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ORIGINAL PAPER

Fine-mapping and molecular marker development for *Pi56(t)*, a NBS-LRR gene conferring broad-spectrum resistance to *Magnaporthe oryzae* in rice

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Abstract The major quantitative trait locus *qBR9.1* confers broad-spectrum resistance to rice blast, and was mapped to a ~69.1 kb region on chromosome 9 that was inherited from resistant variety Sanhuangzhan No 2 (SHZ-2). Within this region, only one predicted disease resistance gene with nucleotide binding site and leucine-rich repeat (NBS-LRR) domains was found. Specific markers corresponding to this gene cosegregated with blast resistance in F_2 and F_3 populations derived from crosses of susceptible variety Texianzhan 13 (TXZ-13) to SHZ-2 and the resistant backcross line BC-10. We tentatively designate the gene as *Pi56(t)*. Sequence analysis revealed that *Pi56(t)* encodes an NBS-LRR protein composed of 743 amino acids. *Pi56(t)* was highly induced by blast infection in resistant lines SHZ-2 and BC-10. The corresponding allele of *Pi56(t)* in the susceptible line TXZ-13 encodes a protein with an NBS domain but without LRR domain, and it was not induced by *Magnaporthe oryzae* infection. Three new cosegregating gene-specific markers, CRG4-1, CRG4-2 and CRG4-3, were developed. In addition, we evaluated polymorphism of the gene-based markers among popular varieties from national breeding programs in Asia and Africa. The presence of the CRG4-2 SHZ-2 allele cosegregated with a blast-resistant phenotype in two BC_2F_1 families of SHZ-2 crossed to recurrent parents IR64-Sub1 and Swarna-Sub1. CRG4-1 and CRG4-3 showed clear polymorphism among 19 varieties, suggesting that they can be used in marker-assisted breeding to combine *Pi56(t)* with other target genes in breeding lines.

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Introduction

Rice blast disease, caused by the fungus *Magnaporthe oryzae,* is one of the most devastating diseases of rice worldwide (Kush and Jena 2009; Liu et al. 2010). Rice yield losses of about 20–50 % occur in the absence of adequate resistance (Savary et al. 2000). Because of the effectiveness of plant resistance (R) genes in preventing diseases, the incorporation of blast resistance genes into cultivars has been the most favored strategy in breeding. A majority of the major resistance genes with strong effects follow a model of gene-for-gene interaction (Jia et al. 2000). Because of this high specificity, when blast resistance of a cultivar is based on a single resistance gene, it can be rapidly overcome by the emergence of compatible races of the pathogen (Hittalmani et al. 2000).

During the past decade, the genetics of blast resistance has been extensively studied. So far, more than 86 major blast R genes have been documented (Huang et al. 2010; Xiao et al. 2010; Liu et al. 2005, 2010, Lin et al. 2007; Ballini et al. 2008; Chen et al. 2006; Xu et al. 2008; Deng et al. 2006). Most of the blast R genes are dominant, except the recessive gene *pi21*, and some are quantitative in nature (Fukuoka and Okuno 2001; Hayashi et al. 2004; Zhou et al. 2004; Xu et al. 2008). Furthermore, many R genes are located as gene clusters with concentrations on chromosomes 6, 9, 11, and 12 (Ballini et al. 2008; Qu et al. 2006; Lin et al. 2007). To date, 18 major R genes, *Pi37*, *Pit*, *Pish*, *Pib*, *Pi9*, *Pi2*, *Piz-t*, *Pi-d2*, *Pid3*, *Pi25*, *Pi36*, *Pi5*, *Pikm*, *Pi54*, *Pia*, *Pik-p*, *Pik*, and *Pita* (Liu et al. 2010; Sharma et al. 2010; Okuyama et al. 2011; Chen et al. 2011; Zhai et al. 2011; Yuan et al. 2011) and two quantitative trait locus (QTL), *pi21* and *Pb1* (Fukuoka et al. 2009; Hayashi et al. 2010) have been cloned.

