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Full Paper

Physical mapping of *Bph3*, a brown planthopper resistance locus in rice

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Abstract: Resistance to brown planthopper (BPH), a destructive phloem feeding insect pest, is an important objective in rice breeding programs in Thailand. The broad-spectrum resistance gene *Bph3* is one of the major BPH resistance genes identified so far in cultivated rice and has been widely used in rice improvement programs. This resistance gene has been identified and mapped on the short arm of chromosome 6. In this study, physical mapping of *Bph3* was performed using a BC_3F_3 population derived from a cross between Rathu Heenati and KDML105. Recombinant BC_3F_3 individuals with the *Bph3* genotype were determined by phenotypic evaluation using modified mass tiller screening at the vegetative stage of rice plants. The recombination events surrounding the *Bph3* locus were used to identify the co-segregate markers. According to the genome sequence of Nipponbare, the *Bph3* locus was finally localized approximately in a 190 kb interval flanked by markers RM19291 and RM8072, which contain twenty-two putative genes. Additional phenotypic experiment revealed that the resistance in Rathu Heenati was decreased by increasing nitrogen content in rice plants through remobilization of nitrogen. This phenomenon should be helpful for identifying the *Bph3* gene.

Keywords: *Nilaparvata lugens*, BPH resistance gene, sequence information, *Oryza sativa*

Introduction

The widespread damage caused by insect pests constitutes the most significant factors leading to substantial and unpredictable decrease in rice yield. Brown planthopper (BPH), *Nilaparvata lugens* Stål, a destructive monophagous insect pest, is one of the main biotic constraints in rice, causing huge yield losses in Asian rice-growing areas every year. The damage caused by BPH feeding has the greatest effect on the growth of rice plant [1]. In addition to feeding on the rice plant directly, BPH also causes indirect damage by transmitting viruses, which cause ragged and grassy stunt diseases [2].

Developing resistant rice cultivars is generally considered to be the most economic and effective way for controlling BPH. Rice plant resistance to BPH is recognized as a qualitative and quantitative trait. The genetic basis of the qualitative and quantitative BPH resistance has been well studied and at least 21 major resistance genes have been discovered from cultivated varieties and wild relatives [3-5]. Of these genes, 17 resistance genes have been assigned to rice chromosomes [3-14]. More than half of the discovered major resistance genes could not be used against some BPH populations found in Thailand [7]. Although BPH resistance genes have been intensively discovered and studied throughout the rice genome, until recently none of the BPH resistance gene has been cloned in rice and our current knowledge about insect resistance genes in rice plant is still limited.

Breeding a resistant cultivar with major resistance genes was highly successful. However, BPH itself also successfully adapts to feed on the resistant cultivars by changing their biotypes. The occurrence of new virulent biotypes has been a serious problem in breeding resistant rice cultivar against BPH. Identification and incorporation of new BPH resistance genes into rice cultivars are important breeding strategies to control the damage caused by new biotypes of BPH [4]. Therefore, selection of BPH resistance genes for improving resistant cultivars needs to be considered carefully.

A local rice cultivar of Sri Lanka, Rathu Heenati, was found to confer a strong and broad-spectrum resistance against BPH populations in Thailand [7]. The resistance in this cultivar was concluded to be controlled by a single dominant gene, *Bph3* [15-16]. The study of genetic analysis by classical genetic approach showed that f *Bph3* was closely linked to *bph4* in rice cultivar Babawee because no recombinants between these genes were observed among nearly 1,200 of F_3 progenies [17]. The chromosomal location of *Bph3* gene was first assigned to rice chromosome 10 based on trisomic analysis [18]. Later, it was suggested that *Bph3* was located on chromosome 4 and *bph4* was located on chromosome 6 [8, 13]. Recently, the chromosomal location of this locus has been confirmed on the short arm of chromosome 6 in two backcross populations derived from crosses of Rathu Heenati/KDML105 and PTB33/RD6. The locus is flanked by simple sequence repeat (SSR) markers RM589 and RM588 [7].

