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Characterization of resistance genes to rice blast fungus *Magnaporthe oryzae* in a "Green Revolution" rice variety

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Abstract The *indica* rice variety Dee Geo Woo Gen (DGWG) was the source of the semidwarf gene (*SD1*) which played an important role in the Green Revolution. In the present study, resistance (*R*) genes to the US race (isolate) IB54 of *Magnaporthe oryzae*, causal agent of rice blast disease, was investigated. Two recombinant inbred line mapping populations, consisting of 175 and 224 individuals derived from crosses of DGWG with the straw hull weedy rice type PI653435 (AR-2001-1135; S population) and the black hull type PI653419 (MS-1996-9; B population), respectively, were used for mapping blast *R* genes and quantitative trait loci (QTLs). Two high-resolution linkage maps with 6,513 (S population) and 14,382 (B population) single nucleotide polymorphic markers derived from genotyping-by-sequencing data were used to map *R* genes. Two partial resistance QTLs, *qBR1.1* and *qBR6.1*, and one major resistance QTL, *qBR11.1*, were identified in the B population. One partial resistance QTL, *qBR6.1*, and one major resistance QTL, *qBR11.1*, were confirmed with the S population. The total phenotypic variation of three resistance QTLs was 51 %, ranging from 1.12 to 47.62 %, in the B population. All three resistance QTLs were localized to relatively small genomic regions. The major resistance QTL, *qBR11.1*, was mapped to a 129-kb region on chromosome 11 near nine known blast *R* genes. Within this 129-kb region, three genes encoding putative nucleotide-binding site and leucine-rich repeat (LRR) disease resistance proteins and three genes encoding WRKY transcription factors WRKY61, WRKY63, and WRKY41 were identified as candidate genes of *qBR11.1* and tentatively designated as *Pi66(t)*. Identification of blast *R* genes in DGWG should help continued deployment of useful genes for improving crop productivity and resistance to rice blast disease.

Keywords DGWG · Rice blast resistance · *R* gene · GBS · Green Revolution · *Pi66(t)*

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Introduction

Rice (*Oryza sativa*), the staple food for human consumption in most of the world, has been constantly

challenged by rice blast, one of the most devastating disease, which is caused by the filamentous ascomycete fungus *Magnaporthe oryzae* (*M. oryzae*). The use of fungicides to manage *M. oryzae* has achieved limited success. However, the continued use of fungicides not only increases production cost but also results in serious environmental impacts. The use of host resistance is the most economical and environmentally favorable means to control rice blast disease. Genetic analysis has revealed that the major resistance (*R*) gene is effective in preventing *M. oryzae* strains that contain the corresponding avirulence gene. Thus far, 86 major *R* genes have been mapped on all of the rice chromosomes except for chromosome 3 (Liu et al. 2013). Most of the blast *R* genes are mapped on a single locus. However, three large gene clusters have been identified including *Pi5*, *Pi3*, *Pi15*, and *Pi56(t)* on chromosome 9, *Pik*, *Pik-h*, *Pik-m*, *Pik-p*, *Pik-s*, *Pi1*, *Pif*, *Pilm2*, *Pi7(t)*, *Pi18(t)*, and *Pi43(t)* on chromosome 11, and *Pi-ta*, *Pi-ta*^{2}, and *Ptr(t)* on the chromosome 12 (Ballini et al. 2008; Sharma et al. 2012; Inukai et al. 1994; Lee et al. 2009; Liu et al. 2013; Rybka et al. 1997; Jia and Martin 2008). Among these, 18 major *R* genes have been cloned, and most of them encode predicted proteins with nucleotide-binding site (NBS) and leucine-rich repeats (LRR), suggesting a conservative mode of pathogen recognition (Moytri et al. 2012).

