Crop Breeding and Applied Biotechnology 13: 99-106, 2013
Brazilian Society of Plant Breeding. Printed in Brazil

ARTICLE

Pyramiding of two *BPH* resistance genes and *Stv-b*^{i} gene using marker-assisted selection in *japonica* rice

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Received 22 March 2012

Accepted 04 February 2013

Abstract – *Rice is affected by several plant diseases and insects that can cause severe yield losses. In this study, it is reported the introgression of resistance genes to brown planthopper (BPH) and rice stripe disease (RSD) in the* japonica *cultivars. Initially, four backcross (BC) lines were obtained using B5 as donor parent. Molecular markers linked to the resistance genes were used to identify the plants containing the genes of interest. In the present research, two BPH resistance genes (*Bph14 *and* Bph15*) and one RSD resistance gene (*Stv-b^{i}*) were successfully transferred into three* japonica *varieties via a marker-aided backcrossing procedure. The progeny lines with* Bph14 *and* Bph15 *genes showed high resistance to BPH, while the progeny lines with* Stv-b^{i} *gene showed high resistance to RSD. Yield research showed this is an effective way in rice breeding to control the damage from BPH and RSD, by using MAS of BPH genes and* Stv-b^{i} *gene.*

Key words: *Molecular marker-assisted selection, brown planthopper, rice stripe disease.*

INTRODUCTION

Rice (*Oryza sativa* L.) is a primary staple food crop for billions of people worldwide. In order to ensure global food security for the continuous population growth, it is vital to control the various insect pests and diseases that damage rice (Normile 2008).

Among the herbivorous rice insects, the brown planthopper (*Nilaparvata lugens*) is one of the most serious insect pests of tropical and temperate rice in Asia. The brown planthopper causes direct damage to crops and indirect damage by acting as a vector for viral diseases, and susceptible rice varieties often suffer severe yield losses annually from BPH infestations (Sogawa et al. 2003).

The genetics of BPH resistance have been well studied, and 19 BPH-resistance genes together with several quantitative trait loci (QTLs) controlling BPH resistance have been identified and assigned to rice chromosomes in *indica* cultivars and wild rice species (Zhang 2007). Some of these BPH-resistance genes have been incorporated mainly into *indica* rice varieties and released in production (Cohen et al. 1997, Cuong et al. 1997). However, the source of BPH resistance genes in temperate *japonica* rice germplasm is very limited because of narrow genetic diversity. Therefore, outbreaks of BPH are a severe problem in *japonica* production. It is imperative to identify BPH resistance genes from alternative sources and incorporate them into *japonica* rice cultivars.

Rice stripe disease is one of the most serious viral diseases in the temperate and subtropical regions of Asia, particularly Japan, China and Korea (Hayano-Saito et al. 2000). Rice stripe disease is caused by the rice stripe virus (RSV), and this virus is transmitted mainly by the small brown planthopper (*Laodelphax striatellus* Fallen). In China, the disease has caused serious grain yield losses of 5-10%, although the loss is usually 20-30% and can even be as high as 100% (Wu et al. 2010). The *Stv-b*^{i} gene, which originated from the *indica* variety Modan, is a widely utilized resistance gene for stripe virus disease. Most *japonica* cultivars, such as Zhendao88 and Xudao 3, have a *Stv-b*^{i} gene derived from Modan through breeding in last dacade. The gene *Stv-b*^{i} has been mapped in about 284 kb region on rice chromosome 11 (Hayano-Saito et al. 1998, Hayano-Saito et al. 2000). As the chromosomal interval of *Stv-b*^{i} was identified, its flanking markers can be used in MAS.

Chemical treatment is the conventional method of controlling BPH and RSD, even though it is expensive and harmful to the environment. Some studies have found that

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plant resistance is the most effective way of controlling pests, including BPH, and thus breeding of insect-resistant cultivars has taken priority in rice improvement programs (Kim and Sohn 2005).

Gene pyramiding, the combination of several resistance genes in a single cultivar conferring resistance to a specific race of the same pathogen, may yield a medium-to-long-term control (Kelly et al. 1995). A well succeeded example of this approach in rice breeding is the pyramiding of three genes (*xa5*, *xa13*, and *Xa21*) which confer resistance to bacterial blight caused by *Xanthomonas oryzae pv. Oryzae* (Xoo) (Sanchez et al. 2000). However, this strategy is difficult to accomplish by conventional breeding methods since the effect of individual resistance gene cannot be easily identified or measured in the presence of other resistance genes in a specific background. This strategy can be aided by molecular markers that allow simultaneous monitoring of several resistance genes (Miklas et al. 1993, Kelly et al. 1995). Using the closely linked or co-segregated molecular markers, the resistance genes could be directly selected. This method has become one of the effective ways in rice varieties breeding of resistance.

