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The Development of Multi-Resistant Rice Restorer Lines and Hybrid Varieties by Pyramiding Resistance Genes against Blast and Brown Planthopper

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Abstract: Rice blast, caused by the fungus *Magnaporthe oryzae*, and brown planthopper (BPH) infestation are two of the most destructive problems of rice production in China. The development of multi-resistant varieties is widely recognized as the most efficient and environmentally friendly approach to controlling crop diseases and pests. Functional molecular markers (FMMs) have been developed from functional variants in the genic region associated with trait variation, greatly enhancing the efficiency of identifying and pyramiding valuable genes in crop breeding. In this study, two FMMs and a multiplex PCR amplification system were developed for two major broad-spectrum BPH resistance genes, *Bph6* and *Bph9*. With the assistance of FMMs in the multi-resistant rice restorer-line development pipeline, two lines (Huahui7713 and Huahui3006) with blast and BPH resistance were developed by pyramiding three resistance genes *Pigm*, *Bph6* and *Bph9*. Two new hybrid rice varieties, Weiliangyou7713 and Xuanliangyou3006, derived from Huahui7713 and Huahui3006, have been developed and commercialized in China. Weiliangyou7713 and Xuanliangyou3006 exhibit enhanced resistance to both blast and BPH, while maintaining optimal yield and grain quality. The adoption of Weiliangyou7713 continues to expand, now being cultivated on a large scale, which is promising for its future role in reducing the dependence on chemical fungicides and pesticides in rice production. This suggests that the implementation of Huahui7713 and Huahui3006 in targeted breeding programs could be highly beneficial for developing rice varieties with strong resistance to blast and BPH.

Keywords: blast; brown planthopper; resistance gene; marker-assisted selection; multiplex PCR; hybrid rice

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1. Introduction

Rice stands as a cornerstone of global agriculture and serves as the primary staple for over 60% of China's population [1]. However, its yield and quality are frequently and severely threatened by various diseases and pests, such as rice blast and brown planthopper (BPH). Rice blast, caused by the filamentous ascomycete fungus Magnaporthe oryzae B.C. Couch (anamorph: Pyricularia oryzae Cavara), is the most devastating rice disease, leading to reduced yields and lower grain quality by damaging leaves, stalks, and spikes. Each year, rice blast affects an estimated global yield of 10–30% of the total harvest, which is enough to feed 60 million people annually [2]. BPH is a notorious rice pest that feeds on the phloem sap of rice leaf sheaths using needle-like mouthparts, causing wilting, yellowing,

and plant death. Moreover, it transmits viral diseases such as rice grassy stunt virus (RGSV), rice ragged stunt virus (RRSV), and rice wilted stunt virus (RWSV), leading to significant yield loss and even complete crop failure [3]. Therefore, developing rice varieties resistant to both pests and diseases is crucial for ensuring food security and enhancing agricultural productivity.

The utilization of resistant germplasm and resistance genes is widely accepted as one of the most efficient approaches to combat crop pests and diseases in crops. To date, more than 40 BPH resistance genes have been identified from cultivated or wild rice germplasm, with 17 of them isolated through map-based cloning [4–6]. These genes serve as invaluable resources for developing BPH-resistant rice varieties. As populations of BPH continue to evolve and new BPH biotypes emerge, rice varieties that possess a single BPH resistance gene tend to lose their resistance over time. For example, the BPH-resistant variety IR26, developed in the 1970s by the International Rice Research Institute, experienced a collapse in its resistance just 3 years after widespread cultivation [7]. This loss of resistance was mainly attributed to the emergence of BPH biotype 2. Similarly, other varieties with single BPH resistance genes, such as *Bph2* or *Bph3*, have also experienced a decline in their resistance effectiveness over time. This decline can be attributed to the adaptation of BPH or the emergence of new biotypes [8]. To address this rapid failure in resistance, it is recommended to pyramid BPH resistance genes or QTLs when developing rice varieties. By incorporating multiple resistance genes, these varieties are better equipped to withstand the evolving BPH populations and the emergence of new biotypes, ensuring long-term effectiveness against BPH infestations [9]. A study conducted by Myint et al. [10] found that the pyramided line carrying both *Bph25* and *Bph26* exhibited significantly higher levels of resistance compared to lines with single resistance genes. Furthermore, the introduction of three BPH resistance genes (*Bph14*, *Bph15*, and *Bph18*) into the elite indica rice variety 93-11 resulted in superior resistance compared to the lines carrying two or single resistance genes [11]. In the case of rice blast disease, more than 100 major resistance genes have been identified and documented on the 12 rice chromosomes, excluding chromosome 3 [12]. Among these genes, at least 39 have been cloned [13,14].

Molecular marker-assisted selection (MAS) is a technique used in rice breeding to help select genotypes based on their genetic composition at the genome level. It offers a fast, efficient, and precise way to combine multiple genes of interest [15]. Effective implementation of MAS relies on the availability of appropriate molecular markers specific to the target genes. The decreasing costs of next-generation sequencing technologies have significantly facilitated the acquisition of genome sequence variations. Numerous rice genome sequencing projects have revealed a wide range of single nucleotide polymorphisms (SNPs) distributed throughout the entire genome [16]. SNP marker systems are particularly attractive and effective when SNPs are present in the target gene region between donor and recipient cultivars. Among the available SNP genotyping methods, the amplification refractory mutation system (ARMS) PCR method stands out due to its ability to detect SNPs with minimal laboratory equipment requirements [17]. It allows the identification of different genotypes through a single PCR amplification, making it a simple, reliable and affordable option for rice molecular breeding [18–20].

