Theor Appl Genet (2004) 110: 182–191
DOI 10.1007/s00122-004-1844-0

ORIGINAL PAPER

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High-resolution genetic mapping at the *Bph15* locus for brown planthopper resistance in rice (*Oryza sativa* L.)

Received: 3 July 2004 / Accepted: 13 October 2004 / Published online: 10 November 2004
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Abstract Resistance to the brown planthopper (BPH), *Nilaparvata lugens* Stål, a devastating sucking insect pest of rice, is an important breeding objective in rice improvement programs. *Bph15*, one of the 17 major BPH resistance genes so far identified in both cultivated and wild rice, has been identified in an introgression line, B5, and mapped on chromosome 4 flanked by restriction fragment length polymorphism markers C820 and S11182. In order to pave the way for positional cloning of this gene, we have developed a high-resolution genetic map of *Bph15* by positioning 21 DNA markers in the target chromosomal region. Mapping was based on a PCR-based screening of 9,472 F_2 individuals derived from a cross between RI93, a selected recombinant inbred line of B5 bearing the resistance gene *Bph15*, and a susceptible variety, Taichung Native 1, in order to identify recombinant plants within the *Bph15* region. Recombinant F_2 individuals with the *Bph15* genotype were determined by phenotype evaluation. Analysis of recombination events in the *Bph15* region delimited the gene locus to an interval between markers RG1 and RG2 that co-segregated with the M1 marker. A genomic library of B5 was screened using these markers, and bacterial artificial chromosome clones spanning the *Bph15* chromosome region were obtained. An assay of the recombinants using the subclones of these clones in combination with sequence analysis delimited the *Bph15* gene to a genomic segment of approximately 47 kb. This result should serve as the basis for eventual isolation of the *Bph15* resistance gene.

Communicated by Q. Zhang

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Introduction

Rice (*Oryza sativa* L.) is a staple food for more than one-half of the world's population. Insect pests are the major biotic constraints in rice production. The brown planthopper (BPH), *Nilaparvata lugens* Stål (Homoptera: Delphacidae), is one of the most destructive and widespread insect pests that can be found throughout the rice-growing areas in Asia, causing significant yield loss in susceptible cultivars every year (Khush et al. 1979; Sogawa et al. 2003). In addition to causing physiological damage to the rice plant, BPH also causes indirect damage by acting as a vector for rice tungro virus, grassy stunt virus and ragged stunt virus (Heinrichs 1979).

The severe damage and frequent outbreaks of BPH, along with the hazardous effects of pesticides, prompted researchers to seek BPH-resistant germplasm from various sources and to utilize the resistant genes for rice improvement against BPH. To date, 17 major effective BPH resistance genes have been identified in *indica* cultivars and four wild relatives, *Oryza australiensis*, *O. eichingeri*, *O. latifolia* and *O. officinalis*. Of these genes, 13—*Bph1*, *bph2*, *Bph3*, *bph4*, *Bph9*, *Bph10*, *Bph11*, *bph12*, *Bph13*, *Bph14*, *Bph15*, *bph16* and *Bph17*—have been assigned to rice chromosomes (Ikeda 1985; Ishii et al. 1994; Hirabayashi and Ogawa 1995; Takita 1996; Hirabayashi et al. 1998; Murata et al. 1998, 2001; Huang et al. 2001; Kawaguchi et al. 2001; Liu et al. 2001; Renganayaki et al. 2002; Yang et al. 2002; Sharma et al. 2003). These genetic mapping studies of BPH resistance genes have led to rice and the brown planthopper becoming ideal model systems for the study of interactions between plants and sucking herbivorous insects.

Two dominant resistance genes, *Bph14* and *Bph15* (previously named *Qbp1* and *Qbp2*, respectively), have been identified in B5, an introgression line of *O. officinalis* collected in China, and found to confer a strong resistance to BPH (Huang et al. 2001). The chromosome locations of these two loci have recently been confirmed in a recombinant inbred line (RIL) population derived

from the same cross combination by quantitative trait locus (QTL) analysis and whole genome mapping (Ren et al. 2004). *Bph14* mapped to the long arm of chromosome 3 and was flanked by restriction fragment length polymorphism (RFLP) markers R1925 and G1318. *Bph15* was located to the short arm of chromosome 4, flanked by RFLP markers C820 and S11182. There is little if any interaction between *Bph14* and *Bph15*. Genetic analysis revealed that in both the F_2 and RIL populations *Bph15* significantly contributed to BPH resistance. Identification of the DNA sequence of *Bph15* should provide clues to the molecular mechanism of the resistance of the gene and could assist in the development of BPH-resistant rice plants via the transgenic approach.

A natural BPH resistance gene has yet to be cloned in rice. As our current knowledge about insect resistance genes in plants is very limited, it is therefore not feasible to adopt a candidate gene approach for cloning the BPH resistance gene in rice. With the development of DNA marker technologies and the progress of the International Rice Genome Sequencing Project (IRGSP), map-based cloning represents the most promising approach to isolating BPH resistance genes and elucidating the molecular mechanism of BPH resistance in rice.

Based on the publicly available genomic sequence data of chromosome 4, markers C820 and S11182 are located around the centromeric region, with the physical distance between them being more than 2 Mb (http://www.gramene.org/). The large physical distance between these markers and the repetitive sequence-rich property of the centromeric region are the main problems hindering the cloning of *Bph15* by chromosome walking. However, the chromosome landing strategy proposed by Tanksley et al. (1995) represents an appropriate approach for isolating *Bph15*. The construction of a high-resolution linkage map with DNA markers is a crucial step in map-based cloning of *Bph15*.

We describe here the development of new DNA markers and the construction of a high-resolution genetic map of the *Bph15* region in a large F_2 population comprising 9,472 plants. Bacterial artificial chromosome (BAC) clones covering the *Bph15* locus were obtained by screening a BAC genomic library of B5 with the most closely linked markers. We were ultimately able to fix the BPH resistance gene *Bph15* to a genomic segment approximately 47 kb long.

