Marker Development for Rice Blast Resistance Gene *Pi66(t)* and Application in the USDA Rice Mini-Core Collection

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ABSTRACT

Molecular markers are useful for the identification of critical genes controlling agricultural traits of interest in crop germplasm and for the use of these genes in crop improvement using marker-assisted selection (MAS). The improvement of blast disease resistance of rice (*Oryza sativa* L.) varieties is one of the major goals for rice breeders. The blast resistance (*R*) gene *Pi66(t)* was previously mapped to a 129-kb genomic region on chromosome 11, where six candidate *R* genes were predicted. To help rice breeders in rice blast resistance MAS breeding, we developed three polymerase-chain-reaction (PCR)-based molecular markers, WRKY41, NBS-LRR-970-1, and NBS-LRR-970-2, for the *Pi66(t)* locus. The validation of two markers, WRKY41 and NBS-LRR-970-1, suggested they cosegregated with the *Pi66(t)* gene in two recombinant inbred line (RIL) populations. The application of two markers in a USDA–ARS rice mini-core collection indicated that NBS-LRR-970-1 was a *Pi66(t)*-linked specific marker. The developed marker not only can be used to identify resistant lines with the *Pi66(t)* gene in mapping populations but also can be used in screening rice germplasm with a broad genetic background. Taken together, these newly developed markers and newly identified rice germplasm lines carrying the *Pi66(t)* gene will provide essential tools and materials for rice breeders in MAS breeding.

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Abbreviations: DGWG, rice cultivar Dee Geo Woo Gen; LRR, leucine-rich repeat; MAS, marker-assisted selection; NBS, nucleotide-binding site; PCR, polymerase chain reaction; QTL, quantitative trait loci; *R*, resistance; RFLP, restriction fragment-length polymorphism; RIL, recombinant inbred line.

As the staple food for humanity, a 50% increase in rice production is required by 2050 to meet the predicted demand of the increasing population (Ray et al., 2013). Increasing rice yield is believed to be one of the most important goals for rice breeding programs worldwide. Rice production, however, has been constantly threatened by diverse abiotic and biotic stresses. Among them, rice blast disease, caused by the filamentous asco-mycete fungus *Magnaporthe oryzae* B.C. Couch, is one of the most devastating diseases and can cause 50 to 100% rice yield losses worldwide (Ou, 1985). The use of resistant varieties is believed to be the most economical and environmentally favorable way to control rice blast disease. However, *M. oryzae* is known to be highly variable with an extraordinary ability to mutate. A rice variety with a single *R* gene is easily overcome by newly emerged, more virulent races in commercial rice fields (Valent and Chumley, 1994). Intense efforts have been undertaken to breed for broad spectra and durable resistant varieties.

One of the effective strategies to develop broad-spectrum resistance varieties is pyramiding of *R* genes or resistance quantitative trait loci (QTL). Traditional breeding is based on phenotypic

selection and often is time and labor intensive and inefficient for pyramiding particular *R* genes or QTLs into a single elite variety. With the increasing availability of diverse molecular markers, including random amplified polymorphic DNA, restriction fragment–length polymorphism (RFLP), single-sequence repeats, single-nucleotide polymorphism, and PCR–based *R*-gene–specific markers, MAS has attracted the breeders' attention in recent decades. Marker-assisted selection can not only assist in the identification of germplasm with agronomically important genes or QTLs, but it can also help in pyramiding those genes or QTLs into new varieties using gene-linked molecular markers without the need of extensive phenotyping. Among them, PCR–based *R*-gene–specific markers are considered to be the most accurate and efficient for MAS (Xu, 2003). Compared with conventional breeding methods, MAS provides a simple and efficient way to identify and transfer the target *R* genes or resistance QTLs to an elite variety (Jia, 2003; Collard and Mackill, 2008). DNA can be prepared from seeds, sheath, root, grain, or leaves, and user-friendly high-throughput methods for large numbers of plant samples are available (Jia, 2003; Zhao et al., 2012). However, specificity of DNA markers at primer sites often limits the accuracy of selection by MAS.

