Identification of novel rice blast resistance alleles through sequence-based allele mining

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Abstract

Background: As rice (*Oryza sativa*) is the staple food of more than half the world's population, rice production contributes greatly to global food security. Rice blast caused by the fungus *Magnaporthe oryzae* is a devastating fungal disease of rice, affecting yield and grain quality and resulting in substantial annual economic losses. Because the fungus evolves rapidly, resistance conferred by most of the single blast race resistance genes is often broken after a few years of intensive agricultural use. Effective resistance breeding in rice therefore requires continual enrichment of the reservoir of resistance genes and alleles. Seed banks represent a rich source of genetic diversity; however, they have not been extensively used to identify novel genes and alleles.

Results: We carried out a large-scale screen for novel blast resistance alleles in 1883 rice varieties from major rice producing areas across China. Of these, 107 varieties showed at least moderate resistance to natural infection by rice blast at rice blast nurseries in Enshi and Yichang, Hubei Province. Using sequence-based allele mining to amplify and clone the allelic variants of major rice blast resistance genes at the Pi2/9/gm/zt locus of chromosome 6 from the 107 blast-resistant varieties, we identified 13 novel blast resistance alleles. We then used controlled infections to assess the resistance of rice varieties carrying the novel alleles to 34 single rice blast isolates from Hubei, Guangdong, Jiangsu, Hunan, Jiangxi, Sichuan, Heilongjiang, and Fujian Provinces. The varieties identified as being resistant in the nursery trials showed varied disease responses when infected with the single blast isolates, suggesting that the novel Pi2/9/gm/zt alleles vary in their blast resistance spectra. Some of the newly identified alleles have unique single nucleotide polymorphisms (SNPs), insertions, or deletions, in addition to polymorphic residues that are shared between the different alleles.

Conclusions: These alleles expand the allelic series of blast resistance genes, enriching the genetic resource for rice blast resistance breeding programs and for studies aimed at deciphering rice–rice blast molecular interactions.

Key words : Pi9 , R-genes, Nucleotide diversity, Gene conversion, Resistance gene alleles, Rice blast

Introduction

Rice blast is an acute and destructive disease that can reduce yield or even wipe out an entire harvest. Grain blast also affects the quality of rice and is a serious food safety problem (Deng et al., 2017; Ishihara et al., 2014). In China, the disease affects more than 3.8 million hectares per year, reducing rice yield by 1 billion kg per year (Jiang et al., 2015; Tian et al., 2016). Rice blast, caused by the fungus *Magnaporthe oryzae*, is the most devastating disease affecting rice, under high temperatures and humidity conditions that favor its spread (Shen et al., 2004; Wang et al., 2017). Rice blast has been reported in almost all rice-producing areas in the world, including the main rice-producing areas of 85 countries and regions (Miah et al., 2013). Effective host resistance, conferred by resistance (R) genes, is considered to be the most economic approach to control plant diseases (Xiao et al., 2017). To date, 86 rice blast R genes have been isolated (Hua et al., 2012; Zhao et al., 2018). While these genes have advanced our understanding of the molecular mechanisms underlying disease resistance, maintaining genetic resistance in rice is challenging, because single varieties of rice are grown over large areas in monoculture and the pathogen evolves quickly. *M. oryzae* is known for its genetic instability and pathogenic variability, leading to rapid breakdown of resistance in rice varieties (Bryan et al., 2000; Jiang et al., 2012). Resistant rice varieties often remain effective for only a few years before new dominant pathogenic races of the fungus emerge (Lee et al., 2009; Li et al., 2017). Plants have evolved various mechanisms that protect them from pathogen invasion and colonization. Previous studies have shown that most R genes, which encode receptors containing a nucleotide-binding site and leucine-rich repeats (NBS-LRR), are organized into tight clusters containing multiple gene copies. Nine of the 13 major rice blast R genes are clustered (Qu et al., 2006; Wu et al., 2012) and most of them are broad-spectrum R genes with variable resistance. The Pi9 locus contains at least six known R genes (Pi2, Pi9, Piz-t, Piz, Pigm and Pi50) and is situated location, close to the centromere of chromosome 6. Four R genes at this locus (Pi2, Pi9, Pigm and Piz-t) have been cloned (Dai et al. 2010; Zhou et al., 2006). Numerous studies have indicated that the clustered arrangement of R genes has contributed to the evolution of novel resistance specificities via gene conversion, recombination, or unequal crossing over (Ashikawa et al., 2008; Dai et al., 2010). Some NBS-LRR gene homologs at the same locus exhibit a different evolutionary pattern. Genomic analysis of the Pi9 locus in rice cultivars and wild rice lines has shown that the copy number and SNP genotypes of Pi9 homologs vary, indicating a complex evolutionary history for this R gene locus..