Bph9, located on chromosome 12, is a dominant BPH resistance gene that confers resistance to BPH biotypes 1, 2, and 3. It encodes a rare type of nucleotide-binding and leucine-rich repeat (NLR) gene. Eight genes (*Bph1*, *bph2*, *bph7*, *Bph9*, *Bph10*, *Bph18*, *Bph21*, and *Bph26*), previously identified and mapped on the long arm of chromosome 12, are in fact multiple alleles of the same locus, which is designated as *BPH1/9*. These alleles can be classified into four allelic types based on their sequences: *BPH1/9-1* (*Bph1*/*Bph10*/*Bph18*/*Bph21*), *BPH1/9-2* (*Bph2*/*Bph26*), *BPH1/9-7* (*Bph7*) and *BPH1/9-9* (*Bph9*) [21]. *Bph6*, which was mapped to the long arm of rice chromosome 4, was later identified as a gene encoding an exocyst-localized protein. It provides resistance to BPH biotype 4 and biotype 2, which are prevalent in Bangladesh and China, respectively [22]. Wang et al. developed a functional dominant marker for *Bph9*, and successfully pyramided *Bph6* and *Bph9* into a 93-11 genetic

background using MAS [23]. The resulting improved line, Luoyang69, exhibited enhanced antixenotic and antibiosis effects on BPH compared to the single-gene introgression lines. Luoyang69 is a valuable resource for developing rice varieties with durable broad-spectrum high resistance to BPH. However, the availability of specific, precise, and efficient molecular markers for utilizing the *Bph6* and *Bph9* genes in BPH-resistant rice breeding is still limited. *Pigm*, a prominent rice blast resistance gene, is located on rice chromosome 6 and consists of multiple NBS-LRR-like disease resistance genes. This gene cluster has been extensively utilized in Chinese rice production [24]. In our previous research, we developed functional markers for the *Pigm* gene and selected a series of rice blast-resistant varieties [25]. The objective of this study is to develop *Bph6* and *Bph9* functional molecular markers (FMMs) and an efficient detection system for breeding practice. Additionally, we aim to select restorer lines with resistance to both rice blast and BPH, and their derived hybrid combinations, by pyramiding *Bph6*, *Bph9*, and *Pigm* through MAS combined with phenotypic selection (PS).

2. Materials and Methods

2.1. Plant Materials

A total of 478 rice varieties were utilized for the development and validation of FMMs. These varieties included BPH resistance germplasm Mudgo (*Bph18*/*Bph1*/*Bph10*/*Bph21*), T12 (*Bph7*), ADR52 (*Bph26*/*Bph2*) and Luoyang69 (*Bph6* and *Bph9*), as well as BPH susceptible varieties TN1, Nipponbare and 02428. R6888, a donor for the rice blast resistance gene *Pigm*, was also used. Two thermo-sensitive genic male sterile (TGMS) lines, Huawei338S and Huaxuan302S, were employed for hybrid rice development. Fengliangyou4 was used as controls in regional trials. The experiment was conducted using a Randomized Complete Block Design (RCBD) at the Guanshan experimental field of Longping Hi-Tech Seed Scientific Research Institute. The materials were randomly allocated across three blocks, serving as replications, to account for potential environmental variability within the experimental site. Each block consisted of individual plots with four rows of eight plants, making a total of 32 plants per plot. Both intra-row and inter-row plant spacing was maintained at 16.5 cm to ensure uniform growth conditions across all experimental units. For newly developed restorer lines, the main agronomic traits were measured, including grain yield (GY), days to heading (DTH), plant height (PH), number of grains per panicle (NGP), panicle length (PL), spikelet fertility (SF), thousand-grain weight (TGW), and rice quality traits such as apparent amylose content (AAC), gel consistency (GC), gelatinization temperature (GT), chalkiness degree (CD), head-rice rate (HRR), and length–width ratio (LWR). These measurements were taken for the five plants in the middle of the central row of each plot.

2.2. Primer Design of the ARMS Markers of Bph6 and Bph9

The genome sequences of *Bph6*, *Bph9*, *Bph7*, *Bph18*, and *Bph26* were obtained from the NCBI (www.ncbi.nlm.nih.gov/ (accessed on 20 June 2016)) database (accession numbers KX818198, KU216221, KU221258, KJ850252, and AB910360, respectively). Additionally, the corresponding genomic sequences of *Bph6* and *Bph9* in the varieties Nipponbare and 93-11 were also obtained. Sequences alignment and analysis of polymorphic sites within genic regions were performed using DNAman software (version 6.0.3.99). Based on ARMS-PCR, two strategies were employed for designing the markers. Strategy A involved using two allelic sequences of a polymorphic site as the 3′ ends of both the forward and reverse inner primers. Two outer primers were designed to pair with the inner primers, forming a four-primer marker system. The genotyping of samples was carried out by detecting the fragment size of the amplification products of the marker system. Strategy B focused on designing two pairs of dominant markers to specifically amplify the two alleles, targeting two co-separated polymorphic sites. These two pairs of markers were then used in combination to identify different genotypes in the same amplification system (Figure 1).