Over the last few decades, the genetic mechanism of rice blast resistance has been extensively studied. To date, over 100 major blast *R* genes have been identified and 23 of them have been cloned including *Pib*, *Pita*, *Pi9*, *Pi2/Piz-5*, *Piz-t*, *Pid2*, *Pi36*, *Pi37*, *Pik-m*, *Pit*, *Pi5*, *Pid3*, *Pi25*, *Pish*, *Pik*, *pi21*, *Pb1*, *Pia*, *Pik-h/Pi54*, *Pik-p*, *Pi1*, *Pi56*, and *Pi64* (Sharma et al., 2012; Liu et al., 2013, 2015; Ma et al., 2015). With the advent of innovative molecular techniques and the availability of *R* gene sequences, different types of molecular markers linked to *R* genes or resistance QTLs have been developed (Hittalmani et al., 1995; Jia et al., 2002; Yi et al., 2004; Fjellstrom et al., 2006; Hayashi et al., 2006; Rathour et al., 2008; Liu et al., 2013), and some of these markers have been used to improve resistance in elite rice breeding lines and cultivars using MAS. Hittalmani et al. (2000) successfully improved the blast resistance of a rice variety CO39 by pyramiding three major *R* genes—*Pi1*, *Piz-5*, and *Pita*—through MAS using RFLP markers. Singh et al. (2011) developed an elite Basmati restorer line for a hybrid rice program through pyramiding the major *R* genes *Piz-5* and *Pi54* using MAS. Jiang et al. (2015) pyramided two *R* genes, *Pi2* and *Xa23*, into an elite rice thermosensitive genic male-sterile line, Guangzhan63-4S through MAS and successfully improved the disease resistance of developed hybrids using Guangzhan63-4S in a hybrid breeding program. Using MAS, Fukuoka et al. (2015) successfully combined four resistance QTLs into one near isogenic line, which exhibited a stable and durable resistance to rice blast.

The rice cultivar Dee Geo Woo Gen (DGWG), the resource of the Green Revolution semidwarf gene (*sd1*), has significantly affected global rice production during the mid-20th century. However, resistance of DGWG to blast disease was only recently explored. A major *R* gene, *Pi66(t)*, controlling complete resistance to rice blast race IB54 was identified in DGWG using next-generation sequencing (Liu et al., 2015). *Pi66(t)* was mapped to a 129-kb region on chromosome 11, where three putative NBS–LRR resistance proteins and three WRKY transcription factors were identified. The blast race IB54 has been commonly found in US rice fields; transferring an *R* gene with resistance to IB54 into elite rice varieties will benefit rice farmers in the United States. To explore resistant germplasm collections with the *Pi66(t)* gene and to facilitate incorporation of this gene using MAS breeding, we aimed to develop *R*-gene-specific molecular markers that are closely linked with *Pi66(t)*. In the present study, we developed three PCR–based molecular markers and used them to identify and verify the presence of *Pi66(t)* with pathogenicity assays in 11 rice germplasm lines from the USDA–ARS rice mini-core collection (Agrama et al., 2009).

MATERIALS AND METHODS

An *indica* rice cultivar, DGWG, and the source of blast resistance gene *Pi66(t)* was used as the resistant control variety (Liu et al., 2015). Two US weedy rice ecotypes, the straw hull type PI653435 (AR–2001–1135; RR9) and the black hull type PI653419 (MS–1996–9; RR20), were used as the susceptible control genotypes, which do not have the blast resistance gene *Pi66(t)* (Liu et al., 2015). Two F_8 RIL populations, designated as the S- and B-populations, were used to verify the newly developed PCR–based markers. The S-population consisted of 152 individuals derived from the cross of DGWG and RR9, while the B-population consisted of 218 individuals derived from the cross of DGWG and RR20. A total of 217 rice germplasm lines were collected from the rice mini-core collection (Agrama et al., 2009) at USDA–ARS Dale Bumpers National Rice Research Center in Stuttgart, AR, USA, and used to identify blast resistant germplasm containing the *R* gene *Pi66(t)* (Supplemental Table S1). The sequenced *japonica* reference rice cultivar Nipponbare was used as the reference genome.

Blast Disease Evaluation

The seeds of the two RIL populations and 217 rice germplasm lines from the mini-core collection were grown in plastic pots. The plastic pots were rearranged into multiple randomized replications in a plastic tray (54 by 28 by 6 cm, 48 pots per plastic tray, and 10 seeds per pot per genotype) with three replicates in the greenhouse at USDA–ARS Dale Bumpers National Rice Research Center. Seedlings were grown under greenhouse conditions at a temperature of 25 to 30°C. After germination, 0.02 g of controlled-release fertilizer (Osmocote Plus, 15-9-12 [N-P-K], Hummert International) was applied to each insert. As in Liu et al. (2015), the rice blast race IB54 was used to evaluate all of the RILs and the mini-core collection in the

Table 1. Information on six candidate genes in the *Pi66(t)* genome region (Liu et al., 2015).

Locus	Chromosome	Physical position	Predicted function
LOC_Os11g45850	11	27740142–27741375	WRKY61, expressed
LOC_Os11g45920	11	27785982–27779324	WRKY63, expressed
LOC_Os11g45924	11	27791943–27789618	WRKY41, expressed
LOC_Os11g45930	11	27793777–27797821	NBS-LRR type disease resistance protein, putative, expressed
LOC_Os11g45970	11	27818431–27812251	NBS-LRR disease resistance protein, putative, expressed
LOC_Os11g45980	11	27820309–27824920	NBS-LRR type disease resistance protein, putative, expressed

Table 2. Primers for the newly developed polymerase-chain-reaction (PCR)-based markers for the resistance gene *Pi66(t)*.

