Identification of a *Pi9*-Containing Rice Germplasm with a Newly Developed Robust Marker

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ABSTRACT

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The *Pi9* gene in rice, originating from *Oryza minuta*, is an effective resistance gene for controlling rice blast disease. However, currently available linked DNA markers do not accurately identify the function of *Pi9*, thus hindering its efficient incorporation into new cultivars through marker-assisted selection (MAS). In addition, no known *Pi9*-containing rice germplasm is available to breeders. In the present study, DNA sequence variation of *Pi9* alleles and their family members was analyzed in 40 diverse rice germplasm accessions from the AA genome to develop a robust

Pi9 marker. In total, 29 DNA primers of 20 to 23 nucleotides were designed and each possible combination of primer pairs was used to detect *Pi9*. Only one combination of DNA primers, KS28/KS6, was identified to specifically detect *Pi9* in the monogenic line IRBL9-W. The presence of *Pi9* was verified with the predicted *Pi9*-specific blast reaction. Subsequently, 201 genetically diverse mini-core rice accessions from 114 countries were screened with KS28/KS6. One germplasm, IR 9660-48-1-1-2, was identified to carry *Pi9* and the function of *Pi9* was verified with pathogenicity assays. This robust *Pi9* marker and a rice germplasm, IR9660-48-1-1-2 (GSOR310687), carrying *Pi9* can be used to improve blast resistance with a MAS approach.

Resistance to rice blast caused by the ascomycete fungal pathogen *Magnaporthe oryzae* is mediated by major and minor resistance (*R*) genes. Thus far, extensive searching of blast *R* genes in rice germplasm worldwide has allowed the identification of effective *R* genes in diverse rice germplasm and some of them, with one or more *R* genes, have been used as a donor for breeding for improved blast resistance. The bottleneck of this approach is that each major *R* gene is only effective against strains of *M. oryzae* that contain the corresponding avirulence (*AVR*) genes. *AVR* genes are random molecules in *M. oryzae* and highly diversified *AVR* variants have been recovered in more virulent field strains worldwide (Dai et al. 2010; Xing et al. 2013; Zhou et al. 2007b). In contrast, most blast *R* genes encode predicted intracellular nucleotide-binding site leucine-rich repeat (NBS-LRR)-type *R* proteins with NBS and LRR domains (Jones and Dangl 2006), suggesting a role in protein–protein interaction and ligand binding.

Up to now, more than 20 blast *R* genes have been cloned. The molecular basis and evolution of blast *R* genes are becoming better understood (Amei et al. 2014; Jia et al. 2000; Roychowdhury et al. 2012). However, only a few of them were analyzed for genomic and expression variation for the development of robust markers from portions of *R* genes (Costanzo and Jia 2009, 2010; Fjellstrom et al. 2004; Jia et al. 2002, 2003, 2004a; Lee et al. 2009, 2011; Li et al.; 2014a,b, 2015; Wang et al. 2008). In the United States, blast races have been characterized with international differentials (Jia et al. 2009; Xing et al. 2013) and common races IA, IB, IC, ID, IE, IG, and IH, of *M. oryzae* have been recovered from the 1950s to the present

(Correll et al. 2000; Xing et al. 2013). Over a dozen blast *R* genes, including *Pi-ta/Pi-ta2*, *Pi-b*, *Pi-kh/m/s*, *Pi20(t)*, and *Pi-z*, have been incorporated into rice varieties through classical plant breeding with and without marker-assisted selection (MAS) over decades (Jia 2003; Li et al. 2008; Wang et al. 2014). *Pi-ta/Pi-ta2* in a single linkage block has been moved into dozens of rice varieties in the southern United States and is still effective in preventing blast disease (Jia 2009; Jia et al. 2004b, 2012). However, *Pi-ta/Pi-ta2* cannot prevent the infections by two races, IE1K and IB33, of *M. oryzae* that do not contain the corresponding *AVR* gene (*AVR-Pita1*) (Dai et al. 2010; Zhou et al. 2007b).

