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Development of rice introgression lines with brown planthopper resistance and low amylose content for germplasm sources through marker-assisted selection

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Abstract

The brown planthopper (BPH) is one of the most serious problems affecting rice production. This study incorporated the BPH resistance gene, *Bph3,* derived from a Rathu Heenati × Pathumthani 1 variety, which is susceptible to BHP and has low amylose content in the endosperm and both of these traits are closely linked on chromosome 6. The objective of this study was to develop introgression lines as germplasm sources with BPH resistance and low amylose content with other removed linkage drag traits and photoperiod insensitivity using marker-assisted selection**.** The newly developed multiplex simple sequence repeat markers specific to both genes were utilized for screening of the F_2 progeny possessing genotypes with breaking linkage drag and selected photoperiod insensitive lines. Then, evaluation of the selected lines for BPH resistance was conducted in the seedling stage of the F_4 progeny and the resistant character was segregated. Selection was made for BPH-resistant plants and their genotype controlling resistance on chromosome 4 was additionally determined using marker RM 16355. The results revealed that all the selected plants had the same genotype as the Rathu Heenati variety. Finally, the elite rice lines could be used as a germplasm source in rice breeding for BPH resistance and low amylose content. Moreover, unfavorable traits were removed by phenotypic selection from the Rathu Heenati variety such as photoperiod sensitivity, non-fragrance and tall plant type. Thus, these rice lines can be used as a germplasm resource for a rice breeding program in the future.

Introduction

Rice (*Oryza sativa* L.) is the major food crop for more than half of the world's population and as the world's population is increasing so rapidly the demand for rice is also accelerating (Food and Agriculture Organization, 2004). However, rice production and rice production capacity are decreasing due to the reduction in available agricultural land and abiotic stresses such as drought and flooding (Farooq et al., 2009). In addition, biotic stresses caused by plant diseases and insects also affect to rice production (Khush, 2005). One of the most serious insect pests of rice is brown planthopper (BPH), *Nilaparvata lugens* Stål (Homoptera: Delphacidae) as it can destroy plants by sucking the phloem sap directly and heavy infestations can cause complete drying conditions known as ''hopper burn'' followed by plant death (Heinrichs, 1979). Moreover, it can destroy plants indirectly as insect carriers can transfer viruses causing ragged stun disease and grassy stunt disease, which likewise affect rice yield (Rivera et al., 1966; Ling et al., 1978). Pesticides are the most common method of controlling BPH, but they are is costly and harmful to the environment (Lakshmi et al., 2010) Utilization of resistant varieties has been considered to be one of the most effective ways to stabilize rice yield production (Jena et al., 2006).

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To date, 28 major BPH resistance genes have been reported in indica cultivars and five wild *Oryza* species (*O. australiensis*, *O. eichingeri*, *O. latifolia*, *O. officinalis*, and *O. minuta*; (Cheng et al., 2013). Several of the BPH-resistance genes have been assigned to rice chromosomes 2, 3, 4, 6 and 12 (Jena and Kim, 2010). *Bph3* is a brown planthopper resistance gene, which shows a broad spectrum of brown planthopper resistance in Southeast Asia including Thailand (Jairin et al., 2007a; Liu et al., 2015. This gene has been widely used in rice breeding programs to control the brown planthopper (Cruz, 2011).

An indica rice landrace variety from Sri Lanka (Rathu Heenati; RH) was found to harbor *Bph3* which is a single dominant gene for brown planthopper resistance located on the short arm of the sixth chromosome (Lakshinarayana and Khush, 1977). In addition, at the same location on the short arm (chromosome 6), there is a gene that controls the quality of the rice flour known as the waxy gene (*Wx*) and this controls the amylose content which in turn is accountable for the quality of the carbohydrate in rice varieties (Itoh et al., 2003; Zhou et al., 2003). Because *Bph3* and the *Wx* gene are closely linked (Jairin et al., 2007b), linkage drag can occur among these two genes which involves a genome fragment of the donor genome surrounding the target gene and being dragged along (Hospital, 2001). However, recombination can occur during the first phase of meiosis, when the homologous pairs of parental chromosomes align and as a result, progenies can have different combinations of genes other than their parents (Hospital, 2001). The recombination frequency depends on the crossing over rate that is related to the distance between genes and thus, genes that are located on the same chromosome have a lower crossing over rate than those on different chromosomes (Hospital, 2001). However, this low rate of recombination can be detected using DNA markers.