The publicly available rice genome sequence information has made map-based cloning in rice much more efficient in getting the target genes. Three BPH resistance genes, *Bph15*, *Bph18* and *bph19*, have been finely mapped on chromosome 3, 4 and 12, respectively [3-5]. *Bph15* was finely mapped to a genomic segment of approximately 47 kb long flanked by RFLP markers RG1 and RG2 [5]. The *bph19* locus was finely defined to an interval of about 60 kb flanked by SSR markers RM6308 and RM3134 [3]. The *Bph18* locus was also physically localized within an 843 kb physical interval that includes three BAC clones between the STS marker R10289S and SSR marker RM6869 [4]. In this study, the construction of a high-resolution linkage map with SSR markers is a crucial step in map-based cloning of *Bph3*. We report the fine mapping of the *Bph3* locus to an approximately 190 kb

target region on rice chromosome 6 using SSR markers. The region contains at least twenty-two putative functional annotation genes.

Materials and Methods

Plant materials

Two *indica* rice (*Oryza stiva* L.) cultivars were used as parents in this study. Rathu Heenati (acc. no. 11730), a landrace variety from Sri Lanka, carries a broad-spectrum resistance against four different BPH biotypes found in Thailand. KDML105, aromatic rice known as Jasmine rice, has a good cooking quality and adapts well in rainfed lowland areas in Thailand. The donor parent, Rathu Heenati, is highly resistant to BPH whereas the recurrent parent, KDML105, is extremely susceptible to BPH.

A backcross population BC_3F_2 consisting of 333 individuals was derived from the cross between Rathu Heenati and KDML105. The population was used to initially locate the *Bph3* in our previous study [7]. A BC_3F_3 population was developed from the BC_3F_2 resistant individual that shows heterozygous in the target region on chromosome 6. A total of 358 BC_3F_3 individuals derived from two families of BC_3F_2 were used to confirm the BPH resistance in the target region on the chromosome 6. From this population, two BC_3F_3 families consisting of 28 individuals were selected as material for physical mapping.

The parents and additional cultivars, TN1 and PTB33, were used in experiments for evaluating BPH resistance in vegetative and reproductive stages of rice plants.

Bioassay for BPH resistance

A BPH population from a single colony of PBH was collected from Ubon Ratchathani province, Thailand, and was grown on a susceptible variety TN1 in a temperature-controlled rearing room. For evaluating the BPH resistance of parents, including resistant and susceptible lines at vegetative and flowering stages, three phenotypic experiments were conducted using standard seedbox screening (SSBS), modified mass tiller screening (MMTS) and semi-field screening (SMFS) methods. The SSBS was used to evaluate the BPH resistance of seedling plants. The pre-germinated seeds of the test lines were sown 5 cm apart in 20 cm rows in seedboxes. The susceptible control, TN1, was sown randomly in all the seedboxes. Seven days after sowing, the seedlings were infested with first to second nymphs of BPH at a number of twenty nymphs per seedling. Damage rating of the test lines was done when 90% of the plants in the susceptible control row were killed. The test lines were graded using the Standard Evaluation System (SES) for rice [19]. The MMTS was used to evaluate the BPH resistance of tillering-stage plants. The pre-germinated seeds of the test lines were individually sown (10×20 cm) in 7×24 m^2 plots. Twenty days after sowing, the seedlings were infested with 3rd-4th instar nymphs of BPH at a number of ten nymphs per seedling. Until TN1 and the susceptible recurrent parents died, we evaluated the severity scores of the test lines. The SMFS was used to evaluate rice plants at vegetative and reproductive stages in the rice field. Ten of twenty-day seedlings were transplanted (20×20 cm) in the rice field, and covered with a nylon net. Fifteen days after transplanting the rice plants were

infested with 3rd-4th nymphs of BPH at a number of five insects per hill. Then, we let the insect population increase for 1-2 generations. When all the TN1 had died, we scored the degree of damage undergone by the test seedlings. The scoring criteria were based on the SES. The remains of resistant lines were scored every ten days until flowering stage.

For evaluating the BPH resistance of BC_3F_3 progenies, the bioassay was done with the MMTS technique at the tillering stage of rice under greenhouse condition. In brief, the seeds of TN1, a susceptible cultivar, PTB33, RD6, parents and each BC_3F_3 progeny were separately sown in seedling plots. When the seedlings had 3-4 tillers (approximately 20-25 days), three similar growth-conditioned tillers were then separated and were transplanted in 7×24 m^2 plots. The leaves of each seedling were clipped for DNA extraction before transplanting. The leaf samples were stored frozen at -80°C prior to extraction. Genomic DNA samples were extracted using the method of Chen and Ronald [20]. Ten days after transplanting, the seedlings were infested with 3rd- 4th instar nymphs of the BPH at the density of 10 insects per tiller. Then, we let the insects feed, mate, lay eggs and hatch freely. Until TN1 and the susceptible recurrent parents died, we evaluated the severity scores of each BC_3F_2 individual on a scale of 1 (very slight damage) to 9 (all plants dead) according to the SES.