In order to gain insight into the origin and evolution of this locus, and to explore new alleles with broad-spectrum resistance for rice molecular breeding, among 1883 rice varieties from different rice regions, 107 varieties resistant to rice blast at least in one rice region were selected, and the genomic sequences of Pi9 homologs were analyzed. We identified 13 novel alleles in these 107 resistant rice varieties. Meanwhile, we inoculated these 13 alleles with *Magnaporthe grisea* single spore. By comparing with the resistance spectrum of the donor materials of Pi2, Pi9, Pigm, Piz-t, we proved that these 13 alleles are new alleles. Moreover, the resistance of Pi9-Type2, Pi9-

Type3 and Pi9-Type5 were better than that of the cloned broad-spectrum rice blast resistance genes. These alleles extend the allele sequence and enrich the genetic resources of rice blast resistance breeding and rice blast interaction research at the molecular level. We plan to further evaluate the resistance level of new alleles by constructing near isogenic lines and transgenic verification. Finally, our goal is to introduce new alleles of broad-spectrum resistance into high-quality rice varieties by molecular breeding, improve the blast resistance of the original varieties, and cultivate new rice varieties resistant to blast.

Materials And Methods

Plant materials

About 2000 rice cultivars was obtained from major rice-growing provinces of China, including indica and Japonica, and then maintained at Huazhong Agricultural University for this study. Rice blast resistant varieties were measured through natural inducement in two rice uniform blast nursery, which are located at Enshi and Yichang in Hubei province. Based on the standard scoring system for leaf blast (scale HS-HR), choosing the varieties that were resistant with a phenotypic score of MR-HR against field mix-inoculum were selected for molecular screening. Lijiangxin Tuan Heigu (LTH), which is highly susceptible to rice blast, was used as a control for disease evaluation.

Pathogen collection, inoculation and disease evaluation

For resistance spectrum analysis, about 34 blast isolates of different races a virulent were used in this study. All these isolates were collected from whole China major rice-growing provinces and have genetic differentiation and belong to different blast lineages (Shen et al. 1998, 2004). We used these isolates to analyze phenotypic of parents with disease resistance genes. The 34 Magnaporthe oryzae isolates, which are highly virulent on most of the rice lines were also been used for phenotypic analysis of Pi9 allele. Twelve day-old seedlings were spray-inoculated with blast spore suspensions (approximately 1×10^5 spores/ml), and then grew in a dark chamber for 24 h (26°C, 90% humidity). After that, the growth conditions were changed to 12 hours of light and 12 hours of dark treatment every day. After 7 days post inoculation, disease reaction (0–5 disease rating scale) of each line was recorded (IRRI, 2002).

PCR for allele mining and blast resistance genes

The rice genomic sequence of the *O. sativa* cv. Nipponbare (www.ncbi.nlm.nih.gov), the BAC clone sequence (DQ454158.1, containing Pi2) of the *O. sativa* cv. C101A51, the sequence (DQ285630.1, containing Pi9) of the *O. sativa* cv. 75-1-127, the BAC clone sequence (KU904633.2, containing Pigm) of the *O. sativa* cv. GM4H and the sequence (DQ352040, containing Piz-t) of the *O. sativa* cv. ZY1H, corresponding to the location of Pi2/9/gm/z-t genes were used for designing PCR primers. This region corresponds to the sequence on TIGR coordinate LOC_Os06g17900. Two sets of primers flanking to the full length gene were designed using Primer 3 software (<http://primer3.ut.ee/>). PCR amplification of alleles was done using genomic DNA extracted from rice leaves using CTAB method (Murray and Thompson 1980). The PCR reaction of 50 µl was setup with 50 ng of template DNA, 50 ng both forward and reverse primers, 5 µl 10X LA Taq Buffer II, 8 µl dNTP Mixture (2.5 mM each) and 2.5 U TaKaRa LA Taq (RR02MQ, TAKARA). The details of various primers used in present study are given in Table S2.

Amplified PCR products were purified and sequenced using Sanger's method based DNA Analyzer Sequencer, ABI 3730XL (ABI, Applied BiosystemsAmersham, USA). Each allele was sequenced three replications using sequencing primers.

DNA sequence analysis

All the sequence reads generated for each allele by sequencing primers were assembled separately for each allele by using Sequencing Analysis Software Version 5.1 (Applied Biosystems). The sequence of high quality was assembled and the assembled DNA sequence of each allele was used to do Blast2Sequences (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch&PROG_DEF=blastn&BLAST_PROG_DEF=blastn&BLAST_SPEC=GlobalAln&LINK_LOC=BlastHomeLink) analysis against the Pi9 genes to check its similarity.

The Pi9 genes from the rice blast resistant lines were used as a reference for comparison studies. Structural analyses of Pi9 alleles were performed to predict gene structure and conserved domains using CDD software (<https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>) and the data were recorded as their position, such as CC, NBS and LRR domain. For Single Nucleotide Polymorphism (SNP) search, multiple sequence alignment was done for all the alleles along with Pi9 alleles from 75-1-127 as references by using Software of

Sequencer ([https://blast.ncbi.nlm.nih.gov/Blast.cgi?](https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch&PROG_DEF=blastn&BLAST_PROG_DEF=blastn&BLAST_SPEC=GlobalAIn&LINK_LOC=BlastHomeLink)

PAGE_TYPE=BlastSearch&PROG_DEF=blastn&BLAST_PROG_DEF=blastn&BLAST_SPEC=GlobalAIn&LINK_LOC=BlastHomeLink). InDels and SNPs were identified, according to Pi9 sequence as reference sequence. Multiple sequence alignment of DNA sequences from amplified gene fragments were done by using CLUSTALW (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>) and MEGA5.0 (<http://www.megasoftware.net>) softwares. The parameters used in MEGA 5.0 were bootstrap (1,000 replications) and neighbour joining with the p-distance model. A phylogenetic tree was constructed with MEGA to analysis the evolution of the Pi9 allele. The average nucleotide diversity (π), the average nucleotide polymorphism (θ) and the Tajima's D significant test value were used for calculating variation among all the alleles isolated from different varieties by using DnaSP 5.0 software (Librado and Rozas 2009). Various domains, such as CC, NBS and LRR, which play vital role in disease resistance were shown on the DNA sequences of all alleles using the customized R scripts.