Except *Pi-d2*, all of the cloned dominant resistance genes, including *Pib, Pita, Piz-5, Piz-t*, and *Pi9*, encode NBS-LRR proteins, the major class of R gene products that interact with the effector genes of the pathogen following a gene-for-gene model of recognition (Bryan et al. 2000; Qu et al. 2006; Jeung et al. 2007). An emerging picture is that resistance against certain pathogen isolates may require pairs of NBS-LRR genes. For example, *Pikm*-mediated resistance is complete only when the two candidate NBS-LRR genes (*Pikm1-TS and Pikm2-TS*) are present in the same plant (Ashikawa et al. 2008). Similarly, two adjacent NBS-LRR genes *Pi5-1* and *Pi5-2* are required for *Pi5*-mediated resistance (Lee et al. 2009).

Because of the specific recognition, many blast resistance genes are not useful in breeding due to the adaptability of the pathogen. In some cases, the donors of these R genes have not been extensively evaluated in agronomically relevant conditions. In other cases, even when the donors have been extensively tested, R genes such as *Pi9, Pi5*, and *Pi3(t)* fail to confer broad-spectrum resistance to the pathogen populations when deployed individually (Variar et al. 2009). For practical breeding, increasing emphasis has been placed on identifying sources of broad-spectrum resistance to blast based on multiple criteria (Jeung et al. 2007). First, the donor germplasm should have a record of blast resistance over a broad area. Second, multiple pathogen isolates should have been used to evaluate the germplasm to establish the specificity of resistance. Third, there is evidence of complementary effects of qualitative or quantitative resistance under polycyclic or sequential infection (Roh et al. 2009).

Sanhuangzhan No 2 (SHZ-2) is an *indica* variety from south China that is renowned for its blast resistance and widely used in breeding programs (Liu et al. 2004). Both historical performance data from the field and the results from artificial inoculation suggest that SHZ-2 possesses broad-spectrum resistance (Chen et al. 1995; Liu et al. 2004, 2011). Through genetic dissection, we have identified a blast resistance QTL, *qBR9.1*, that is a key factor responsible for the broad-spectrum resistance observed in SHZ-2 (Liu et al. 2011). The resistance conferred by *qBR9.1* was strong but not absolute; hence, it was considered a QTL that confers quantitative resistance. In this study, we mapped *qBR9.1* into an interval of ~69.1 kb on chromosome 9. Through genetic recombination and sequence analysis, we identified a member of the NBS-LRR gene family as the likely candidate for *qBR9.1*, and tentatively named it as *Pi56(t).* To aid introgression into elite lines, we developed polymorphic gene-based markers for *Pi56(t).* Because extensive evaluation of blast resistance has already been done on SHZ-2 and its derivatives (Liu et al. 2004, 2011), *Pi56(t)* is expected to be a valuable addition to the pool of blast resistance genes used in rice breeding programs.

Materials and methods

Plant materials and mapping populations

Five rice cultivars, Sanhuangzhan No 2 (SHZ-2), Texianzhan 13 (TXZ-13), Lijiangheituanxingu (LTH), IR64 and CO39, as well as two backcross breeding lines BC-10 and BC-116 selected from BC_3F_2 with indica cultivar TXZ-13 background were used for fine mapping in this study (Table 1). SHZ-2 and BC-10 a backcross line (Table 1) exhibit broad-spectrum resistance to rice blast in China and the Philippines (Liu et al. 2011). For fine mapping the major QTL, SHZ-2 and BC-10 were used as donor parents and crossed to TXZ-13, the susceptible parent. Each of these crosses was performed to develop segregating F_2 populations for DNA marker and phenotype segregation analysis. The F_2 population of SHZ-2 × TXZ-13 consists of 343 individuals and the F_2 population of BC-10 × TXZ-13 consists of 600 individuals. F_3 families were developed from each F_2 population to verify the mapped gene using a single-seed descent method (Snape and Riggs 1975).

Inoculation and disease evaluation

To evaluate the phenotypes of F_2 and F_3 progeny, blast isolate PO6-6, which has a broad range of virulence to rice genotypes in the Philippines, was used in greenhouse inoculations. Seedlings at 21 days after sowing (or the 3–4 leaf stage) were spray-inoculated with a suspension of PO6-6 ($5–7 \times 10^5$ spores/ml) as described (Liu et al. 2011). The inoculated plants were placed in darkness in