Fine genetic mapping of the Bph3 locus

Initial localization of the *Bph3* locus was based on the recent report of mapping on the short arm of rice chromosome 6 [7]. The linkage analysis was performed using 333 BC_3F_2 individuals from the cross between Rathu Heenati and KDML105. The resistance gene was located between the flanking markers RM589 and RM588. In this study, 16 additional SSR markers covering the BPH resistance gene region were used to screen Rathu Heenati and KDML105. The SSR markers were obtained from the public database released by Gramene (http://www.gramene.org/). PCR was performed in a 10 µl reaction mixture containing 25 ng of template DNA, 0.5 µM of each primer, 250 µM of each dNTP, 1.5 mM $MgCl_2$, 1 unit *Taq* polymerase and 2 µl of ×10 PCR reaction buffer. Amplification was performed for 35 cycles (1 min at 94°C, 1 min at 55°C and 2 min at 72°C) followed by 5 min at 72°C. The amplified product was electrophoresed on a 4.5% denaturing silver-stained polyacrylamide gel. The polymorphism between Rathu Heenati and KDML105 was screened using 16 SSR markers covering the target region. The polymorphic markers were used to assay 28 BC_3F_3 plants for the fine genetic and physical mapping of *Bph3*. The physical location of the *Bph3* locus in the *japonica* cultivar Nipponbare was determined. A physical map spanning the resistance gene locus was constructed *in silico*, based on the contig map. The prediction of candidate resistance genes with the conserved structures in the target region anchored by tightly linked markers was then analyzed according to the sequences of Nipponbare and was based on the TIGR prediction method (http://www.tigr.org).

Results

Evaluation of BPH resistance

The resistance of the parents and the BPH resistant cultivar PTB33 was studied using SSBS, MMTS and SMFS methods. At the seedling and tillering stage, Rathu Heenati and PTB33 expressed

strong resistance to BPH in both the SSBS and MMTS (Table 1). Although Rathu Heenati and PTB33 showed high resistance to BPH in the vegetative stage (seedling to tillering stages) of heavy BPH infestation (Figure 1a, Table 1), they showed susceptibility during the reproductive stage (flowering to grain filling stage) when the remaining BPH in the field moved to feed on the panicles and panicle necks until plants died (Figure 1b,c, Table 2). Similar to Rathu Heenati, the resistant BC_3F_2 lines from a cross between Rathu Heenati and KDML105 were also susceptible to the BPH at flowering and grain filling stages (Figure 1d). The result indicated that Rathu Heenati and introgression lines were susceptible to BPH at the flowering stage and BPH can feed and grow well on the panicle of the resistant plants carrying *Bph3*.