Crossing and selection scheme

Thirteen Pi9 allele genes were crossed with J23B, and then their F1 hybrids were backcrossed with J23B to obtain the BC1F1 populations. Markers closely linked with the Pi9 resistance genes were used to check the corresponding Pi9 allele genes among the above BC1F1 populations. Six plants with the target Pi9 allele gene from each BC1F1 populations were selected and genetic background of these BC1F1 were profiled by RICE6K (with 5102 SNP and InDel markers), a whole-genome single nucleotide polymorphism (SNP) array (Yu et al. 2014). Only one plant with the target Pi9 allele gene and background closest to J23 was selected to backcross with J23B up to BC2F1. Similarly, BC3F1 was obtained from BC2F1 populations by backcrossing the plant with the target gene and the background closest to J23B. After selfing, the BC1F2, BC2F2 and BC3F2 populations were obtained, which were then used to evaluate the effects of individual Pi9 alleles genes in J23B backgrounds.

Scoring rice blast

The BC3F2 families and control varieties were planted in a randomized complete block design in 2017 in Enshi and Yichang, Hubei Province, China. Enshi and Yichang are both mountainous areas, showing high humidity and heavy fog annually. The tests were performed in three replications. In each replication, each of the plots consisted of 4 rows with 6 plants per row at a planting density of 16 cm between plants and 16 cm between rows. To adequately induce blast disease infection, LTH was planted at both sides of each row and around the population. Field management essentially followed normal agricultural practices with the exception of not using bactericides.

All the plants were scored for leaf blast severity at the tillering stage and for neck blast severity at maturity stage using the HS-HR (HS, S, MS, MR, R and HR) scale rating system from IRRI (2002). The most seriously infected leaf among the top two or three new leaves was scored for each plant at the tillering stage as leaf blast rate. And the percentage of the infection on the neck was scored for each plant at physiological maturity stage as neck blast rate.

Results

Selection of varieties resistant to *Magnaporthe oryzae*

We collected 1883 rice varieties originating from regions across China, including 729 farm-cultivated varieties, 485 varieties with core germplasm resources, 514 japonica varieties from northern China, and 155 varieties from other sources (Fig. 1). Among these varieties, 72.7% are indica and 27.3% are japonica.

Evaluate the blast resistance of these varieties, plants were grown at Enshi and Yichang, Hubei Province, where test nurserie have been established to evaluate the blast resistance of rice varieties in regional trials. We classified the disease resistance of cultivar into six categories: HR, R, MR, MS, S, and HS, according to formula: disease resistance of cultivar = Leaf blast grade $\times$ 0.25 + Disease grade of ear blast $\times$ 0.25 + Loss rate of ear blast $\times$ 0.5. When the disease resistance rate of variety is less than 0.1, it is determined as HR; and when it is between 0.1 and 2.0, it is called R. We identified 361 varieties that displayed HR or R resistance phenotypes in Enshi or Yichang (Fig. 1). A PCR-based screen for the presence of Pi2, Pi9, Pigm, or Piz-t (Table S1; Table S2) identified 107 varieties as candidates for allele mining,, including 31 varieties for Pi2, 4 varieties for Pi9, 6 varieties for Pigm and 18 varieties for Piz-t (Table S1).

Isolation of Pi9 alleles

Pi9 genomic sequences of about 2 kb, including the promoters and full-length coding regions, were amplified from all 107 resistant varieties. Based on our analysis of these sequences, we identified 13 Pi9 alleles from these 107 varieties. The obtained sequences were compared with the reported Pi9 gene sequence specifically in the coding region. The 13 new alleles are novel and contain unique SNPs, insertions, and deletions. Figure 1 diagrams the sequence alignment of the identified Pi9 alleles. Among the 107 varieties, 4 varieties as honor of Pi9 gene (Table S2). The Pi9-Type5 allele was the most widespread Pi9 allele, appearing in 56 rice varieties. Moreover, the donors of Pi2 and Piz-t genes all had the Pi9-5 allele at this locus; therefore, Pi9-Type5 is considered to be the allele of Pi2 or Piz-t genes at this locus. Similarly, the donor of the Pigm gene had the Pi9-Type4 allele at this locus, which was detected in eight rice varieties (Table S2).

Sequence analysis of the Pi9 alleles

Among the 13 Pi9 alleles identified, Pi9-Type08 had the lowest level of amino acid sequence identity (92%) with the reference allele Pi9 and five alleles had more than 99% identity (Pi9-Type1, Pi9-Type5, Pi9-Type6, Pi9-Type8, Pi9-Type10, Pi9-Type12 and Pi9-Type13). These 13 alleles differed from Pi9 by many nucleotide polymorphisms, insertions and deletions, either uniquely or shared among the different alleles.