Table 1 Plant materials used in this study

Designation	Gene	Remarks
SHZ-2	*Pi56(t)*	The donor parent of BC-10 and BC-116, *Indica-type* variety
TXZ-13	–	The recurrent parent of BC-10 and BC-116*, Indica-type* variety
BC-10	*Pi56(t)*	BC_3F_2 breeding line with TXZ-13 background and the introgression of complete R gene from SHZ-2
BC-116	–	BC_3F_2 breeding line with TXZ-13 background, sister line of BC-10 without *Pi56(t)* gene
LTH	–	*Japonica-type* variety with no blast resistance gene
IR64	–	*Indica-type* variety as check
CO39	–	*Indica-type* variety as check
Nipponbare	–	*Japonica-type* variety as check
IRBL5-M	*Pi5*	Monogenic line only with *Pi5* gene in LTH background
IRBL3-CP4	*Pi3(t)*	Monogenic line only with *Pi3(t)* gene in LTH background
Moroberekan	*Pi5*	*Pi5-donor parent*, a tall West African upland genotype
BC_4F_5-10-19	*Pi56(t)*	Pre-NIL only with *Pi56(t)* gene in TXZ-13 background (BC_4F_5)
BC_4F_5-10-4	*Pi56(t)*	Pre-NILs only with *Pi56(t)* gene in TXZ-13 background (BC_4F_5)
BC_4F_5-22-4	*Pi56(t)*	Pre-NIL with *Pi56(t)* + *qBR2.1* + *qBR6.1* in TXZ-13 background (BC_4F_5)

dew chambers at 100 % relative humidity for 24 h at 25 °C, and then transferred to a semi-temperature-controlled greenhouse, maintained at approximately 25 °C. Diseased leaf area (DLA) of inoculated plants was scored 7 days after inoculation as described by Liu et al. (2011). TXZ-13, BC-116, LTH, IR64, and CO39, described in Table 1, were used as susceptible checks for phenotype assays using *M. oryzae* isolate PO6-6 and 20 additional standard differential blast isolates from the Philippines (Table 2; Telebanco-Yanoria et al. 2008).

To determine the resistance spectrum of *Pi56(t)*, the 20 standard blast differential isolates (Table 2; Telebanco-Yanoria et al. 2008) were used to evaluate disease reaction of two BC_4F_5 lines, BC_4F_5-10-19 and BC_4F_5-10-4 [designated as pre-near isogenic lines (pre-NILs) in Table 1], which carry the *Pi56(t)* but no other known R genes from SHZ-2. The two pre-NILs were evaluated together with the parents SHZ-2 and TXZ-13. Two R lines, BC-10 and one BC_4F_5 lines BC_4F_5-22-4 (Table 1) which have *Pi56(t)* plus two other resistant QTLs from SHZ-2 were used as resistance controls. CO39 and LTH were used as the susceptible controls. *Pi56(t)* and the major genes *Pi3(t)* and *Pi5* belong to a gene cluster on chromosome 9. To differentiate the three genes, the disease reaction patterns of the *Pi56(t)* pre-NILs, *Pi5* monogenic line IRBL5-M, and *Pi3(t)* monogenic line IRBL3-CP4 were compared. Moroberekan, a cultivar

Table 2 The disease reaction pattern of the monogenic lines of *Pi5, Pi3(t)* and pre-isogenic lines of *Pi56(t)* to 20 standard differential blast isolates in the greenhouse at IRRI, Philippines

Lines	Target gene or QTLs	20 isolates																			
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
IRBL3-CP4	*Pi3(t)*	MR	S	R	S	S	MR	MR	R	R	R	S	R	S	R	S	S	S	S	S	S
IRBL5-M	*Pi5*	R	S	MR	S	S	R	R	R	R	R	S	R	S	R	S	S	S	S	S	S
BC_4F_5-10-19	*Pi56(t)*	R	S	R	R	R	R	MR	R	R	R	R	R	MR	R	MR	R	R	R	R	R
BC_4F_5-10-4	*Pi56(t)*	R	S	R	R	R	R	MR	R	R	R	R	R	MR	R	MR	R	R	R	R	R
BC_4F_5-22-4	*qBR2.1* + *qBR6.1* + *Pi56(t)*	R	S	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
SHZ-2	*qBR2.1* + *qBR6.1* + *Pi56(t)*	R	S	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
BC-10	*qBR2.1* + *qBR6.1* + *Pi56(t)*	R	S	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Moroberekan	*The donor of Pi5*	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
TXZ-13	Recurrent parent of *Pi56(t)*	S	S	R	R	R	R	S	R	R	R	R	S	R	R	R	R	R	R	R	R
CO39	Susceptible control	S	S	S	S	S	S	S	R	R	S	S	S	S	R	S	S	S	S	R	R
LTH	Susceptible control	S	S	S	S	S	S	S	R	R	S	S	S	S	R	S	S	S	S	R	R