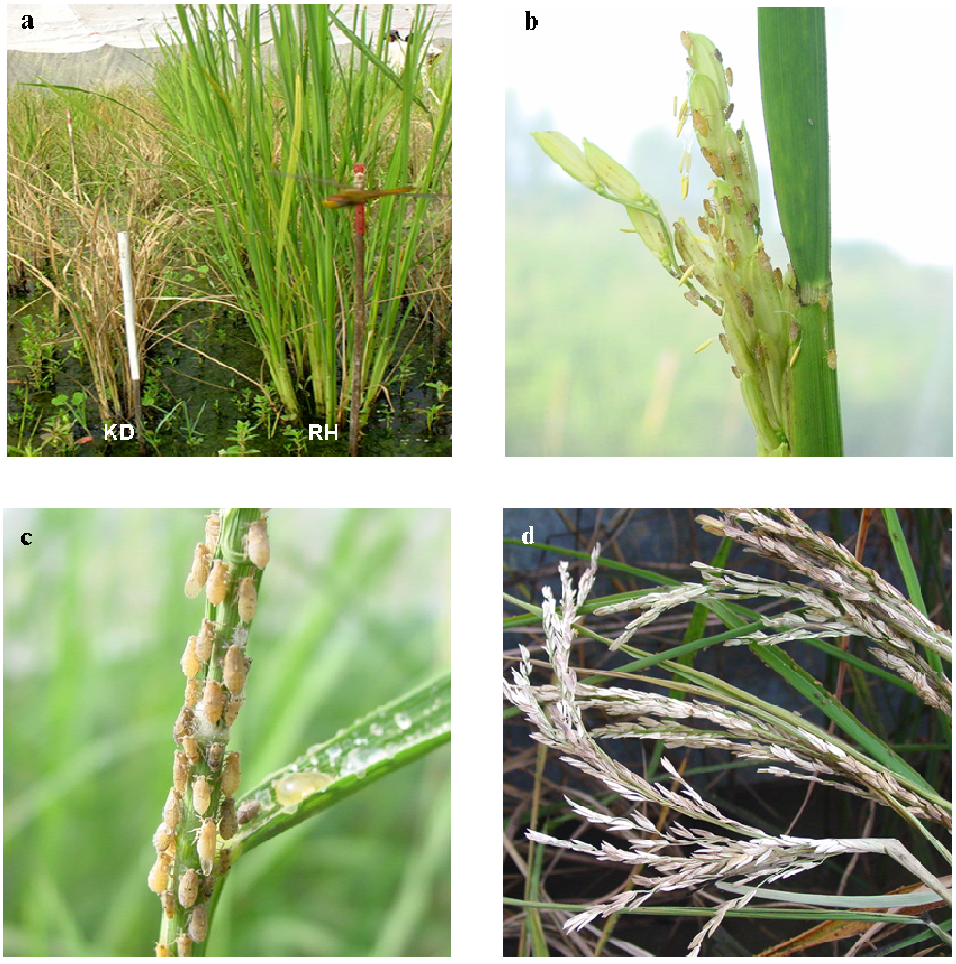

Figure 1. Evaluation of BPH resistance of Rathu Heenati in the rice field using semi-field screening **a** Rathu Heenati (RH) is highly resistant while KDML105 (KD) is susceptible to BPH at vegetative stage, **b** BPH nymphs can feed on the panicle at the flowering stage, **c** BPH feeding on the panicle neck at grain filling stage, **d** The feeding of BPH causes the unfilled grains before the rice plant dies.

Table 1. Average damage score of the parents and controls by BPH at the vegetative stage (seedling and tillering stages) of rice plants

Cultivar	Seedling stage by SSBS			Tillering stage by MMTS		
	7 DAI	10 DAI	14 DAI	7 DAI	15 DAI	23 DAI
Rathu Heenati	1.0	2.2	2.4	1.0	1.0	1.0
PTB33	1.0	2.4	3.5	1.0	1.0	1.0
KDML105	6.5	8.9	9.0	5.0	9.0	9.0
TN1	7.0	9.0	9.0	5.0	9.0	9.0

DAI=Days after infestation
Damage score: 1=very slight damage, 9=all plants dead

Table 2. Average damage score of the parents and controls by BPH. The evaluation was conducted in the rice field using semi-field screening method (SMFS)

Cultivar	30 DAI	40 DAI	50 DAI	Flowering stage
Rathu Heenati	1.0	1.0	1.6	9.0
PTB33	1.0	1.1	2.0	9.0
KDML105	9.0	9.0	9.0	9.0
TN1	9.0	9.0	9.0	9.0

DAI=Days after infestation
Damage score: 1=very slight damage, 9=all plants dead

In the previous study, we used a backcross population consisting of 2,343 BC_3F_2 plants derived from a cross between Rathu Heenati (donor parent) and KDML105 (recurrent parent), in which 333 random plants were used to evaluate and locate a major BPH resistance gene [7]. In this study, we selected two BC_3F_2 plants that were heterozygous on the short arm of chromosome 6 where *Bph3* is located. A total of 358 BC_3F_3 plants derived from the selected two BC_3F_2 were randomly selected and used to confirm the inheritance of BPH resistance in Rathu Heenati at the vegetative stage. Phenotypic evaluations of BPH resistance for the BC_3F_3 and the parents were conducted using the MMTS. Rathu Heenati expressed strong resistance to a Thai biotype of BPH whereas KDML105 was completely susceptible to BPH (Figure 2). Segregation of resistant and susceptible plants (265 resistant plants, 93 susceptible plants) fits in a 3:1 ratio in the 358 random BC_3F_3 plants ($\chi^2 = 0.18$, $p<0.67$) indicating the presence of a major dominant gene conferring resistance to BPH.

