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Development and Application of *Pi-k*^{h} Co-dominant Marker for Rice Blast Resistance Gene

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Abstract Rice, as one of the staple food crops in the world, is reduced by 10~30% every year due to the damage of rice blast. However, the application of marker assistant selection (MAS) with rice disease resistance genes can be effectively, accurately and rapidly used to breed rice blast resistant varieties. The rice blast resistance gene *Pi-k*^{h} has been proved to have broad-spectrum resistance to the rice blast. Therefore, the selection and utilization of *Pi-k*^{h} gene by MAS in rice disease resistance breeding possess high application value for improving rice blast resistance. In this study, based on two InDel differences in Coding sequence (CDS) region of *Pi-k*^{h} gene, a co-dominant functional molecular marker was developed at the conservative position of the sequence, which was named ZJPikh. The marker was used in the disease resistance breeding with rice blast susceptible variety “Yudao 518” as female parent and rice blast resistant variety “Shida 68” as male parent. The genotype of gene *Pi-k*^{h} related with rice blast resistance in F2 isolated population and high generation plants were verified by pedigree method and MAS. A new rice blast resistant third-grade variety “Yudao 58” with *Pi-k*^{h} was successfully bred. At the same time, the *Pi-k*^{h} genotypes of 21 main rice varieties were identified, which provided technical support for the targeted and efficient breeding of blast resistant varieties carrying *Pi-k*^{h} gene through MAS technology. Our research provides convenience for identifying rice blast resistance germplasm resources and molecular marker-assisted disease resistance breeding.
Keywords Rice blast; MAS; *Pi-k*^{h}; Yudao 58; Breed

Rice is one of the main food crops in the world, the world’s arable land area is declining, and the population is increasing. It is very important to reduce the impact of disease on rice production and improve rice yield, which has become a critical scientific problem that breeders need to solve urgently. However, the current annual reduction in rice production due to rice blast damage in the world is still as high as 10%~30%, and even no harvest in extreme cases (Fernandez and Wilson, 2014). Rice blast is also known as rice fever, commonly known as fire blast, neck blast, hanging head blast, rice cancer, etc. It is one of the most serious fungal diseases that affect the safety of rice production (Zhao et al., 2019). Rice blast is a fungal disease caused by *Magnaporthe oryzae* produced by *Gascony fungi* infecting rice (Dean et al., 2012). Rice blast can occur during the arbitrary growth period of rice, and the field disease often manifests as a panicle blast and leaf blast. Due to the frequent variation of the pathogenicity of the rice blast flora, the resistance of bred varieties is difficult to maintain. Therefore, continuously excavate new sources of resistance and resistance genes and utilizing them to breed new disease-resistant varieties has become an important research goal of rice blast resistance breeding (He et al., 2020).

So far, at least 35 rice blast resistance genes have been cloned in rice (Wang et al., 2017a; Zhao et al., 2018), of which the rice blast resistance genes have been successfully utilized through MAS technology and new blast-resistant lines or varieties have been selected, such as applying the KASP marker developed based on *Pi9*, and quickly screened homozygous lines containing *Pi9* resistance genes through MAS (Wei et al., 2019); The developed *Pigm* marker was used to identify disease-resistant plants in the F2 population of Huaidao 9/Gumei 4,

and high-resistant rice blast lines were obtained (Chen et al., 2020). Similar to *Piz-t* (Liu et al., 2016), *PigmR* (Zeng et al., 2018), etc. The rice blast resistance gene *Pi-k*^{h} possess broad-spectrum resistance to rice blast fungus (Ma et al., 2018). Previous study indicated that the *Pi-k*^{h} gene showed significant resistance to more than 81% of 445 races in Yunnan and Guangdong provinces of China (Li et al., 2005; Yang et al., 2008). Therefore, the *Pi-k*^{h} gene is selected by MAS and used in rice disease resistance breeding, which has high application value for improving rice blast resistance.