Materials and methods

Plant materials and insects

B5 is an introgression line of *Oryza officinalis* and shows high resistance to BPH biotypes 1 and 2 and some natural BPH populations (Huang et al. 2001). A RIL population consisting of 187 lines, as described previously by Ren et al. (2004), was developed through successive selfing for up to eight generations from a cross between B5 and a susceptible *indica* cultivar, Minghui 63 (MH63). RI93, a selected RIL carrying a single *Bph15* resistance gene, was crossed with a BPH-susceptible variety, Taichuang Native 1 (TN1), to develop an F_2 population. A total of 9,472 F_2 plants were subjected to simple sequence repeat (SSR) analysis, and 48 recombinant individuals were identified that contained recombination events within the *Bph15* region spanning SSR markers RM261 to MS1. The 48 recombinant F_2 individuals were used to develop a high-resolution map of the chromosome region around the *Bph15* locus. The BPH insects used for infestation were maintained in the Genetics Institute of Wuhan University on TN1 plants.

BPH resistance scoring and sorting

The *Bph15* genotypes (R: homozygous resistant, H: segregating heterozygous, S: homozygous susceptible) of F_2 individuals were determined by assaying the phenotypes of their tillers and of those of the corresponding F_3 progenies. We employed the modified tiller testing technique (Fu et al. 1994) to evaluate the BPH resistance of each F_2 plant. Four tillers of each F_2, RI93 and TN1 line were planted in a plastic pot 30 cm in diameter. Ten days later, 180 insects at the second- to third-instar nymphs were placed in each pot. The degree of damage conferred to each F_2 plant was evaluated when the tillers of the TN1 had died. Accordingly, the bulked seedling test was performed on the F_3 families as described by Huang et al. (2001). For evaluating the BPH resistance of each F_3 family, we sowed 20 seeds harvested from each F_2 individual in a 20-cm-long row in a plastic box. The distance between rows was 2.5 cm. Two lines of RI93 and two lines of TN1 were randomly planted among the F_3 families as controls. At the second-leaf stage, the seedlings were infested with second- to third-instar nymphs of BPH at a concentration of ten insects per seedling. When all of the TN1 seedlings had died, we scored the degree of damage undergone by the test seedlings. The scoring criteria were based on the Standard Evaluation System for Rice (IRRI 1988), with 0 indicating no symptoms and 9 indicating that the seedling was dead. The resistance scoring experiments were repeated three times.

RFLP analysis

Total cellular DNA was isolated by the CTAB method as described by Murray and Thompson (1980) with one additional purification step using chloroform/isoamyl alcohol to obtain high-quality DNA. Five restriction enzymes, namely, *Bam*HI, *Hin*dIII, *Eco*RI, *Eco*RV and *Dra*I, were used for surveying RFLPs. Restriction digestion, electrophoresis, hybridization and detection followed procedures described previously (Liu et al. 1997).

SSR analysis

The SSR analysis was carried out in a total volume of 12.5 µl containing 1× PCR buffer, 20 ng genomic DNA, 0.2 μM of each primer, 200 μM of each dNTP and 0.5 U *Taq* DNA polymerase. The amplification reactions were carried out using the following profile: 1 cycle of 94°C for 5 min; 35 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 2 min; a final extension step of 72°C for 5 min. The amplification products were separated through a 6% sequencing gel and detected by silver staining according to Panaud et al. (1996).

Bulked segregant analysis and AFLP analysis

Ten resistant and ten susceptible RILs were selected by the molecular markers flanking the BPH resistance genes *Bph14* and *Bph15* to form the resistant bulk (B_R) and susceptible bulk (B_S). The resulting DNA concentrations of the bulks and of the parents were adjusted to 100 ng/µl.

We essentially followed the AFLP protocol developed and described by Vos et al. (1995) with minor modifications. Primers that detected qualitative polymorphisms between the parents and the bulks were further tested in ten resistant and ten susceptible individuals composed of the bulks. Following this, the linked AFLP markers were employed to genotype the remainder of the RIL population individually and were integrated into the molecular genetic map constructed by Ren et al. (2004). Loci linkages and mapped distances were determined using MAPMAKER/EXP 3.0 at an LOD score of 3.0 (Lincoln et al. 1992).

Conversion of AFLP markers

AFLP bands were eluted from the polyacrylamide gels, re-amplified and cloned into plasmid vector pGEM-T. Plasmid preparations containing the appropriate insert fragments were completely sequenced using pUC/M13 primers. Primers for sequence-tagged site (STS) markers were designed on the basis of sequences of AFLP fragments using PRIMER3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi/). RFLP markers were derived from the cloned AFLP bands. Each AFLP fragment insert was amplified from the recombinant pGEM-T plasmid using its corresponding AFLP primers. The amplified DNA fragment was used as a probe for RFLP analysis following the procedure described by Liu et al. (1997). For probe labeling, the procedure described by Frohman and Mizobuchi (1992) was employed.

Development of co-dominant DNA markers from the sequence information of the *Bph15* region

DNA sequence information of the target chromosome region flanked by RFLP markers C820 and S11182 (http://www.gramene.org/) was used to generate PCR-based markers for genetic and physical mapping. Primers were designed using PRIMER3 software. The protocol described by Temnykh et al. (2001) was employed to detect the putative SSR loci. STS markers were designed to PCR-amplify single-copy fragments that were 350–500 bp in length. The PCR products of RI93 and TN1 were electrophoresed through a 4% sequencing gel and detected by silver staining. The polymorphic primers were then used as STS markers. The PCR products of RI93 derived from monomorphic primers were then used as probes for RFLP analysis following the procedure described by Liu et al. (1997).

Genetic analysis

We determined the *Bph15* genotypes of each F_2 plant in which recombination had occurred around the *Bph15* locus by means of progeny testing. The markers developed in this study were used to assay 48 recombinant F_2 plants for the fine genetic and physical mapping of *Bph15*. Chi-square tests were used to evaluate segregation distortion for the *Bph15* gene in both the F_2 and F_3 generations and for the markers linked to this resistance gene.