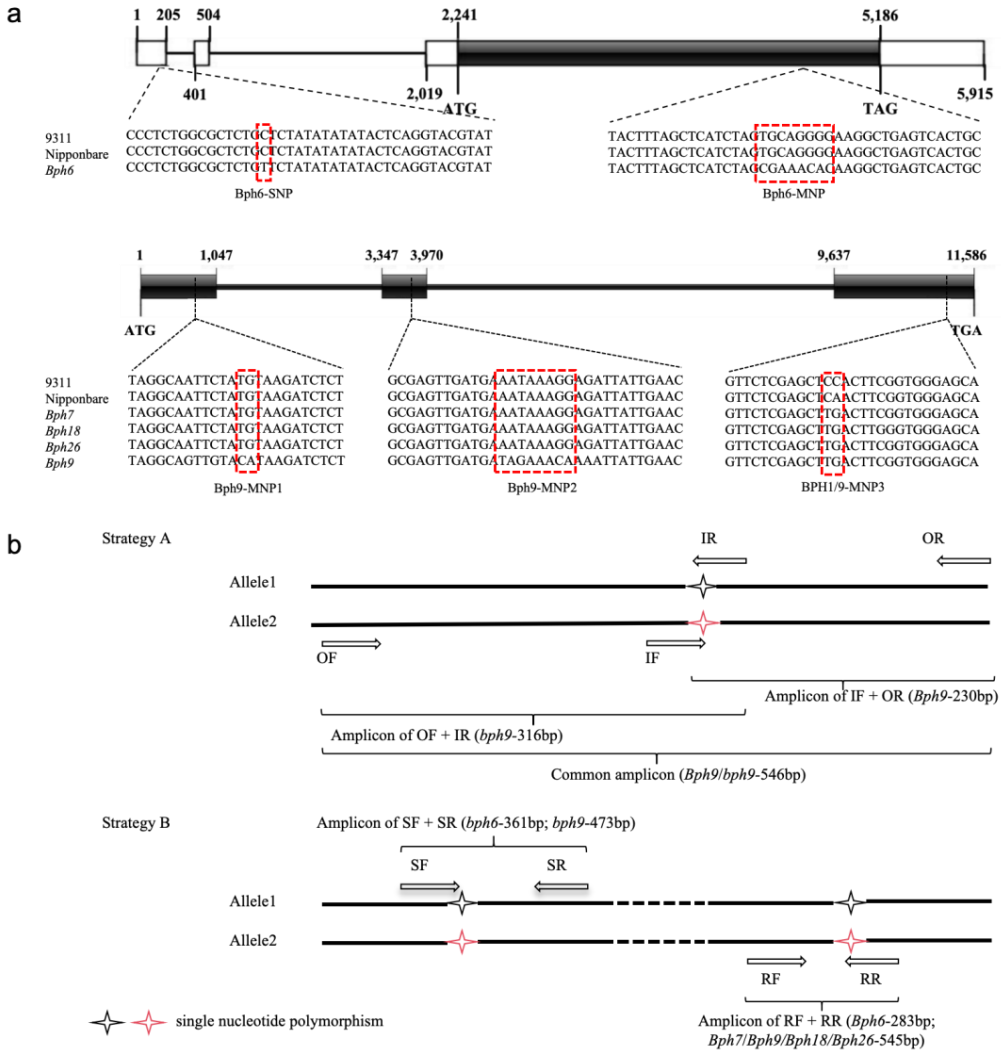


Figure 1. Development of molecular markers targeting *Bph6* and *Bph9* genes. Identification of nucleotide polymorphisms in *Bph6* and *Bph9* (**a**). Schematic illustration of two strategies for ARMS primers design for *Bph6* and *Bph9* (**b**). Red boxes represent polymorphic regions.

2.3. Evaluation of BPH and Blast Resistance

BPH resistance was assessed using the modified bulked seedling test [23], with Luoyang69 and TN1 serving as the resistant and susceptible controls, respectively. The resistance level of a seedling was determined by the resistance score (RS = 0, 1, 3, 5, 7, or 9), and the resistance level of a line was determined by the average RS of 16 seedlings. The new restorer lines were subjected to blast resistance evaluation at the Dawei Mountain Rice Breeding Station located at 28°49′ N, 113°99′ E in Liuyang, Hunan Province, China. The site offers environmental conditions conducive to rice blast outbreaks, including optimal temperatures, elevated humidity, and limited sunlight. Rice blast resistance was evaluated following the method described by Qin et al. [25]. For leaf blast resistance, a scoring system ranging from 0 to 9 was utilized. A score of 0 to 3 indicated a resistant reaction, 4 to 5 was considered moderately resistant, 6 indicated moderate susceptibility, and a score of 7 to 9 represented susceptibility.

2.4. MAS of New Hybrid Rice Restorer Lines with High-Level BPH and Blast Resistance

In order to pyramid rice blast and BPH resistance genes into the same rice variety, R6888 (*Pigm*) and Luoyang69 (*Bph6* and *Bph9*) were crossed. The resulting F_1 plants were self-crossed to obtain the F_2 segregating population. We genotyped the F_2 population for *Pigm*, *Bph6* and *Bph9* using the functional marker GM [25] and the newly developed markers

in this study. Additionally, we conducted phenotypic evaluations of agronomic traits to further screen the population. When the population reached the advanced generation F_6, lines were evaluated for BPH and blast resistance, as well as for the main agronomic and grain quality traits. The genetically stable and best performing lines were then crossed with TGMS lines to produce hybrids.

2.5. Evaluation of Main Agronomic and Grain-Quality Traits

To evaluate the F_1 hybrid combinations, newly selected rice restorer lines were crossed with commonly used sterile lines Huawei338S and Huaxuan302S. The multi-location evaluation was conducted by the Research and Development Center of Yuan Longping High-Tech Agriculture Company (Changsha, China), following the guidelines provided by the National Committee of Crop Variety Certification (https://www.natesc.org.cn/, accessed on 24 March 2024).