Magnaporthe oryzae is known to be a highly mutable pathogen partially due to avirulence gene instability (Valent and Chumley 1994). Consequently, resistance mediated by a single major blast *R* gene can be overcome in a few years after deployment. Instead, partial resistance is mediated by multiple genes each providing minor effects that are less easily overcome. Such forms of resistance can endure longer after deployment. To date, over 350 partial blast *R* quantitative trait loci (QTLs) have been identified (Ballini et al. 2008); however, only a few have been deployed for crop protection. The *indica* variety Dee Geo Woo Gen (DGWG) was one of the most important resources of the semidwarf gene (*SD1*) and has significantly impacted global rice production during the "Green Revolution" of the mid-twentieth century. The development of semidwarf rice varieties using DGWG doubled rice yields within 15 years (Sasaki et al. 2002). To date, over 90 % of high-yielding rice varieties carry *SD1* from DGWG (Spielmeyer et al. 2002). In contrast, the content of blast *R* genes in DGWG is still unknown.

Here, we report the use of genotyping-by-sequencing (GBS) technology and phenotypic analysis of two recombinant inbred line (RIL) populations using DGWG as a parent to identify blast *R* genes/QTLs in DGWG. Our analysis suggests that DGWG has strong resistance to the US *M. oryzae* race IB54. The major resistance QTL was initially identified in proximity to a cluster of blast *R* genes, *Pik/piks/pikm*. Further analysis indicates that genetic resistance in DGWG is conferred by a new major *R* gene, herein named *Pi66(t)*. These findings will greatly help to improve blast resistance and semidwarf breeding program in the future.

Materials and methods

Plant materials

DGWG was used as a donor parent to cross with two US weedy rice ecotypes, straw hull weedy rice type PI653435 (AR-2001-1135; RR9) and black hull type PI653419 (MS-1996-9; RR20), to generate two RIL populations using the single seed descent breeding method in the greenhouse at the USDA Agricultural Research Service, Dale Bumpers National Rice Research Center (USDA-ARS DB NRRC) in Stuttgart, Arkansas (Y. Jia, unpublished data). S population was derived from the cross of DGWG and RR9 and consists of 175 F_5 individuals. B population was derived from the cross of DGWG and RR20 and consists of 224 F_5 individuals. F_5 RILs of the two mapping populations were genotyped using the GBS method described by Liu et al. (2014, in preparation). F_6 RILs were grown in the field during the summer of 2012 at USDA-ARS DB NRRC to increase seed number. Seeds of F_7 RILs of the two populations were used for the evaluation of blast disease in the greenhouse.

Evaluation of blast disease reaction

Rice seeds were sown in plastic trays (54 cm × 28 cm × 6 cm) with three replications of 10 plants per RIL line in the greenhouse. Seedlings were grown under greenhouse conditions at a temperature of 25–30 °C. Blast isolates (Table 1) were prepared following the procedure described by Jia and colleagues (Jia et al. 2009; Jia and Liu 2011). The

Table 1 Disease reactions of three parental rice lines to 14 US blast races (isolates) of *Magnaporthe oryzae*

Race (isolate) of *M. oryzae*	Rice and weedy rice genotype		
	DGWG	RR9	RR20
IB33 (FN9)	5	5	1
IC1 (BRFD-2F-2)	5	5	1
IC17 (ZN57)	5	1	2
IE1 K (TM2)	5	4	2
ID1 (ZN42)	5	2	2
IB54 (isolate unnamed)	0	2	5
IB49 (ZN61)	5	4	4
IB1 (isolate unnamed)	5	4	4
IA1 (BRFD-21-2)	5	5	4
IG1 (ZN39)	5	4	4
IE1 (ZN13)	5	4	5
IB45 (isolate unnamed)	5	5	5
IA45 (75L14)	5	5	5
IH1 (isolate unnamed)	5	3	5