The 20 isolates were used in this experiment listed as following: 1, PO6-6; 2, CA89; 3, 43; 4, CA41; 5, M64-1-3-9-1; 6, M39-1-3-8-1; 7, M39-1-2-21-2; 8, M36-1-3-10-1; 9, JMB8401; 10, IK81-25; 11, IK81-3; 12, BN111; 13, V850256; 14, V850196; 15, V86010; 16, JMB840610; 17, BN209; 18, M101-1-2-9-1; 19, B90002; 20, C923-49

R resistance to an isolate, *S* susceptible to an isolate, *MR* moderate resistance to an isolate

that exhibits durable resistance (Table 1), was also included in the disease evaluation.

Marker development

Total genomic DNA of the F_2 and F_3 individuals as well as other lines and varieties was extracted from young leaves following the protocol by Murray and Thompson (1980) after scoring the disease reaction. For fine mapping the *qBR9.1* locus, additional markers were developed flanking the target region defined by previous work (Liu et al. 2011). Three types of markers, SSR markers, candidate resistance gene (CRG) specific markers, and SNP markers were used in this study. The primer pairs of SSR markers were directly adopted from the Gramene marker database (http://www.gramene.org. 2006). For the CRG and SNP markers, the reference sequences of the candidate genes were downloaded from the TIGR web site (http://www.tigr.org/tigr. 2007), and primers were designed using Oligo Primer Analysis software (version 5.0). Each CRG and SNP primer was confirmed to be unique by BLAST analysis in NCBI (http://www.ncbi.nlm.nih.gov). The primers' sequences, their expected PCR product sizes, and annealing temperatures are listed in Table 3. All the primers were synthesized by SBS Company (Beijing, China).

The PCR conditions included pre-denaturation for 4 min at 94 °C, followed by 35 cycles of the polymerization reaction, each consisting of denaturation for 40 s at 94 °C, annealing for 30 s at annealing temperature, and an extension step for 1–2 min at 72 °C. A final extension step was run for 7 or 10 min at 72 °C. The SSR PCR products were separated on 3.0 % agarose gels, and gene-specific PCR products were separated on 1.2 % agarose gels. Gels were stained with 10 % SYBR Safe DNA gel Stain (Invitrogen, San Diego, CA, USA) to detect the amplicons. The PCR conditions and products of SNP markers followed the TILLING method of Raghavan et al. (2007).

Physical map construction

The physical locations of SSR markers were determined in the Gramene marker database (http://www.gramene.org, Gramene Annotated Nipponbare Sequence. 2006) and the physical locations of the CRG markers were determined on the basis of the Nipponbare genome database from the Gramene *Oryza sativa japonica* ContigView. Finally, a physical map flanking the target locus was constructed based on the Nipponbare genome sequence.

Candidate gene prediction and sequencing

A candidate gene for *qBR9.1* was identified from the output of the gene prediction by Gramene and TIGR 5.0, based on the Nipponbare sequence for the 69.1 kb genomic region defined by the flanking markers RM24022 and CRG5. The candidate gene was amplified from SHZ-2, BC-10, and the susceptible parent TXZ-13 using primers CRG4-1, CRG4-2, and CRG4-3. The PCR products were purified from agarose gels (Gel Extraction Kit, Qiagen, Mainz, Germany), and the purified PCR products were inserted into pCR®-XL-TOPO vector prior to transformation into competent *Escherichia coli* cells (Invitrogen, San Diego, USA). Cloned fragments were sequenced (Macrogen, Seoul, Korea) and the sequences were aligned using BLAST analysis in NCBI. Sequence comparison was performed between the resistant and susceptible genotype by ClustalW (http://www.ebi.ac.uk/Tools/clustalw).