Physical mapping of the Bph3 locus

The published genetic mapping data from our previous study was used as a starting point for this study. Using a backcross population, BC_3F_2, derived from a cross between Rathu Heenati and KDML105, *Bph3* was mapped to about 1.4 cM interval between SSR markers RM588 and RM589 on the short arm of chromosome 6 [7]. The co-segregation marker RM589 explained 80.4% of the phenotypic variance of BPH resistance in the 358 random BC_3F_3 individuals.

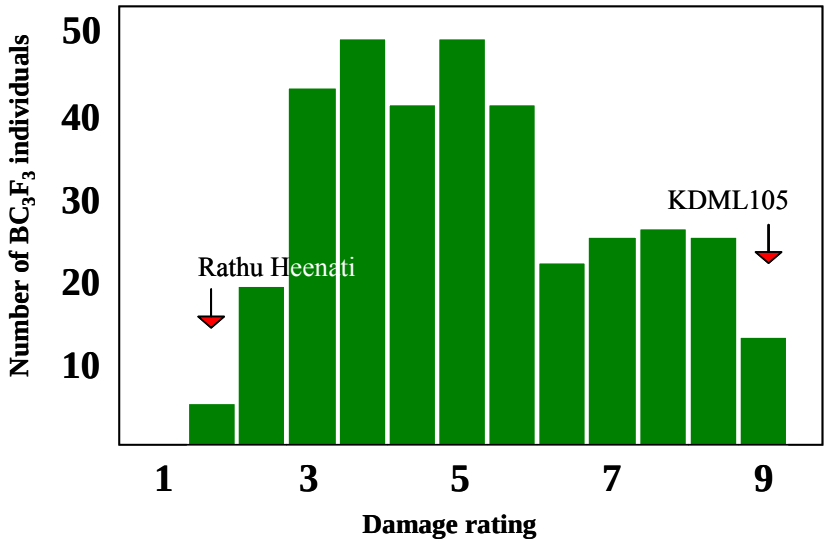

Figure 2. Frequency distribution of BPH resistance scores based on the overall average of four scoring periods from the modified mass tiller screening method at tillering stage of rice plants. The mean scores of Rathu Heenati and KDML105 are indicated by arrows

The location of the *Bph3* resistance gene on the map was determined on the basis of the resistance scores of the 28 recombinant plants. MMTS was employed to distinguish resistant plants from susceptible ones among the recombinant plants. A number of 16 SSR markers located around this genomic region were selected to screen polymorphism between Rathu Heenati and KDML105. Seven SSR markers (RM19291, RM19295, RM19296, RM8072, RM8074, RM19310, and RM19311) detected polymorphisms between the two parents. These seven markers were used to narrow down the region encompassing *Bph3* locus between the two flanking markers RM589 and RM588. The resulting high-resolution map of *Bph3* showed that RM19291 and RM8072 were flanking the *Bph3* resistance gene (Figure 3a). Twenty-eight plants were then identified with recombination break points between the SSR markers RM19291 and RM8072. Of these, three recombinant events were detected with marker RM19291, and five were found with marker RM8072. No recombinants were detected with the other three markers, RM19295, RM19296, and RM589. These three markers were identified to co-segregate with the *Bph3* locus. According to the genome sequence of a japonica rice cultivar Nipponbare, the *Bph3* locus was finally localized to approximately 190 kb interval flanked by markers RM19291 and RM8072 (Figure 3b).

Putative genes in the 190 kb region

Based on the available sequence annotation database of the *japonica* rice Nipponbare (http://www.rgp.dna.afrc.go.jp; http://www.tigr.org), there are twenty-two predicted putative genes in the 190 kb target region. Of these genes, seven had unknown functions, seven were hypothical proteins, and the functional annotation of the remaining eight genes encoded one NBS-LRR disease resistance protein (LOC_Os06g03500), two pentatricopeptides (LOC_Os06g03530 and LOC_Os06g03570), two oligopeptide transporters (LOC_Os06g03540 and LOC_Os06g03560), one

zinc finger, C3HC4 type family protein (LOC_Os06g03580), one transcriptional co-regulator family protein (LOC_Os06g03600), and one protein kinase family protein (LOC_Os06g03610).