In this study, based on the two InDel differences in the coding sequence (CDS) region of the *Pi-k*^{h} gene, a co-dominant functional molecular marker was developed at a conservative position in the sequence, and the marker was applied to the breeding of new resistant lines or varieties with "Yudao 518" as female parent and "Shida 68" as male parent by molecular marker assistant select (MAS). A new rice blast resistant line "Yudao 58" with *Pi-k*^{h} resistant genotype was successfully bred by tracking and testing the genotype of *Pi-k*^{h} associated gene in F2 population and higher generation plants. At the same time, the *Pi-k*^{h} genotypes of 21 rice varieties were accurately and efficiently identified. This study provided technical support for the cultivation of *Pi-k*^{h} gene resistant rice varieties by molecular marker assisted selection.

1 Results and Analysis

1.1 Development the *Pi-k*^{h} marker

Two genotypes were found in detection the sequence variation of rice blast associated gene *Pi-k*^{h}. We amplified, sequenced and analyzed the *Pi-k*^{h} genotype of Zhengdao 18 and Nipponbare respectively. According to the sequencing results, it was identified as an allelic variation of the rice blast related gene *Pi-k*^{h} by comparing with the homologous sequence in GenBank. 10 homologous sequences from GenBank were retrieved by blast and compared them with sequencing sequences (Figure 1). The result showed that there was no intron in *Pi-k*^{h} of rice blast resistant materials (varieties/lines), which encoded an NBS-LRR resistance protein with 330 amino acids. There was a 143 bp deletion mutation of *Pi-k*^{h} at 33 position from the start codon, and a 37 bp deletion at the C-terminal of the coding amino acid in the resistant materials compared with the *Pi-k*^{h} in the susceptible materials such as INRC-779, Nipponbare and CN-1789 etc. At the same time, combined with our analysis of *Pi-k*^{h} gene sequence, many SNPs were found. In order to develop a specific primer containing two indel mutations, a pair of primers were designed at the conservative sequence positions according to the sequence alignment results, and marked (Figure 2). The primer sequence information was shown (Table 1). Theoretically, 722 bp and 543 bp target bands could be amplified in susceptible and resistant materials respectively.

1.2 Reliability test of *Pi-k*^{h} mark

A fragment about 750 bp in size was detected in Nipponbare and Xindao 18, and about 500 bp in Zhengdao 18 and Xinkedao 21 using ZJPIKH marker, which were consistent with the conclusion detected by Wang et al. (2017b) that Xindao 18 was *Pi-k*^{h} susceptible genotype, Zhengdao 18 and Xinkedao 21 were *Pi-k*^{h} resistant genotypes. The results showed that ZJPIKH marker could effectively distinguish *Pi-k*^{h} resistant genotype from susceptible genotype (Table 2).

1.3 Molecular marker *Pi-k*^{h} assisted breeding of "Yudao 58"

Next, ZJPIKH marker was employed in breeding of new rice lines and varieties with resistance to rice blast. The F2 generation segregation population were obtained by artificial cross, and bagging and selfing with "Shida 68" carrying *Pi-k*^{h} resistant genotype as male parent and "Yudao 518" carrying rice blast *Pi-k*^{h} susceptible genotype as female parent. ZJPIKH marker was used to identify the *Pi-k*^{h} genotype of segregant population plants in F2 generation. The plants with *Pi-k*^{h} resistance genotype were further selected by pedigree method, and F3 generation were obtained by selfing, and *Pi-k*^{h} genotype was further identified in F3 generation. According to the results of partial electrophoresis bands (Figure 3), about 500 bp bands were amplified from “Shida 68, F3-1, F3-3, F3-8 and F3-9, signified that it carried disease resistance genotypes; and 750 bp bands were amplified from Yudao 518, F3-2, F3-5, F3-6 and F3-7, signified that it carried susceptible genotypes. Two bands of about 500 bp and 750 bp were amplified from F3-4 and F3-10, indicating that *Pi-k*^{h} was a heterozygous genotype. After multiple

generations of molecular marker selection, a new rice blast resistant line "Yudao 58" with homozygous *Pi-k*^{h} resistance genotype and elite comprehensive characters was selected. At present, the line has entered the second year regional test and production test in the national Huang Huai rice region of China, and is expected to become a new rice variety with high yield and good quality.