Physical mapping using a BAC library and BAC DNA pools

A genomic library of B5 comprising 5.1 haploid rice genome equivalents (Shi et al. 2003) was used in this study. High-density filters representing the entire library were prepared according to Shi et al. (2003). The BAC DNA pools were prepared following the protocol described by Xu et al. (1998), and candidate clones were screened by PCR analysis with the co-segregating primer M1. The BAC-end DNA fragments of positive clones were isolated by thermal asymmetric interlaced (TAIL)-PCR (Liu and Whittier 1995) and used for fine-scale mapping. The specific primers used to amplify the left end were LS1 (5′-AGCGCTTACGTACATGGTC-GAT-3′), LS2 (5′-ATGTGCTGCAAGG CGATTA AGT-3′) and LS3 (5′-AACGACGGCCAGTG AATTGTAA-3′), while the right end was amplified with RS1 (5′-GAATGGCAAAAGCTCCAATACG-3′), RS2 (5′-CAAAAG CTCCAATACGCAAACC-3′) and RS3 (5′-TTTATGCTTCCGGCTCGTATGT-3′). The insert sizes of the BAC clones were determined by pulse field gel-electrophoresis (PFGE) following digestion with restriction enzyme *Not*I.

Results

Identification of AFLP fragments tightly linked to *Bph15* using targeted BSA

To detect polymorphic AFLP fragments tightly linked to *Bph15*, we adopted targeted bulked segregant analysis

Table 1 Molecular marker genotypes of RILs forming the brown planthopper (BPH)-resistant bulk and BPH-susceptible bulk

Marker[a]s	RILs for BPH-susceptible bulk										RILs for BPH-resistant bulk									
	18	51	96	120	124	129	142	143	149	191	47	58	60	89	93	99	105	114	147	184
RM261	S[b]	S	S	S	S	S	S	S	S	S	R	R	R	R	R	R	R	R	R	R
C820	S	S	S	S	S	S	S	S	S	S	R	R	R	R	R	R	R	R	R	R
R288	S	S	S	S	S	S	S	S	S	S	R	R	R	R	R	R	R	R	R	R
S11182	S	S	S	S	S	S	S	S	S	S	R	R	R	R	R	R	R	R	R	R
R1925	R	S	S	S	S	R	S	S	S	S	R	R	R	R	S	S	S	R	S	S
G1318	R	S	S	S	S	R	S	S	S	S	R	R	R	R	S	S	S	R	S	S

[a]Markers RM261, C820, R288 and S11182 are tightly linked to *Bph15*. Markers R1925 and G1318 that flank *Bph14* are also noted
[b]*R*, Homozygous for the allele from B5; *S*, homozygous for the allele from MH63

(BSA) (Dussle et al. 2003). The markers used for selecting the RILs to form the resistant bulk (B_R) and the susceptible bulk (B_S) were R1925, G1318, RM261, C820, R288 and S11182. The genotypes of these markers of the individual lines comprising the two bulks are shown in Table 1. A total of 225 AFLP primer combinations (+3/+3) were tested on the two bulks, B5 and MH63. On average, 35 AFLP bands could be reliably scored for each of the 225 AFLP primer combinations. These AFLP primer combinations showed qualitative differences between the parents, and the two bulks were further tested for the individuals constituting the bulks. Nineteen AFLP fragments generated by 18 pairs of primers were confirmed as being tightly linked with the *Bph15* resistance gene, which represents 2% of the bands polymorphic between B5 and MH63. Eleven fragments were amplified from B5 and the resistant bulk, and eight from MH63 and the susceptible bulk. An example of an R-associated AFLP band (E-AGT/M-CTT) detected in a single gel is shown in Fig. 1. These 19 tightly linked AFLP fragments were further used to genotype the 187 RILs and integrated into the genetic map constructed by Ren et al. (2004). All of these AFLP fragments showed normal segregation in the RIL population. The results of mapping these AFLP fragments are presented in Fig. 2a. Of these 19 AFLP fragments, nine co-segregated with C820 and two co-segregated with S11182. We ultimately used 11 AFLP fragments that mapped in the chromosome region containing *Bph15* for further analysis.

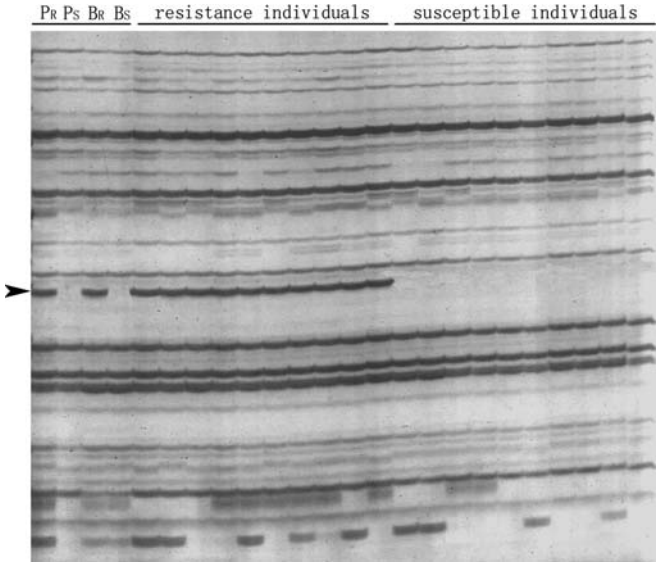

Fig. 1 Portion of an AFLP gel showing the banding pattern produced by the primer combination E-AGT/M-CTT in the brown planthopper (BPH)-resistant parent B5 (P_R), BPH-susceptible parent MH63 (P_S), two RIL bulks (resistant bulk, B_R; susceptible bulk, B_S) and ten resistant and ten susceptible RILs forming the bulks. The *arrowhead* indicates the AFLP fragment linked to the *Bph15* resistance gene

Conversion of AFLP markers

The 11 AFLP fragments were cloned and sequenced to develop co-dominant markers. Sequence alignment with the rice genome sequences by BLASTN searches revealed that these AFLP fragments were physically located in the chromosome region around *Bph15* (data not shown). Sequence-specific primers for these AFLP fragments were then designed. Two primer pairs derived from E-ATG/M-CAC and E-ATC/M-CGT revealed polymorphism between the B5 and MH63; these were consequently converted into STS markers, designated as M1 (F-5′-TCAAGCCTGTCTACTTCAGCA-3′; R-5′-AAATCCGTAGAAATTCTTGTCC A-3′) and M2 (F-5′-CATCTATGTTGGGCATGTGG-3′ and R-5′-ATGAGCCC ATCACGG ATAAT-3′). M1 and M2 were validated in the whole RIL population. The remainder of the AFLP fragments were converted into RFLP probes to detect polymorphism between RI93 and TN1. Four AFLP fragments were polymorphic between RI93 and TN1—E-ATT/M-CGT, E-AAG/M-CAA, E-ATG/M-CAA and E-AGT/M-CTT. Thus, two STS markers and four RFLP markers derived from the 11 AFLP fragments could be used to fine map *Bph15* in the large F_2 population.