The alleles include several large insertions/deletions in the nucleotide sequence between – 728 and 2844, which encompasses the promoter region from about 728 bp upstream of the start codon, the first exon, and part of the first intron (Fig. 1).

Two alleles, Pi9-type10 and Pi9-type11, have insertions at different positions in the promoter region (Fig. 2, Table 1). In the first intron, we identified three large insertion regions located at 149–514 bp, 1099–1234 bp, and 2796–2844 bp. The insertions at 14–514 bp and 2796–2844 bp were present in only one allele each: Pi9-Type08 has the insertion at 149–514 bp and Pi9-Type09 has the insertion at 2796–2844 bp. The insertion at 1099–1234 bp was present in Pi9-Type02, Pi9-Type04, Pi9-Type05, Pi9-Type08, and Pi9-Type09. All Pi9 alleles have conserved CC and NBS domains, suggesting that these domains are important for Pi9 function. By contrast, the LRR domain is a highly polymorphic SNP-rich region. The allelic variation in the LRR domain indicates that this region is under less selective pressure than the other domains (Fig. 1; Table 2). We also performed nucleotide polymorphism analyses for all thirteen Pi9 alleles by DnaSP5.10. The average nucleotide diversity (π) of the alleles was 0.01674. Sliding window analysis of Pi9 allele nucleotide diversity showed that the diversity rate was higher in regions with abundant nucleotide polymorphisms and that there were more deletion/insertion in the first intron than elsewhere in the allele (Fig. 2; Table 2). The D test value of Tajima was less than 1 (–0.64099), indicating that the Pi9 locus was under positive selection, especially the conservative domains of CC and NBS (Fig. 2; Table 2).

Analysis of the derived Pi9 protein sequences

Pi9 proteins are composed of three conserved domains: CC, NBS and LRR. Eight of the new alleles (Pi9-Type1, Pi9-Type5, Pi9-Type6, Pi9-Type8, Pi9-Type10, Pi9-Type12 and Pi9-Type13) have complete open reading frames (ORFs) similar to that of Pi9. The remaining six alleles (Pi9-Type2, Pi9-Type3, Pi9-Type4, Pi9-Type7, Pi9-Type9 and Pi9-Type11) appear to have shorter ORFs due to sequence changes resulting in early termination codon. Among these six alleles, Pi9-Type2, Pi9-Type3, Pi9-Type4, and Pi9-Type7 have an SNP deletion in the CC domain that changes the reading frame, such that the translated protein has only 100 amino acids. Pi9-Type9 and Pi9-Type11 have some insertions/deletions in the LRR domain, which make the translated protein shorter than that of Pi9 (Table 1).

Phylogeny and distribution of the new Pi9 alleles in rice subspecies

In addition to the cloned Pi9 gene, we identified thirteen Pi9 alleles in this chromosomal region.. The reference allele Pi9 from the donor 75-1-127 is widespread among indica varieties and was also identified in some japonica varieties through genomic breeding. The presence of other Pi9 alleles varied among rice subspecies. Three of the thirteen new Pi9 alleles (i.e., Pi9-Type01, Pi9-Type06 and Pi9-Type12), were found in only one rice line each. The donor of Pi9-Type06 is japonica subspecies and the other two alleles are derived from indica subspecies. Pi9-Type02, Pi9-Type09 and Pi9-Type11 are present only in the indica subspecies, whereas Pi9-Type0 and Pi9-Type13 exist only in the japonica subspecies. However, the remaining alleles are distributed in both indica and japonica (Fig. 3).

To establish the genetic relatedness among the Pi9 alleles, we analyzed the phylogeny of the thirteen new alleles and the cloned Pi9 reference gene. A phylogenetic tree was constructed using nucleotide sequences that included the complete ORF and 840 bp of the promoter sequence upstream of the start codon. Three major clusters were observed. Among them, cluster I and cluster III were composed

of Pi9 alleles from indica rice, while cluster II was divided into three clear sub-clusters (Fig. 3). Only the Pi9 alleles in cluster II-subgroup III, including Pi9-Type06 and Pi9-Type13, were derived from japonica rice. The Pi9 alleles in the other two subgroups of cluster II are distributed between both indica and japonica.

Four established broad-spectrum R genes, Pi9, Pi2, Piz-t, and Pigm, are located in the same ~ 10.38-Mb region on the short arm of chromosome 6. The thirteen new alleles of Pi9 were sequenced using sequencing primers for Pi2, Piz-t and Pigm. The donor of the Pi2 gene is the Pi9-Type05 allele, the donor of the Pigm gene is the Pi9-Type04 allele, and the donor of the Piz-t is either the Pi9-type05 or Pi9-type08. Due to genomic breeding, these Pi9 alleles are widely present in both indica and japonica varieties.