3. Results

3.1. Development and Validation of Molecular Markers for Bph6 and Bph9

Sequence alignment revealed the presence of two polymorphic regions within *Bph6* (Bph6-SNP and Bph6-MNP) and three within *Bph9* (Bph9-MNP1, Bph9-MNP2 and Bph9-MNP3) (Figure 1a). Two strategies were employed for the design of ARMS-PCR primers, contingent on the compatibility of the sequences flanking polymorphic loci with ARMS-PCR primer design requirements (Figure 1b). In strategy A, a set of tetra-primers (B9) was designed to target Bph9-MNP1. Amplification of the *Bph9* allele resulted in a 230-bp band, while the *bph9* allele produced a 316-bp band (Table 1, Figure 2b,e). In strategy B, two polymorphic sites, Bph6-SNP and Bph6-MNP, were used as targets for the development of a set of tetra-primers (B6). B6 enabled the amplification of the *Bph6* allele, generating a 283-bp band, while the *bph6* allele produced a 361-bp band (Figure 2a,d). Additionally, Strategy B was also used to develop tetra-primers (B1/9) for BPH1/9, targeting the polymorphic Bph9-MNP2 and Bph9-MNP3. Primers B1/9-RR and B1/9-RF were capable of specifically amplifying *BPH1/9*-resistant alleles (*Bph7*, *Bph9*, *Bph18* and *Bph26*), generating a 545-bp band (Figure 2c,f). The tetra-primers B1/9 and B6 can be used for multiplex PCR to simultaneously detect *Bph6* and *Bph9* genotypes (Figure 2g). These tetra-primers are applicable for *Bph6* and *Bph9* genotyping in breeding programs.

Table 1. Primers for *Bph6*, *Bph9* and *Pigm*.

Target Gene	Marker Name	Primer Name	Sequence (5′-3′)	Product Size (bp)
Bph6	B6	SF	AGGGCCTCTGGCGCTCTAC	361(−)/283(+)
		SR	AATGTGAAAGTGCAATTAGAAGGT	
		RF	ATAGTGAAGTTGAATCCGAAGG	
		RR	AGTGACTCAGCCTTGTGTTTCG	
Bph9	B9	IF	ACCATTGTTAGGCAGTTGTTCA	316(−)/230(+)
		OR	ATTCGACTCCCTTTCTTGTTATCT	
		OF	AATGTCGCACCCAGCAGC	
		IR	CAGCCTCCTGAAGAGATCTTTCA	
BPH1/9	B1/9	SF	GGCGAGTTGATGAAATAAAGG	473(−)/545(+)
		SR	GTTCTTTGCATCGCTGTCTC	
		RR	TTGCTCCCACCGAAGTCA	
		RF	AGGGAATGGTGTAGTAGCA	
Pigm	GM	OF	TGCTGAACAAGGTGTAGAGGTA	448(−)/621(+)
		OR	GCCAGGTCCTACTTCCACAAAA	
		IF	GTGACATCCAGTCCTACACAATCTAA	
		IR	CACGGAACTCGTTTTCGTC	

"+" indicates the size of the PCR product of the resistant allele; "−" indicates the size of the PCR product of the susceptible allele; the underlined primers B1/9-RR and B1/9-RF can be used as universal dominant markers for *Bph7*, *Bph9*, *Bph18* and *Bph26*.