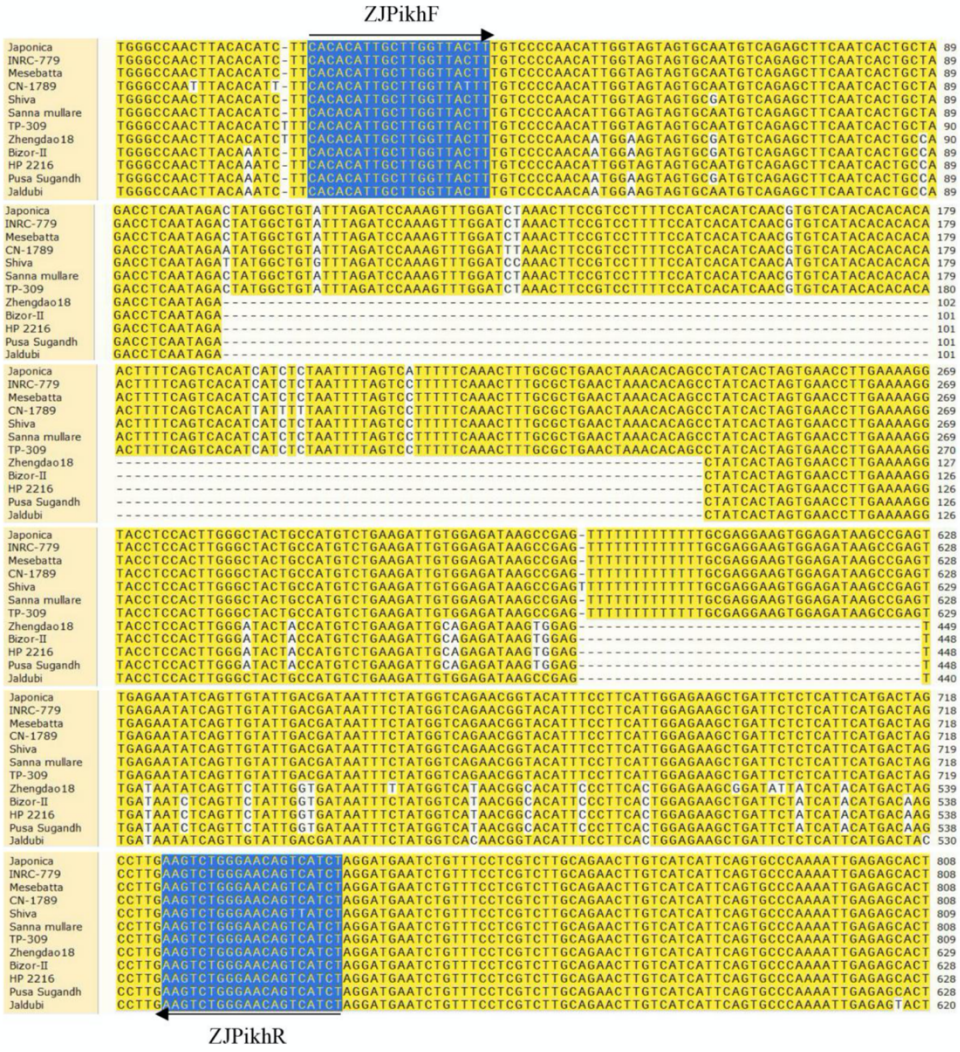

Figure 1 Alignment analysis of sequences in gene banks and sequencing sequences
Note: The forward and reverse primer sequence was marked with black arrow

