/9/6/05/spl_table1/table1.pdf). Amplification reactions were carried out in a 10 μl mixture containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 50 μM each of dNTPs, 0.2 μM of each primer, 0.5U of *Taq* polymerase (TaKaRa, Dalian) and 20 ng of DNA template. Reactions were performed using a PTC-200 thermal cycler (MJ Research Inc.) programmed as follows: 94°C for 4 min, followed by 35 cycles of 1 min at 94°C, 1 min at 55°C 1.5 min at 72°C with a final extension of 7 min at 72°C. Amplification products were separated on a 8% non-denaturing PAGE gel, and observed by the silver staining method based on the report of Sanguinetti *et al*. (1994). Then, the bands were scored on a light box with fluorescent lamps.

QTL analysis and gene mapping

Linkage groups and the order of the markers were determined using MAPMAKER/EXP 3.0 (Lander *et al*. 1987). The Kosambi mapping function was used to transform the recombination frequency to genetic distances (cM). Composite interval mapping (CIM) was performed using Windows QTL Cartographer V2.0 (Wang *et al*. 2003) to detect QTLs for BPH resistance in the F_2 population. The permutation method (Churchill and Doerge 1994) was used to obtain the empirical thresholds for determining the main-effect QTL of the experiment based on 1000 runs of random shuffling of the trait values at the experiment-wise significance level of 0.05, (2.21). Thus, this LOD threshold was used for determining the significant main-effect QTL in the present study. The additive effects and the percentage of variation explained by individual QTLs were estimated. Inheritance of BPH resistance was analyzed by the Chi-square test. Genotypes for the BPH response (RR: homozygous resistant, Rr: segregating heterozygous, rr: homozygous susceptible) of 156 F_2s were determined by assaying the phenotype of the corresponding $F_{2:3}$ lines, which was inferred based on the weighted average of the seedlings tested. The RS (Resistance Score) 1–2 were categorized as RR, RS 2–6 for Rr, and RS 6–9 for rr. The phenotype and the SSR data were combined for linkage analysis using the MAPMAKER program and a linkage map of the specific chromosome region surrounding the BPH resistance gene was constructed.

Results

Evaluation of BPH resistance

In the bulk seedling test, the resistance scores of Rathu Heenati and 02428 were 0.3 and 8.1, respectively, which indicated that Rathu Heenati was resistant to the BPH while 02428 was susceptible. The F_1 plants with a resistance score of 1.1 were resistant to the BPH. It seemed that Rathu Heenati was more resistant than ASD7 whose resistance score was 1.3 (Table 2).

The resistance scores of the 156 F_3 lines infested with a BPH population with a mixture of biotypes 1 and 2 showed a continuous distribution, ranging from the low value of 0.1 to the high value of 9.00, with three apparent peaks around 1, 5 and 8 in the distribution curve (Fig. 1). Genotypes for the BPH response of 156 F_2s were determined by examining the phenotypes of the corresponding $F_{2:3}$ lines in the BPH bioassay. All the 156 $F_{2:3}$ lines were classified into three categories based on the resistance scores as resistant, segregating and susceptible and the corresponding F_2 plants were genotyped as RR (homozygous resistant), Rr (segregating heterozygous) and rr (homozygous susceptible). The segregation of the F_2 population showed a good fit to the expected ratio of 1RR: 2 Rr: 1 rr ($\chi^2 = 1.69$, $\chi^2_{0.05} = 5.99$).

Construction of rice linkage map

A total of 548 SSR primer pairs on all the chromosomes were surveyed for polymorphisms between Rathu Heenati and 02428, of which 178 (32.5%) markers showed polymorphism. In the F_2 population consisting of 156 individuals, the framework of the linkage map with 124 SSR markers was constructed to identify the genes controlling the BPH resistance. The map covered 1499.1 cM on all the 12 chromosomes, with an average interval of 12.09 cM. The orientation of the 120 markers out of 124 determined was consistent with that of the maps published by Temnykh *et al*. (2000) and McCouch *et al*. (2002).

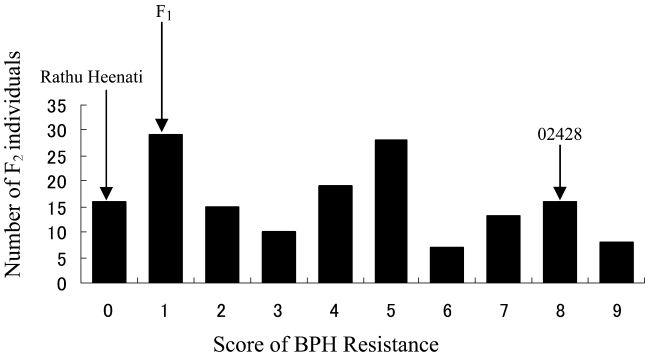

Fig. 1. Frequency distribution of BPH-resistant $F_{2:3}$ lines from 156 F_2 individuals derived from a cross between Rathu Heenati and 02428. The scores of Rathu Heenati, 02428 and F_1 were 0.3, 8.1 and 1.1, respectively.

