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Identification of a Rice Gene (*Bph 1*) Conferring Resistance to Brown Planthopper (*Nilaparvata lugens* Stal) Using STS Markers

Suk-Man Kim and Jae-Keun Sohn*

Department of Agronomy, College of Agriculture, Kyungpook National University, Daegu 702-701, Korea.

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This study was carried out to identify a high-resolution marker for a gene conferring resistance to brown planthopper (BPH) biotype 1, using japonica type resistant lines. Bulked segregant analyses were conducted using 520 RAPD primers to identify RAPD fragments linked to the BPH resistance gene. Eleven RAPDs were shown to be polymorphic amplicons between resistant and susceptible progeny. One of these primers, OPE 18, which amplified a 923 bp band tightly linked to resistance, was converted into a sequence-tagged-site (STS) marker. The STS marker, BpE18-3, was easily detectable as a dominant band with tight linkage (3.9cM) to *Bph1.* It promises to be useful as a marker for assisted selection of resistant progeny in backcross breeding programs to introgress the resistance gene into elite japonica cultivars.

Keywords: *Bph1*; Marker Assisted Selection; *Oryza sativa* L.; STS Marker.

Introduction

The brown planthopper (BPH), *Nilaparvata lugens*, is one of the most serious insect pests throughout rice growing areas in Asia. This is especially true in countries mainly culturing Japonica rice cultivars that do not have a gene conferring resistance to BPH, and where outbreaks of BHP are therefore a severe problem. The BPH causes direct damage to crops and indirect damage by acting as a vector for viral diseases. Chemical treatment is the conventional method of controlling pests such as BPH, even though it is expensive and harmful to the environment. Many researchers have reported that host plant resistance is the most effective way of controlling pests including BPH, and thus breeding of insect resistance has taken priority in rice improvement programs. Until now, 13BPH resistance genes, together with several quantitative trait loci (QTLs) controlling BPH resistance (Alam and Cohen, 1998; Xu *et al.*, 2002), have been reported in two wild relatives and indica cultivars (Murai *et al.*, 2001). Diverse sources of BPH resistance have been identified and genetic analysis has revealed 6 dominant [*Bph1*, *3*, *6*, *9*, *10*, and 13(*t*)] and 7 recessive [*bph2*, *4*, *5*, *7*, *8*, *11*(*t*), and *12*(*t*)] genes controlling BPH resistance (Ishii *et al.*, 1994; Khush *et al.*, 2001). *Bph1*, *bph2*, *Bph9*, and *Bph10*(*t*) were assigned to rice chromosome 12 (Ishii *et al.*, 1994; Murata *et al.*, 2000). *Bph1* and *bph2* confer resistance to biotypes 1, 3 and 1, 2 which are widely distributed in Southeast Asia (Khush and Brar, 1991). Many studies aimed at identifying BPH resistance genes have been conducted over the years in order to develop a resistant cultivar; however, a japonica cultivar with a BPH resistance gene has not yet been developed. Thirteen of the BPH resistance genes identified so far are not from japonica rice, but from indica rice. In bioassays, it has been reported that the reaction of early rice seedlings to BPH differed between japonica and indica (Yeo and Sohn, 1995). Japonica introgression lines with BPH resistance genes exhibited undesirable characteristics such as poor grain quality and lodging-related traits to which the resistance genes seemed to be highly linked (Yeo and Sohn, 2001). The undesirable linkage drag between a BPH resistance gene and genes conferring agriculturally important characters may be removed by intensive work to select recombinants between the traits, and a molecular marker tightly linked to the target gene could be useful for selecting the desired recombinants.

The use of tightly linked genetic markers for resistance genes offers great scope for improving the efficiency of conventional plant breeding by allowing selection to be

* To whom correspondence should be addressed.
Tel: 82-53-950-5711; Fax: 82-53-958-6880
E-mail: jhsohn@knu.ac.kr

Abbreviations: BPH, brown planthopper; STS, sequence-tagged-site.

based on molecular markers linked to a trait at an early stage of growth, rather than being based on the trait itself. Resistance genes to gall midge and blast have been identified in rice (Han *et al.*, 2004), and linkage between DNA markers and these resistance genes has been analyzed (Nair *et al.*, 1996; Zenbayashi *et al.*, 2002). In this way linkage maps of genes associated with resistance to diseases and pests have been constructed in wheat, barley, and other economically important crops (Tóth *et al.*, 2003; Weerasena *et al.*, 2004).