Marker-assisted selection (MAS) may greatly increase the success of selection of desirable lines as it directly targets the genotype without the influence of the environment and thus speeds up conventional selection procedures (Collard et al., 2005). Multiplex primers can be mixed with more than a primer pair within one reaction considering primer specificity, non-primer cross dimer, melting temperatures balance and polymerase chain reaction (PCR) product-size differences to save time and cost of the operation (Sint et al., 2012).

The development of brown planthopper resistant lines having the *Bph3* allele with low amylose content is quite difficult even using the backcross breeding method because it requires a long time to remove the linkage drag (Hospital, 2001; Jairin et al., 2009). Thus, an objective of this study was to solve this problem by breaking the linkage drag between the *Bph3* and Wx^a alleles of the *Waxy* locus through MAS using multiplex simple sequence repeat (SSR) primers with PCR and the main objective was to develop introgression lines (ILs) as germplasm sources with brown planthopper resistance and low amylose content with other removed linkage drag traits and photoperiod insensitivity for a rice breeding program in the future.

Material and Methods

Plant materials and development of rice population

The cross used was Pathumthani 1 (PTT 1) × Rathu Heenati (RH) (acc. No.11730). PTT 1 is a rice cultivar which is susceptible to brown planthopper (Chaiyawat et al., 2009) and has low amylose content (Wx^b), whereas RH is a locally cultivated rice from Sri Lanka, carrying BPH resistance (*Bph3*) and having high amylose content (Wx^a). These two were used as the female and male parents, respectively. The experiments were conducted from 2013 to 2017 in the Department of Agronomy, Faculty of Agriculture, Kasetsart University, Bangkok, Thailand. The F_1 seeds were grown in plots containing rice field soil. Then, 1,213 F_2 seeds were obtained from self-pollination of F_1 plants (three plants) at the flowering stage and were grown in 220-holed seed trays and then later put in 38 cm width × 53 cm length × 12.5 cm depth plastic trays.

Selection for photoperiod insensitive character

The major constraint to using photoperiod sensitive rice as germplasm in a rice breeding program is that it can only be transplanted under suitable field conditions for one crop per year, which affects the period of breeding and management as this becomes complicated when planting off-season. In total, 1,213 F_2 plants from the Pathumthani 1/Rathu Heenati cross were grown individually under long day conditions (daylength > 12 hr/d) in a nursery. Flowering plants were selected and leaf tissue was kept for marker-assisted selection of the genotypes involved in BPH resistance and low amylose content.

Bioassays for brown planthopper resistance

Two BPH bioassays, namely the virulence of the BPH female test modified from Tanaka and Matsumura (2000) and the standard seed-box screening test, were used to determine the reaction of the rice genotypes to the BPH populations at the seedling stage of rice under greenhouse conditions (Heinrichs et al., 1986). To ensure that all the seedlings were at the same growth stage for BPH infestation, each genotype was germinated on moist tissue paper in a Petri dish and seedlings of each genotype were then transplanted. After inspecting all plants to ensure there were no other predators and insects, approximately 10 nymphs (second to third instar) per seedling were used to infest plants. Scoring was started when the susceptible check (Taichung Native1; TN1) had 90% mortality due to the nymphs or 1 wk after infestation. The reaction of each genotype to BPH was given a score of 0, 1, 3, 5, 7 or 9 according to the criteria of the standard evaluation system for rice (International Rice Research Institute, 2002).

DNA extraction and polymerase chain reaction amplification

PCR amplifications were performed with a Phire® Plant Direct PCR Mastermix (Thermo Scientific; Foster City, California, USA), designed to amplify DNA directly from rice leaf samples. DNA extraction was not required prior to the PCR. The PCR reactions were performed using a 10.0 µL volume containing five pieces of 0.5 mm leaf sample used as the DNA source together with 5.0 µL 2 × plant PCR buffer and 3.0 µL and 4.0 sterile distilled H_2O for multiplex SSR primers and normal PCR reaction, respectively, together with 1.0 µM of forward and reverse primers for 0.5 µL of each primer. The amplification was performed in a T100 machine (Bio-Rad Laboratories, Inc.; Hercules, California, USA) and the program was set at 94 ºC for 3 min, followed by 40 cycles at 94 ºC for 40 s, 59 ºC for 1 min and 72ºC for 1 min, with a final extension at 72ºC for 5 min. The PCR products were separated on 6% polyacrylamide gel electrophoresis using silver staining ($AgNO_3$) following the method of Benbouza et al. (2006).