Designation	Forward Primer	Reverse Primer	Annealing Temperature	Expected PCR product
			°C	bp
WRKY63-1	TGGGTAACAATGACGGCACC	CACACCTGTAGTAACCCCTG	55	851
WRKY41	CATTTGTGCGGACATCCCAG	TGCTAGACTTGTTACCCTCC	55	981
NBS-LRR-930-1	CAAGGACAAGAGGTGACTGC	CAGATGAGAGCCCAGAGTAC	55	806
NBS-LRR-930-2	CTCTATCGAACCTCGTCTCG	ACCCTGAGGCCTGAACATAC	55	730
NBS-LRR-970-1	CAGCAGCCACCTGAAAGTTG	TGGGATATGCATGCCACTCG	55	927
NBS-LRR-970-2	GTAGTTTTCGGATCACCGAG	CCAAAGAAGGCAAGTGTTCG	55	1099

greenhouse. The preparation of the blast isolate, inoculation, and disease assays were modified based on the method described by Liu et al. (2015). The concentration of the spore suspension was measured using a hemocytometer and then adjusted to ~1 × 10^5 spores mL^−1 in 0.25% gelatin solution. Twenty–one–day–old rice seedlings at the 3 to 4 leaf stage were sprayed with 30 mL of the spore suspension per tray in a black plastic bag (0.99 by 1.47 m). The inoculated seedlings were sealed in the inoculation bags, maintained at a temperature of 25°C with a relative humidity of >90% for 24 h, and then transferred to the greenhouse and maintained at 25 to 28°C and 60 to 90% relative humidity with a 12–h–day and 12–h–night light cycle. The disease reactions were scored at 7 d after inoculation using a scale of 0 to 5, where 0 to 2 and 3 to 5 indicated resistant and susceptible plants, respectively. Disease reaction scores were determined based on all 10 plants. For most of the RILs, the same disease reaction was obtained for all 10 plants per RIL. However, the average disease reaction score was used when the 10 plants did not exhibit the same reaction.

Marker Development for Pi66(t)

The *Pi66(t)* gene in DGWG was previously mapped to a 129-kb genomic region (Liu et al., 2015). Within this region, three genes encoding putative nucleotide-binding site (NBS) and leucine-rich repeat (LRR) disease resistance proteins and three genes encoding WRKY transcription factors (WRKY61, WRKY63, and WRKY41) were predicted as the candidate genes for *Pi66(t)* (Table 1). To develop *Pi66(t)*–linked molecular markers, sequences of the six candidate genes, based on the Nipponbare reference genome sequences, were downloaded from Rice Genome Annotation Project–Missouri State University Rice Genome Annotation (Osa1) Release 7 (http://rice.plant-biology.msu.edu/cgi–bin/gbrowse/rice) and used for designing primers. Primers were designed with the primer analysis software Oligo 5.0 (http://www.oligo.net). The sequences of six primer pairs are listed in Table 2. The specificity of primer pairs was verified using BLAST on the database at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) and then tested by PCR amplification using genomic DNA of Nipponbare as the template. Primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, Iowa, USA).

DNA Isolation and Polymerase Chain Reaction Amplification

Total DNA was isolated from leaf tissue of plant materials DGWG, Nipponbare, RR20, RR9, the individuals of two RIL populations, and 217 lines from the mini–core collection using the CTAB method (Murray and Thompson, 1980) with modifications. The quality and the concentration of DNA were determined using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific). The final concentration of DNA was adjusted to 50 ng μL^−1 with sterilized distilled water for PCR analysis.

The PCR amplifications were set up in a volume of 20 μL using Taq PCR Master Mix Kit (Qiagen). Each PCR reaction contained 10 μL Taq PCR master mix, 0.4 μL forward primer (5.0 uM), 0.4 μL reverse primer (5.0 uM), 2.0 μL DNA (50 ng μL^−1), and 7.2 μL water. The amplification program was 94.0°C for 5 min for initial denaturation followed by 35 cycles of 94.0°C for 45 s, 55°C for 45 s, 72°C for 1 min, and a final extension at 72°C for 10 min. All PCR reactions were performed in a PTC–225 Peltier Thermal Cycler (MJ Research). The PCR products were separated in a 1.2% agarose gel and visualized using Bio-RAD ChemiDoc imaging system (Bio-Rad Laboratories).