Table 2. Scores of BPH resistance in parents, F_1 and control varieties

Variety[a)]	Number of seedlings tested	Resistance score (0–9)	Significance level[b)]
Taichung Native 1	60	9.0	A
02428	60	8.1	B
ASD7	60	1.3	C
F_1	26	1.1	C
Rathu Heenati	60	0.3	D

a) ASD7 and Taichung Native 1 were used as resistant and susceptible controls, respectively.
b) Significance at 1% level.

Table 3. Segregation of BPH resistance in F_2 population derived from the cross between Rathu Heenati and 02428

F_2 genotype	Number of F_2 individuals[a)]	Phenotype of corresponding $F_{2:3}$ family[a)]
RR	44	RS < 2
Rr	70	2 ≤ RS < 6
rr	42	RS ≥ 6

a) χ^2 value for 1 RR: 2 Rr: 1 rr is 1.69 ($\chi^2_{0.05,2} = 5.99$).
b) Number in this column correspondes to the range of resistance scores; RS, Resistance Score.

Identification of the BPH resistance genes

QTL analysis using Windows QTL Cartographer V2.0 at a LOD threshold of 2.21 and significance level of 0.01 enabled to detect three QTLs for BPH resistance (Table 4 and Fig. 2). The first, designated as *Qbph3*, which was detected with a LOD score of 2.32, was located between RM313 and RM7 on chromosome 3. This QTL explained 6.5% of the phenotypic variance of BPH resistance in the population. The second QTL, *Qbph4*, was detected with a high LOD score of 63.7 between RM8213 and RM 5953 on chromosome 4. This QTL accounted for 83.9% of the phenotypic variance of BPH resistance in the population. The third QTL, *Qbph10* controlling the BPH resistance with a LOD score of 2.74 and PVE (phenotypic variation explained) of 10.1% was identified between markers RM484 and RM496 on chromosome 10.

It should be noted that the major QTL on chromosome 4, *Qbph4*, was detected with a high LOD score of 63.7 and it explained 83.9% of the phenotypic variance of BPH resistance in this population. Linkage analysis further indicated that the BPH resistance gene was mapped between the two SSR markers RM8213 and RM5953 with map distances of 3.6 cM and 3.2 cM, respectively (Fig. 3).

The resistance score in the F_2 population was significantly differentiated by the genotypes at markers RM5953 and RM8213 (Table 5), which further indicated that this gene for BPH resistance was linked to the two markers. As indicated by the effects estimated, this gene was derived from the resistant parent Rathu Heenati and reduced the damage caused by BPH feeding.

Table 4. QTLs identified for BPH resistance using the F_2 population of Rathu Heenati /02428

QTL	Interval	Chromosome	LOD score	PVE (%)[a)]	Additive effect[b)]
Qbph3	RM313–RM7	3	2.32	6.5	–0.52
Qbph4	RM8213–RM5953	4	63.7	83.9	–3.63
Qbph10	RM484–RM496	10	2.74	10.1	–0.81

a) Percentage of variance explained (%).
b) Additive effect of "Rathu Heenati".

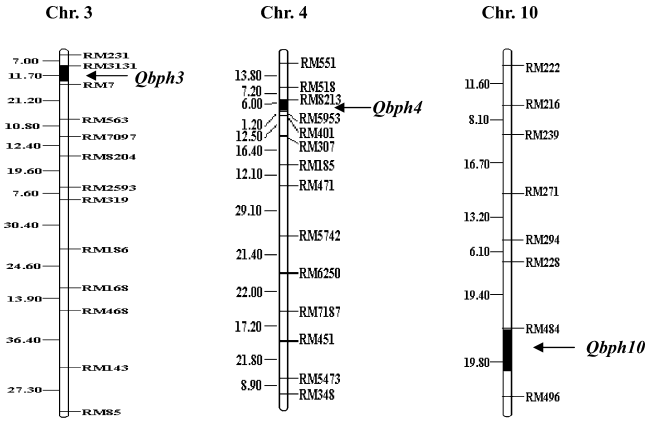

Fig. 2. Location of three BPH resistance QTLs identified by QTL analysis. Marker names are listed on the right hand side of the chromosome with the distances (in cM) indicated on the left. The *solid bars* indicate the locations of the three quantitative trait loci for BPH resistance, designated as *Qbph3*, *Qbph4* and *Qbph10*.