Evaluation of blast resistance with *Magnaporthe oryzae*

Leaf blast resistance of the 13 allele of Pi9 donors and their Pi2, Pi9, Pigm and Piz-t donors were assessed in green house using 34 isolates of *Magnaporthe oryzae* from Hubei, Jiangxi, Hunan, Fujian and Guangdong provinces, China. The donors of Pigm, Pi2, Pi9 and Piz-t genes are Gumei4, C101A51, 75-1-127 and Dianyu1, which showed broad-spectrum resistance to rice blast with resistance frequencies ranging 58.8 to 94.1%. The resistance frequencies of donors of the Pi9 allele ranged from 23.5 to 100%. The donor GD-1S, (containing Pi9-Type5 allele) and the donor THAVALU (containing Pi9-Type9 allele) were resistant to all 34 blast isolates with a resistance frequency of 100%, an even higher resistance frequency than GM4H. The donors YD4038 and ZWH210, containing the Pi9-Type6 and Pi9-Type10 allele, were resistant to more than 30 of 34 blast isolates with a resistance frequency more than 91.2% (Table 3; Table S3).

Evaluation of blast resistance by field test

Six Pi9 allele genes with a resistance ratio greater than 85% (Pi9-Type3, Pi9-Type5, Pi9-Type6, Pi9-Type9, Pi9-Type10, Pi9-Type11) and three cloned genes (Pigm, Pi2 and Pi9) were introduced into recurrent parent J23B. The resistance to leaf blast and neck blast of Pi9 allele genes and three cloned genes introduced lines were tested in 2017 in Enshi and Yichang. Among the three cloned genes, Pigm showed greatest resistance to both leaf blast and neck blast in the background of J23B or donor parent, at Enshi or Yichang (Table 4). Like cloned genes, among six Pi9 allele genes, Pi9-Type6, Pi9-Type10 and Pi9-Type11 showed significantly enhanced resistance to leaf blast and neck blast in the background of J23B than the control, recurrent parent J23B, at Enshi and Yichang (Table 4). Pi9-Type3, Pi9-Type5 and Pi9-Type9 showed significantly enhanced resistance to leaf blast at Enshi and significantly enhanced resistance to neck blast at Yichang (Table 4). Pi9-Type3 showed significantly enhanced resistance to neck blast at Enshi. All of the Pi9 allele genes showed enhanced resistance to leaf or neck blast at least at one place. Moreover, the Pi9 allele genes of Pi9-Type6 and Pi9-Type11 were greater resistance to both leaf blast and neck blast than cloned gene Pi2 or Piz-t (Table 4).

Discussion

Current Status of Cloning and Evolutionary Analysis of Rice Blast R Genes

Identifying and cloning novel broad-spectrum blast-R genes is critical for breeding resistant rice varieties and has been a major focus of rice genome research. With the development of molecular marker technology, the construction of a high-density genetic linkage map and the improvement of related molecular techniques, great progress has been made in the cloning of rice blast R genes (Chen et al., 2006; Hayashi and Yoshida, 2009; Hittalmai et al., 2000; Lin et al., 2007; Sharma et al., 2005). Since Sasaki first presented the theory of rice blast resistance genes in 1922, initiating nearly 100 years of discovery and utilization of these genes in rice breeding. In 1966, Yamasaki cloned the rice blast R genes Pia, Pii and Pik (Yamasaki and Kiyosawa, 1966) from Aichi Asahi, Ishikari Shiroke and Kanto 51. In 1999, Wang cloned the R gene Pib (Wang et al., 1999) by map-based cloning. With improvements in technologies in the 21st century, the cloning and exploitation of rice blast R genes has seen explosive development. To date, 119 blast R genes and more than 400 quantitative trait loci (QTLs) have been mapped, and more than 30 blast resistance genes have been mapped and cloned (Bryan et al., 2000; Chen et al., 2018; Chen et al., 2006; Chen et al., 2018; Chujo et al., 2014; Fukuoka et al., 2009; Inoue et al., 2010; Hayashi et al., 2010; Hayae et al., 2017; Ishihara et al., 2014; Jia et al., 2011; Li et al., 2017; Lin et al., 2007; Liu et al., 2002; Liu et al., 2007; Zhai et al., 2011; Zhao et al., 2018).

Most blast R genes, except for a few genes such as pi21, Pid2, Pid3, and Ptr, encode nucleotide-binding site leucine-rich repeat (NBS-LRR) proteins (Chen et al., 2006; Fukuoka et al., 2009; Liu et al., 2007; Xu et al., 2014). Analysis of the cloned blast R genes revealed that most of the broad-spectrum R genes are located in tandemly repeated gene clusters on chromosomes 6, 9, 11 and 12. Generally, rice blast genes that have an NBS-LRR structure and occur in tandem repeat regions have broad-spectrum resistance. The structures of tandem repeat

regions differ greatly in different rice varieties, harboring various inversions and deletions. Furthermore, many R genes with highly similar structures are tandemly duplicated with pseudogenes (Dai et al., 2010).

The short arm of chromosome 6 contains at least 10 blast R genes (Pigm, Pi2, Pi9, Piz-t, Piz, Pi22, Pi25, Pi26, Pi40, Pi42) in tandem repeats. Pi2 was cloned from the variety "C101 A51" and encodes an NBS-LRR protein composed of 1032 amino acids (Hittalmani et al., 2000; Zhou et al., 2006). Pi9 is highly similar to Pi2 but has a different resistance profile (Queet al., 2006). Piz-t, derived from Xiushui 209, differs from Pi2 at eight amino acids (Fig. 1) due to differences at more than 20 bases in the coding regions of the two genes. Pigm is an unusual R gene formed by one copy each of PigmR and PigmS in series. PigmR imparts broad-spectrum disease resistance but reduces yield. PigmS does not confer resistance but improves seed setting rate. Because of its high concentration of R genes, we focused our analysis on this region of chromosome 6 and found 13 new alleles.