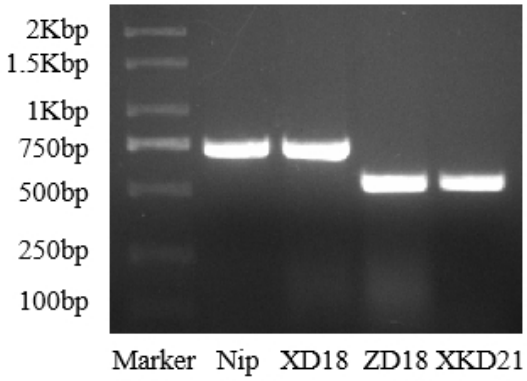

Figure 2 PCR detection of ZJPikh marker on 4 known resistant/ susceptible varieties
Note: M: DNA Maker DL2 000; Nip, XD18, ZD18, XKD21 correspond to rice varieties Nipponbare, Xindao 18, Zhengdao 18, and Xinkedao 21, respectively

Table 1 The Designed primer sequence information of ZJPikh marker

ZJPikhF	5‘-CACACATTGCTTGGTTACTT-3’
ZJPikhR	5‘-AGATGACTGTTCCCAGACTT-3’

Table 2 The polymorphism detection of *Pi-k*^{h} co-dominant marker ZJPikh

Variety	Resistance
Zhengdao 18	+
Xindao 18	-
Subei No. 9	-
Shengdao 806	-
Zhendao 99	+
01D41LB88	+
Jindao 1007	-
Xinkedao 21	+
Yandao 334-6	+
Jinxing No. 1	+
Yudao 518	-
Zhendao 8	-
Daliang 203	-
YD518a	-
Xinkedao 31	+
Xindao 69	-
Xinkedao 29	+
Xindao 89	+
YD518b	-
Yudao 58	+
Hongdao 59	-

Note: "+" means carrying *Pi-k*^{h} resistance genotype, while "-" means not

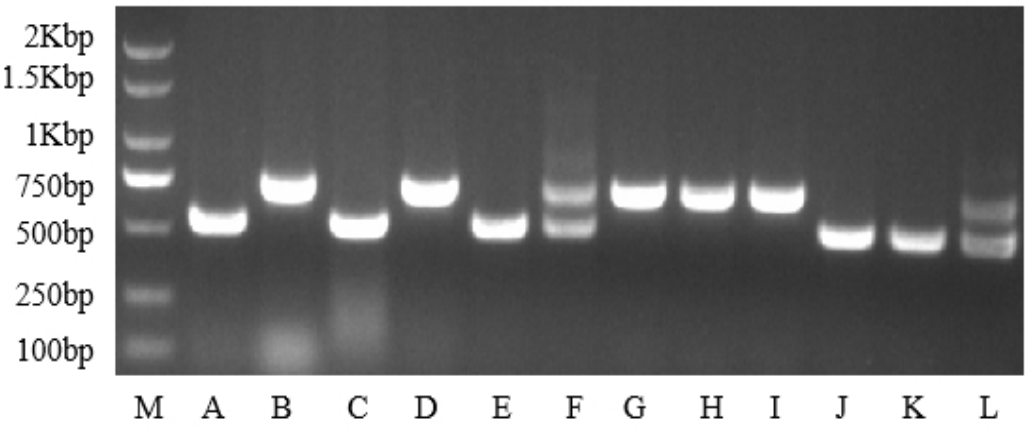

Figure 3 Identification the genotype of segregated populations of Yudao 518, Shida 68 and their hybrid offspring by ZJPikh marker
Note: M: DNA Maker DL2000; Lane A~L are “Shida 68”, “Yudao 518”, F3-1, F3-2, F3-3, F3-4, F3-5, F3-6, F3-7, F3-8, F3-9, F3-10

1.4 Identification of Yudao 58’s disease resistance

In order to further determine the rice blast resistance of "Yudao 58" bred by molecular marker selection, the blast resistance identification tests were carried out in the institute of Plant Protection of Jiangsu Academy of Agricultural Sciences and the institute of Plant Protection of Tianjin Academy of Agricultural Sciences (designated by the country as the identification institution of rice variety test). Natural induction and artificial inoculation were applied to identify the disease resistance. In 2019, the disease resistance identification was conducted in Jiangsu and Tianjin, with Tianjin seedling leaf blast grade 2, the loss of panicle neck blast grade 3,

and Jiangsu seedling leaf blast grade 4, the loss of panicle neck blast grade 3, comprehensive rice blast index 4.3, and medium resistance rice blast grade 3.