Recently, Wang et al. (2015) characterized commonly found blast races in the United States with International Rice Research Institute (IRRI) monogenic lines carrying 24 major blast *R* genes verifying the effectiveness of *Pi-ta2* and identified another useful *R* gene, *Pi9*, for breeders in the United States to use. The IRRI monogenic line with *Pi9* was resistant to all common blast races except race IE1k, which was unstable and gave an ambiguous disease reaction (Wang et al. 2015). *Pi9* introduced from *Oryza minuta* into cultivated rice was also predicted to be effective to all tested blast strains in China and the Philippines (Wu et al. 2015). Identification of *Pi9* using a linked genetic marker has been undertaken worldwide; however, the accuracy of linked markers is limited.

In the present study, we analyzed DNA sequence variation among members of *Pi9*, *Pi9* in ‘Nipponbare’ rice, and 40 diverse rice germplasms in the AA genome (Liu et al. 2011) and identified one set of DNA primers that specifically amplify the *Pi9* gene. This set of primers was used to screen a mini-core collection that was selected from the United States Department of Agriculture (USDA) core collection from 114 countries, and found one germplasm (IR 9660-48-1-1-2) that contains *Pi9*. We then performed pathogenicity assays with 14 commonly found races on *Pi9*-containing rice germplasm and an IRRI monogenic line with *Pi9*, and the disease reaction results support the idea that this new germplasm does, indeed, contain *Pi9*, and also contains additional blast *R* genes. We suggest that the DNA marker we developed is a robust marker

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*The e-Xtra logo stands for “electronic extra” and indicates that one supplementary figure and three supplementary tables are published online.

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for *Pi9* and this new germplasm is useful for marker-assisted incorporation of *Pi9* for improving blast resistance using MAS (Jia 2003).

MATERIALS AND METHODS

Pi9 marker development. DNA sequences of 76 kb obtained from GenBank (accession number DQ285630) were used to identify six members of the *Pi9* gene family (Liu et al. 2011; Qu et al. 2006). Multiple DNA sequence alignments were performed using ClustalW2 (Larkin et al. 2007) to identify polymorphisms at the coding region of the *Pi9* gene. From 29 primers developed (KS5 to KS33) (Table 1; Supplementary Table S1), DNA sequence assemblies were constructed with Nucleotide Blast of the National Center for Biotechnology Information supported by the ClustalW2 software (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>).

Plant materials. Rice germplasms used in the study were obtained from the Genetic Stocks *Oryza* (GSOR) collections at the USDA Agricultural Research Service (ARS) Dale Bumpers National Rice Research Center (DB NRRC). Seed were sown in plastic trays divided into wells (5.5 by 5.0 by 5.5 cm [length by width by height]) containing sterilized local soil. Rice lines were grown in a greenhouse at 20 to 30°C for 3 weeks until plants were at three- to four-leaf stages for DNA extraction and pathogenicity assays.

DNA extraction and polymerase chain reaction amplification. Rice leaves were collected and DNA was prepared using the Qiagen DNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA) following the manufacturer’s instructions. Each polymerase chain reaction (PCR) was performed at 20 µl and consisted of 20 to 50 ng of total DNA, 10 µl of 2× Taq PCR Master Mix (Qiagen), and 0.4 µl of each 10 µM primer. PCR was performed in a Peltier Thermal Cycler PTC-200 (MJ Research, Waltham, MA). The program used for KS28 and KS6 primers was 3 min at 95°C for initial denaturation; followed by 30 cycles of 30 s at 94°C, 45 s at 57°C, and 1 min at 72°C; and then a final extension at 72°C for 5 min. Aliquots (10 µl) of the PCR products were separated by electrophoresis on 1.5% agarose gels in 1× Tris-acetate-EDTA buffer containing 0.001% of SYBR safe DNA gel stain (Invitrogen, Carlsbad, CA) and were visualized by the ChemiDoc MP Imaging System (Bio-Rad, Hercules, CA).