Current Situation of Molecular Breeding of Rice Blast Resistance Genes

Rice blast, caused by the fungus *Magnaporthe oryzae*, is one of the most important rice diseases in the world (Ashkani et al., 2015; Shen et al., 2004). Rice blast has been reported in almost all rice-producing areas in the world, including the main rice-producing areas of 85 countries and regions (Mgonja et al., 2017; Miah et al., 2013; Zhu et al., 2000). High temperatures and humidity favor the development of rice blast. Rice blast is an acute and destructive disease that can reduce yield or even wipe out an entire harvest. Grain blast also affects the quality of rice and is a serious food safety problem (Miah et al., 2013).

In China, the disease affects more than 3.8 million hectares per year, reducing rice yield by 1 billion kg per year (Deng et al., 2017). At present, fungicides are the main method used to control rice diseases in rice production. From 1980 to 1990, the area requiring rice blast control in China increased from 2.4 million hectares to 8.9 million hectares (Shen et al., 2004). Over the past 20 years, the use of pesticides in China has increased each year. Although chemical control can reduce diseases and yield losses to a certain extent, the use of chemicals is a burden to farmers and pollutes the environment (Mi et al., 2018). Therefore, the best way to prevent rice blast is to breed blast-resistant rice varieties (Wu et al., 2016; Zhou et al., 2018).

Blast R genes (Mi et al., 2018; Zhou et al., 2018) can be introduced into high-quality susceptible rice varieties by molecular marker-assisted selection (MAS). Locating and cloning genes with broad-spectrum resistance to rice blast is of great significance for rice blast resistance breeding at this stage. In order to identify the resistance spectrum of the cloned rice blast resistance genes, we used the donor materials "Tetep", "TSUYUAKE", "Gumei No. 4", "C101 A51", "75-1-127", "Xiushui 209", "RIL260", "Jin23B", "Jin23B", "Tetep", "K59" corresponding to the genes Pi1, Pikm, Pigm, Pi2, Pi9, Piz-t, Pi5, Pid2, Pid3, Pikh, Pit, Pita, Pib and pi21. The donor varieties containing Pigm and Pi9 genes had the best resistance to 94.1% and 91.4% of 34 monospores. Donor varieties containing Pi2 and Piz-t genes could resist most of the monospores of rice blast in northern China (Jiang et al., 2015; Tian et al., 2016), while the donor varieties containing Pi1 and Pikm genes showed better resistance to monospores from southern China. Non-tandemly repeated blast R genes were not associated with broad-spectrum resistance.

Because the physiological races of rice blast pathogen are highly variable and change rapidly, any gene conferring resistance to a single race is easily overcome. The breakdown of resistance can be avoided by developing rice varieties with a large number of broad-spectrum R genes associated with strong resistance. This is of great importance for breeding disease-resistant rice varieties and preventing rice blast.

In this study, we examined more than 1883 rice germplasm resources in China. We screened 107 rice varieties with broad-spectrum resistance to rice blast and sequenced their Pi9 alleles. Seventeen alleles of Pi9 were obtained, including Pi9, Pigm, Piz-t, and Pi2. Although these alleles were isolated from rice germplasm resources that showed resistance to rice blast in our own tests, the resistance in these varieties could also be influenced by the presence of other major R genes and QTLs. To exclude this possibility, we used a BSA strategy to identify plants in the F2 populations and BC3F2 near-isogenic lines that were resistant to rice blast. After further functional testing, such as by complementation experiments or gene silencing, the new broad-spectrum resistance alleles and previously cloned rice blast R genes can be used in molecular marker-assisted breeding of different gene combinations to improve the durability of rice blast resistance.

Declarations

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Availability of data and materials

The data sets supporting the results of this article are included within the article and its supporting files.

Authors contributions

YZ and WY designed the experiments. YZ, FL, QW and WH performed the experiments. BY and YZ helped with field management. YZ and FL analyzed the data. YZ and WY wrote the manuscript. All authors approved the manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests

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Tables

Table 1 Summary of SNP and different alleles of the *Pi9* gene in different species

<i>Pi9</i> allele	No.	Varieties	Haplotype	Identity to <i>Pi9</i>	Number of SNP sites	Number of SNP sites			Number of inserts/deletions	Number of inserts/deletions		
						Pt	Ex	In		Pt	Ex	In
<i>Pi9</i>		75-1-127	2	-	-	-	-	-	-	-	-	-
<i>Pi9</i> -Type01	GRP00494	Heo Trang	1	99%	79	29	47	3	17	9	0	8
<i>Pi9</i> -Type02	GRP00617	IR64	2	95%	443	246	94	103	259	10	0	249
<i>Pi9</i> -Type03	GRP01331	HC1H	13	98%	134	33	61	40	26	20	3	3
<i>Pi9</i> -Type04*	GRP00328	PBB	11	96%	410	253	56	101	266	10	0	256
<i>Pi9</i> -Type05*	GRP00574	XS209	56	95%	451	259	57	135	320	9	0	311
<i>Pi9</i> -Type06	GRP00234	YD4038	1	99%	104	25	31	48	241	9	3	12
<i>Pi9</i> -Type07	GRP00537	KAUKKYI ANI	2	99%	105	30	36	39	23	20	0	3
<i>Pi9</i> -Type08*	GRP00713	DY1H	6	92%	710	519	56	135	581	9	0	572
<i>Pi9</i> -Type09	GRP00499	THAVALU	1	93%	608	312	103	193	342	109	3	230
<i>Pi9</i> -Type10	GRP00249	ZWH210	6	97%	316	282	16	18	269	259	0	10
<i>Pi9</i> -Type11	GRP00077	R03138	3	96%	387	339	22	26	339	234	0	105
<i>Pi9</i> -Type12	GRP01188	JP-5	1	99%	32	22	4	6	9	7	0	2
<i>Pi9</i> -Type13	GRP01106	ZD5H	2	99%	105	26	30	49	26	9	3	14