Development of marker-assisted selection for multiplex simple sequence repeat primers in polymerase chain reaction

The selected F_2 plants for photoperiod insensitivity were used for genotyping. Marker-assisted selection (MAS) may greatly increase

the success of selection of desirable lines as it directly targets to the genotype without the influence of environment and thus speeds up the conventional selection procedures (Collard et al., 2005). Furthermore, multiplex primers can be mixed using more than one primer pair within one reaction to save time and cost (Sint et al., 2012). A database of sequences and genes from NCBI (https://www.ncbi.nlm.nih.gov/mapview/) was used to develop SSR markers on chromosome 6 of rice between the RM589 to RM190 markers which have a distance of about 380 kb (Jairin et al., 2007b). Sequence alignments were performed using the WEBSAT software (http://wsmartins.net/websat/) to develop multiplex SSR primers in PCR, which resulted in 128 markers, but just 40 markers were synthesized**.**

DNA markers for marker-assisted selection

The multiplex SSR primers to identify the recombination were KRNP6 and KRNP123 markers. KRNP6 (the tightly-linked marker for BPH resistance trait detected by microsatellite Agn alleles) was used to determine the presence of the *Bph3* allele of RH on chromosome 6, while KRNP123 (the tightly linked marker for the amylose content trait detected by microsatellite Atn alleles) was used to select the presence of the *Wx* allele of PTT1 (Wx^b). The functional marker Naro1 (Rattanapol et al., 2011) was used to select the fragrance allele of PTT1. Another 11 markers on chromosome 4 were synthesized from the Oryzabase database (http://shigen.nig.ac.jp/rice/oryzabase/) following the report of Liu et al.(2015) and Chumwong et al. (2016) to screen for polymorphism between parental lines. Finally, four markers had polymorphism to distinguish genotypes for BPH resistance genes, amylose content (AC) and fragrance (FR) as shown in Table 1.

Evaluation of agronomic and grain quality traits

The selected F_4 plants were transplanted into the paddy field for individual yield testing with a row spacing of 20 cm × 20 cm. Parents and check varieties (RD31 and RD49) were also grown in the same field using the same spacing. The plant height, flag leaf length and panicle length (all measured in centimeters), panicle number, number of grains per panicle, 100-grain weight (measured in grams) and grain yield per plant (measured in grams/plant) were collected from individual plants. The trait of amylose content in the endosperm was determined using the colorimetric method with iodine-potassium iodide, modified from Juliano (1971). In addition, the fragrance of the rice was determined using sensory evaluation based on a simple laboratory technique developed by International Rice Research Institute (1971).

Results

Evaluation of parents for brown planthopper resistance

The virulence of the BPH female test modified from Tanaka and Matsumura (2000) was used to evaluate the BPH resistance of parental cultivars and compared with the susceptible check (TN1) at the seedling stage of the rice plants in the laboratory. RH expressed resistance to the BPH, while PTT1 was susceptible to the BPH (Fig. 1).

Table 1 Target molecular markers for marker-assisted selection

Marker	Type	Chr.	Trait	Primer sequence	
				Forward primer sequence (5′-3′)	Reverse primer sequence (5′-3′)
KRNP6	SSR	6	BPH	TCGAGGATTTGGTTTAGAGA	CAACAGAGAGAGAGATGCCT
KRNP123	SSR	6	AC	GCTGATTTCATCCTACCAAG	GAGGGTGTTGTTTCTGTGTT
RM16533	SSR	4	BPH	CCACTGAACAAGTGTTGTAGTTCTGC	CTAGCTGACCACATGCTTCATCC
Naro1	F	8	FR	AGGTTGCATTTACTGGGAG	TGGCTACTAGAATGATGCT

F = functional marker; BPH = brown planthopper; AC = amylose content; FR = fragrance.