RESULTS
Development of Polymorphic Polymerase Chain Reaction Markers for *Pi66(t)*

The specificity of the six primer pairs was verified by PCR using Nipponbare genomic DNA, and all PCR products were of anticipated sizes (Supplemental Fig. S1).
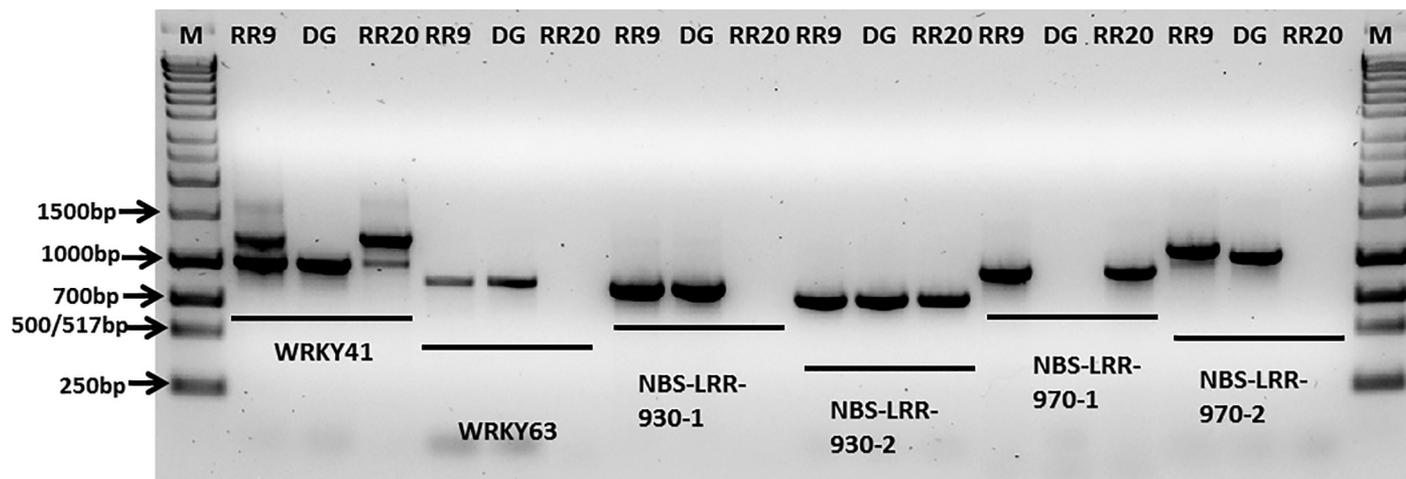
Fig. 1. Polymorphism test among the parental lines. M, 1-kb DNA ladder; DG, cultivar Dee Geo Woo Gen, resistant parental line; RR9, straw hull awnless susceptible parental line; RR20, black hull awned susceptible parental line.

Table 3. Chi-square test for the segregation of resistance and susceptibility in two F_8 recombinant inbred line (RIL) populations inoculated with the rice blast isolate IB54.

Cross	Number of total RILs	Number of resistant lines	Number of susceptible lines	χ^2	P-value†
DGWG × RR9	151	72	79	0.324	0.75 > P > 0.5
DGWG × RR20	217	117	100	1.33	0.25 > P > 0.1

† P-value > 0.05; indicates the goodness of fit to a 1R/1S ratio.

The polymorphism of primer pairs among three parental genotypes—DGWG, RR9, and RR20—was identified using a PCR method (Fig. 1). Specifically, the primer pairs WRKY41, NBS-LRR-970-1, and NBS-LRR-970-2 exhibited polymorphism between the blast resistant parent, DGWG, and susceptible parents, RR9 and RR20. The primers WRKY63, NBS-LRR-930-1, and NBS-LRR-930-2 showed polymorphism between DGWG and RR20 but not between DGWG and RR9. Because NBS-LRR-970-1 and NBS-LRR-970-2 were designed for the same candidate gene in the Pi66(t) region, NBS-LRR-970-1 was selected for marker validation and future use. Thus, two markers, WRKY41, which can amplify two bands in RR9 and RR20 but one in DGWG, and NBS-LRR-970-1, which can amplify one band in RR9 and RR20 but is absent in DGWG, were selected as the markers for the Pi66(t) locus.

Verification of the Markers using Two Recombinant Inbred Line Populations

Results of the disease reactions of the two RIL populations are listed in Supplemental Tables S2 and S3. Recombinant inbred lines of the two populations segregated at a ratio of 1:1 (1R/1S) (Table 3), suggesting that Pi66(t) is a single dominant nuclear R gene and responsible for blast resistance to IB54 in DGWG. To determine whether the two newly developed markers were associated with the resistant phenotype, two RIL populations were genotyped with the newly developed markers, WRKY41 and

NBS-LRR-970-1 (Supplemental Tables S2, S3). The genotypes of 36 individuals from the S-population are shown in Fig. 2 and 3 using the two markers. The RILs with the same genotype as DGWG were resistant to IB54, while RILs with the same genotype as RR9 or RR20 were susceptible to IB54 in the two RIL populations, suggesting that WRKY41 and NBS-LRR-970-1 cosegregated with the resistant phenotype in the RIL populations.