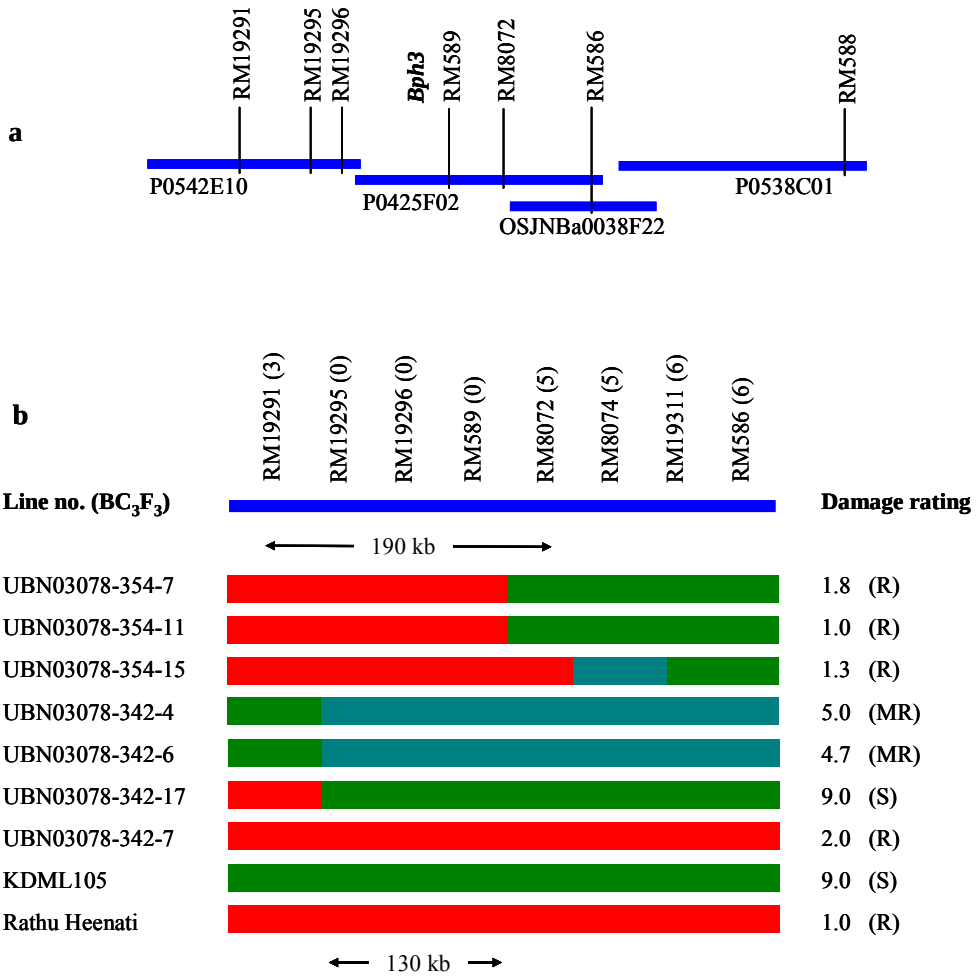

Figure 3. Physical map of the *Bph3* locus. **a** Physical mapping of *Bph3* locus showing four Nipponbare BAC clones interval delimited by RM19291 and RM588, **b** Genotypes and phenotypes of the recombinants between RM19291 and RM586. Red bars = homozygous Rathu Heenati allele; green bars = homozygous KDML105 allele; blue bars = heterozygous. The numerals in parentheses indicate the recombination events occurred at the corresponding marker loci. The BPH resistance score is on the right, R = resistant, MR = moderately resistant, and S = susceptible

Discussion

In our earlier study, a major resistance gene *Bph3* was identified using two backcross populations, which were derived from crosses of Rathu Heenati/KDML105 and PTB33/RD6, at the vegetative stage of the rice plant with heavy infestation of the second generation of BPH. The broad-spectrum BPH resistance gene was mapped within a 1.4 cM interval between SSR markers RM589 and RM588 onto the short arm of rice chromosome 6. Starting from the flanking markers, we were able to locate the gene to a 190 kb segment of genomic DNA. The fragment contains twenty-two putative genes, which encode fourteen proteins (seven hypothetical and seven expressed) of unknown function, an NBS-LRR

disease resistance protein, two pentatricopeptides, two oligopeptide transporters, a zinc finger protein, a transcriptional co-regulator protein, and a protein kinase protein. This result should be helpful for cloning the *Bph3* gene, which is now in progress. The closely linked molecular markers found in this study should be also useful in the marker-assisted breeding programs against BPH.