The current study was conducted to identify *Bph1*-related DNA markers in rice, and thus to permit the establishment of a marker-assisted breeding program to introgress the BPH-resistance gene into japonica rice cultivars.

Materials and Methods

Plant material A cross of Samgangbyeo (BPH-resistant Tongil-type cultivar) and Nagdongbyeo (BPH-susceptible japonica) was used for this study. Samgangbyeo has a gene that confers resistance to BPH biotype 1 derived from either (or both) Mudgo and TKM6 (Ha *et al.*, 1999; Kim *et al.*, 2004). 64 anther culture-derived DH lines were used for bulked segregant analysis (BSA), and a segregating F_2 population consisting of 158 plants was screened for resistance to BPH biotype 1 and used for linkage analysis of RAPD markers.

Culture procedures for the DH population F_1 plants of a cross of Samgangbyeo and Nagdongbyeo were grown in a paddy-style rice field at Kyungpook National University (Korea). Anther culture methods were applied to the F_1 plants; chilled anthers were cultured by a two-step method. The inflorescences, incubated at 12°C for 10 d, were surface-sterilized using 70% ethanol for 20 s, followed by rinsing three times with sterile distilled water. The anthers were cultured on a Chu medium supplemented with 1 mg/L 2-4-D, 0.1 mg/L zeatin, 20 g/L sorbitol, and 5 g/L gelrite, according to the one-step culture method (Kwon *et al.*, 2002). After 30 days, calli derived form the cultured anthers were transferred to a regeneration medium consisting of 2 mg/L kinetin, 0.2 mg/L IAA 30 g/L sucrose and 5 g/L gelrite.

Screening for reaction to BPH The parents and the DH lines were screened in five replications for their reaction to BPH biotype 1 provided by the National Yeongnam Agricultural Research Institute (YARI), Korea, following the standard bulk seedling test procedure (Kalode and Krishna, 1979). A bioassay was conducted to compare the levels of BPH resistance between Samgangbyeo and Hangangchalbyeo, and Nagdongbyeo and TN1 were used as susceptibility controls. To screen resistant and susceptible plant, we established a checkpoint at which most controls (susceptible cultivar, Nagdongbyeo) died, three to four leaves shrank, or two to four leaves shriveled. Each of the 158 F_2 plants was grown for 25 d in a plug box with 72 holes (3 × 3 × 3 cm) before sampling for DNA extraction and subsequent rationing for 7 d. After inoculation at the rate of 15–20 nymphs per seedling for about 2 weeks, F_2 plants were examined and each seedling was given an R or S classification according to the above criteria (Ha *et al.*, 2000).

RAPD analysis Equal amounts of DNA from 10 resistant and 10 susceptible DH plants were pooled to form the resistant and susceptible bulks. These were used together with the parental DNA in the following BSA. 520 random primers from Operon Tech were used to identify closely linked markers. Amplification reactions were carried out in a total volume of 12 μl containing 20 ng of genomic DNA, 50 mM Tris-HCl, pH 8.5, 2 mM $MgCl_2$, 50 mM KCl, 200 μM dNTP, 0.2 μM of each primer, and 0.5 units of *Taq* DNA polymerase. The initial step of amplification was denaturation at 94°C for 5 min, followed by 40 cycles of 94°C for 30 s, 40°C 30 s, 72°C 1 min, and a final extension at 72°C for 5 min. The PCR products were resolved by electrophoresis on 1.2% agarose gels.

Development of an STS marker from the polymorphic band A RAPD marker linked to *Bph 1* was isolated from the agarose gels using a gel extraction kit. The purified amplicon was cloned into plasmid vector pGEM-T (Promega, USA), which was then transformed into competent *E. coli* JM 109 cells. Based on sequence data, we developed a PCR-based STS marker, BpE18-3, using the site-specific oligonucleotide primers BpE18-3F (5′-CGCTGCGAGAGTGTGACACT-3′) and BpE18-3R (5′-TTGGGTTACACGGGTTTGAC-3′).