Application of Two Markers in the USDA–ARS Rice Mini-Core Collection

The disease reactions to IB54 of the USDA–ARS rice mini-core collection were evaluated in a greenhouse. The phenotypic data of 217 rice accessions are summarized in Supplemental Table S1. Among 217 accessions, 39 showed completely resistant to IB54, which had the same disease reaction score as DGWG. These 217 rice accessions were genotyped by a PCR method using the markers WRKY41 and NBS-LRR-970-1 (data not shown). The results suggested that there were no relatively close correlation between the phenotypic data and the genotypic data of 217 accessions using WRKY41 (some genotypic data not recorded). However, using NBS-LRR-970-1, 178 rice accessions showed a susceptible allele, and we only identified 11 rice accessions that had the same genotype as the resistant cultivar DGWG; there was a good, but not perfect; correlation between the phenotypic data and the genotypic data (Table 4; Fig. 4). Eight of these 11 rice accessions (Thang 10, PD46, IR 9660-48-1-1-2,
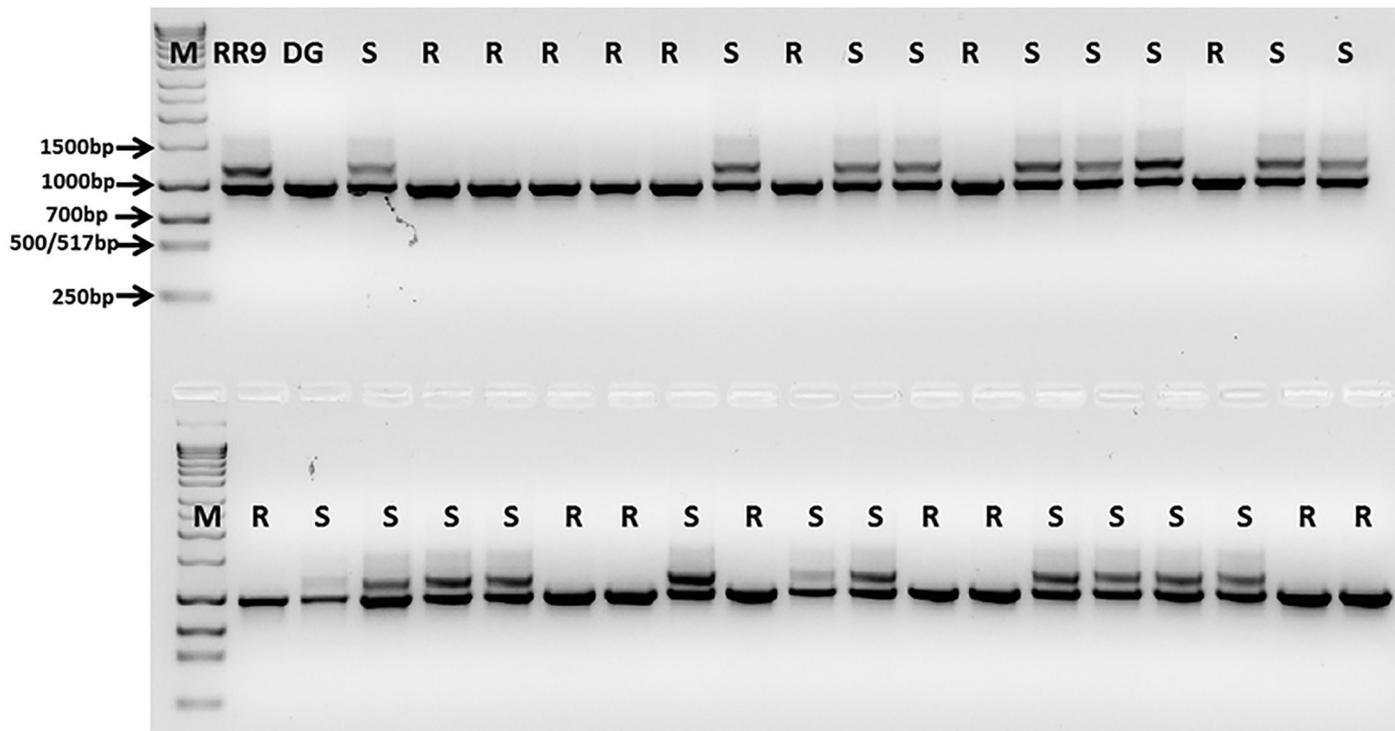
Fig. 2. Verification of the newly developed polymerase chain reaction marker WRKY41 using the S-population. M, 1-kb DNA ladder; DG, cultivar Dee Geo Woo Gen, resistant parent line; R, resistant line; S, susceptible line.
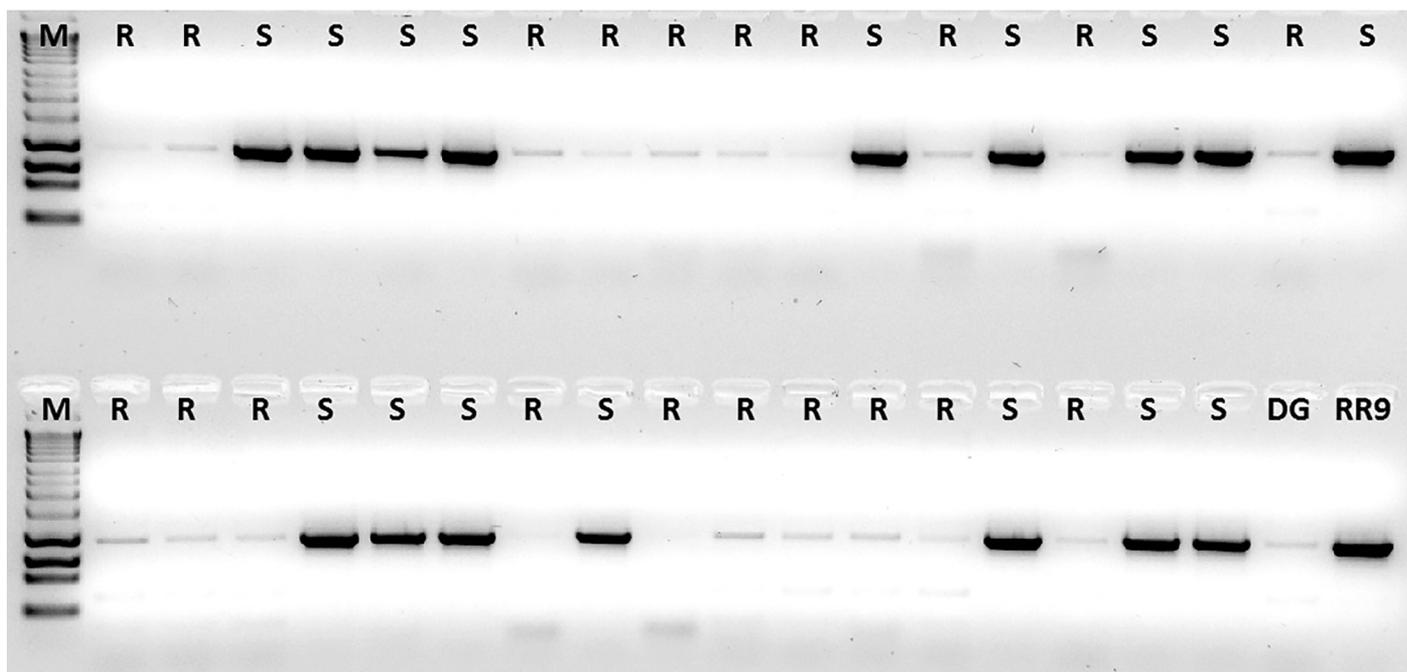