Development of DNA markers from rice genome sequence information

Based on the physical map data of rice chromosome 4 (http://www.gramene.org/), nine SSR markers (RM4853, RM7472, RM3536, RM2530, RM5009,

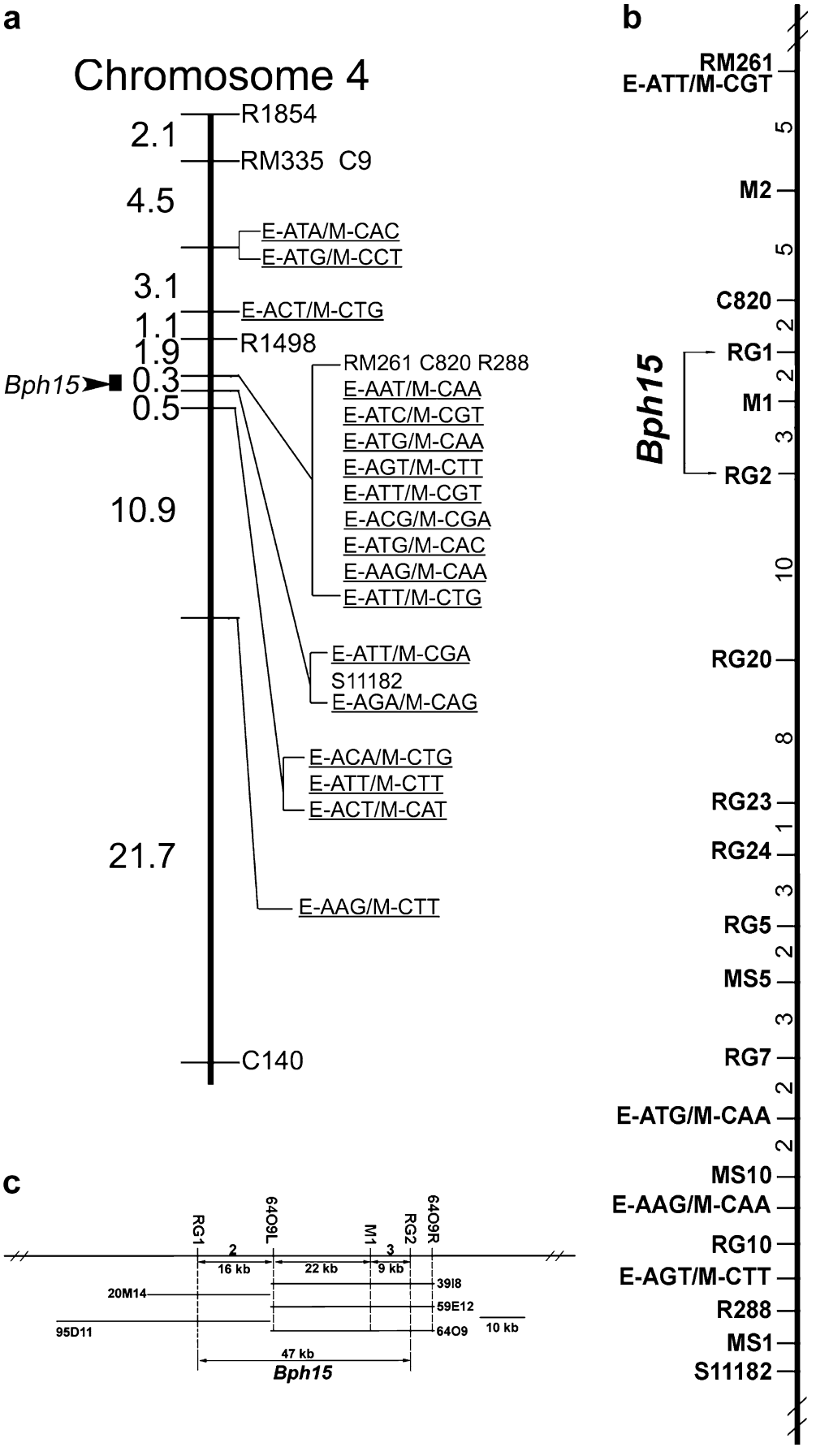

Fig. 2 **a** Integration of the newly identified AFLP markers into the genetic map (Ren et al. 2004) of the *Bph15* region. The AFLP fragments identified in this study are *underlined*. One primer combination, E-AGA/M-CAG, generated two fragments mapped in the same position on the map. **b** Fine-scale linkage map around the *Bph15* locus made with 9,472 segregating plants. *Vertical bars* represent the positions of molecular markers, *numbers between markers* indicate the numbers of plants in which recombination occurred between each pair of adjacent markers. **c** Physical map encompassing the *Bph15* locus. *Long horizontal line* indicates the chromosome region containing the *Bph15* gene, *short horizontal lines* represent BAC clones, *dashed vertical lines* indicate that markers are contained in each clone. The physical distances between markers were deduced by analyzing the sequences of the BAC clones

RM3917, RM6156, RM2848 and RM5953) are distributed in the *Bph15* region. Unfortunately, all of these nine SSR markers were either monomorphic between the resistant RIL RI93 and susceptible variety TN1 or they amplified poorly and could not be used for genetic mapping. An additional 47 new primer pairs were generated on the basis of the DNA sequences of the *Bph15* region, of which 22 bracketed SSR markers and 25 STS markers were designed to amplify the single-copy genomic fragments. Of the newly designed SSR markers, three (MS1, MS5 and MS10) were polymorphic between RI93 and TN1. All of the 25 STS markers amplified PCR products of the same sizes in the RI93 and TN1 genotypes. These STS markers were then converted into RFLP markers, of which eight (RG1, RG2, RG5, RG7, RG10, RG20, RG23 and RG24) were polymorphic between RI93 and TN1. In total, 11 newly developed markers from the genomic sequence information were usable for fine mapping of the *Bph15* resistance gene. The primers for the 11 newly designed markers, including three SSR markers and eight STS-derived RFLP markers, are shown in Table 2.