1.5 Identification of marker resistance to breeding materials

Based on ZJPIKH marker, we further identified the *Pi-k*^{h} genotype of 21 breeding or variety materials collected by our research group (Figure 4). The results showed that about 500 bp bands were amplified from 10 varieties including Zhengdao 18, which carried the *Pi-k*^{h} resistance genotype, and about 750 bp bands were amplified from 11 varieties including Xindao 18, which carried the *Pi-k*^{h} susceptible genotype (Table 2).

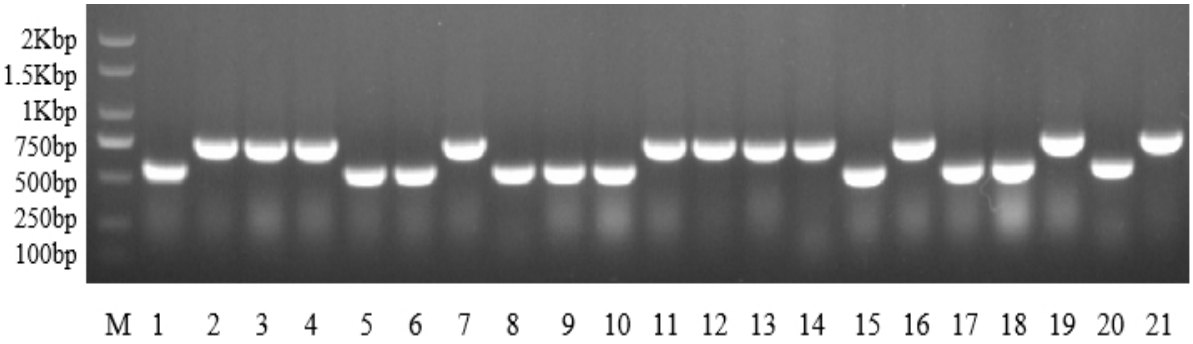

Figure 4 Polymorphism detection of Pi-kh co-dominant marker ZJPikh. M: DNA Maker DL2 000; Lane A-L: Zhengdao 18, Xindao 18, Subei No. 9, Shengdao 806, Zhendao 99, 01D41LB88, Jindao 1 007, Xinkedao 21, Yandao 334-6, Jinxing No. 1, Yudao 518, Zhendao 88, Daliang 203, YD518a, Xinkedao 31, Xindao 69, Xinkedao 29, Xindao 89, YD518b, Yudao 58, Hongdao 59

2 Discussion

Breeding new disease-resistant varieties is the most direct and effective method to control rice blast. However, the cycle of breeding disease-resistant varieties through traditional breeding methods is very long and requires a lot of labor for phenotypic identification. It usually takes at least 6 years to improve the disease resistance of an old variety, and the identification of rice blast resistance is time-consuming and laborious, and the phenotypic identification lacks certain accuracy. Mining disease resistance genes and utilizing them through molecular marker-assisted selection technology is an effective way to quickly improve the disease resistance of varieties and extend the life of old varieties. For example, Jiang et al. (2019) used molecular marker-assisted breeding to simultaneously improve the resistance to brown planthopper and rice blast of the two-line sterile line with 75-1-127 as the resistance source, and obtained homozygous disease resistance genotype in the first self-crossing generation. Zhang et al. (2019) introduced the brown planthopper resistance gene into the water-saving and drought-resistant rice restorer line Hanhui 3 through a combination of molecular marker-assisted selection and conventional backcross breeding. After 6 generations of selection, a series of single genes, double gene, 3 gene and 4 resistant gene improved line were obtained. Tu et al. (2019) applied molecular MAS technology combined with traditional breeding methods to breed a new conventional early rice variety with medium resistance to rice blast, suitable for direct seeding, good plant shape and leaf shape, and superior quality.