Fig. 3. Verification of the newly developed polymerase chain reaction marker NBS-LRR-970-1 using the S-population. M, 1-kb DNA ladder; DG, cultivar Dee Geo Woo Gen, resistant parent line; R, resistant line; S, susceptible line.

Kin Shan Zim, Hsin Hsing Pai Ku, Shui Ya Jien, 10340, and UZ ROS 59) were rated as 0, which had the same disease resistance rate as DGWG to IB54. Two rice accessions, Chunjiangzao NO. 1 and Juppa, were rated as 1 or 2, which are also resistant lines but not rated the same resistance rate as DGWG. One cultivar, 4484, was rated as 3 (different from the disease score of DGWG to IB54) indicating that it's moderately susceptible to IB54 despite having the NBS-LRR-970-1 resistance genotype. The success rate for identifying *Pi66(t)* in the 11 rice accessions from the rice mini-core using NBS-LRR-970-1 was 72.7% (eight divided by 11) for *Pi66(t)*. When evaluating the genotypes of these 11 accessions using the marker WRKY41, we found that nine accessions (Thang 10,

Table 4. Phenotype and genotype information of 11 selected germplasm lines from the USDA–ARS rice mini-core collection.

Designation	Name of germplasm	Disease reaction to IB54†	Genotype of germplasm‡ NBS-LRR-970-1	WRKY41
Parental line	DGWG§	0 (R)	A	A
Parental line	RR9	4 (S)	B	B
Parental line	RR20	5 (S)	B	B
310420	Thang 10	0 (R)	A	A
310442	PD46	0 (R)	A	A
310687	IR 9660-48-1-1-2	0 (R)	A	A
310791	Kin Shan Zim	0 (R)	A	A
310901	Juppa	2 (R)	A	A
311105	Hsin Hsing Pai Ku	0 (R)	A	A
311113	Shui Ya Jien	0 (R)	A	A
311123	10340	0 (R)	A	A
311286	UZ ROS 59	0 (R)	A	A
311483	4484	3 (S)	A	C
311497	Chunjiangzao NO. 1	1 (R)	A	C

† Disease reactions were scored using a scale of 0 to 5, where 0, 1, and 2 were resistant (R) and 3, 4, and 5 were susceptible (S).

‡ Genotype of germplasm using marker NBS-LRR-970-1 and WRKY41; A, resistant genotype; B, susceptible genotype; C, genotype different from the parental lines.

§ Rice cultivar Dee Geo Woo Gen.