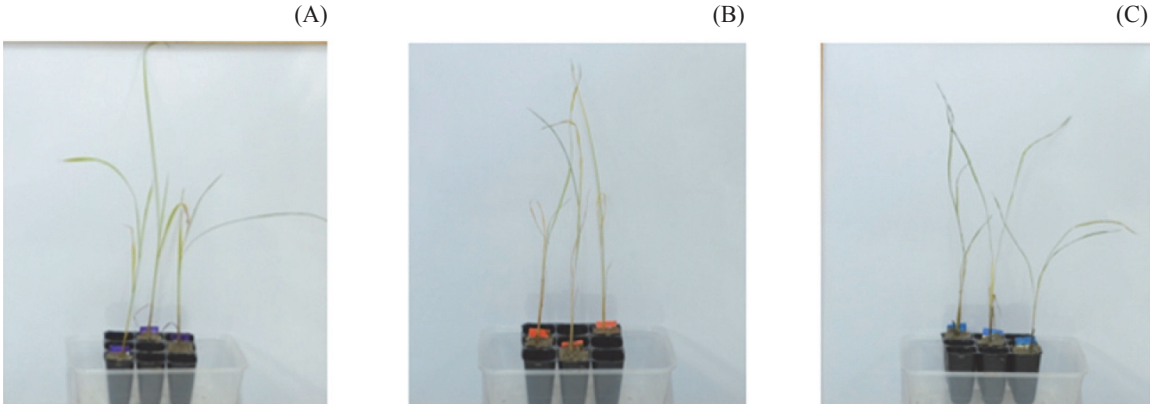

Fig. 1 Levels of resistance to brown planthopper at seedling stage of parents and susceptible check: (A): Rathu Heenati variety, (B): Pathumthati 1 variety, (C) Taichung Native1 variety

Photoperiod insensitivity in F_2 population

The F_2 population was derived from a cross between a photoperiod insensitive variety (PTT1) and a photoperiod-sensitive variety (RH). In total the 1,213 plants were divided into two groups (Table 2) of 882 plants with photoperiod sensitivity, and 331 plants with photoperiod insensitivity. The chi square test was accepted at a 3:1 ratio.

Table 2 Phenotypic selection of photoperiod insensitive trait in F_2 population

Population	Number of plants		
	Photoperiod sensitive	Photoperiod insensitive	$x^2_{(3:1)}$ test
F_2 (1,213 plants)	882	331	3.39^{ns}

^{ns} = non-significant.

Multiplex simple sequence repeat primers for Bph3 allele and Wx^b allele in F_2 population

The F_2 population was prepared from the cross between PTT1 and RH. Screening in F_2 individuals derived from F_1 had a linkage phase which is a coupling phase that used multiplex SSR primers (KRNP6 and KRNP123). These markers could identify the recombination between both genes (Fig. 2). Among the 331 plants from photoperiod insensitive lines, selected from F_2 plants, there were 88 plants which had a homozygous parental type of RH and 68 plants had homozygous parental type of PTT1. However, the target genotype (the *Bph3* allele for RH and the *Wxb* allele for PTT1) could not be found. Nonetheless, the recombinations from six plants were identified with the homozygous *Bph3* allele as RH, but the heterozygous *Wx* allele and seven plants were identified with the heterozygous *Bph3* allele, whereas the homozygous Wx^b allele was identified as PTT1 (Table 3).

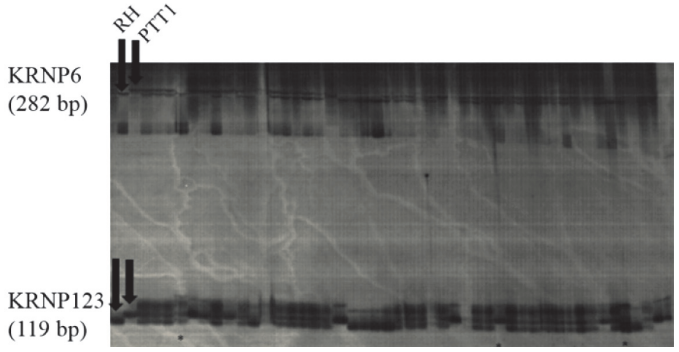

Fig. 2 Polymerase chain reaction amplification of multiplex primers, KRNP6 and KRNP123, in F_2 population,where progenies with an asterisk were selected to develop the F_3 population

Validation for amylose content in F_3 seeds

Validation of the amylose content in F_3 seeds from the four lines that had genotype of $Bph3Wx^a/Bph3Wx^a$ (AABB), $Bph3Wx^a/bph3Wx^b$ (AaBb) and $bph3Wx^b/bph3Wx^b$ (aabb) showed that the samples were scored homozygous Wx^a as in RH, and heterozygous and homozygous Wx^b as in PTT1, respectively (Table 3) compared with parents (Table 4). The results showed that KRNP123 was tightly linked with the amylose content and can be used to identify these three genotypes. Therefore, the accuracy of marker-assisted selection was confirmed by the phenotypic screening.