To validate the *Pi9* marker in an automated system, KS6 primer was labeled with 6FAM (Integrated DNA Technologies, Coralville,

IA). PCR products were run against the GeneScan 500 LIZ dye size standard (GS500LIZ; Thermo Fisher Scientific, Grand Island, NY). The samples were separated on an ABI Prism 3730 DNA analyzer (Applied Biosystems, Foster City CA) using methods as described by the manufacturer. The sizes of fragments were estimated using the software GeneMapper v 3.7 (Applied Biosystems).

Pathogenicity tests. To verify the functionality of the *Pi9* gene, the near-isogenic line IRBL9-W, containing *Pi9*, was used as a positive control and M202 as a negative control due to its susceptibility to predominant *M. oryzae* races in the United States. Plants were inoculated with the same 14 isolates described by Wang et al. (2015). Oatmeal agar (BD Difco, Franklin Lakes, NJ) plates were inoculated with the isolates and allowed to sporulate for 7 to 10 days at 24 to 25°C. Each isolate suspension was prepared using 0.25% gelatin with a spore concentration of 2 to 5 × 10⁵ spores/ml. Plants were inoculated inside a plastic bag which was then sealed to maintain high humidity. After 24 h, plants were removed from the bags and returned to the greenhouse and kept at 25°C, with a photoperiod of 12 h. Disease reactions were determined 7 days after inoculation on the basis of reaction type, using a 0-to-9 standard evaluation scale (International Rice Testing Program 1988). Resistance was scored based on no visible infection and no conidia produced from inoculated tissue (scores 0 to 3), and susceptibility was scored with a lesion ≥3 mm in length (score 4 to 9). These reactions were determined using three to five plants in each pot with at least three repeats for each isolate.

Fig. 2. Validation of specific molecular marker (KS28/KS6) to the *Pi9* gene. IRBL9-W = *Pi9* monogenic line, M202 = susceptible line, Katy = rice variety containing *Pi-ta/Pita2* gene, GSOR310199 = K12 C/53, GSOR310200 = Chin Chin, GSOR310237 = Jappein Tunkungo, GSOR310276 = Khao Khao, GSOR310336 = Chao Puak Deng, GSOR310351 = Warrangal culture 1252, GSOR311174 = Vary Vato 275, GSOR311205 = CTG 1516, and GSOR311634 = Patchaipurumal. Size of polymerase chain reaction amplicon is indicated with a DNA ladder.

TABLE 1. DNA sequences, annealing temperature (T_m), predicted polymerase chain reaction (PCR) amplicon, and location of primers for the *Pi9* gene

Primer	Primer sequence (5'–3')	T _m (°C)	PCR product (bp)	Location of primer (bp) ^a
KS28	GCCCTGCAAGGACCCTAATA	57	699	33,466–33,485
KS6	GATGTTTCGTGGATGAGATC	...	...	34,164–34,145

^a Primer location was based on GenBank accession no. DQ285630.

Fig. 1. Sequence alignment between Nipponbare (DQ454158) and *Pi9*-carrying line 75-1-127 (DQ285630) for developing the *Pi9* marker. Forward primer was located in the position –260 to –241 and reverse primer in the position +440 to +421 of the *Pi9* gene. Alignment was produced with Nucleotide Blast, National Center for Biotechnology Information, supported by the ClustalW2 software.

RESULTS

Development of a dominant *Pi9* marker from the *Pi9* gene. The primer combinations KS19/KS21 and KS24/KS25 distinguished *Pi9* from the monogenic line IRBL9-W from 'Katy' and M202, which does not contain *Pi9*. However, both KS19/KS21 and KS24/KS25 failed to distinguish IRBL9-W from nine susceptible lines previously identified as *Pi9*-containing rice lines with a linked marker, suggesting that these nine rice lines are false positives for *Pi9* (data not shown; Supplementary Table S2). DNA sequences at the 5' leader region of the *Pi9* gene were aligned with that of Nipponbare (accession number DQ454158), with polymorphic regions indicated in Figure 1. Polymorphic regions between the positions -260 and -241 (before the translation start codon ATG) were used to design primer KS28, which was combined with primer KS6 in the position +440 to +421 (after the start codon ATG) of *Pi9* and distinguished IRBL9-W from Katy, M202, and nine false-positive lines to *Pi9*, suggesting that this marker is a robust dominant marker for *Pi9* (Table 1; Fig. 2).