Disease reaction scale is 0–5; 0–2, resistance; 3–5, susceptible

DGWG Dee Geo Woo Gen, *RR9* straw hull weedy rice ecotype PI653435 (AR-2001-1135), *RR20* black hull weedy rice ecotype PI653419 (MS-1996-9)

concentration of the spore suspension was adjusted to 1×10^5 spores/mL with a 0.25 % gelatin solution. A tray of rice seedlings at the 3–4 leaves stage, approximately 21 days old, were placed in a plastic bag and sprayed with 30 mL of the spore suspension. The inoculated seedlings were kept at a temperature of 25 °C with high humidity for 24 h. The seedlings were then transferred to a greenhouse (25–30 °C) with 60–70 % humidity. Disease reactions were determined at seven days after inoculation using a scale of 0–5; where 0–2 is considered to be resistant and 3–5 is susceptible. The same disease reaction was obtained for all 10 plants per RIL.

Genotype analysis of parents and mapping populations

Parental lines and all individuals of the two F_5 RIL populations were genotyped using GBS as described previously (Liu et al. 2014, in preparation). Plant genomic DNA was extracted using QIAamp DNA Mini Kit following the manufacturer's protocol (Qiagen Inc., Valencia, CA, USA). Genomic DNA from a subset of 175 and 224 F_5 RILs was digested using the restriction enzyme *Ape*KI and bar-coded following the protocol as described by Elshire et al. (2011). Sets of 96 samples per lane were sequenced with an Illumina HiSeq DNA sequencer at Cornell University, Ithaca, New York. Raw data were processed using the standard Tassel GBS pipeline. Single nucleotide polymorphism (SNP) calls were based on the Nipponbare reference genome sequence.

Construction of genetic maps and QTL mapping

Genetic positions of the SNPs were identified using cM Converter (http://mapdisto.free.fr/cMconverter) based on the physical positions of the SNPs in the MSU Rice Genome Annotation Release 6 (MSU 6.0). The genetic linkage map was generated using the R/qtl software package (Broman and Sen 2009; Liu et al. 2014). QTLs were analyzed using both single-marker analysis (SMA) and composite interval mapping (CIM) methods of the R/qtl package. Haley–Knott regression (HK) and multiple imputation (IMP) (Haley and Knott 1992) programs were used for data analysis (Sen and Churchill 2001), respectively.

Prediction of candidate *R* genes

To predict candidate *R* genes, the physical positions of SNPs flanking the target QTL regions were converted from MSU 6.0 to MSU Rice Genome Annotation Release 7 (MSU 7.0) (http://rice.plantbiology.msu.edu/cgi-bin/gbrowse/rice/). The QTL intervals were delimited by the flanking SNP markers. Candidate *R* genes were predicted within the mapped QTL regions based on the output of gene prediction by MSU 7.0.

Results

Disease reactions of parents and individuals of two mapping populations

Disease reactions of three parental lines, DGWG, RR9, and RR20, to 14 US blast races (isolates) were evaluated in the greenhouse (Table 1). DGWG was resistant to the race IB54 (isolate unnamed) and susceptible to the remaining 13 races including IB33 (FL9, a laboratory strain), IC1 (BRFD-2F-2), IC17 (ZN57), IE1 K (TM2), ID1 (ZN42), IB49 (ZN61), IB1 (isolate unnamed), IA1 (BRFD-21-2), IG1 (ZN39),