Regional high-resolution map and placement of the *Bph15* resistance gene

To precisely determine the genetic distance between molecular markers in the region of *Bph15*, we used flanking markers RM261 and MS1 to screen for recombinant individuals in a large F_2 population derived from a cross between RI93 × TN1. The two marker genotypes were analyzed and compared in 9,472 plants, corresponding to a genetic resolution of 0.005 cM, and 48 recombinants were identified.

The location of the *Bph15* resistance gene on the map was determined on the basis of the resistance score of the 48 recombinant plants. Special attention was paid to the phenotyping of these 48 recombinant plants. We evaluated the resistance scores for both the recombinant F_2 individuals and their corresponding F_3 progenies. The tiller testing technique (Fu et al. 1994) was employed to distinguish resistant plants from susceptible ones among the recombinant plants. The bulked seedling test of the F_3 progeny confirmed the phenotype of susceptible F_2 plants and further distinguished the heterozygous from the homozygous among the resistant plants. In the F_2 bioassay, 11 F_2 plants died 7 days following infestation with BPH insects, while 37 recombinant F_2 individuals survived for 15 days after infestation. Consequently, the 11 F_2plants were classified as susceptible (S) and the remaining 37 F_2 plants were categorized as resistant (R). The ratio of resistant to susceptible recombinant F_2 plants fitted the expected 3(R):1(S) ratio ($\chi^2 = 0.11$, $P > 0.7$). In the F_3 experiment, all of the seedlings of the 11 susceptible parent F_2 plants were given a score of 9 within 10 days after infestation. This result confirmed that the *Bph15* genotypes of these 11 F_2 plants were BPH susceptible (S). The heterozygous *Bph15* genotype showed an obvious segregation of BPH resistance in the F_3 family, with the proportion of resistant progenies ranging from 60% to 75%. The segregation of R and S plants in the complete set of F_3 progenies deviated from the 3(R):1(S) ratio, with there being more susceptible progenies than expected for the single dominant gene-control of resistance. Homozygous resistance was thus judged on the basis of more than 90% resistant progenies because occasionally less than 10% of the seedlings of the resistant parent F_2 plants showed a susceptible-like response. Murai et al. (2001) observed a similar phenomenon when they studied the *bph2* resistance gene. Subsequently, nine F_2 plants were classified as BPH-resistant (R), 28 as segregating (H) and 11 as susceptible (S) types, showing a good fit to the expected ratio of 1(R):2(H):1(S) ($\chi^2 = 1.5$, $P > 0.3$) (Table 3).

The markers around the *Bph15* chromosome region were assayed in the 48 recombinants, and a local linkage map was constructed (Fig. 2b). This map contains 21 DNA markers spanning the region from RM261 to MS1. No double recombinants were detected by intervening markers in the chromosome region flanked by RM261 to MS1. Normal segregation was observed for the marker loci around the *Bph15* gene from this flanking region as indicated by chi-square tests for goodness-of-fit to a 1:2:1 ratio. The resulting high-resolution map of *Bph15* showed that RG1 and RG2 were

Table 2 Markers used in fine genetic mapping of the *Bph15* gene together with their locus name, PCR product size, primer sequences and marker type. These markers were generated from the publicly available sequence information on Nipponbare BAC clones spanning the *Bph15* locus

Marker name	PCR product length (bp)	Forward primer (5′–3′)	Reverse primer (5′–3′)	Marker type
MS1	197	catggacccacttgtcatcc	agcatgagagactgccaagg	SSR
MS5	215	ttgtgggtcctcatctcctc	tgacaacttgtgcaagatcaaa	SSR
MS10	168	caatacgagaagcccctcac	ctgaaggaacacgcggtagt	SSR
RG1	355	tgaaggatcagtttggcaat	catccagtctccaccaggac	STS-RFLP
RG2	434	ttcaccaaccctctcctc	acttggcaacctctttcatc	STS-RFLP
RG5	360	tgtctacccctctttcttgttc	ctataatagtggcagatgcacc	STS-RFLP
RG7	445	ttaactcggcacgcaatg	agacaccagatcccaacac	STS-RFLP
RG10	393	atgctgccactaccaaatac	gcagaaggacaaaccaacac	STS-RFLP
RG20	363	gctccacgatgaggttcttg	acaaagcttctggcggttc	STS-RFLP
RG23	361	acctagctcaagttactgcc	tccacctcttcttttctcacc	STS-RFLP
RG24	362	ctccagaaagctcctagcttg	cgactcaaaccttggtcagtg	STS-FLP

Table 3 Segregation of BPH resistance conferred by *Bph15* in the recombinant F_2 and F_3 population derived from the cross between RI93 and TN1

F_2 genotype of BPH resistance	Number of F_2 plants[a]	Number of F_3 progenies
R	9	642
H	28	2,146[b]
S	11	846
Total	48	3,634

[a] χ^2 value for 1(R):2(H):1(S) = 1.5, $P > 0.3$
[b] χ^2 value for 3(R) (1,382) : 1(S) (764) = 129, $P < 0.001$

the most closely linked markers flanking the *Bph15* resistance gene. Two recombinants were identified between RG1 and *Bph15*; three recombinants were identified between RG2 and *Bph15*. The STS marker M1, derived from AFLP marker E-ATG/M-CAC, co-segregated with *Bph15* (Fig. 2b).

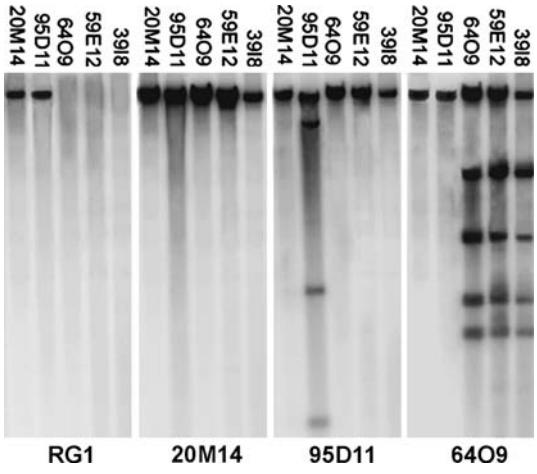

Fig. 3 Southern blot analysis of *Bam*HI-digested BAC clones covering the *Bph15* region. Five positive clones (20M14, 95D11; 64O9, 59E12, 39I8) were *Bam*HI-digested and transferred onto the filter. The filters were hybridized with RG1, and with *Bam*HI-digested 20M4, 95D11 and 64O9, respectively