Fig. 3. Polymerase chain reaction (PCR) assay of a group of germplasms analyzed for *Pi9* presence with the KS28/KS6 marker. IRBL9-W = monogenic line containing *Pi9*, GSOR310598 = Red, GSOR 310630 = BKN 6987-68-14, GSOR310670 = Kubanets 508, GSOR310687 = IR 9660-48-1-1-2 (positive line for *Pi9* gene), GSOR310693 = Bakiella 1, GSOR 310702 = Jumli dhan, GSOR310714 = Tchampa, GSOR310715 = Phudugey, GSOR310723 = WIR 3039, GSOR310724 = Ak Tokhum, GSOR310747 = Bhim Dhan, GSOR 310757 = RP2151-173-1-8, GSOR310767 = HB-6-2, GSOR310773 = ECIA76-S89-1, GSOR310777 = WC 3532, GSOR310779 = GPNO 1106, and GSOR 310788 = Toga. Size of the PCR amplicon is indicated with a DNA ladder.

Identification of a *Pi9*-containing rice germplasm. To test the specificity of the *Pi9* marker and identify a publically available rice germplasm for breeders to use, 201 rice accessions from the mini-core collection (Supplementary Table S3), representing the genetic diversity of the USDA core collection (Li et al. 2010), were screened with the KS6/KS28 primer pair. A PCR amplicon of 699 bp was expected from the *Pi9* gene in IRBL9-W, and was used as the positive control. PCR amplicons larger than 699 bp in a few germplasms were detected (Fig. 3), suggesting that they are homologs to *Pi9*. Luckily, out of 201 rice accessions, IR9660-48-1-1-2 (GSOR 310687) yielded the expected size of 699 bp for the *Pi9* gene, suggesting that the KS6/KS28 combination is specific for *Pi9*.

Functional confirmation of *Pi9* by pathogenicity assay. To confirm the functionality of *Pi9* in the rice line IR9660-48-1-1-2 that contains the *Pi9* gene, pathogenicity assays were performed with the susceptible M202 as a negative control and IRBL9-W as a positive control. Both controls and IR9660-48-1-1-2 were inoculated with 14 U.S. races of *M. oryzae*. M202 was susceptible against all the isolates inoculated (Fig. 4). IRBL9-W was resistant against 10 races of *M. oryzae*. The data were consistent, with minor

TABLE 2. Disease reactions of three germplasms inoculated with 14 U.S. races of *Magnaporthe oryzae*

Races	Disease reactions for rice lines ^a		
	IR 9660-48-1-1-2	IRBL9-W	M202
IA1	0.0 ± 0.0	1.0 ± 2.0	6.6 ± 1.5
IA45	0.0 ± 0.0	0.4 ± 0.9	5.8 ± 1.5
IB1	0.0 ± 0.0	1.0 ± 0.0	5.5 ± 1.3
IB33	0.0 ± 0.0	0.3 ± 0.5	6.0 ± 1.4
IB45	0.0 ± 0.0	0.3 ± 0.5	6.6 ± 1.1
IB49	0.0 ± 0.0	2.5 ± 1.3	6.8 ± 1.6
IB54	0.0 ± 0.0	0.0 ± 0.0	6.8 ± 1.0
IC1	1.0 ± 1.7	2.8 ± 2.2	4.8 ± 0.8
IC17	0.0 ± 0.0	0.8 ± 1.8	5.7 ± 1.2
ID1	0.0 ± 0.0	3.8 ± 0.5	7.5 ± 1.0
IE1	0.0 ± 0.0	1.0 ± 0.8	5.4 ± 1.1
IE1K	0.3 ± 0.5	2.2 ± 1.8	7.3 ± 1.6
IG1	0.0 ± 0.0	2.8 ± 1.9	7.8 ± 0.4
IH1	0.0 ± 0.0	1.0 ± 1.7	7.0 ± 1.1

^a Disease reactions were evaluated as 0 to 3 = resistant and 4 to 9 = susceptible (International Rice Testing Program 1988). Averages of disease reactions ± standard errors are shown.