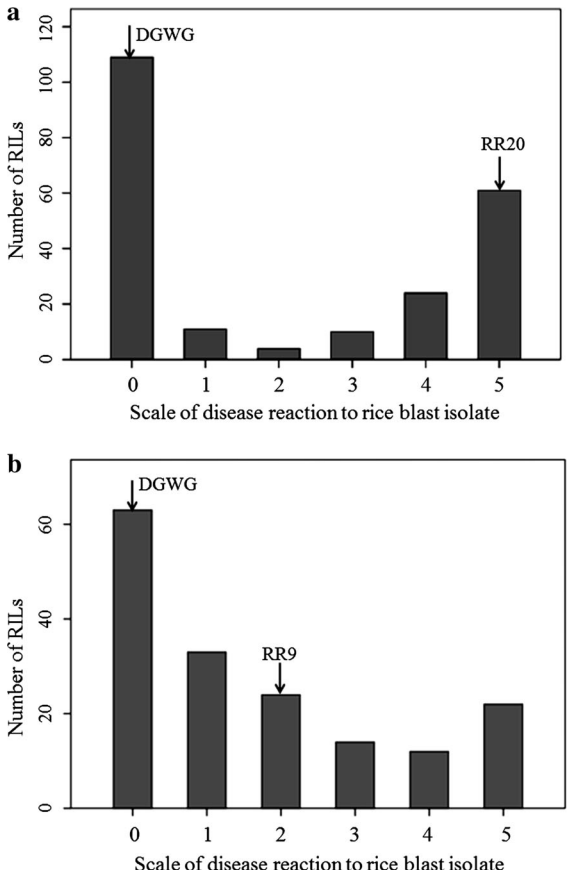

Fig. 1 Disease reaction distribution of two F_7 recombinant inbred line (RIL) populations to the US blast race IB54. **a** Disease reaction distribution of F_7 RILs derived from DGWG and RR9 (S population). **b** Disease reaction distribution of F_7 RILs derived from DGWG and RR20 (B population). DGWG is the *indica* variety Dee Geo Woo Gen; RR9 is the straw hull weedy rice ecotype PI653435 (AR-2001-1135); RR20 is the black hull weedy rice ecotype PI653419 (MS-1996-9)

IE1 (ZN13), IB45 (isolate unnamed), IA45 (75L14), and IH1 (isolate unnamed) (Table 1). RR9 was resistant to three races, IC17, IB54, and ID1, and susceptible to 11 races, IB33, IC1, IE1 K, IB49, IB1, IA1, IG1, IE1, IB45, IA45, and IH1. RR20 was resistant to the five races, IB33, IC1, IC17, IE1 K, and ID1, and susceptible to the remaining nine races, IB49, IB54, IB1, IA1, IG1, IE1, IB45, IA45, and IH1. Subsequently, IB54 was selected to evaluate disease reactions of all individuals in the two F_7 RIL populations since the parental lines (DGWG, resistant; RR9; moderately resistant; RR20, susceptible) demonstrated differential reactions to this race.

The distributions of disease reactions of the two RIL populations are shown in Fig. 1. The frequency of RILs in both B and S populations was skewed toward resistance (Fig. 1a, b). This result suggests that there is a major resistance gene effect to IB54.

Mapping blast resistance QTLs

A total of 6,513 SNPs were identified from the S population and 14,382 SNPs from the B population. High-resolution linkage maps for each population were generated by Liu et al. (2014, in preparation). The genetic map for the S population was 1,402 centimorgans (cM) and that of B was 1,536 cM. The average inter-marker distance for S and B populations was 0.227 ± 0.01 and 0.107 ± 0.01 cM, respectively (Liu et al. 2014, in preparation).

Using the B population, we identified three resistance QTLs against IB54 (Table 2; Supplementary Fig. 1a). The three resistance QTLs were named as *qBR1.1*, *qBR6.1*, and *qBR11.1* following the recommended nomenclature for rice QTLs (McCouch 2008). The QTL *qBR1.1*, accounting for 2.35 % of the phenotypic variance, is located within a genomic interval of 0.12 cM on chromosome 1 with a logarithm of odds (LOD) score of 8.58. This QTL is flanked by two SNP markers, S1_16414192 and S1_16507769. The QTL *qBR6.*1, accounting for 1.12 % of the phenotypic variance, is located within a genomic interval of 1.68 cM on chromosome 6 with a LOD score of 7.02. This QTL is flanked by SNP markers, S6_22799025 and S6_23199833. The major resistance QTL, *qBR11.1*, is located within a genomic interval of 1.15 cM on chromosome 11 with a high LOD score of 57.5. This locus accounts for 47.62 % of the phenotypic variance and is flanked by the SNP markers S11_27011388 and S11_27359640 (Table 2). The additive effect of *qBR1.1* and *qBR11.1* to the phenotypic variance was 1.72 %.