PD46, IR 9660-48-1-1-2, Kin Shan Zim, Juppa, Hsin Hsing Pai Ku, Shui Ya Jien, 10340, and UZ ROS 59) had the same genotype as DGWG, whereas 4484 and Chunjiangzao NO. 1 did not (Table 4; Fig. 4). This suggests that nine out of 11 selected accessions may contain *Pi66(t)*; however, 4484 and Chunjiangzao NO. 1 may not contain *Pi66(t)*. Combining with the marker WRKY41, the success rate for *Pi66(t)* identification in the rice mini-core using NBS-LRR-970-1 was increased to 88.9%.

DISCUSSION

In rice breeding programs, MAS has been successfully used to improve blast disease resistance of numerous contemporary rice cultivars (Jia et al., 2002; Hittalmani et al., 2000; Singh et al., 2011; Jiang et al., 2015; Koide et al., 2010). The specificity of the gene-linked markers is crucial to improve the efficiency of selection in a broad genetic background. The development of specific molecular markers linked with *R* genes is the prerequisite of disease resistance breeding with MAS.

A novel major blast *R* gene, *Pi66(t)*, was recently identified in a "Green Revolution" cultivar DGWG at a 129-kb genomic region on chromosome 11 (Liu et al., 2015) and exhibits complete resistance to blast race IB54, which has been commonly found in the United States. To facilitate the use of *Pi66(t)* in rice resistance breeding using MAS, six user-friendly PCR-based markers were developed for the *Pi66(t)* locus targeting the candidate genes within this region (Table 2; Fig. 1). Results of the polymorphic analysis of the six newly developed markers suggested that

straw hull awned weedy rice, RR9, has a closer relationship with *indica* rice than the black hull awned weedy rice RR20, which is consistent with several previous studies (Londo and Schaal, 2007; Gealy et al., 2009; Reagon et al., 2010). Phenotypic analysis and genotype screening of two RIL populations using three newly developed markers, WRKY41, NBS-LRR-970-1, and NBS-LRR-970-2, suggested that these three markers in DGWG cosegregated with the blast resistance to IB54. Because the size of the two RIL populations was limited, we could not exclude any candidate gene from the previously mapped *Pi66(t)* (129-kb) genomic region with newly developed markers. Thus, these three newly developed markers can be directly used to select resistant lines from the two RIL populations. In return, the results in this study validated the candidate genes previously identified for *Pi66(t)* (Liu et al., 2015).

The USDA–ARS rice mini-core is representative of the genetic diversity of over 10,000 rice germplasm lines collected from over 100 countries (Agrama et al., 2009). To examine the specificity of the new developed markers, 217 rice germplasm lines in the USDA–ARS mini-core collection were analyzed with the markers WRKY41 and NBS-LRR-970-1. NBS-LRR-970-1 was specific to *Pi66(t)* in 217 diverse accessions but not WRKY41. This was unexpected, since WRKY41 segregated perfectly with the phenotype in the RIL populations. Subsequently, using the marker NBS-LRR-970-1, 11 rice accessions with *Pi66(t)* were identified. Among the 11 accessions, eight showed complete resistance to IB54 similar to DGWG. The efficiency of selection for *Pi66(t)* mediated resistance was 72.7% using NBS-LRR-970-1. These results suggest that the *Pi66(t)* genomic region in diverse rice germplasm is relatively conserved. This observation may reflect the fact that DGWG was widely used by rice breeders worldwide during the "Green Revolution" to incorporate the semidwarf *sd1* gene, resulting in unintentional introgression of conserved genomes. The majority of the accessions identified as having the *Pi66(t)* gene were improved resistant cultivars, thus they might have been developed using IR8.