Fig. 4. Pathogenicity assays of three germplasms inoculated with 14 U.S. races of *Magnaporthe oryzae*. M202 = variety susceptible to *M. oryzae*, IRBL9-W = *Pi9* monogenic line, and IR 9660 corresponds to IR 9660-48-1-1-2 selected with KS6/KS28. Races of IA1 to IH1 of *M. oryzae* are indicated. Pictures were taken 1 week after inoculation with indicated pathogen races.

differences of resistant reactions in comparison of IR9660-48-1-1-2 with IRBL9-W. Variability of the disease reactions with the races IC1, ID1, IE1K, and IG1 of *M. oryzae* were observed (Table 2). Regardless, disease reactions of IR 9660-48-1-1-2 largely agreed with that of IRBL-9, and the resistance responses of IR 9660-48-1-1-2 was more complete than that of IRBL-9, suggesting that IR 9660-48-1-1-2 has other blast *R* genes.

Testing the *Pi9* marker in a DNA analyzer. To test whether the *Pi9* marker can be used in an automated system, the KS6 primer, labeled with 6FAM, and KS28 primer, not labeled, were tested in a DNA analyzer. A product of 699 bp was visible from PCR amplicons from IRBL9-W and IR 9660-48-1-1-2 (Fig. 5). As negative controls, a product of 767 bp was observed from the susceptible M202, and no PCR amplicon was observed from the resistant Katy that contains the

Fig. 5. Validation of KS28/KS6 marker to *Pi9* for its application in the automated system. KS6 primer was labeled with 6FAM and run in an ABI Prism 3730 DNA analyzer. Rice germplasms used are indicated; sizes of fragments were predicted based on peaks of polymerase chain reaction amplicons.

Fig. 6. Greenhouse-grown plants of IR 9660 (IR9660-48-1-1-2) versus IRBL9-W. Plants shown at tillering stage, panicles, and seed with and without hulls.

Pi-ta gene but not *Pi9*. These results were reproducible, indicating that the *Pi9* marker can be used in an automated system for high-throughput germplasm screening.

DISCUSSION

In the present study, we developed a new marker to tag the function of the *Pi9* gene by aligning DNA sequences of the members of the *Pi9* family with that of the Nipponbare reference genome (<http://rice.plantbiology.msu.edu/>). The initial efforts to develop a robust *Pi9* marker with DNA primers designed from the open reading frame (ORF) of *Pi9* were not successful due to sequence homology. Knowledge of the evolutionary dynamics of the *Pi2/9* locus in the AA genome (Liu et al. 2011; Zhou et al. 2007a) allowed us to identify polymorphic DNA sequences at the 5' leader region of *Pi9*, which made it possible to successfully develop the dominant marker KS28/KS6 for *Pi9*. With the new *Pi9* marker, we only found IR 9660-48-1-1-2 carrying *Pi9* in the USDA rice mini-core collection (Li et al. 2010), and this germplasm was resistant to 14 isolates of *M. oryzae*. The USDA mini-core captured the genetic diversity of the USDA world rice collection of 18,000 accessions from 116 countries (Li et al. 2010, Yan et al. 2007), which suggests that the *Pi9* marker was highly specific in diverse germplasms. Completion of genotyping analysis of the entire USDA world collection with KS28/KS6 will help to determine the extent of *Pi9* deployment at a global scale.