* *Pigm* is contained in the donor of *Pi9*-Type04; *Pizt* is contained in the donor of *Pi9*-Type08 and a part of *Pi9*-Type05; *Pi2* is contained in the donor of a part of *Pi9*-Type05.

Table 2 Summary of the natural variation of different *Pi9* allele genes in different species

Allele	π	θ	Tajima's D
CC	0.00670	0.01475	-2.23881
NBS	0.01035	0.01574	-1.49472
LRR	0.03311	0.03529	-0.27483
Promoter	0.01401	0.01823	-0.94844
Exson1	0	0	-
Intron1	0.01499	0.01665	-0.45014
Exson2	0.02137	0.02604	-0.80967
Intron2	0	0	-
Part of Exson3	0	0	-
Total	0.01674	0.01950	-0.64099

Table 3 Disease reactions of *Pigm*, *Pi2*, *Pizt*, *Pi9* and *Pi9* alleles honor plants to *Magnaporthe grisea* isolates.

Gene	No.	Varieties	Resistance ratio	Resistance
CK1	GRP00704	LTH	0.00%	4.82±0.39
CK2	GRP02083	J23B	0.00%	3.76±0.70
<i>Pigm</i>	GRP02064	GM4H	91.20%	1.50±1.02
<i>Pi2</i>	GRP02074	C101 A51	67.60%	2.29±1.12
<i>Pi9</i>	GRP02075	75-1-127	94.10%	1.62±0.60
<i>Pizt</i>	GRP00713	DY1H	58.80%	2.53±1.58
<i>Pi9</i> -Type1	GRP00494	Heo Trang	26.50%	3.09±1.26
<i>Pi9</i> -Type2	GRP00617	IR64	23.50%	3.38±1.04
<i>Pi9</i> -Type3*	GRP01331	HC1H	85.30%	1.76±1.21
<i>Pi9</i> -Type4- <i>Pigm</i> *	GRP00328	PBB	88.20%	1.71±1.03
<i>Pi9</i> -Type5*	GRP00727	GD-1S	100.00%	1.15±0.36
<i>Pi9</i> -Type5- <i>Pi2</i>	GRP00884	CT 18664-9-18-1-3-2	70.60%	1.91±1.14
<i>Pi9</i> -Type5- <i>Pizt</i>	GRP01232	ASD 18	67.60%	2.15±1.05
<i>Pi9</i> -Type6*	GRP00234	YD4038	91.20%	1.29±0.72
<i>Pi9</i> -Type7	GRP00537	KAUKKYI ANI	61.80%	2.44±1.44
<i>Pi9</i> -Type8- <i>Pizt</i>	GRP00713	DY1H	58.80%	2.53±1.58
<i>Pi9</i> -Type9*	GRP00499	THAVALU	100.00%	1.00±0.00
<i>Pi9</i> -Type10*	GRP00249	ZWH210	94.10%	1.32±0.91
<i>Pi9</i> -Type11*	GRP00077	R03138	88.20%	1.68±0.91
<i>Pi9</i> -Type12	GRP01188	JP-5	79.40%	1.68±0.94
<i>Pi9</i> -Type13	GRP01106	ZD5H	55.90%	2.68±1.41

*When Resistance Ratio is greater than 85% and Resistance value is less than 2, NIL of allele material is done.

Table 4 Disease reactions of *Pigm*, *Pi2*, *Pizt*, *Pi9* and *Pi9* alleles honor plants to *Magnaporthe grisea* isolates.