In this study, it was reported the successful use of SSR and STS markers in pyramiding two BPH resistance genes and one *Stv-b*^{i} gene into three elite *japonica* varieties (Shengdao 15, Shengdao 16, Xudao 3) through marker-assisted backcrossing.

MATERIAL AND METHODS

Genetic material

The *indica* variety 'B5' was used as a donor in this research. It is a long cultivated *indica* cultivar, but not a commercial cultivar in China. B5 is a highly resistant variety with two anti-BPH genes (*Bph14*, *Bph15*) derived from the wild rice (*Oryza officinalis*). Both genes had large effects on BPH resistance and acted essentially independent of each other in conferring the resistance. *Bph14* and *Bph15* are located on the long arm of chromosome 3 and the short arm of chromosome 4, respectively (Huang et al. 2001). They are both dominant BPH resistance genes, and *Bph14* was the first BPH-resistance gene that has been cloned (Du et al. 2009). Molecular markers closely linked with *Bph15* were also found, such as MRG4319, which is located in a genetic distance of 0.3 cM (Yang et al. 2004). The fidings above laid a foundation for the use of molecular marker assisted selection of *Bph14* and *Bph15*.

The *japonica* cultivars Shengdao 15, Shengdao 16 and Xudao 3 were used as the recurrent parents in this work. They are major cultivars in Huang-Huai area of China (Shandong province, north of Jiangsu province, north of Anhui province, hanzhong plain in Shanxi province, north and middle of Henan province) in recent years. The three cultivars were medium maturing *japonica* varieties, with the growing period of about 150 days. Shengdao 15 and Shengdao 16 were bred by the Shandong Rice Research Institute, while Xudao 3, by the Xuzhou Academy of Agricultural Sciences, in Jiangsu province. These three cultivars harbour the *Stv-b*^{i} gene, a widely utilized resistance gene for stripe virus disease, originated from the *indica* variety Modan.

Crosses

The three independent F_1 plants were obtained between the donor and the three recurrent parents. Three separate backcross programmes were conducted using three *japonica* cultivars as recurrent parents and 'B5' as donor parent. Starting from the BC_1F_1 to other following BCF_1 generations, approximately 50 plants were genotyped by molecular markers linked to the genes of interest. From these, plants carrying resistant alleles of the three target resistance genes (based on their markers genotyped) and which were phenotypically similar to the recurrent parents were selected as the parents for the next backcross, and the procedures lasted to BC_4F_1 or BC_nF_1 generations. The selected BC_4F_1 or BC_nF_1 plants of each recurrent parent were selfed to produce BC_4F_2 and BC_nF_2 seeds. Based on phenotypic similarity to their recurrent parents, 22, 20 and 21 BC_4F_2 plants in the genetic backgrounds of Shengdao 15, Shengdao 16, and Xudao 3, respectively, were selected for homozygosity at the marker genotypes. The F_3, F_4 and F_5 plants derived from the BC_4F_2 were selected with the molecular markers and phenotypic selection. Finally, the stable lines were evaluated for resistance to BPH and RSD in field and yield components.

These materials were all planted in experiment field of Shandong Rice Research Institute (Jining, Shandong, China) in summer, and planted in Nanbin farm of Sanya city (Hainan, China) in winter. All of the materials were planted with single plant per hill, spaced at 15 × 25 cm.

Target genes and markers for marker-assisted selection

The target genes selected for the MAS experiment are three dominant genes conferring resistance to brown planthopper and rice stripe virus. The primers linked to the three resistance genes were listed (Table 1). *Bph14* was cloned in 2009 (Du et al. 2009). Several Insertion/Deletions locations were detected by comparing the sequence of *Bph14* with the sequence of Nipponbare in NCBI. The

InDel marker B14, which co-segregated with the *Bph14*, was developed by primer3.0 (http://frodo.wi.mit.edu/). The closely linked marker used in the MAS of *Bph15* was previously designed in the course of the gene fine mapping (Yang et al. 2004). The *Stv-b*^{i} gene was previously mapped in approximately 286 kb physical distance on chromosome 11 (Hayano-Saito et al. 1998, Hayano-Saito et al. 2000, Wu 2009). The STS primers S1 for *Stv-b*^{i} were designed based on the genomic sequences in the target regions using Premier 5.0.