We also noticed pathogenicity instability when IRBL9-W was inoculated with the races IC1, ID1, IE1K, and IG1 of *M. oryzae*. For example, Wang et al. (2015) reported that IRBL9-W was weakly susceptible to IE1K. The inconsistencies in the pathogenicity tests of IRBL9-W may be due to mutations in the isolate during subculturing and variations in environmental conditions during pathogenicity assays. IR 9660-48-1-1-2 was resistant to the same isolate when compared with IRBL9-W, with fewer infection lesions, possibly from additional *R* genes, suggesting that IR 9660-48-1-1-2 may contain *Pi9*-independent *R* genes in addition to *Pi9*. When we applied KS28/KS6 to control lines and IR 9660-48-1-1-2 in a DNA analyzer, PCR amplifications were reproducible when compared with results obtained in a conventional agarose gel, proving that this marker is useful in the automated system. KS28/KS6 proved to be robust even when annealing temperature was increased to 65°C, and was optimized at 57°C (data not shown). A *Pi9* linked marker was effective in positional cloning of *Pi9* (Qu et al. 2006). However, KS28/KS6 can overcome the shortcomings of a *Pi9* linked marker, such as false positives identified with the linked markers.

The absence of *Pi9* in germplasm was verified with the absence of PCR amplification or amplicons bigger than 699 bp expected for *Pi9*, suggesting different genetics rearrangements in the 5' leader region of the *Pi9* gene, because KS28 was designed to detect sequence polymorphism while KS6 was designed from a highly homologous region in the ORF of the *Pi9* gene. The genus *Oryza* has high genetic variability, with 22 wild species (Joshi et al. 2000; Vaughan 1989), and *Pi9* was originally introgressed into indica rice lines from *O. minuta* (Amante-Bordeos et al. 1992). Therefore, natural hybridization among rice species and random mutation in the rice genome may have interfered with *Pi9*, which explains different PCR amplification patterns observed in the screening of the mini-core collection with the KS28/KS6 marker.

Blast disease can cause annual food losses that can feed up to 60 million people (Pennisi 2010). Management of rice blast disease through resistant varieties is the most desirable means. However, resistance in newly released rice varieties may soon be lost due to high genetic variability of the fungus *M. oryzae* (Ebbole 2007). Continuing the search for more blast *R* genes and accurately incorporating them into breeding programs are critical to maintaining rice production stability for global food security. The availability of the robust DNA marker derived from a portion of the *Pi9* gene and a rice germplasm carrying *Pi9* with suitable agronomic characteristics

can now be used to improve blast resistance with MAS. MAS accelerates *R* gene incorporation (Jia 2003) but relies on accuracy of DNA markers and availability of donor germplasm. Cloning of blast *R* genes has allowed us to understand the molecular basis of blast resistance (Jia et al. 2000; Qu et al. 2006), and also allowed us to develop robust markers from the *R* gene itself (Jia et al. 2002). The robust markers for *R* genes are important for obtaining effective blast resistance to rice blast (Miah et al. 2013). The dominant and codominant markers for *Pi-ta* and *Pi-b* were the first such markers that have been effectively used in rice breeding programs worldwide (Jia et al. 2002, 2004a,b; Thakur et al. 2013; Wang et al. 2007; Wang et al. 2010, 2014).

Pi9 is an important gene in rice breeding because it confers resistance to a broad range of rice blast strains (Liu et al. 2002; Qu et al. 2006; Wang et al. 2015). However, *Pi9* has not been widely adopted into rice breeding programs worldwide (Y. Jia, unpublished data). The monogenic line IRBL9-W carrying *Pi9* has undesirable agronomic traits such as poor tillering, weak culm, and grain with a red pericarp, whereas IR 9660-48-1-1-2 has modern plant architecture with acceptable tillers and produces white, medium grains (Fig. 6), which makes it more desirable as a donor for *Pi9* than IRBL9-W for rice breeding. Taken together, we have developed a new toolbox consisting of a user-friendly DNA marker and an acceptable donor rice germplasm for a blast *R* gene for developing improved resistance to *M. oryzae* using a MAS approach in the United States and worldwide.

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