Physical mapping of the *Bph15* locus

To identify BAC clones covering the *Bph15* gene, markers RG1 and RG2 were used to screen the BAC library for B5 by hybridizing to high-density filters on which the entire library was arrayed. Two positive clones, 20M14 (27 kb) and 95D11 (47 kb), were identified by RG1. Three positive clones, 39I8, 59E12 and 64O9, each 36 kb long, were identified by RG2. These BAC clones were digested by *Bam*HI and transferred to a nylon filter. The filter was sequentially hybridized with the markers that identified the positives clones and also with the BAC clones that were included in the filter. Three positive clones, 39I8, 59E12, 64O9, showed the same *Bam*HI fingerprint (Fig. 3). 20M14 possessed only one *Bam*HI digestion fragment, which had the same size as the vector pCLD04541. 95D11 possessed all of the fragments generated by *Bam*HI digestion of 20M14, plus three additional fragments (Fig. 3). This led to the conclusion that 20M14 is included in 95D11 and that 39I8, 59E12 and 64O9 are identical. Comparison of the *Bam*HI fingerprints of 95D11 and 64O9 did not reveal any physical relationship between them. The co-segregating marker M1 was then used to identify BAC clones filling this chromosome region by screening the BAC DNA pools. 39I8, 59E12 and 64O9 were confirmed to be positive for M1 (data not shown).

The BAC ends of clone 20M14, 95D11 and 64O9 were isolated by TAIL-PCR. The ends of 20M14 and 95D11 were monomorphic between RI93 and TN1 and, consequently, could not be mapped. The ends of 64O9 (64O9L and 64O9R) showed polymorphism and were used to assay the 48 recombinant individuals. Three recombinant events were detected between 64O9R and *Bph15*, and no recombination was detected between 64O9L and *Bph15*. The 64O9L end was used to hybridize the high-density filters, and no additional positive clone was identified. The ends of 95D11, 20M14 and 64O9 were then sequenced and compared. BLASTN searches against the rice genome showed that each of these BAC ends produced a single hit. The sequence of 20M14R and that of 95D11R were identical . Sequence alignment results indicated that the 20M14R and 64O9L sequences are contiguous within the rice genome, overlapping at the *Bam*HI restriction site (data not shown). In order to confirm that there was no gap between 20M14R and 64O9L, two primers (F-5′-cgtccctcttatcaccgtca-3′ and R-5′-cgcatatgcatctcacatgg-3′) were designed, based on the sequences of 20M14R and 64O9L, to amplify the B5 genomic DNA. The sequencing result of the corresponding amplified genomic DNA fragment validated that 20M14R and 64O9L were contiguous DNA sequences within the B5 genome. All of this evidence led to the conclusion that clones 20M14 and 64O9 covered the *Bph15* locus.

The RG1, M1 and RG2 sequences were used for BLASTN searches against the Nipponbare genomic sequence database (http://www.gramene.org/). The results revealed that they were single-copy fragments in the rice genome and that the physical distances between RG1, 64O9L, M1 and RG2 were about 22, 18 and 12 kb, respectively. The BAC clones 20M14, 95D11 and 64O9 were sequenced at the National Center for Gene Research of China. Sequence analysis of these BAC clones (Yang et al., unpublished observations) revealed that genomic segment flanked by RG1 and RG2 was approximately 47 kb, which is 5 kb shorter than the sequence delimited by the same markers in Nipponbare. The physical distances in B5 between RG1, 64O9L, M1 and RG1 can be deduced to be approximately 16, 22 and 9 kb, respectively (Fig. 2c).

Discussion

In this study we present a genetic fine mapping and physical delineation of the BPH resistance gene *Bph15* on chromosome 4. Starting from the flanking markers C820 and S11182 that are more than 2 Mb physically apart, we ultimately were able to locate the gene to a 47-kb segment of genomic DNA. This work represents a prerequisite step for positional cloning of the BPH resistance gene. The closely linked molecular markers developed in this study should be very useful in marker-aided breeding programs against BPH.

Map-based cloning is applicable to any gene known only by its phenotype, especially those with characteristics of QTLs (Takahashi et al. 2001). However, *Bph15* is located close to the centromere of chromosome 4, a position that is difficult to map. The centromeric region of rice chromosomes is characterized by a number of clusters of CentO satellite repeats and centromeric retroelements (CRs) (Wu et al. 2004; Zhang et al. 2004). Because of the highly heterochromatic structure of the centromeres, the complete cloning, sequencing and assembling of the genomic components of this region have remained a significant challenge. Meiotic recombination is generally suppressed across the centromere of eukaryotic chromosomes. However, a low level of recombination in or near centromeres has been detected in plants (Copenhaver et al. 1998; Anderson et al. 2003). In the rice genome, the ratio of the physical distance to genetic distance is on average approximately 260 kb per centiMorgan (Chen et al. 2002). According to published data, the ratio of physical distance to genetic distance around the region of *Bph15* is more than 1Mb per centiMorgan. The suppression of recombination frequency at the *Bph15* locus was observed in this study for only 48 recombinants within the *Bph15* region that were identified by the analysis of 9,472 F_2 plants. Isolating *Bph15* via map-based cloning is a challenge for which the chromosome landing strategy would be feasible, as proposed by Tanksley et al. (1995).

To find markers tightly linked to the *Bph15* gene, we used saturation mapping with large segregating populations. BSA combined with the AFLP technique has been proven to be an effective method for detecting molecular markers tightly linked to genes of interest (Murai et al. 2001; Dussle et al. 2003). We successfully identified 18 primer combinations that produced 19 fragments closely linked to the *Bph15* locus. Based on the sequence data of rice chromosome 4 (Feng et al. 2002), we further developed 22 SSR markers and 25 STS markers around *Bph15*. Using this approach, the genetic map developed in this study is easily tied to the physical and sequence map developed by the IRGSP (http://rgp.dna.sffrc.go.jp/; http://www.gramene.org/). Three SSR markers detected polymorphism between RI93 and TN1, and eight STS markers showed polymorphism when converted into RFLP markers. SSR markers are relatively abundant and polymorphic in the rice genome (Temnykh et al. 2001; Goff et al. 2002). However, in this study RFLP markers derived from STS markers were more polymorphic (32%) than SSR markers (13.6%), indicating that the polymorphic rate of different marker types might vary in different chromosome regions. We ultimately successfully saturated the *Bph15* region with 21 informative markers at a resolution of 0.005 cM.