DNA extraction and molecular marker detection

Genomic DNA for each plant was isolated from fresh leaves using the cetyltrimethylammonium bromide (CTAB) method. The PCR reaction mixture (total volume of 20 μL) contained 20 ng template DNA, 2.0 mmol L^{-1} 1× PCR buffer, 1.5 mmol L^{-1} $MgCl_2$, 2.0 mmol L^{-1} dNTP mixture, 2.0 mmol L^{-1} primer combinations, and 0.2U Taq DNA polymerase. The protocol for PCR was as follows: predenaturation at 94 °C for 5 min, followed by 35 cycles at 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1.5 min, and a final extension at 72 °C for 10 min. The PCR products were separated on a 3% agarose gel or polyacrylamide gel electrophoresis.

Identification of resistance to BPH

The standard seedbox screening test (SSST) was used to measure the levels of resistance of parents and backcross populations at seedling stage (Heinrichs et al. 1985). About 20 pre-germinated seeds of tested lines were sown 5 cm apart in 20 cm rows in enamel trays (60 × 50 × 10 cm). The parents "B5" and "Shengdao 15, Shengdao 16 or Xudao 3", and the susceptible control 'Taichung Native 1' were grown at random, together with the progenies of backcross populations in each tray. At the three-leaf stage, the seedlings were infested with ten BPH per seedling. When all of the seedlings of TN1 were completely killed, each seedling of the lines was examined and given a score of 0, 1, 2, 3, 5, 7 or 9 according to the criterion. The resistance level of each stable line was inferred based on the average value of the seedlings.

Identification of resistance to RSD

The seedling nursery was located in the neighborhood of a wheat field, where *L. striatellus* was rich in number (3000 head m^{-2}). After wheat harvest, late in May, a large number of *L. striatellus* migrated to the rice seedling nursery, and the insect population was sufficient for full occurrence of RSV. In the middle of July, the infection rate (IR) and the disease rating index (DRI) of the populations were investigated. Infection rate is the ratio of the number of plants showing RSV symptoms to the total number of plants, and DRI is calculated as described by Washio et al. (1968). The susceptible and resistant controls are Wuyujing 3 and Zhendao 88, respectively. The experiments were repeated twice in two consecutive years.

Evaluation of main agronomic charateristics

Agronomic traits of the backcross lines and three recurrent parents (Shengdao 15, Shengdao 16 and Xudao 3) were evaluated at the experiment field in two consecutive years. Heading date, plant height, neck-panicle length, number of effective panicle, panicle length, filled grain number per panicle, 1000-grain weight, leaf blade length and leaf blade wide were measured.

RESULTS

Molecular marker detection and gene pyramiding

After three BC generations of MAS, the selected BC_4F_1 plants for each of recurrent parents were selfed one more generation in order to produce BC_4F_2 progeny. A total of 63 BC_4F_2 plants showing the recurrent phenotype and presumably possessing one to three target resistance genes in various combinations were selected. Among them, 23 plants were derived from Shendao 15; 19 plants, from Shengdao16; and the remaining 21 plants, from Xudao3. The selected BC_4F_2 plants together with the parents were genotyped for the markers linked to the three resistance genes (Figure 1).

The closely linked molecular markers were used to detect the two BPH resistance genes and *Stv-b*^{i} gene in the

Table 1. Markers used for marker-assisted selection of resistance genes to *Bph14*, *Bph15*, and *Stv-b*^{i}

Marker	**Chromosome**	**Primer sequence** (5'→3')	**Linked gene**	**Distance** (cM)	**Reference**
B14	3	F: ATCGAAGCCACTTGGTGAAC R: CCTCTGATTCTGGCAAACAA	*Bph14*	0.0	
B15	4	F: TTGTGGGTCCTCATCTCCTC R: TGACAACTTTGTGCAAGATCAAA	*Bph15*	0.3	Yang et al. (2004)
S1	11	F: GAGGTAGTATATTGGCAGG R: AGGGATGTAAGTGTGGAG.	*Stv-b*^{i}	1.0	Chen et al. (2009)