In the S population, we identified two resistance QTLs against IB54 which are located on chromosomes 6 and 11 (Table 2; Supplementary Fig. 1b). These two resistance QTLs are the same as those identified in the B population and were designated as *qBR6.1* and *qBR11.1*. In the S population, *qBR6.1*, accounting for 1.89 % of the phenotypic variance, is located within a 0.72-cM genomic interval on chromosome 6 with a LOD score of 8.72, flanked by the SNP markers S6_22095798 and S6_22967097. The other resistance QTL, *qBR11.1*, accounted for 30.91 % of the phenotypic variance with a LOD score of 24.8, is located

Table 2 Chromosome locations, flanking markers, and additive effects of three resistance (*R*) gene QTLs identified in DGWG

Mapping population	QTL	Chr	Flanking SNP markers (MSU 6.0)		Nearest SNP marker	Interval (cM)	LOD	R^2 (%)	Additive effects R^2 (%)
B population	*qBR1.1*	1	S1_16414192	S1_16507769	S1_16477038	0.12	8.58	2.35***	1.72* (*qBR1.1*qBR11.1*)
	qBR6.1	6	S6_22799025	S6_23199833	S6_22967097	1.68	7.02	1.12**	
	qBR11.1	11	S11_27011388	S11_27359640	S11_27025241	1.15	57.49	47.62***	
S population	*qBR6.1*	6	S6_22095798	S6_22967097	S6_22488722	0.72	8.72	1.89*	2.07*** (*qBR6.1*qBR11.1*)
	qBR11.1	11	S11_27229912	S11_27373217	S11_27327632	3.11	24.81	30.91***	

B mapping population: RIL mapping population derived from the cross of DGWG and the black hull type PI653419 (MS-1996-9; RR20); S mapping population: RIL mapping population derived from the cross of DGWG and the straw hull weedy rice type PI653435 (AR-2001-1135; RR9)

Chr chromosome, *LOD* means logarithm of the odds

*** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$

within a 3.11-cM genomic interval on chromosome 11 and flanked by the SNP markers S11_27229912 and S11_27373217. The additive effect of *qBR6.1* and *qBR11.1* to phenotypic variance was 2.07 %.

Physical mapping of *Pi66(t)* and prediction of candidate resistance genes

The major resistance QTL, *qBR11.1*, was identified in DGWG using two populations derived from the crosses of DGWG with two weedy rice ecotypes. *qBR11.1* was flanked by two SNP markers, S11_27011388 and S11_ 27359640, using B population and located in a region of 348,252 base pairs (bp). In S population, we found that *qBR11.1* was flanked by the SNP markers S11_27229912 and S11_27373217 and located in a region of 143,305 bp. Combining this information, *qBR11.1* should be located in the overlap of these two regions and was delimited by two SNP markers, S11_27229912 and S11_ 27359640, a relatively fine region of 129,728 bp.

The physical positions of SNP markers in our study were based on the MSU 6.0 version. We then converted physical positions of each flanking markers into MSU 7.0 using assembly converter function in Gramene (http://ensembl.gramene.org/tools.html), and *qBR11.1* was located in a 129,728-bp region from 27,831,254 bp to 27,701,526 bp (Fig. 2). Within the 129,728-bp region, 21 genes were predicted. Among them, three putative NBS-LRR disease-resistant proteins and three WRKY transcription factors, WRKY61, WRKY63, and WRKY41, were identified (Table 3). All six were considered candidate genes for the major QTL *qBR11.1* and renamed as *Pi66(t)*.