Results

Reaction of plants to BPH 158 F_2 and DH plants from a cross Samgangbyeo/Nagdongbyeo were screened for resistance to BPH biotype 1. The F_1 of the cross was resistant to BPH biotype 1, indicating that BPH resistance is controlled by a dominant gene(s). Segregation analysis of the 158 F_2 population gave a good fit to the 3 (resistance):1 (susceptibility) ratio ($x^2 = 0.3046$, $P < 0.50$) expected for a single dominant gene. The 64 DH lines also fitted the expected 1R: 1S ratio ($x^2 = 0.25$, $P < 0.50$).

Bulked-segregant RAPD analysis The two parental DNAs along with the resistant and susceptible bulks were screened using 520 RAPD primers to identify RAPD fragments linked to the BPH resistance gene. About 2,450 discrete products were generated by the 520 primers. The majority gave the same pattern in the two bulks, but eight primers generated specific dominant amplicons in either the resistant group, including the resistant parent (RP) and resistant bulk (RB), or the susceptible group, including the susceptible parent (SP) and susceptible bulk (SB). The markers were tested preliminarily using 46 randomly selected DH plants. Of these markers, OPE18 showed tight linkage to BPH resistance in the DH lines. Further analy-

Table 1. Segregation of BpE18-3 and BPH resistance in 158 F_2 plants derived from the cross Samgangbyeo/Nagdongbyeo.

		Phenotype of F_2 plants		
		Total	R	S
Polymorphism pattern of BpE18-3	+[a]	126	124	2
	–	32	4	28
	Total	158	128	30

[a] '+' and '–'denote presence and absence of BpE18-3, respectively.

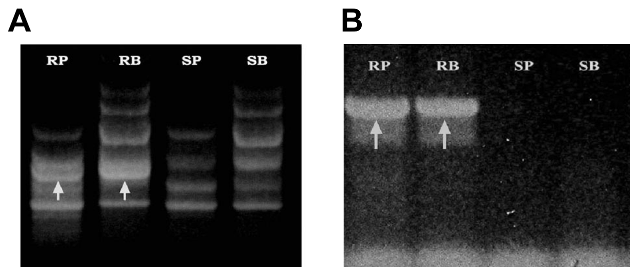

Fig. 1. Conversion of the RAPD marker into BpE18-3. **A.** The polymorphic band amplified by PCR with the OPE 18 primer. **B.** The single band of 523 bp amplified by STS primers designed from the sequence of the OPE 18_{923} fragment. Arrow indicates the polymorphic band closely related to *Bph 1.*

sis using the F_2 confirmed that OPE18 was tightly linked to the resistance gene (Table 1). A BHP-specific dominant fragment of 923 bp ($OPE18_{923}$), was amplified (Fig. 1A). This polymorphic band was isolated and cloned for further analysis.

Conversion of the RAPD band into an STS marker, and linkage analysis $OPE18_{923}$ was cloned and sequenced (GenBank accession No. AB200354), and three primer sets were synthesized based on the sequence. After performing PCR with these primer sets, a sequence-specific primer set, BpE18-3, was selected. PCR using BpE18-3-F and BpE18-3-R yielded a single 523 bp fragment in both the resistant parent and RB, but not in the susceptible parent or SB, indicating that BpE18-3 is a dominant marker (Fig. 1B). Blast search using the sequence of BpE18-3 (www.gramene.org) revealed that it had significant similarity (91.2%) to a BAC clone, AL 732381, on chromosome 12 (Fig. 2). Based on the map position of this BAC clone, we analyzed linkage relationships with RFLP markers located in the adjacent chromosomal region (Fig. 3). In the F_2 population of 128 resistant and 30 susceptible plants, two resistant plants did not yield the marker and two susceptible plants did yield the marker (Table 2). Figure 4 shows the linkage map constructed for *Bph1* and BpE18-3, together with the 5 RFLP markers located on rice chromosome 12. BpE18-3 mapped 3.9cM from *Bph1*, midway between RFLP markers, RG336 and RG901.

Table 2. Linkage between BPH resistance and four markers in the F_2 of Samgangbyeo/Nagdongbyeo.

Markers	Recombination value (%)	x^2	LOD	P
G 402 – RG 4	22.2	2.36^{ns}	10.7	0.05
RG 4 – G 261	7.6	1.50^{ns}	28.1	0.05
G 261 – RG 336	31.1	2.64^{ns}	4.7	0.05
RG336 – BpE18-3	47.1	2.84^{ns}	0.1	0.05
BpE18-3 – *Bph1*	3.9	1.10^{ns}	35.6	0.05
Bph1 – RG 901	32.3	2.70^{ns}	4.0	0.05

Discussion

In this study, a genetic factor for BPH resistance was found in the Tongil-type rice cultivar Samgangbyeo using a DH and F_2 population derived from a cross of Samgangbyeo and Nagdongbyeo. Through bulked segregant RAPD analysis, we developed an STS marker, designated BpE18-3, linked (3.9cM) to the BPH resistance gene, *Bph1*. It is easily detected by amplification and hence appears to be ideal for marker-aided selection of individuals in a breeding program.