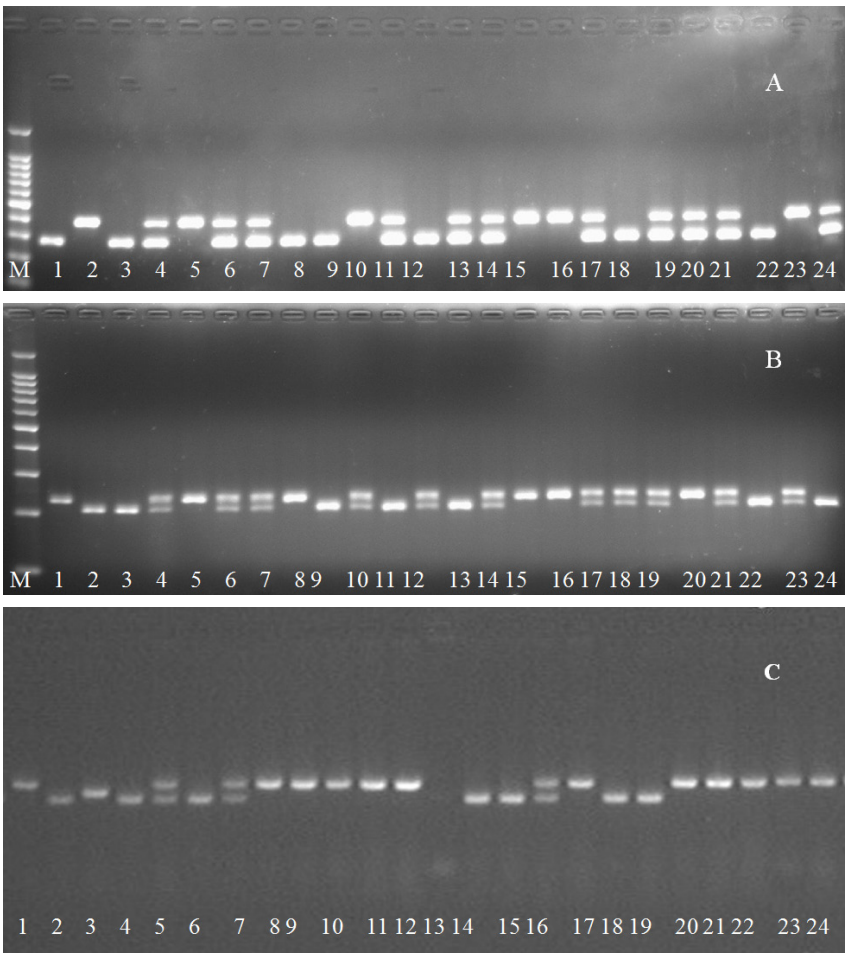

Figure 1. Markers banding pattern of selected rice plants and their corresponding parents for markers linked to *Bph14*, *Bph15* and *Stv-b*^{i} in Shengdao 16/B5 population. (**A**) PCR using B14 as primers for *Bph14*, (**B**) PCR using B15 as primers for *Bph15*, (**C**) PCR using S1 as primers for *Stv-b*^{i}. M: DL2000 Marker; 1: B5; 2: Shengdao 16; 3-24: Plants of BC segregating populations.

63 plants (Table 2). Based on the marker genotype of two BPH resistance genes, the plants were found to carry one or two BPH resistance genes. There were 5 plants with two BPH resistance genes, 8 plants with *Bph14*, and 10 plants only with *Bph15* in the 23 progenies of Shengdao 15. In the population from Shengdao 16, 3 plants carried the double homozygous genes, and the other 16 plants, only the single resistance gene. There were 4 plants with two BPH resistance genes, 7 plants with *Bph14*, and 8 plants with *Bph15* in the progeny population of Xudao 3, and 2 plants without resistance genes. There were 21 plants with homozygous RSD resistance gene in three BC_4F_2 progeny populations. Through the genotype analysis of closely linked molecular markers, 8 BC_4F_2 plants were identified to carry all of the three homozygous resistance genes. In other words, the three resistance genes were pyramided into these plants.

Resistance evaluation

The disease reactions of the 63 plants to the BPH and RSV were verified by inoculation. The reactions to brown planthopper were verified by inoculation in nethouse (Figure 2). Three plants only with *Bph15* were susceptible to BPH, according to their resistance score of '7'. Two plants with *Bph15* and one plant with *Bph14* were moderately susceptible to BPH, according their score of '5'. All the other plants with single or two resistance genes showed resistance or high resistance to BPH. Their resistance spectra were comparable to those of the donor parents. The results of phenotyping and genotyping indicated that the accuracy of MAS for *Bph14* and *Bph15* were 96.3%, 92.5%, respectively (Table 2).