Discussion

As the source of the semidwarf phenotype for over 90 % of semidwarf rice varieties worldwide, the DGWG rice variety has been investigated with respect to its blast resistance genes in the present study. DGWG was highly resistant to the blast race IB54 but susceptible to the remaining 13 races assayed (Table 1). Two minor blast resistance QTLs, *qBR1.1* and *qBR6.1*, on chromosomes 1 and 6, accounting for 2.35 and 1.12 % disease reduction to IB54, respectively, and one major blast resistance QTL, *qBR11.1*, on the chromosome 11, accounting for 47.62 %

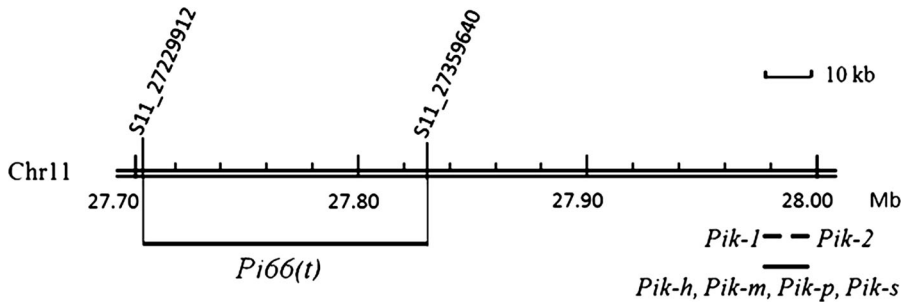

Fig. 2 Integrated physical map of a blast resistance (*R*) gene cluster on chromosome 11 including *Pi66(t)*, *Pik*, *Pik-h*, *Pik-m*, *Pik-p*, and *Pik-s.* Five blast *R* genes, *Pik*, *Pik-h*, *Pik-m*, *Pik-p*, and *Pik-s*, are allelic on chromosome 11. The positions of the *R* genes, *Pik*, *Pik-h*, *Pik-m*, *Pik-p*, and *Pik-s*, were integrated based on the nucleotide blast results of their genomic sequences in NCBI using Nipponbare as a reference (MSU 7.0)

Table 3 Candidate resistance (*R*) genes within the *qBR11.1* region on chromosome 11 based on the Nipponbare reference database in MSU 7.0

Locus	Chr	Physical position	Predicted function
LOC_Os11g45780	11	27701883–27701070	RGH2B, putative, expressed
LOC_Os11g45790	11	27707310–27703761	NB-ARC domain-containing protein, expressed
LOC_Os11g45800	11	27715402–27715668	Expressed protein
LOC_Os11g45809	11	27717452–27721100	Expressed protein
LOC_Os11g45820	11	27723836–27726283	Expressed protein
LOC_Os11g45840	11	27734572–27737316	Expressed protein
LOC_Os11g45850[a]	11	27740142–27741375	WRKY61, expressed
LOC_Os11g45864	11	27749419–27743718	Transposon protein, putative, CACTA, En/Spm subclass, expressed
LOC_Os11g45880	11	27755233–27752701	Transposon protein, putative, CACTA, En/Spm subclass, expressed
LOC_Os11g45890	11	27761647–27759845	Expressed protein
LOC_Os11g45899	11	27764931–27767518	Retrotransposon protein, putative, unclassified, expressed
LOC_Os11g45908	11	27768003–27771893	Retrotransposon protein, putative, unclassified, expressed
LOC_Os11g45920[a]	11	27785982–27779324	WRKY63, expressed
LOC_Os11g45924[a]	11	27791943–27789618	WRKY41, expressed
LOC_Os11g45930[a]	11	27793777–27797821	NBS-LRR type disease resistance protein, putative, expressed
LOC_Os11g45940	11	27799133–27800295	Expressed protein
LOC_Os11g45950	11	27804967–27804177	NAC domain-containing protein 90, putative, expressed
LOC_Os11g45960	11	27806906–27807244	Expressed protein
LOC_Os11g45970[a]	11	27818431–27812251	NBS-LRR disease resistance protein, putative, expressed
LOC_Os11g45980[a]	11	27820309–27824920	NBS-LRR type disease resistance protein, putative, expressed
LOC_Os11g45990	11	27828621–27835246	Von Willebrand factor type A domain-containing protein, putative, expressed