The identification of BACs carrying the most closely linked markers is a crucial step towards the cloning of a gene. Using the tightly linked markers RG1 and RG2, we successfully obtained the BAC clones spanning the *Bph15* locus without chromosome walking. Two and three positive clones were identified by markers RG1 and RG2 in the B5 BAC library, respectively. Given the fivefold redundancy of the B5 BAC library (Shi et al. 2003), the number of positive clones identified by these two single-copy probes was fewer than expected. This may be due to the DNA fragments of the centromeric region being difficult to clone. Southern blotting and sequencing of the positive clones showed that two clones, 20M14 and 64O9, covered the *Bph15* locus. The *Bph15* region, delimited by RG1 and RG2, is approximately a 47-kb genomic segment.

Recombination is apparently not evenly distributed along the segment of the genome encompassing the *Bph15* locus. No crossover was observed between 64O9L and M1, which is a 22-kb segment. In contrast, three recombinant events took place between M1 and RG2, a 9-kb segment. The genomic segment between RG1 and RG2 is about 52 kb in *japonica* rice Nipponbare, while in B5 it is 5 kb shorter. This difference in length could arise from varietal genomic diversity.

GENSCAN analysis indicated that the 47-kb genomic segment contained 11 open reading frames. Rice has become a model plant for structural and functional genomic studies, and *Agrobacterium*-mediated transformation is a routine practice in rice gene manipulation. We are initiating complementary experiments to test which gene present in the B5 genome flanked by RG1 and RG2 will confer resistance against BPH when introduced into BPH-susceptible plants.

Acknowledgements We sincerely thank Dr. B. Han for sequencing the BAC clones. This research was supported by grants from the National Special Key Project on Functional Genomics and Biochip of China, the National Program of High Technology Development (2001AA227151) and the National Natural Science Foundation of China (30170085). The work has been carried out in compliance with the current laws governing genetic experimentation in China.

References

Anderson LK, Doyle GC, Brigham B, Carter J, Hooker KD, Lai A, Rice M, Stack SM (2003) High-resolution crossover maps for each bivalent of *Zea mays* using recombination nodules. Genetics 165:849–865

Chen M, Presting G, Barbazuk W, Goicoechea J, Blackmon B, Fang G, Kim H, Frisch D, Yu Y, Sun S, Higingbottom S, Phimphilai J, Phimphilai D, Thurmond S, Gaudette B, Li P, Liu J, Hatfield J, Main D, Farrar K, Henderson C, Barnett L, Costa R, Williams B, Walser S, Atkins M, Hall C, Budiman M, Tomkins J, Luo M, Bancroft I, Salse J, Regad F, Mohapatra T, Singh N, Tyagi A, Soderlund C, Dean R, Wing R (2002) An integrated physical and genetic map of the rice genome. Plant Cell 14:537–545

Copenhaver GP, Browne WE, Preuss D (1998) Assaying genome-wide recombination and centromere functions with *Arabidopsis* tetrads. Genetics 95:247–252

Dussle C, Quint M, Melchinger A, Xu M, Lübberstedt T (2003) Saturation of two chromosome regions conferring resistance to SCMV with SSR and AFLP markers by targeted BSA. Theor Appl Genet 106:485–493

Feng Q, Zhang YJ, Hao P, Wang SG, Fu G, Huang YC, Li Y, Zhu JJ, Liu YL, Hu X, Jia PX, Zhang Y, Zhao Q, Ying K, Yu SL, Tang YS, Weng QJ, Zhang L, Lu Y, Mu J, Lu Q, Zhang LS, Yu Z, Fan DL, Liu XH, Lu TT, Li C, Wu YR, Sun TG, Lei HY, Li T, Hu H, Guan JP, Wu M, Zhang RQ, Zhou B, Chen ZH, Chen L, Jin ZQ, Wang R, Yin HF, Cai Z, Ren SG, Lv G, Gu WY, Zhu GF, Tu YF, Jia J, Zhang Y, Chen J, Kang H, Chen XY, Shao CY, Sun Y, Hu QP, Zhang XL, Zhang W, Wang LJ, Ding CW, Sheng HH, Gu JL, Chen ST, Ni L, Zhu FH, Chen W, Lan LF, Lai Y, Cheng ZK, Gu MH, Jiang JM, Li JY, Hong GF, Xue YB, Han B (2002) Sequence and analysis rice chromosome 4. Nature 420:316–320

Frohman LA, Mizobuchi M (1992) A rapid and simple method for labeling short DNA fragments using *Taq* polymerase. Biotechniques 12:350–354

Fu Q, Chen W, Zhang ZT (1994) Evaluation for resistance of the interspecific somatic rice hybrids to *Nilaparvata lugens*. Chin J Rice Sci 8:247–249

Goff SA, Ricke D, Lan TH, Presting G., Wang R, Dunn M, Glazebrook J, Sessions A, Oeller P, Varma H et al (2002) A draft sequence of the rice genome (*Oryza sativa* L. ssp. *japonica*). Science 296:92–100

Heinrichs EA (1979) Control of leafhopper and planthopper vectors of rice viruses. In: Moramorosch K, Arris KF (eds) Leafhopper vectors and planthopper disease agents. Academic Press, New York, pp 529–558

Hirabayashi H, Ogawa T (1995) RFLP mapping of *Bph-1* (the brown planthopper resistance gene) in rice. Jpn J Breed 45:369–371

Hirabayashi H, Angeles ER, Kaji R, Ogawa T, Brar DS, Khush GS (1998) Identification of the brown planthopper resistance gene derived from *O. officinalis* using molecular markers in rice (abstract in Japanese). Breed Sci 48[Suppl1]:82

Huang Z, He GC, Shu LH, Li XH, Zhang QF (2001) Identification and mapping of two brown planthopper resistance genes in rice. Theor Appl Genet 102:929–934

Ikeda R (1985) Studies on the inheritance of resistance to the rice brown planthopper (*Nilaparvata lugens* Stål) and the breeding of resistant rice cultivars. Bull Nat Agric Res Cent 3:51–54

IRRI (1988) Standard evaluation system for rice. International Rice Research Institute, Manila