Plants with homozygous genotypes of *Stv-b*^{i} gene were evaluated for the RSD resistance in the field. The infection rate

Table 2. Accuracy of marker-aided selection for the BPH resistance genes, *Bph14* and *Bph15*, based on SSR marker genotypes (B14 for *Bph14*, and B15 for *Bph15*) verified by progeny testing

Cross	Resistance genes		Number	Resistance level				Accuracy (%)[1]	
	Bph14	*Bph15*		HR	R	MS	S	*Bph14*	*Bph15*
	+	+	5	5					
Shengdao 15/B5	+		8	6	2			100	90
		+	10	5	4		1		
	+	+	3	3					
Shengdao 16/B5	+		9	5	3	1		88.9	100
		+	7	4	2				
	+	+	4	4					
Xudao 3/B5	+		7	5	2			100	87.5
		+	8	5	2	1			

The accuracy of MAS for *Bph14* was 96.3% according to the data of three populations.

The accuracy of MAS for *Bph15* was 92.5% according to the data of three populations.

range of the 20 plants was from 0.5% to 2.2 %, whereas the infection rate of susceptible contrast (Wuyujing 3) was 72%. One plant with the resistance gene was susceptible to RSV. Therefore, the consistency rate of MAS of *Stv-b*^{i} was 95.2%.

Importantly, the 8 plants which carried all of the three homozygous resistance genes showed high resistance to BPH and RSD in the resistance assay. Due to the intentional selection for the phenotype of the recurrent parent in every BC generation, the final 8 selected plants showed a high degree of similarity to their respective recurrent parents.

Yield evaluation

The 8 selected plants with three resistance gene were tested for traits related to yield. These plants performances were very similar to those of the recurrent parents. Table 3 shows that some of the selected plants were better than the recurrent parents in agronomic traits. It is expected to choose better lines than control from the 8 plants in yield traits.

DISCUSSION

After yield, disease resistance and quality are the main focus of rice breeder throughout the world. Along with the change of the planting model, plant diseases and insect and pests have become the important factors affecting the rice yield in China.

In this work, it was reported the development of conventional *japonica* rice lines simultaneously carrying genes conferring resistance to BPH and RSV. The concept of gene pyramiding assisted by molecular markers has been success-

Table 3. Comparison of the agronomic traits among part selected lines and each parent

Line	HD (d m^{-1})	**PH** (cm)	**NPL** (cm)	PN	PL (cm)	FN	**GW** (g)	**BL** (cm)	**BW** (cm)
15-1	25/8	93.3	1.2	13	17.2	162	26.0	22.3	1.72
15-2	25/8	93.5	1.4	14	16.7	143	25.1	19.2	1.89
15-3	25/8	94.8	1.2	13	18.1	169	27.2	21.9	1.76
CK1	24/8	92.2	1.1	14	16.9	156	26.1	20.6	1.72
16-1	24/8	100.2	2.8	13	15.8	176	27.6	26.8	1.73
16-2	25/8	96.3	3.5	15	17.5	192	26.2	25.7	1.86
CK2	24/8	95.2	3.2	13	17.2	183	26.8	27.1	1.81
3-1	28/8	98.2	1.9	16	16.8	152	25.8	23.3	1.45
3-2	28/8	100.5	2.1	15	17.6	130	27.1	25.4	1.58
3-3	27/8	102.3	2.5	15	14.3	136	26.1	25.2	1.39
CK3	26/8	97.5	1.7	17	16.5	140	26.3	24.0	1.51

HD: Heading date; PH: plant height; NPL: Neck-panicle length; PN: Number of effective panicle; PL: Panicle length; FN: Filled grain number per panicle; GW: 1000-grain weight; BL: Blade leaf length; BW: Blade leaf wide; 15-1, 15-2, 15-3: Lines from Shengdao 15; 16-1, 16-2, 16-3: Lines from Shengdao 16; 3-1, 3-2, 3-3: Lines from Xudao 3; CK1: Shengdao 15; CK2: Shengdao 16; CK3: Xudao 3.