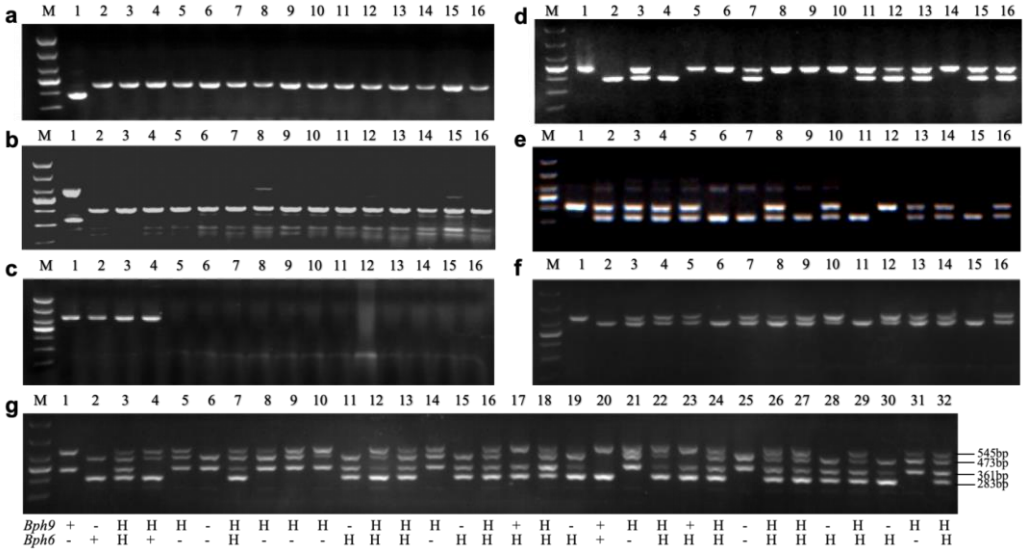


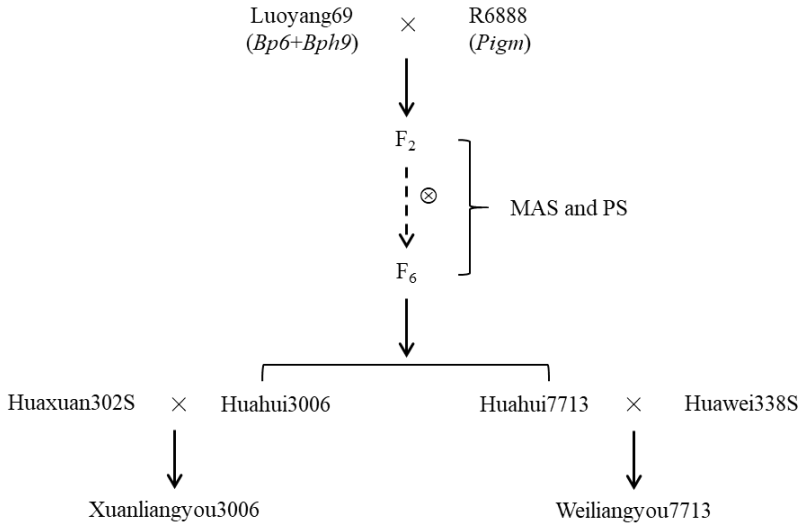
Figure 2. Validation of the developed markers. Validation of markers B6 (**a**), B9 (**b**) and B1/9 (**c**) with rice varieties. M: DL1000 DNA marker, lane 1–16: Luoyang69, Mudgo, T12, ADR52, 9311, Nipponbare, Huazhan, 02428, IRTA129, Huabao, Qiuguang, Chujing28, Chenghui19, Mianhui725, Nongxiang39, Fudao88. Validation of markers B6 (**d**), B9 (**e**) and B1/9 (**f**) using segregating population. Lane 1–16: individual plants from the F_2 of R6888/Luoyang69. (**g**) Amplification pattern of *Bph6* and *Bph9* multiplex PCR amplification system in F_2 population; "+" indicates carrying *Bph6* or *Bph9*, "−" indicates without *Bph6* or *Bph9*, H indicates heterozygous genotype.

We conducted a comprehensive analysis of 478 core rice varieties using newly developed markers. Surprisingly, we found that only Luoyang69 possessed the *Bph6* and *Bph9* genotypes, while the other varieties did not have these genotypes. Furthermore, by utilizing the B1/9 dominant marker B1/9-RF/B1/9-RR, we identified 115 out of the 478 rice varieties that carried BPH1/9-resistant alleles (*Bph7*, *Bph9*, *Bph18*, and *Bph26*). Through further re-sequencing analysis, we determined that all 115 rice cultivars carried one of the four resistance genes, with *Bph18* being the most prevalent (71), followed by *Bph26* (42), and only one instance each of *Bph7* and *Bph9* (Supplementary Table S1). These findings align with the widespread use of *Bph1* and *Bph2* in rice breeding programs.

3.2. Development of New Restorer Lines with BPH and Blast Resistance by MAS

Luoyang69, a BPH resistance improved line, incorporates the *Bph6* and *Bph9* gene into the background of 93-11, an elite indica restorer line of two-line hybrid rice in China. R6888, harboring the *Pigm* gene, is an elite indica restorer line of two-line hybrid rice developed by Yuan Longping High-Tech Agriculture Co., Ltd. A cross (Luoyang69/R6888) was generated to produce an F_2 population. Out of approximately 2400 F_2 plants, a subset of 560 plants exhibiting homozygous resistance alleles for *Pigm*, *Bph6* and *Bph9* by MAS were specifically selected for transplanting. The plants with superior performance in terms of yield and ideotype were harvested for subsequent selfing, enabling further detailed observation of agronomic characteristics, yield potential, resistance, and grain quality. By the F_6 generation, 24 outstanding new lines exhibiting enhanced resistance against blast and BPH, along with superior agronomic traits, were selected for test-crossing trials. These lines were categorized into three groups: Gm69, Gm6 and Gm9. The Gm69 group consisted of six lines that were pyramided with *Bph6*, *Bph9*, and *Pigm*. The Gm6 group consisted of eight lines pyramided with *Bph6* and *Pigm*, while the Gm9 group consisted of ten lines pyramided with *Bph9* and *Pigm* (Table 2). The average seedling blast resistance score for the Gm69, Gm6 and Gm9 group lines was 3.76, 3.62 and 3.43, respectively, which was significantly higher than that of the parental Luoyang69 with a score of 8.23. Similarly, the average seedling BPH resistance score for the Gm69, Gm6 and Gm9 group lines was 2.75, 3.56 and 3.26, respectively, showing significant enhancement compared to the parental

R6888 with a score of 8.20. Furthermore, the BPH resistance of the Gm69 group lines was significantly higher than that of the Gm6 and Gm9 group lines, indicating that pyramiding *Bph6* and *Bph9* can more effectively enhance BPH resistance than pyramiding a single *Bph6* or *Bph9* gene. After test-crossing trials on these lines, two elite Gm69 lines, namely Huahui3006 and Huahui7713, demonstrated the highest combining ability and heterosis, displaying outstanding agronomic performance in hybrid-inbred lines (Table 3, Figure 3). Both Huahui3006 and Huahui7713 carry *Pigm*, *Bph6* and *Bph9*, and exhibit high-level resistance to both blast disease and BPH. In contrast, the parental lines, Luoyang69 and R6888, were highly susceptible to blast disease and BPH, respectively (Table 4, Figure 4).



Note: PS stands for phenotypic selection

Figure 3. The breeding process of new restorer lines and hybrids with improved blast and BPH resistance. Symbol ⊗ stands for self-crossing.