Simple sequence repeat marker-assisted selection for homozygous of target genotype

Thirteen F_2 progenies were selected (those carrying heterozygous or homozygous on *Bph3* and Wx^b region). Afterward, those plants were self-pollinated to generate the F_3 population. Then, all F_3 plants were genotyped using the same marker that had been previously used to identify heterozygous loci. F_3 individual plants were selected using the KRNP6 marker for the BPH resistance trait. Nine homozygous genotypes of the RH rice variety were selected from 55 plants which had the low amylose content gene. The chi square test was accepted at a 1:2:1 ratio (Table 5). For the amylose content trait, F_3 individual plants were selected using the KRNP123 marker. One homozygous genotype of the PTT1 rice variety was selected from two plants having the BPH resistance gene. Thus, in total, there were 10 plants that had the target genotype.

Validation of brown planthopper resistance in seedling stage

Two lines of the F_3 progeny were obtained after selection of the same flowering date as PTT1**.** Then, for evaluation of brown planthopper resistance, standard seed-box screening was used to determine the reaction of the rice genotypes to the BPH populations. The experiment was conducted in the seedling stage of the F_4 progeny. The segregation of any resistant character was shown**.** Liu et al. (2015) and Chumwong et al. (2016) reported finding the BPH resistance gene on chromosome 4. Thus, in this experiment, selection for resistant plants and their genotypes controlling resistance on chromosome 4 was additionally determined using the RM 16355 SSR marker. The results revealed that all the selected plants had the same genotype as the RH variety.

Marker-assisted selection for fragrant trait

The functional marker Naro1 was used to select the fragrant allele of PTT1. The result showed that all resistant plants had the same fragrant allele as PTT1.

Table 3 Segregation of simple sequence repeat (SSR) markers tightly linked to brown planthopper resistant alleles (*Bph3*) and low amylose content alleles (*Wx*) in the F_2 populations derived from PTT1 × RH

Population	Number of plant[a]								
	AABB	AABb	AAbb	AaBB	AaBb	Aabb	aaBB	aaBb	aabb
F_2 (331 plants)	88	6	-	5	154	7	-	3	68

A and a = alleles of SSR marker KRNP6 (tightly linked *Bph3*); B and b = alleles of SSR marker KRNP123 (tightly linked *Wx*) on chromosome 6; uppercase and lowercase letters represent RH and PTT1 alleles, respectively.

Table 4 Amylose content of four lines validated using KRNP123 markers compared with the parents

Line/Variety	Score	Amylose content
21–17	A	Low
21–7	H	Intermediate
21–9	H	Intermediate
21–46	B	High
PTT1	A	Low
RH	B	High

A = homozygous genotype (*Wx^bWx^b)* as in PTT1variety; H = heterozygous genotype (*Wx^aWx^b);* B = homozygous genotype (*Wx^a Wx^a)* as in RH variety.

Table 5 Segregation of alleles associated with resistance to brown planthopper in F_3 population

Population	Number of plants[a]			
	AAbb	Aabb	aabb	$x^2_{(1:2:1)}$ test
F_3 (45 plants)	9	21	15	3.36^{ns}

[ns] = non-significant.

A and a =alleles of simple sequence repeat marker (SSR) KRNP6 (tightly linked *Bph3*); b = alleles of the SSR marker KRNP123 (tightly linked *Wx*) on chromosome 6, where uppercase (A) and lowercase (a) letters represent alleles from RH and PTT1, respectively.

The lowercase "b" indicated low amylose content as in the PTT1.

Agronomic performance and grain quality traits of introgression lines

The results of agronomic performance and grain quality traits of ILs evaluated in the field and laboratory compared with the parents and check varieties showed segregation in the morphological traits of ILs such as plant type. The 20-67-4-2 and 20-67-4-4 lines had the same plant type as PTT1, but the grain quality of all ILs was similar to PTT1 (Table 6). These results showed the efficiency and accuracy of MAS for the AC and FR traits.