Figure 2. Resistance test of the lines with two BPH resistance genes or one BPH resistance gene at the seedling stage 6: susceptible control 'Taichung Native 1', 7: resistance control 'RHT', 1, 2, 3, 4, 5, 8, 9, 10, 11, 12, 13: the obtained lines with BPH resistance genes. Plants after infested by BPH.

fully used to create advanced lines with wide and potentially durable resistance. Many data showed that varieties with multiple insect resistance genes generally presented a higher level of resistance than the varieties with a single resistance gene. For example, two resistant varieties Pbt33 with *Bph2*, *Bph3* and IR64 with major gene and minor gene for BPH resistance have higher resistance level to BPH than other varieties (Sidhu and Khush 1978, Alam and Cohen 1998). According to the inoculation experiment, the resistance level of lines with both *Bph14* and *Bph15* were grade 1, higher than the lines which presented only single resistance gene. BPH was not inhibited by pesticides in the field where the lines carrying BPH resistance gene were planted. The lines carrying single or two resistance gene showed certain resistance to BPH in the natural condition, while those without the resistance gene obviously were harmed by BPH. The results of this study further confirm the ideas above.

The identification of resistance to BPH and RSD is considerably difficult due to large influence of environmental condition on the incidence of the disease. Thereby, molecular markers closely linked to the resistance genes are of great importance for screening resistant lines.

The key issue in MAS is the accurate selection of the target gene, which is determined by the cosegregation of the target gene and the marker. According to the results of inoculation and field resistance assessment, the accuracy of selection for three resistance genes of the markers were approximately above 90%, indicating that the markers are effective. As the marker B14 was located within the *Bph14* gene, the accuracy of selection for *Bph14* reached almost 100%. These markers were effectively used to aid the pyramiding process and the development of advanced lines simultaneously resistant to BPH and RSV. Development of PCR-based markers for the target genes allowed a large number of progeny to be genotyped in the generations with minimum costs, so that intensive selection for new potential type was simultaneously practiced. The use of markers based on the gene internal difference can significantly improve the efficiency and accuracy of MAS, such as the marker in MAS for *Bph14*. The disagreement between the marker genotype and the field performance in this study is probably due to the incomplete linkage between the markers and the target gene.

The lines obtained are now being evaluated in the field. If the superior agronomic performance of these lines is confirmed, they will be subjected to the Shandong regional test or national regional test. In addition, the resistance gene to BPH can be convenient transferred to other *japonica* cultivars.

The present study clearly testified the usefulness of the genes *Bph14* and *Bph15* in the *japonica* rice to improve BPH resistance. Rice black streaked dwarf (RBSD) is a serious disease that has occurred in *japonica* rice area in recent year. This virus is also transmitted mainly by the small brown planthopper. The infection rate of lines with BPH resistance gene to RBSD was lower than those varieties without resistance gene in the field. So far, no rice genetic resource resistant to RSDV has been identified. The harm of rice disease from RBSD can be reduced by decreasing the harm of rice planthopper.

The use of *Bph14*, *Bph15* and *Stv-b*^{i} greatly facilitates the development of rice varieties with resistance to BPH and RSV; thus, it simultaneously reduces pesticide usage and decreases economic and environmental costs. The *japonica* lines with improved BPH and RSV resistance were obtained by pyramiding the BPH and RSV resistance genes into the same background. The results showed that the developed molecular markers of target genes for MAS is effective and can be used for rice resistance breeding.

ACKNOWLEDGEMENTS

This work was supported by Agricultural Seed and Germplasm Innovation Project of Shandong Province.

Piramidação de dois genes de resistência *BPH* e gene *Stv-b*^{i} em arroz *japonica* usando seleção assistida por marcador

Resumo – *Arroz é afetado por diversas doenças e insetos-praga causadores de grandes perdas de produção. Este estudo reportou a introgressão de genes de resistência a cigarrinha parda (BPH) e a virose do enrolamento (RSD) em cultivares* japonica*. Inicialmente, quatro linhagens retrocruzadas foram obtidas usando o parental doador B5. Marcadores moleculares ligados aos genes de resistência foram usados para identificar plantas contendo genes de interesse. Dois genes de resistência a BPH (*Bph14 *e* Bph15*) e um gene de resistência a RSD (*Stv-b^{i}*) foram transferidos para três variedades* japonica *por retrocruzamento assistido por marcador (SAM). A linhagem com genes* Bph14 *e* Bph15 *e aquela com gene* Stv-b^{i} *mostraram alta resistência a BPH e a RSD, respectivamente. A pesquisa demonstrou que este é um caminho efetivo no melhoramento do arroz visando controle de danos a BPH e RSD, por uso de SAM com os genes BPH e* Stv-b^{i}.

Palavras-chave: *Seleção assistida por marcador, cigarrinha parda, virose do enrolamento do arroz.*

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