Sharma et al. (2005) cloned the *Pi-k*^{h} gene in disease-resistant rice materials, and found that *Pi-k*^{h} possessed only one exon in the disease-resistant materials, encoding a 330 amino acid NBS-LRR disease-resistant protein. In the susceptible material Nipponbare, *Pi-k*^{h} possessed an intron with the length of 212 bp, which was significantly different from the *Pi-k*^{h} gene of the disease-resistant genotype. In this study, based on the sequence alignment and analysis of the cloned broad-spectrum rice blast resistance gene *Pi-k*^{h}, we developed a codominance marker in the conservative position of the *Pi-k*^{h} gene sequence according to the InDel in the *Pi-k*^{h} gene sequence. The functional molecular marker ZJPikh, which can accurately identify the resistant and susceptible genotypes of the *Pi-k*^{h} gene by agarose gel electrophoresis. By using this molecular marker, homozygous disease-resistant plants can be obtained through MAS in F2 isolation. Compared with traditional phenotypic observation and identification, it effectively shortens the time of variety breeding and reduces the labor intensity of field selection. Based on the ZJPikh marker, we have used MAS technology to breed continuously for multiple generations, and efficiently

bred a new medium-resistant rice blast line "Yudao 58", which has now entered the second-year regional test of the national Huanghuai rice area in China. It is expected to become a new high-yield, high-quality, and resistant rice blast variety. Our results showed that molecular marker-assisted selection breeding can effectively improve the selection efficiency of target genes and traits, and accelerate the process of breeding.

At the same time, we used this marker to further identify the *Pi-k*^{h} genotypes of 21 key breeding materials or popularized japonica rice varieties collected in the laboratory, and found that many bred varieties lack *Pi-k*^{h} resistance genes, and it can be considered to introduce the *Pi-k*^{h} resistance gene through recurrent breeding to improve the resistance of varieties and extend the production life of old varieties. In addition, the resistant materials screened in this study can be used as the source of resistance, providing us with rice blast resistant germplasm resources for the selection of rice blast resistant varieties and selection of resistant sources in the future. The research and development of molecular markers provide convenience for the development of molecular marker-assisted disease resistance breeding.

3 Materials and Methods

3.1 Material

Rice blast susceptible material Nipponbare, breeding materials "Yudao 518" and "Normal University 68" were all provided by Henan Normal University. The 21 breeding or line materials identified by the marker were: Zhengdao 18, Xindao 18, Subei 9, Shengdao 806, Zhendao 99, 01D41LB88, Jindao 1007, Xinkedao 21, Yandao 334-6, Jindao Xing 1, Yudao 518, Zhendao 88, Daliang 203, YD518a, Xinkedao 31, Xindao 69, Xinkedao 29, Xindao 89, YD518b, Yudao 58, Hongdao 59, the specific information of material breeding or providing institute (Table 3).

Table 3 21 varieties/line material breeding (provided) institute identified by ZJPikh mark

Variety/Strain	Breeding / Provided Institutions	Variety/Strain	Breeding / Provided Institutions
Zhengdao 18	Institute of Food Crops, Henan Academy of Agricultural Sciences	Zhendao 88	Zhenjiang Institute of Agricultural Sciences, Jiangsu Hilly Region
Xindao 18	Xinxiang Academy of Agricultural Sciences	Daliang 203	Linyi Daliang Seed Industry Co., Ltd.
Subei No. 9	Jiaxing Academy of Agricultural Sciences	YD518a	Henan Normal University
Shengdao 806	Shandong Rice Research Institute	Xinkedao 31	Xinxiang Academy of Agricultural Sciences
Zhendao 99	Zhenjiang Institute of Agricultural Sciences, Jiangsu Hilly Region	Xindao 69	Xinxiang Academy of Agricultural Sciences
01D41LB88	Xinxiang Academy of Agricultural Sciences	Xinkedao 29	Xinxiang Academy of Agricultural Sciences
Jindao 1007	Tianjin Rice Research Institute	Xindao 89	Xinxiang Academy of Agricultural Sciences
Xinkedao 21	Xinxiang Academy of Agricultural Sciences	YD518b	Henan Normal University
Yandao 334-6	Agricultural Science Research Institute of Yandu District, Yancheng City, Jiangsu Province	Yudao 58	Henan Normal University
Jinxing No. 1	Institute of Crops, Tianjin Academy of Agricultural Sciences	Hongdao 59	Henan Normal University
Yudao 518	Henan Normal University		