Ishii T, Brar DS, Multani DS, Khush GS (1994) Molecular tagging of genes for brown planthopper resistance and earliness introgressed from *Oryza australiensis* into cultivated rice, *O. sativa*. Genome 37:217–221

Kawaguchi M, Murata K, Ishii T, Takumi S, Mori N, Nakamura C (2001) Assignment of a brown planthopper (*Nilaparvata lugens* Stål) resistance gene *bph4* to the rice chromosome 6. Breed Sci 51:13–18

Khush GS (1979) Genetics of and breeding for resistance to brown planthopper. Threat to rice production in Asia. International Rice Research Institute, Los Banos

Lincoln S, Daly M, Lander ES (1992) Constructing genetic maps with MAPMAKER/EXP 3.0, 3rd edn. Whitehead Institute Technical Report, Cambridge, Massachusetts

Liu GY, Yan HH, Fu Q, Qian Q, Zhang ZT, Zhai WX, Zhu LH (2001) Mapping of a new gene for brown planthopper resistance in cultivated rice introgressed from *Oryza eichingeri*. Chin Sci Bull 46:1459–1462

Liu KD, Wang J, Li HB, Xu CG, Liu AM, Li XH, Zhang Q (1997) A genome-wide analysis of wide compatibility in rice and the precise location of the *S5* locus in the molecular map. Theor Appl Genet 95:809–814

Liu Y, Whittier R (1995) Thermal asymmetric interlaced PCR: Automatable amplification and sequencing of insert end fragments from P1 and YAC clones for chromosome walking. Genomics 25:674–681

Murai H, Hashimoto Z, Sharma PN, Shimizu T, Murata K, Takumi S, Mori N, Kawasaki S, Nakamura C (2001) Construction of a high-resolution linkage map of a rice brown planthopper (*Nilaparvata lugens* Stål) resistance gene *bph2*. Theor Appl Genet 103:526–532

Murata K, Fujiwara M, Kaneda C, Takumi S, Mori N, Nakamura C (1998) RFLP mapping of a brown planthopper (*Nilaparvata lugens* Stål) resistance gene *bph2* of *indica* rice introgressed into a *japonica* breeding line "Norin-PL4". Genes Genet Syst 73:359–364

Murata K, Fujiwara M, Murai H, Takumi S, Mori N, Nakamura C (2001) Mapping of a brown planthopper (*Nilaparvata lugens* Stål) resistance gene *Bph9* on the long arm of rice chromosome 12. Cereal Res Commun 29: 245-250

Murray MG, Thompson WF (1980) Rapid isolation of high molecular-weight plant DNA. Nucleic Acids Res 8:4321–4325

Panaud O, Chen X, McCouch SR (1996) Development of microsatellite markers and characterization of simple sequence length polymorphism (SSLP) in rice (*Oryza sativa* L.). Mol Gen Genet 252:597–607

Ren X, Weng QM, Zhu LL, He GC (2004) Dynamic mapping of quantitative trait loci for resistance to brown planthopper in rice. Cereal Res Commun 32:31–38

Renganayaki K, Fritz AK, Sadasivam S, Pammi S, Harrington SE, McCouch SR, Kumar SM, Reddy AS (2002) Mapping and progress toward map-based cloning of brown planthopper biotype-4 resistance gene introgressed from *Oryza officinalis* into cultivated rice, *O. sativa*. Crop Sci 42:2112–2117

Sharma PN, Ketipearachchi Y, Murata K, Torii A, Takumi S, Mori N, Nakamura C (2003) RFLP/AFLP mapping of a brown planthopper (*Nilaparvata lugens* Stål) resistance gene *Bph1* in rice. Euphytica 129(1):109–117

Shi Z, Ren X, Weng Q, Li X, He GC (2003) Construction of a genomic library from a brown planthopper-resistant rice line using a transformation-competent vector and identification of clones spanning the *Qbp1* locus. Plant Sci 165:879–885

Sogawa K, Liu GJ, Shen JH (2003) A review on the hyper-susceptibility of Chinese hybrid rice to insect pests. Chin J Rice Sci 17:23–30

Takahashi Y, Shomura A, Sasaki T, Yano M (2001) *Hd6*, a rice quantitative trait locus involved in photoperiod sensitivity, encodes the α subunit of protein kinase CK2. Proc Natl Acad Sci U S A 98:7922–7927

Takita T (1996) A new dominant gene for brown planthopper resistance found in an improved Japanese rice strain (abstract in Japanese). Breed Sci 46 [Suppl 1]:211

Tanksley SD, Ganal MW, Martin GB (1995) Chromosome landing: a paradigm for map-based gene cloning in plants with large genomes. Trends Genet 11:64–68

Temnykh S, Declerck G, Lukashova A, Lipovich L, Cartinhour S, McCouch S (2001) Computational and experimental analysis of microsatellites in rice (*Oryza sativa* L.): frequency, length variation, transposon associations, and genetic marker potential. Genome Res 11:1441–1452

Vos P, Hogers R, Bleeker M, Reijans M, Van De Lee T, Hornes M, Fritjers A, Pot J, Peleman J, Kuiper M, Zabeau M (1995) AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res 23:4407–4414

Wu J, Yamagata H, Hayashi-Tsugane M, Hijishita S, Fujisawa M, Shibata M, Iton Y, Nakamura M, Sakaguchi M, Yoshihara R, Kobayashi H, Ito K, Karasawa W, Yamamoto M, Saji S, Katagiri S, Kanamori H, Namiki N, Katayose Y, Matsumoto T, Sasaki T (2004) Composition and structure of the centromeric region of rice chromosome 8. Plant Cell 16:967–976

Xu J, Yang D, Domingo J, Ni J, Huang N (1998) Screening for overlapping bacterial artificial chromosome clones by PCR analysis with an arbitrary primer. Proc Natl Acad Sci USA 95:5661–5666

Yang HY, Ren X, Weng QM, Zhu LL, He GC (2002) Molecular mapping and genetic analysis of a rice brown planthopper (*Nilaparvata lugens* Stål) resistance gene. Hereditas 136:39–43

Zhang Y, Huang Y, Zhang L, Li Y, Lu T, Lu Q, Feng Q, Zhao Q, Cheng Z, Xue Y, Wing RA, Han B (2004) Structural features of the rice chromosome 4 centromere. Nucleic Acids Res 32:2023–2033