A number of efforts have been made over the years to determine the chromosomal locations of BPH resistance genes. Four of these genes, *Bph1*, *bph2*, *Bph9*, and *Bph10*(*t*), have been mapped on chromosome 12 (Ishii *et al.*, 1994; Murata *et al.*, 2000); the additional resistance genes *bph4*, *bph11*(*t*), *bph12*(*t*), and *Bph13*(*t*) have been assigned to chromosome 6, 3, 4, and 2, respectively (Hirabayashi and Ogawa, 1999; Kawaguchi *et al.*, 2001; Liu *et al.*, 2001). However, since these results were produced by analyzing relationship between DNA markers and responses to BHP without using the GenBank database, their chromosomal locations were unclear, and information for map-based cloning was lacking. In this study, to identify more precisely the chromosomal location of *Bph1*, we developed BpE18-3 using BSA, and aligned it with the Nipponbare BAC clone AL732381 (Fig. 2).

Recently, the Rice Genome Research Program (RGP) has released most of the rice genome sequence and constructed the Rice Annotation Database (RAD). The availability of this database provides a promising means for identifying genetic loci and isolating interesting genes (Sasaki *et al.*, 2005).

In East Asia, including Korea and Japan, a resistance breeding program has been initiated to develop cultivars resistant to *Bph1* or *bph2*, the major rice insect pests. *Bph1* is derived from Mudgo and TKM6, and *Bph1*-introgressed cultivars were used to develop resistant cultivars as resistant parents. BPH-resistance genes are only defined for BPH biotypes that have the ability to feed on rice plants and confer monogenic resistance. It is hard to

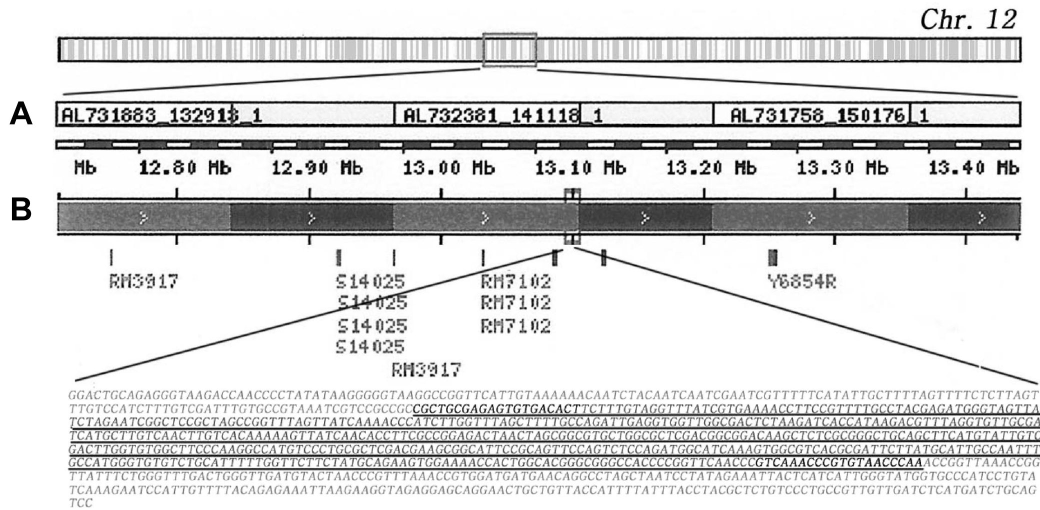

Fig. 2. Alignment of OPE18$_{923}$ with the BAC clone, AL732381 (japonica), of rice chromosome 12. The underlined portion indicates the sequence of the amplicon amplified by BpE18-3. The sequences shown in bold are present in the primer set, BpE18-3F and BpE18-3R. (A) Chromosome band; (B) DNA (contigs).