[a] Candidate *R* genes

Chr chromosome

disease reduction to IB54, were identified using the B mapping population. These three resistance QTLs were mapped to 0.12, 1.68, and 1.15 cM, respectively. The same resistance QTL, *qBR6.1*, on the chromosome 6, accounting for 1.89 % disease reduction, and one major resistance QTL, *qBR11.1*, on chromosome 11, accounting for 30.91 % disease reduction to IB54, were identified using the S mapping population. These two resistance QTLs were mapped to 0.72 and 3.11 cM, respectively, and the additive effects were positive and accounted for 2.07 % of disease variance. We do not know the reason why *qBR1.1* was not identified in S population. One plausible explanation is that the relative minor effect of *qBR1.1* was masked by epistatic interactions among major *R* genes in this mapping population since two parental lines DGWG and RR9 are resistant to IB54 (Supplementary Fig. 2).

In the present study, the major *R* gene *Pi66(t)* was mapped to a 129-kb region on chromosome 11 where nine blast *R* genes, *Pik*, *Pik-h*, *Pik-m*, *Pik-p*, *Pik-s*, *Pi1*, *Pilm2*, *Pi7(t)*, and *Pi43(t)*, are located (Ballini et al. 2008; Lee et al. 2009). To differentiate *Pi66(t)* from those *R* genes, an integrated physical map of these *R* genes was constructed by analysis of position of these *R* genes' sequences and the linked markers based on the Nipponbare reference genome sequence in MSU 7.0 (Fig. 2). In this map, *Pi66(t)* was delimited by the flanking SNP markers S11_27229912 and S11_27359640, which were located from 27,701,526 to 27,831,254 bp region on the chromosome 11. *Pik*, *Pik-h*, *Pik-m*, *Pik-p*, *Pik-s*, *Pi1*, and *Pi7(t)* are allelic and located at the genomic region of 27,983,597–27,989,134 bp on chromosome 11. *Pi43(t)* was flanked by simple sequence repeat markers, RM224 and RM1233, and located at a genomic region of 21,849,538–26,137,496 bp. Moreover, in previous studies, the blast resistance spectra of *Pik*, *Pik-h*, *Pik-m*, *Pik-p*, *Pi-lm2*, *Pi1/Pi7(t)*, and *Pi43(t)* are different from that of *Pi66(t)* (Lee et al. 2009; Tabien et al. 2000; Jia et al. 2009; Yuan et al. 2011). We suggest that *Pi66(t)* is a novel blast *R* gene.

Compared to conventional QTL mapping, GBS technology has facilitated the identification of candidate genes for rice blast resistance QTLs without additional fine-mapping. Since *Pi66(t)* was mapped to a 129-kb region using GBS, three putative NBS-LRR proteins and three WRKY transcription factors, WRKY61, WRKY63, and WRKY41, were identified within the 129-kb region. Because the majority of the plant *R* genes are NBS-LRR family members and WRKY transcription factors (WRKY TFs) are important for biotic and abiotic stresses (Belkhadir et al., 2004; Wu et al. 2005), we consider them as candidate genes for *Pi66(t)*. We do not exclude others genes from the candidate genes of *Pi66(t)* without further study. In summary, the identification of candidates *R* genes for *Pi66(t)* set an important stage for map-based cloning of the *R* gene. The flanking SNP markers for *Pi66(t)*, identified in the present study, can be used to tag these new blast *R* genes in DGWG and other rice germplasm. The resulting knowledge will facilitate the deployment of user-friendly DNA markers to monitor the blast *R* genes whenever *SD1* in DGWG is used for the development of semidwarf rice variety.

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