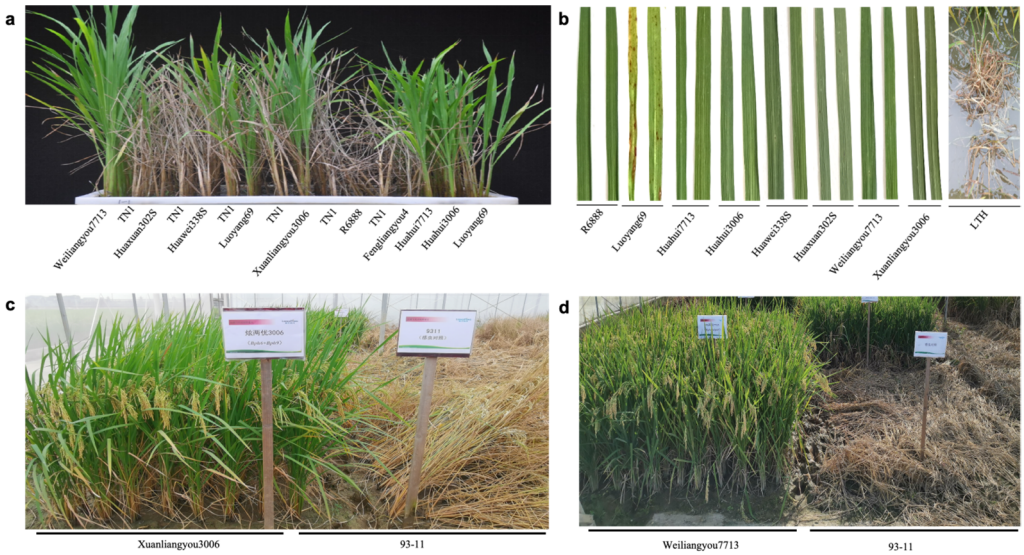


Figure 4. The BPH- and blast-resistance performance of improved restorer lines and their hybrid combinations. Performance of new restorer lines for resistance to BPH (**a**) and rice blast (**b**) at seedling stage. BPH-resistance performance of hybrid combinations derived from Xuanliangyou3006 (**c**) and Weiliangyou7713 (**d**).

Table 2. Resistance analysis of pyramiding lines and their parents to rice blast and BPH.

Lines	Genes of Pyramiding	Number of Lines	Seedling BPH Resistance Score	Seedling Blast Resistance Score
Gm69 lines	*Bph6* + *Bph9* + *Pigm*	6	2.75 ± 0.37 c	3.76 ± 0.41 a
Gm6 lines	*Bph6* + *Pigm*	8	3.56 ± 0.54 b	3.62 ± 0.42 a
Gm9 lines	*Bph9* + *Pigm*	10	3.26 ± 0.19 b	3.43 ± 0.52 a
R6888	*Pigm*		8.20 ± 0.26 a	3.63 ± 0.41 a
Luoyang69	*Bph6* + *Bph9*		2.73 ± 0.15 c	8.23 ± 0.15 b
9311			7.90 ± 0.16 a	8.33 ± 0.33 b

Different characters of superscripts (a, b and c) indicate significant differences by one-way ANOVA analysis.

Table 3. The main agronomic traits and rice quality of parents and improved new lines.

Line	GY (t/ha)	DTH (d)	PH (cm)	PL (cm)	NGP	SF (%)	TGW (g)	AAC (%)	GC (mm)	GT	HRR (%)	LWR	CD (%)
Huahui3006	8.43 ± 0.2 b	125 ± 0.6 c	114.7 ± 2.6 b	23.8 ± 0.7 a	220.6 ± 27.4 b	88.9 ± 1.9 a	27.5 ± 1.9 a	16.1 ± 0.1 c	62 ± 2.0 b	6.0 ± 0.2 a	64.2 ± 1.2 b	3.4 ± 0.08 a	3.6 ± 0.2 c
Huahui7713	8.67 ± 0.2 ab	126 ± 1.4 b	114.3 ± 1.2 b	23.2 ± 0.9 b	230.6 ± 9.7 b	88.2 ± 1.8 a	27.8 ± 1.8 a	16.4 ± 0.1 b	63 ± 1.7 ab	6.0 ± 0.2 a	63.1 ± 2.5 b	3.3 ± 0.03 b	4.6 ± 0.3 b
R6888	8.31 ± 0.2 b	129 ± 1.2 a	117.6 ± 1.6 a	22.3 ± 1.0 b	258.2 ± 20.2 a	85.8 ± 1.6 b	27.3 ± 1.1 a	15.5 ± 0.2 d	65 ± 1.5 a	3.0 ± 0.1 b	64.1 ± 1.8 b	3.3 ± 0.07 b	4.41 ± 0.3 b
Luoyang69	8.52 ± 0.3 a	120 ± 0.8 d	112.3 ± 3.0 b	23.8 ± 0.9 a	171.4 ± 14.2 c	88.01 ± 1.1 ab	29.4 ± 1.4 a	17.3 ± 0.2 a	65 ± 1.4 a	6.2 ± 0.3 a	69.5 ± 1.7 a	2.9 ± 0.12 c	7.05 ± 0.2 a

Abbreviations: GY, grain yield; AAC, apparent amylose content; CD, chalkiness degree; DTH, days to heading; GC, gel consistency; GT, gelatinization temperature; GY, grain yield; HRR, head-rice rate; LWR, length–width ratio; NGP, number of grains per panicle; PH, plant height; PL, panicle length; SF, spikelet fertility; TGW, thousand-grain weight. Different characters of superscripts (a, b, c and d) indicate significant differences by one-way ANOVA analysis.