Discussion

BPH is a major biotic stress limiting rice production in most Asian countries. Improvement of rice resistance is one of the best methods to maintain rice yield despite BPH (*Nilaparvata lugens* Stål). *Bph3*, one of the major resistance genes derived from the RH, has been mapped to the short arm of chromosome 6 of rice (Jairin et al., 2007b). Later, Liu et al. (2015) found that this *Bph3* gene encoded for lectin receptor kinase. More recently, Chumwong et al. (2016) reported that identification of quantitative trait loci involved the BPH resistance gene in the RH variety and that these loci were on chromosomes 4 and 6 which was consistent with the current study. The various gene positions may be due to the diversity of the rice varieties and BPH populations used in each experiment.

'Linkage drag' is a common phenomenon in breeding programs (Lewis et al., 2007; Liu et al., 2009). Normally, breaking linkage drag would depend on the size of the population, as the larger the population, the greater the opportunity to break the linkage drag; however, this makes management difficult. When the unexpected linkage drag occurred in the current study because the *Bph3* and *Waxy* genes are closely linked (Jairin et al., 2007b), MAS was used to select rice lines carrying recombinants in a small population which could not be selected as the target genotype at once because the target genotype could ,at least, occur for one plant in 4,444 plants in the population. Thus, the current study used stepwise screening to reduce the population size and to increase the number of selected lines in the screening (Sreewongchai et al., 2010, Min et al., 2012) with the integration of multiplex SSR primers to identify the recombination identification between genes controlling BPH resistance and high amylose content in rice, thus saving time and cost. In addition, the reduction of other linkage drag from the RH variety (such as tall plant type and photoperiod insensitivity by phenotypic selection) was performed in this study.

Table 6 Agronomic and grain quality traits of the selected lines from F_4 lines compared with parents and check varieties

Line/Variety	Agronomic trait							Grain quality trait	
	PH	FL	PL	PN	NP	GW	GY	FR	AC
20-67-4-2	56.00	35.00	20.00	8.00	107.00	2.43	11.75	+	LAC
20-67-4-3	102.00	47.00	27.00	5.00	196.00	2.61	16.65	+	LAC
20-67-4-4	52.00	31.00	23.00	6.00	131.00	2.46	13.62	+	LAC
21-13-24-1	120.00	52.00	30.00	4.00	243.00	2.01	11.98	+	LAC
21-13-24-2	125.00	38.00	35.00	4.00	231.00	2.23	14.25	+	LAC
21-13-24-3	126.00	52.00	30.00	11.00	246.00	2.03	23.10	+	LAC
21-13-24-4	113.00	47.00	32.00	9.00	192.00	2.40	22.07	+	LAC
21-13-24-5	118.00	50.00	36.00	13.00	201.00	2.28	31.81	+	LAC
21-13-24-6	110.00	39.50	33.00	7.00	170.00	2.52	21.03	+	LAC
21-13-24-7	118.00	41.00	28.00	7.00	153.00	2.29	15.69	+	LAC
PTT1	40.67	22.50	18.33	4.00	37.67	2.17	2.77	+	LAC
RH	125.00	44.33	23.00	11.00	315.67	2.46	36.39	-	HAC
RD31	55.67	40.00	22.00	5.00	63.33	2.44	5.71	-	HAC
RD49	51.67	28.33	28.00	6.00	91.67	2.89	9.92	-	HAC

PH = plant height (cm); FL = flag leaf length (cm); PL = panicle length (cm); PN = panicle number; NP = number of grain per panicle; GW = 100-grain weight (g); GY = grain yield per plant (g/plant); FR = fragrant; AC = amylose content; LAC = low amylose content; HAC = high amylose content.

The results indicated that the BPH resistance level of ILs found in this study was the same as that in RH, which confirmed that marker-assisted introgression of the *Bph3* allele from RH is effective. Moreover, the grain quality of the IL developed in this study were almost same as those of PTT1. Consequently, this IL can be used as genetic material for resistance to BPH and for low amylose content in rice breeding by backcrossing with PTT1 to increase the genetic background of PTT1 or it can be used as the donor parent for improvement of the amylose content trait in a rice variety, which has resistance to BPH but a high amylose content to avoid the phenomenon of linkage drag.

Elite lines derived from a cross between PTT1 and RH can be used as germplasm sources for brown planthopper resistance and for a low level of amylose content together with removing removed linkage drag from the RH variety.

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Conflicts of Interest

The authors declare that there are no conflicts of interest.

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