3.2 Development functional marker of *Pi-k*^{h}

The gene sequence of *Pi-k*^{h} rice blast susceptible cultivar were retrieved and download from that genbank (https://www.ncbi.nlm.nih.gov/genbank/) database (Table 4). The Muscle program in Mega X software was used to compare the sequences, and the comparison parameter was the default value. Based on the InDel sequence region, conservative sequence sites were selected from the sequence to develop co-dominant markers.

3.3 DNA extraction

The genomic DNA of rice leaves was extracted by CTAB from the top green leaves of normal growing plants (Doyle and Doyle, 1990). The extracted DNA was diluted with TE buffer, and the final concentration was 200 ng/μL. the extracted DNA was sub packed and stored at -20℃.

3.4 *Pi-k^h* Marker amplification and identification

PCR reaction system (Table 5).

Reaction parameters and product detection:

The PCR cycle conditions were set according to the optimal Taq reaction temperature and primer annealing temperature (Table 6).

The band sizes of *Pi-k^h* disease resistance and susceptibility gene amplification were 543 bp and 722 bp, respectively. The PCR products were detected by 1% agarose gel electrophoresis with a voltage of 150 V, and the disease-resistant genotype of the material was judged according to the target band shown by agarose gel electrophoresis.

Table 4 Sequence information of *Pi-k^h* gene in cultivated species

Varieties	Serial number	Resistance
Japonica	AC104846.2	Susceptible
INRC-779	HE586181.1	Susceptible
Mesebatta	HE586173.1	Susceptible
CN-1789	HE586205.1	Susceptible
Shiva	HE586210.1	Susceptible
Sanna mullare	HE586188.1	Susceptible
TP-309	HE586157.1	Susceptible
Bizor-II	GU258499.1	Resistance
HP 2216	HE589458.1	Resistance
Pusa Sugandh 3	HE586249.1	Resistance
Jaldubi	HE586197.1	Resistance

Table 5 PCR reaction system

Component	Volume (10 μL)
2x Taq MasterMix (Dye)	5.0
ddH2O	3.8
Template DNA	0.4
Forward Primer	0.4
Reverse Primer	0.4

Table 6 PCR cycle conditions

Reaction step	Reaction temperature (℃)	Reaction time (min)	Number of cycles
Predenaturation	94	2	-
Denaturation	94	0.5	-
Annealing	54.2	0.5	34
Extension	72	0.5	-
Terminal Extension	72	2	-

3.5 Identification of rice blast

The rice blast identification was carried out at the Plant Protection Institute of the Jiangsu Academy of Agricultural Sciences and the Plant Protection Institute of the Tianjin Academy of Agricultural Sciences (the national rice variety test designated rice blast resistance identification institute). Artificial inoculation and natural induction methods were used, and the disease nursery was set up in the experimental field where the occurrence of

rice blast was more serious over the years. The sowing and management were consistent with those in the normal experimental field. The strains were inoculated artificially in seedling and booting stage (Xiao et al., 2013). The disease was investigated at the seedling stage of 7~8 leaves and the later stage of grain filling (Gao and Yan, 2006).

Authors' contributions

Sang Shifei, Wang Junyi and Zhou Jing were the experimental design and experimental research executors of this study; Sang Shifei and Wang Junyi completed the data analysis and the first manuscript of the paper; Zhou Jing, Cao Mengyu, Wang Yanan and Zhang Jianqiang participated in the experimental design and the results analysis; Ji Shengdong and Zhang Wenling are the designers of the project. They guide the experimental design, data analysis, thesis writing and revision. All authors read and approved the final manuscript.

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