<i>Pi9</i> allele	No.	Varieties	Generation	Enshi		Yichang	
				Lr	Nr	Lr	Nr
CK1	GRP00704	LTH	F0	S	S	HS	S
CK2	GRP01884	J23B	F0	MS	S	HS	MS
<i>Pigm</i>	GRP02064	GM4H	F0	MR	R	HR	HR
<i>Pi2</i>	GRP02074	C101 A51	F0	R	MR	MR	MR
<i>Pi9</i>	GRP02075	75-1-127	F0	MR	R	HR	R
<i>Pizt</i>	GRP00713	DY1H	F0	MR	MR	R	MR
<i>Pi9</i> -Type1	GRP00494	Heo Trang	F0	HR	R	HR	R
<i>Pi9</i> -Type2	GRP00617	IR64	F0	MS	MS	R	R
<i>Pi9</i> -Type3*	GRP01331	HC1H	F0	R	MR	R	R
<i>Pi9</i> -Type4- <i>Pigm</i> *	GRP00328	P ₀ B	F0	R	R	HR	HR
<i>Pi9</i> -Type5*	GRP00727	GD-1S	F0	HR	HR	HR	HR
<i>Pi9</i> -Type5- <i>Pi2</i>	GRP00884	CT 18664-9-18-1-3-2	F0	MR	MR	MR	MR
<i>Pi9</i> -Type5- <i>Pizt</i>	GRP01232	ASD 18	F0	MR	MS	MR	MR
<i>Pi9</i> -Type6*	GRP00234	YD4038	F0	R	HR	HR	HR
<i>Pi9</i> -Type7	GRP00537	KAUKKYI ANI	F0	MR	HR	HR	R
<i>Pi9</i> -Type8- <i>Pizt</i>	GRP00713	DY1H	F0	S	HS	HR	HR
<i>Pi9</i> -Type9*	GRP00499	THAVALU	F0	HR	R	HR	R
<i>Pi9</i> -Type10*	GRP00249	ZWH210	F0	R	HR	HR	MR
<i>Pi9</i> -Type11*	GRP00077	R03138	F0	HR	HR	HR	HR
<i>Pi9</i> -Type12	GRP01188	JP-5	F0	R	HR	HR	MS
<i>Pi9</i> -Type13	GRP01106	ZD5H	F0	MR	MR	R	HS
<i>Pigm</i>	GRP02064	GM4H	BC3F2	MR	R	R	HR
<i>Pi2</i>	GRP02074	C101 A51	BC2F2	MR	MS	MR	R
<i>Pi9</i>	GRP02075	75-1-127	BC3F2	MR	MR	R	R
<i>Pi9</i> -Type3	GRP01331	HC1H	BC3F2	MR	MR	MS	R
<i>Pi9</i> -Type5	GRP00727	GD-1S	BC3F2	MR	MS	R	R
<i>Pi9</i> -Type6	GRP00234	YD4038	BC3F2	R	MR	R	R
<i>Pi9</i> -Type9	GRP00499	THAVALU	BC3F2	MR	MS	R	MR
<i>Pi9</i> -Type10	GRP00249	ZWH210	BC2F2	R	MR	R	MR
<i>Pi9</i> -Type11	GRP00077	R03138	BC3F2	R	MR	R	R

Additional Files

Additional file 1 :

Table S1. List of 107 rice varieties as candidates for allele mining

About 2000 varieties were grown at test nurserie of Enshi and Yichang, Hubei Province. We identified 361 varieties that displayed HR or R resistance phenotypes in Enshi or Yichang. A PCR-based screen for the presence of *Pi2*, *Pi9*, *Pigm*, or *Piz-t* identified 107 varieties as

candidates for allele mining. The sequencing results of all 107 materials are shown in this table.

Table S2. Primers for sequence analysis of *Pi9*, *Pi2*, *Pigm* and *Pizt* allele genes in rice.

Among them, the alleles of *Pi2*, *Pigm* and *Piz-t* share a set of primers for amplification and sequencing; the alleles of *Pi9* use a unique set of primers for amplification and sequencing. The amplified primers are labeled amplification in the table, and the sequenced primers are labeled sequence in the table.

Table S3. Disease reactions of *Pigm*, *Pi2*, *Pizt*, *Pi9* and *Pi9* alleles honor plants to *Magnaporthe grisea* isolates.

The sheet of resistance ratio and resistance shown the identification results of all materials to *Magnaporthe grisea* isolates. The information about of *Magnaporthe grisea* isolates, collected from all provinces of China, is also shown in the table. The resistance value of HR, R, MR, MS, S, showed in sheet of resistance ratio, is corresponding as 0-5 showed in sheet of resistance.

Figures

Figure 1

chematic map for sequence alignment of newly identified Pi9 alleles. Thirteen alleles of Pi9 were isolated from the studied rice accessions and compared with the total sequence of all alleles. The domain regions of the Pi9 alleles are shown as gray frames (CC, NBS and LRR) at the bottom. Boxes indicate exons and lines indicates introns, and the start codon and the termination codon are labeled with ATG and TGA, respectively, in the figure above. The unit scale indicates the location of nucleotides. The black line on the bar indicates the nucleotide polymorphism compared with the reference sequence. The gap in the bars for alleles indicates deletions. The gap between allele strips indicates deletion, and the size of gap indicates the length of deletion sequence.

Figure 2

NP rich region in the Pi9 alleles. Sliding-window analysis of nucleotide diversity (π) about new Pi9 alleles are shown above. The gene structure is described at the unit scale of nucleotide location, and the domains (CC, NBS and LRR) are illustrated as gray frames.

Figure 3

Phylogenetic relationship among Pi9 allele sequences. Thirteen new Pi9 allele sequences found in our research materials and previously reported Pi9 sequences from rice materials 75-1-127 were used for analysis. A. The genomic sequences (from ATG to TGA, including the introns) for 13 new Pi9 alleles based on our sequencing results and Pi9 gene reference sequence.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [supplementarytable2.xlsx](#)
- [supplementarytable1.xlsx](#)
- [supplementarytable3.xlsx](#)