Table 4. The genotypic and resistance performances of parents, improved lines and derived hybrid combinations.

Line	Genotype			Blast Resistance				BPH Resistance
	Bph6	*Bph9*	*Pigm*	Seedling	Panicle Blast Severity Index	Incidence of Infected Panicles	Integrated Resistance Index	
R6888	−	−	+	3	3	3	3	HS
Luoyang69	+	+	−	9	9	9	9	R
Huahui3006	+	+	+	3	3	3	3	R
Huahui7713	+	+	+	3	3	3	3	R
XuanLiangYou3006	+/−	+/−	+/−	3	3	3	3	R
WeiLiangYou7713	+/−	+/−	+/−	3	3	3	3	R

Abbreviations: HS, highly susceptible; R, resistant. + stands for resistant genotype, − stands for susceptible genotype; +/− stands for heterozygous genotype.

3.3. New Hybrid-Rice Variety Development with Blast and BPH Resistance

Huahui7713 and Huahui3006 were crossed with the elite sterile lines Huawei338S and Huaxuan302S, respectively, which were newly developed by Yuan Longping High-Tech Agriculture Co., Ltd. The derived hybrid rice varieties, named as Weiliangyou7713 and Xuanliangyou3006, were evaluated in the state's regional trials for new variety of middle-season indica in the middle and lower reaches of the Yangtze River in 2019 and 2020, respectively. In the state's regional trials, both Weiliangyou7713 and Xuanliangyou3006 showed significantly improved resistance to rice blast and BPH. Additionally, both hybrid rice varieties displayed superior yield performance compared to the control variety. Notably, Weiliangyou7713 showed a remarkable yield increase, of 7.52% (Table 5). Furthermore, both varieties showed good rice quality with second-class grain quality according to the standard of superior quality rice set by the industry in China (NY/T593-2013). Due to the outstanding performance, Weiliangyou7713 and Xuanliangyou3006 received new variety certifications in 2021 and 2022, respectively.

Table 5. The agronomic trait performance, and BPH and blast resistance of new hybrid rice varieties in state new-variety trials.

Variety	Year	Agronomic Traits		Grain Quality Traits				Blast Resistance		BPH Resistance
		GY (t/ha)	DTM (d)	AAC (%)	GC (mm)	CD (%)	HRR (%)	IDI	HSPBS	
Weiliangyou7713	2019	10.22 (CK + 6.27%)	138.0 (CK + 4.6)	15	82	0.8	68.9	3.5	5	R
	2020	9.61 (CK + 8.76%)	135.9 (CK + 2.8)	14.3	82	0.9	65.9	3	5	R
Xuanliangyou3006	2020	9.13 (CK + 3.24%)	137.5 (CK + 5.6)	16.8	60	1.4	66.3	3.4	3	MR
	2021	9.39 (CK + 1.89%)	139.0 (CK + 8.1)	15.4	61	7	64.9	2.6	3	MR

Abbreviations: GY, grain yield; DTM, days to mature; CK, control-check variety in state new-variety trial; AAC, apparent amylose content; GC, gel consistency; CD, chalkiness degree; HRR, head-rice rate; IDI, integrated disease index (IDI = disease score of seedling × 0.25 + disease score of incidence of panicle blast × 0.25 + disease score of panicle blast severity × 0.5, described by the National Committee of Crop Variety Certification in China (https://www.natesc.org.cn/ (accessed on 20 October 2021)); HSPBS, highest scale of panicle blast severity; R, resistant; MR, medium resistant.

Additionally, Weiliangyou7713 was recognized as super-rice by the Ministry of Agriculture and Rural Affairs of China (MARAC) in 2023, and it is also the first super-rice conferring both blast and BPH resistance. Weiliangyou7713 achieved an average yield of 16.26 tons/ha and 15.55 tons/ha in demonstration plot trials over 66.67 hectares in Hunan province in 2021 and 2022, respectively. By 2023, the planting area of Weiliangyou7713 had surpassed 53.3 thousand hectares.

4. Discussion

Developing rice varieties with broad-spectrum or persistent resistance has been a significant goal for breeders. However, it has been revealed that BPH resistance genes, like disease resistance genes, encode immune receptors to perceive the insect effector [26]. It is commonly observed that one resistance gene provides resistance to a specific pathogenic microbe or insect biotype [27,28]. As pathogenic microbes mutate or insect biotypes evolve, the resistance of rice varieties carrying a single resistance gene may diminish over time. Therefore, breeders are actively seeking ways to reduce selection pressure on specific pathogens or insects. One approach is to utilize broad-spectrum resistance or to pyramid multiple resistance genes, which can reduce the frequency of pathogen variation or insect evolution. The ultimate aim is to develop rice varieties with durable and stable resistance. In recent years, most studies on improving rice resistance through MAS have focused on either diseases or insects, with fewer studies targeting both disease and insect resistance [29–32]. There are also limited reports on the commercial-scale promotion of varieties resistant to both rice blast and BPH. In this study, we successfully pyramided the BPH resistance genes *Bph6*, *Bph9*, and the broad-spectrum rice blast resistance gene *Pigm*, resulting in the

development of two new restorer lines with high-level resistance to both blast disease and BPH. Their derived hybrid combinations also exhibit resistance to rice blast and BPH, as well as excellent performance in terms of yield and rice quality. Up to now, two derived new hybrid rice varieties (Weiliangyou7713 and Xuanliangyou3006) have been certificated and commercially released in China. Notably, both hybrid rice varieties demonstrated enhanced resistance against rice blast and BPH, without compromising yield and grain quality. The cultivation of Weiliangyou 7713, extending over 53,300 hectares by 2023, is expected to markedly decrease fungicide and pesticide usage, significantly contributing to the control and prevention of rice blast and BPH outbreaks. This provides strong evidence that the utilization of Huahui7713 and Huahui3006 is highly effective in rice breeding programs aimed at improving resistance against blast and BPH.