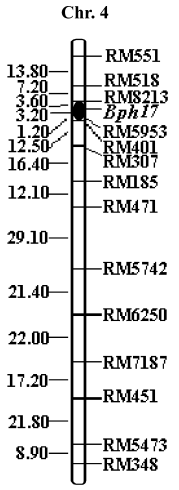

Fig. 3. Molecular mapping of rice chromosome 4 showing the location of the *Bph17* gene. Marker names are listed on the right hand side of the chromosome with the distances (in cM) indicated on the left.

Discussion

The threat of BPH to rice has resulted in a constant search for resistance genes and an accelerated effort to develop resistant varieties (Khush 1979, Bonman *et al*. 1992). However, the difficulty in breeding for resistance was markedly exacerbated by the ability of the BPH to develop new biotypes for the "breakdown" of resistant varieties and to become virulent on novel plant genotypes rapidly. The first BPH-resistant variety IR26 harboring *Bph1*, which was released in 1973, initially enabled to control the BPH over large areas. However some BPH populations became adapted to IR26 within as few as 2 years. This pattern was repeated with subsequent varieties harboring the *bph2* gene. At present, four BPH biotypes have been identified. Biotype 1 and biotype 2 are widely distributed in Southeast Asia, biotype 3 is a laboratory biotype produced in the Philippines, and biotype 4 occurs in the Indian subcontinent (Khush *et al*. 1991).

To address the problem of resistance breakdown associated with outbreaks of new biotypes, the identification of additional BPH resistance genes was required for widening the genetic base. Therefore, the major BPH resistance gene at the locus recognized here should be useful as an additional source of BPH resistance, in marker-assisted selection. In the present study, we constructed the framework of a linkage

Table 5. Distribution of resistance scores by genotypes of markers RM5953 and RM8213 in F_2 population

Marker	Genotype[a)]	Number of F_2s in resistance score class			Total	Mean[b)]
		RS<2	2≤RS<6	RS≥6		
RM5953	1/1		1	38	39	8.23
	1/2	2	69	4	75	4.67
	2/2	42			42	1.41
RM8213	1/1		1	37	38	8.12
	1/2	4	68	5	77	4.92
	2/2	40	1		41	1.49

a) 1/1denotes the genotype of 02428, 1/2 denotes the genotype of the heterozygote, 2/2 denotes the genotype of Rathu Heenati; RS, Resistance Score.
b) Shows a significant difference between means of three genotypes at 1%.

map with an F_2 population and also attempted to analyze QTLs for BPH resistance. QTL analysis revealed the presence of three loci on chromosomes 3, 4 and 10. It should be noted that one major QTL, *Qbph4*, which was detected with a high LOD score of 63.7 and PVE of 83.9%, was located on the short arm of chromosome 4. Comparison of the chromosomal locations and reactions to the BPH biotypes showed that the gene was different from sixteen previously identified BPH resistance genes. Therefore, the major BPH resistance gene in Rathu Heenati was tentatively designated as *Bph17*. Linkage analysis indicated that Bph17 was mapped between the two SSR markers RM8213 and RM5953 at distances of 3.6 cM and 3.2 cM, respectively (Fig. 3). The QTL on chromosome 10, *Qbph10*, detected in the present study, with a LOD score of 2.74, explained 10.1% of the phenotypic variance of BPH resistance in the population. Comparison of our results with those reported by Su *et al*. (2002), revealed that *Qbph10* was mapped at similar genomic locations with the BPH resistance QTL segregating in a population of backcross inbred lines derived from Nipponbare/Kasalath//Nipponbare. The other QTL, *Qbph3*, could only explain 6.5% of the phenotypic variance of BPH resistance in the F_2 population of Rathu Heenati/02428. The results indicated that the BPH resistance in Rathu Heenati is likely to be controlled by the major resistance gene *Bph17* as well as by two minor genes. Therefore, we considered that *Bph3* in Rathu Heenati did not represent a single dominant gene but was associated with multiple loci affecting the resistance to the BPH.

To slow down the progression of the BPH population, several approaches have been proposed, such as rotation of different cultivars, planting of lines with multiple resistance within the same field, and combination of different resistance genes within the same cultivar (Pathak and Khush 1979, Khush 1984, Heinrichs 1986, Saxena and Khan 1989). Pyramiding of *Bph17* and the other BPH resistance genes through molecular breeding can be proposed to enhance the resistance to the BPH. In order to combine the resistance genes, it is necessary to identify plants harboring multiple genes from plants containing either one or the other genes. The most reliable method for producing cultivars harboring more than one resistance gene is to perform gene tagging using molecular markers. Cloning and incorporation of these resistance genes into susceptible breeding lines using technology at a molecular level could be suitable for enhancing the BPH resistance of rice cultivars. The molecular markers linked to *Bph17*, especially the time- and money-saving SSR markers identified in this study, would definitely promote the use of this resistance gene in a marker-assisted selection program for the breeding of new BPH-resistant cultivars.

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