For the NBS-LRR-970-1 marker, which is absent in resistant lines but present in susceptible lines, any single-nucleotide polymorphism in the priming sites will lead to a failed PCR. This could be the cause of the difference between resistance genotype (i.e., no PCR product) and susceptible genotype. Evidently, out of 11 accessions identified using NBS-LRR-970-1 marker, two were resistant to IB54, with a score of 1 or 2, and one was moderately susceptible to IB54 with a score of 3. When we applied the codominant marker WRKY41 to these 11 accessions, 4484 and Chunjiangzao NO. 1, with scores of 3 and 1, respectively, were excluded because their genotypes were inconsistent with the resistant parent, DGWG. Therefore, the efficiency of selection for *Pi66(t)* was increased to 88.9% using the two newly developed markers WRKY41
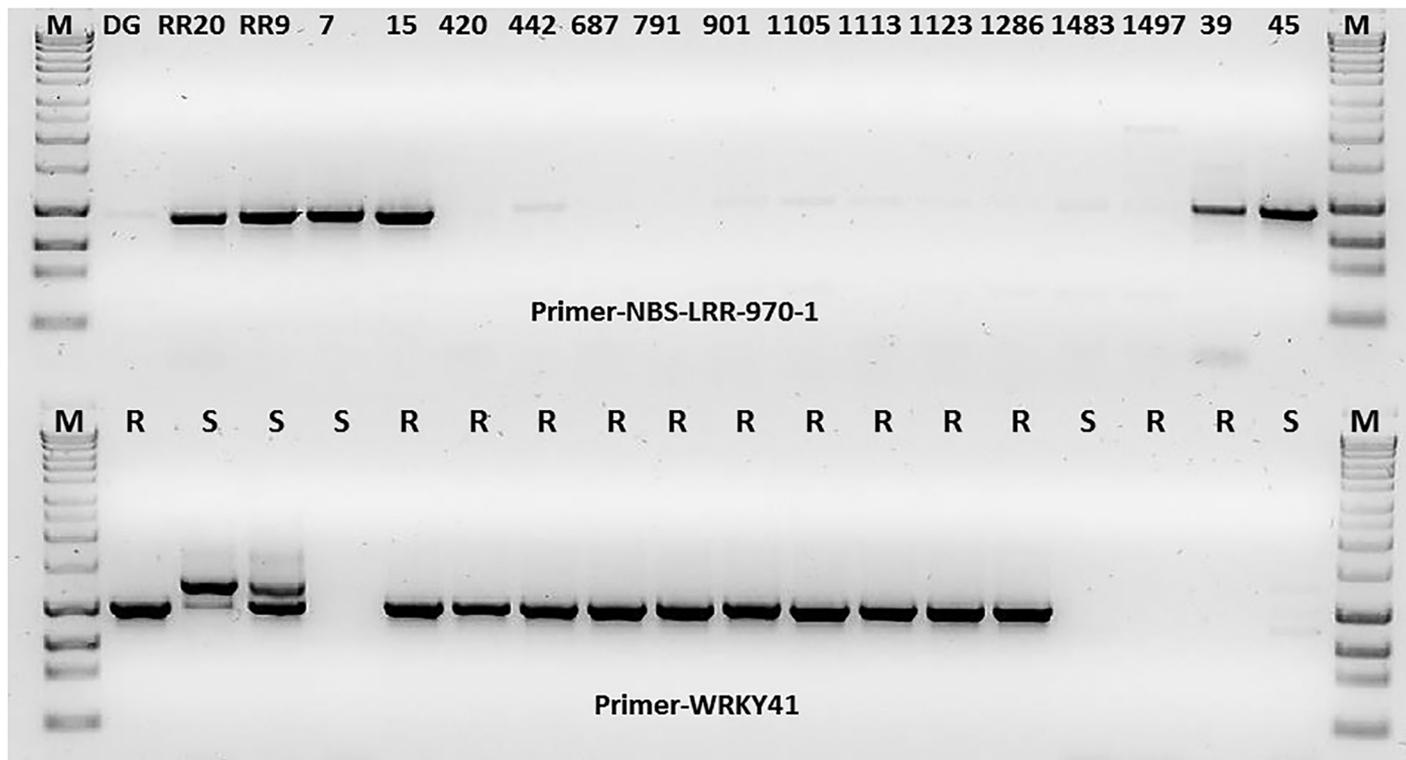
Fig. 4. Genotypes of 11 selected lines from USDA–ARS rice mini-core collection using two newly developed polymerase chain reaction marker markers NBS-LRR-9701 (the top row) and WRKY41 (the bottom row). M, 1-kb DNA ladder; DG, cultivar Dee Geo Woo Gen, resistant control. RR9 and RR20 are the susceptible control. The numbers 7, 15, 39, and 45, which were randomly chosen from 217 germplasm lines, represent lines 310007, 310015, 310039, and 310045 respectively; The numbers 420, 442, 687, 791, 901, 1105, 1113, 1123, 1286, 1483, and 1497 represent 310420, 310442, 310687, 310791, 310901, 311105, 311113, 311123, 311286, 311483, and 311497, respectively; Additional information on germplasm is listed in Table 4 and Supplemental Table S1.

and NBS–LRR–970–1; although, WRKY41 cosegregated with *Pi66(t)* in RIL populations but not in rice mini-core collection. This result suggests that a combination of more molecular markers linked with target genes can increase prediction accuracy for *Pi66(t)*. Further analysis of genomic regions nearby these two markers in diverse rice accessions may lead the development of more powerful markers for *Pi66(t)*.

Among the 217 accessions, 39 accessions were completely resistant to IB54. However, using the markers NBS-LRR–970–1 and WRKY41, eight out of 39 accessions were verified to carry *Pi66(t)*. These results suggest that the other 31 accessions may contain *R* genes other than *Pi66(t)*.

The PCR–based markers developed in this study provide rice breeders and researchers a valuable tool for blast disease resistance selection of *Pi66(t)* using MAS. The *Pi66(t)*–linked gene-specific marker, NBS-LRR–970–1, in combination with WRKY41, will facilitate the introgression or pyramiding of blast *R* genes that will be useful in the development of high-yielding and blast-resistant varieties through MAS breeding without the need for disease evaluation in fields or greenhouses.

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