The *Bph6* and *Bph9* genes, found in tropical rice cultivars, have less undesirable linkage drag and are more likely to be effectively used in rice breeding. These genes are dominant, which means they provide a higher level of resistance in heterozygous genotypes, making them valuable for hybrid rice breeding. However, our analysis of 478 rice varieties suggested that *Bph6* and *Bph9* have not been utilized in breeding. To control BPH damage, it is important to accelerate the application of *Bph6* and *Bph9* in breeding and to pyramid them to develop BPH-resistant rice varieties. A routine breeding method for pyramiding multiple genes based on phenotype evaluation of insect resistance is impracticable, due to a huge amount of work and difficulties in distinguishing gene effects. In our study, the combination of MAS with conventional breeding methods has successfully pyramided two BPH resistance genes along with a blast resistance gene into the high yield and superior-quality background. Although molecular marker technology has been extensively used in rice breeding practice, breeders are still primarily concerned with improving the accuracy and efficiency of molecular markers and reducing the cost of detection. One approach to achieving this is through the development of functional markers or gene-specific molecular markers, which are based on gene-specific sites. Here, we developed functional molecular markers for *Bph6* and *Bph9*. These markers are capable of distinguishing the allele variation of the selected genes, and consequently enhance the accuracy and efficiency of MAS [33–35]. Another strategy for improving the efficiency of molecular marker detection is the utilization of multiplex PCR technology. This technology allows for the simultaneous identification of multiple markers in a single PCR amplification system, and is therefore an important avenue for enhancing the efficiency of molecular marker detection [36–38]. In this experiment, ARMS-PCR was applied for detecting SNPs, based on allele-specific amplification PCR. The conventional strategy, known as strategy A, involves designing four primers with a single site as the target. However, this approach has limitations as both the upstream and downstream sequences of the target site need to satisfy the primer design, resulting in a limited success rate. In contrast, strategy B offers better flexibility and a higher success rate by using dual targets to design allele-specific amplification primers separately. Additionally, strategy B allows for the design of amplification products of different sizes for multiple sites of different genes, making it relatively easy to achieve multiplex PCR amplification and improve detection efficiency. In this study, we obtained several representative variations of *Bph6* and *Bph9*, but most of them did not meet the requirements for primer design. Among the selected representative variations, we designed gene-specific markers for *Bph9* and *Bph6* based on strategy A and strategy B, respectively. These markers can accurately identify rice varieties carrying *Bph6* or *Bph9*, making them valuable for screening BPH-resistant germplasm resources. Additionally, we developed a multiplex PCR amplification genotyping system for *Bph6* and *Bph9*, which allows for the simultaneous detection of their genotypes through 1.5–3% agarose gel electrophoresis. This system is fast, simple, and cost-effective, and will contribute to pyramiding multiple genes and breeding varieties of high yield, superior quality and durable resistance.

5. Conclusions

The development of multi-resistant varieties is widely recognized as an efficient and environmentally friendly approach to controlling crop diseases and pests. In this study, we reported the development of two functional molecular markers for two major BPH resistance genes (*Bph6* and *Bph9*), which can be used in molecular design breeding. Two new elite male lines (Huahui7713 and Huahui3006) for two-line hybrid rice were also developed by pyramiding *Bph6, Bph9* and the major blast resistance gene *Pigm*. Huahui7713 and Huahui3006 exhibit enhanced resistance to both BPH and blast, along with high-yielding and superior quality performance. Furthermore, we have successfully developed two Huahui7713- and Huahui3006-derived two-line hybrid rice varieties (Weiliangyou7713 and Xuanliangyou3006) with BPH and blast resistance. These hybrids performed excellently in state-wide trials in China, have been approved for commercial release, and have gained acceptance in the market with an annual promotion area of 53.3 thousand hectares in the three years since their release. Notably, these varieties provide the dual benefits of high yield and improved resistance to both BPH and blast, thereby potentially minimizing the use of pesticides and fungicides in rice production. This study exemplifies the practical application of molecular design in rice breeding programs, particularly for enhancing disease and pest resistance. The developed markers and breeding strategies provide valuable tools for future BPH- and blast-resistance breeding in rice, and the commercial release of resistance varieties can contribute significantly to ensuring food security.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy14050878/s1, Table S1: Information of 478 rice varieties used for evaluating the markers in this study.

Author Contributions: Y.Y. and K.W. conceived and designed experiments. Z.D. and P.Q. contributed to data analysis and the first draft of the manuscript. K.L. and N.J. and contributed to data collection. T.Y. and X.Z. were in charge of file management. K.W., Y.Y., C.F. and G.H. supervised and complemented the manuscript writing. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: Author Zhao Deng, Peng Qin, Kaiyu Liu, Nan Jiang, Tianze Yan, Xuanwen Zhang, Chenjian Fu, Kai Wang and Yuanzhu Yang are employed by the company Yuan Longping High-Tech Agriculture Co., Ltd. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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