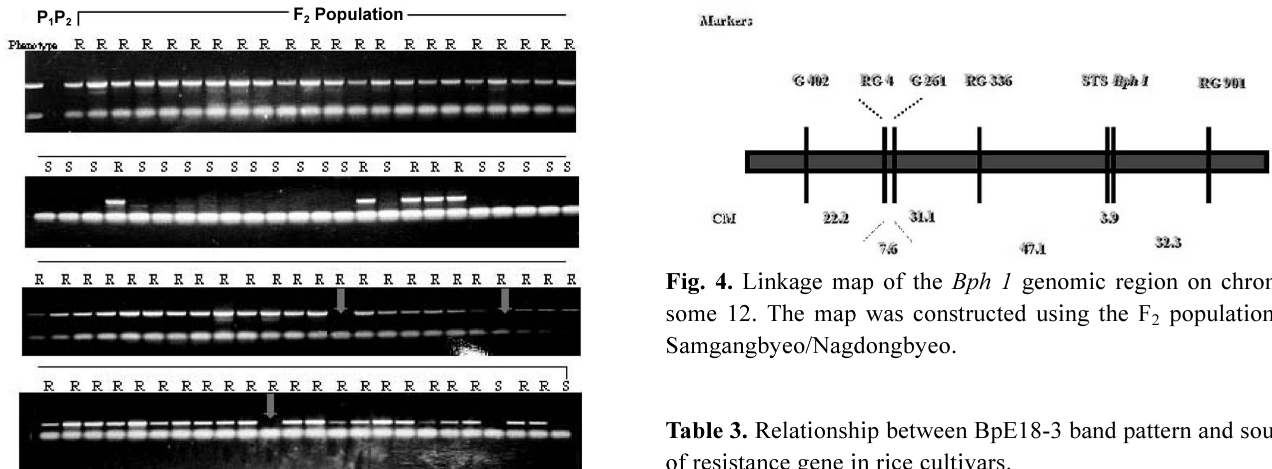

Fig. 3. Linkage analysis using the BpE18-3 marker. F$_2$ individuals shown as S are SS (homozygous susceptible), and all others are RR (homozygous resistant) or RS (heterozygous resistanct). Arrows (⇩) designate recombinants produced by crossing over in the DNA region. P$_1$, Samgangbyeo; P$_2$, Nagdongbyeo; R, resistance; S, susceptibility.

Fig. 4. Linkage map of the *Bph 1* genomic region on chromosome 12. The map was constructed using the F$_2$ population of Samgangbyeo/Nagdongbyeo.

classify new resistance genes by established methods depending on diet preference of them. However, the availability of tightly linked genetic markers will help in identifying plants carrying new genes conferring resistance against the same BPH biotype. In the current study, the BpE18-3 band depended on the resistant source (Table 3). All cultivars with the amplicon defined by this marker had TKM6 as the resistance donor in their parentage. This suggests that cultivars resistant to biotype1 can be classified according to source of resistance at the DNA marker level. Considering the fact that the occurrence of new virulent biotypes has been a serious problem in protecting rice against BPH, we believe that classification of the sources of resistance, and development of highly linked DNA markers, will be very important in developing a BPH-

Table 3. Relationship between BpE18-3 band pattern and source of resistance gene in rice cultivars.

Origin of BPH resistance	BpE18-3 reaction	Cultivar	BPH reaction
TKM6	+[1]	Baegunchalbyeo	R[3]
TKM6	+	Hangangchalbyeo	R
Mudgo, TKM6	+	Samgangbyeo	R
Mudgo, TKM6	–[2]	Andabyeo	R
Mudgo, TKM6	–	Namyoungbyeo	R
Mudgo	–	Cheongcheongbyeo	R
Mudgo	–	Nampungbyeo	R
Mudgo	–	Changseongbyeo	R
-	–	Nagdongbyeo	S

[1] – indicates absence of PCR product using BpE18-3.
[2] + indicates presence of PCR product using BpE18-3.
[3] R, resistance; S, susceptibility.

resistance breeding system.

We are currently developing mid-parent lines with BPH resistance using BpE18-3. 643 crossed seeds were produced from 27 crosses between several resistant DH lines and 6 japonica-type elite cultivars in 2003. Moreover, the backcrossed populations, BC_3F_1, BC_2F_1, and BC_2F_2, have been developed with the MAS system using the crossed seeds and the marker. We plan to use BpE18-3 for marker-assisted selection of BPH resistant progeny in backcross breeding programs.

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