According to previous studies, some of the major and minor BPH resistance genes tend to be clustered in particular regions of the rice chromosomes. There are four main clusters of BPH resistance genes on chromosomes 3, 4, 6 and 12. Of the twenty-one major BPH resistance genes reported to date, six resistance genes are derived from wild species of *Oryza* and the remaining fifteen are derived from native *indica* cultivars [5-14, 21-23]. Among the resistance genes from cultivated rice, *Bph3* has the most broad-spectrum resistance against BPH biotypes found in Thailand [7]. *Bph3* has been used extensively in rice breeding programs throughout Asia as well as in Thai breeding programs. Although breeding resistant cultivars with major resistance genes has been highly successful, BPH itself has also successfully adapted to feed on resistant cultivars. Therefore, understanding the gene function or mechanism of BPH resistance gene may provide information on how BPH can overcome the resistant cultivars. BPH resistance genes have been intensively studied but none of the gene has been cloned in rice. Until now, up to five resistance genes, *Bph1*, *bph2*, *Bph15*, *Bph18* and *bph19* have been finely mapped and were about to be cloned [3-5, 24].

BPH resistance in rice cultivars carrying *Bph3* was reported to govern an antixenotic reaction to BPH [25]. Rathu Heenati has no repellent chemical against planthoppers and only has common volatiles as released by susceptible cultivars. The feeding inhibition of this cultivar occurred when the insect started to ingest phloem sap [26]. In the present study, Rathu Heenati showed high resistance to BPH at the vegetative stage; only a few numbers of BPH could survive on the resistant plants. The surviving insects had light body weight, slow development and low fecundity (data not shown). On the other hand, Rathu Heenati was susceptible to BPH at the flowering and grain filling stages. BPH could feed and grow well on panicle necks and panicles of the resistant plants (Figure 1). This phenomenon may affect the expression of BPH resistance gene in Rathu Heenati. Further studies are needed to clarify this event especially the chemical analysis of the phloem sap from resistant and susceptible isogenic lines [27]. Comparison of phloem sap components by using a chemically defined diet [28] will also provide information to clarify the phenomenon.

The mechanism of plant resistance to phloem sap-feeding insects has been reported to involve the balance of the amino acid composition of the phloem sap [29-30]. Variation of phloem amino acid composition has been implicated in the nitrogen quality of the phloem sap for phloem feeders [29-31]. It plays a major role in the performance and fitness of insects [32]. The susceptibility of Rathu Heenati at the flowering stage observed in this study may probably involve in the nutritional quality of the phloem sap. In the rice panicles, the total nitrogen arises from remobilization of glutamine synthetase through the phloem from senescing organs [33-35]. The major forms of reduced nitrogen in the phloem sap of rice plants are glutamine and asparagine [36]. Application of a nitrogen fertilizer can dramatically increase the amount of total nitrogen and free amino acids available in the phloem sap [37], especially glutamine and asparagine [38-39]. Therefore, the remobilization of nitrogen in rice plants can increase the total free amino acids in the phloem sap, which may affect the BPH resistance in rice plants and insect performance. Currently, three possible hypotheses can explain how BPH resistance gene is involved in the phenomena: (i) a resistance gene(s) may be poorly expressed in the

upper internodes of heading rice plants, (ii) the amount of the reduced nitrogen forms or nitrogenous compounds in the phloem sap may affect the expression of the BPH resistance gene, and (iii) a resistance gene(s) may involve the phloem nitrogen quality, which affects the activities of symbiotic micro-organisms in BPH. However further studies are needed to investigate the mechanism of BPH resistance in Rathu Heenati and should elucidate which gene present in the 190 kb segment confers resistance against BPH when introduced into BPH-susceptible plants.

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