RNA isolation, mRNA purification, and reverse transcriptase-PCR

For semi-quantitative RT-PCR assay of *Pi56(t)* expression, 21-day-old seedlings of the parents (SHZ-2 and TXZ-13) and BC-10 were spray-inoculated with a suspension of PO6-6 (5–7 $\times$ 10^5 spores/ml) as described above. Mock

Table 3 The sequence information of PCR-based markers used for fine mapping the *qBR9.1* locus

Marker	Forward primer (5′–3′)	Reverse primer (5′–3′)	PCR predicted length (bp)	Annealing temperature (°C)
RM7364	TTTCGTGGATGGAGGGAGTACG	TGGCGACTTATGAGCGTTTGTAGG	184	55.0
CRG 1	TCCTCCCAATCATCGCCTAC	CAAGTAACAGATGCAGCCAG	294	58.0
CRG 2	TGGGATTGAACTCATGGGGA	ACATCCCAGCTCTCAAACAG	951	63.0
CRG 3	TGCTGGTCGTTTGAAGCTGC	TCAGCACGGTCCATGTTTCC	1,594	66.0
CRG 4-1	TCTACGAGCTGGAGGATCTG	CTGCAGCAAGCCAAGTTTCC	1,521	66.0
CRG 4-2	CCTGTCAGTCTTTCCGAGAG	GAATCCGGTAGCTCAAGGTG	1,358	63.5
CRG 4-3	TGCAAGAACCCTCCTCCTAC	CCTGCCATCTCAAGACTCTC	725	63.0
CRG 5	AGGGTGCTGTACGAGGTATG	CGTTGCACCTCTTCTTCTCC	709	66.0
RM24022	TCCTCTCAACCATGTACGGAATAGC	CGATGTGTTTATGTCCGCAATCC	299	55.0
RM24031	AAGGTGGTAGCTGCTTCATTCG	GGAGATCATATGGAGGGAAAGG	447	55.0
F-cDNA	GATCGCAACCTCCCCTTCTCTACCGCTC	GATTCACCTCTTGCTATTGCCCATTC	3,285	58.0

inoculation was done by spraying sterile distilled water. Inoculated plants were maintained in the dark for 24 h at 25 °C and 100 % humidity, and then were transferred to 25 °C with 100 % humidity. Each treatment has three biological replicates. A pool of five seedlings was harvested at 0, 12, 24, 48, and 72 h after inoculation, frozen in liquid nitrogen, and stored at −80 °C.

Total RNA was extracted from seedlings using the TRIzol method (Invitrogen, San Diego, USA). RNA samples were treated with DNase I (Invitrogen, Amplification Grade) to remove any contaminating genomic DNA. First-strand cDNA was synthesized using a SuperScript II Reverse Transcriptase kit (Invitrogen, San Diego, USA). The cDNA was used as a template, and amplification was done by pre-denaturation for 4 min at 94.0 °C, followed by 25–35 cycles (30 s at 94 °C, 30 s at 58 °C, and 1 min at 72 °C), and extension for 7 min at 72 °C. A primer pair, CRG4-3 (Table 3), was designed for *Pi56(t)* expression analysis using Oligo Primer Analysis Software Version 5.0 based on the Nipponbare gene sequence from TIGR. Primers were synthesized by SBS Company (Beijing, China).

High fidelity PCR was used for sequencing the full-length coding region of the candidate gene *Pi56(t)*; the amplification was performed in 50 µl volume containing 1 × 5 × KAPAHiFi GC Buffer; 0.3 mM dNTP; 0.3 µM forward primer and reverse primers; and KAPAHiFi Hot-Start DNA Polymerase (1 U/µl). The amplified PCR fragments were purified (Gel Extraction Kit, Qiagen, Germany), inserted into the pCR®-XL-TOPO vector, and transformed into competent *E. coli* cells (Invitrogen, San Digeo, USA). The cloned fragment was sequenced using primer walking (Macrogen, Seoul, Korea).

Polymorphism assay of newly developed markers for *Pi56(t)*

To test the polymorphism of the newly developed markers for *Pi56(t)*, two sister backcross lines, BC-10 and BC-116, four common rice varieties, LTH, CO39, IR64, and Nipponbare, and thirteen popular varieties from national breeding programs were screened by CRG4-1, CRG4-2, and CRG4-3 by conventional PCR. The disease reactions of these varieties to PO6-6 were determined by inoculation as described above.

Marker validation in breeding populations

To validate the usefulness of the *Pi56(t)*-derived markers, we applied the markers towards the development of near-isogenic lines with individual resistance genes from SHZ-2. Two BC_2F_1 populations were developed to introgress *Pi56(t)* from SHZ-2 into IR64-Sub1 and Swarna-Sub1 using CRG4-2. Cosegregation of the blast disease reaction and CRG4-2 alleles was assayed in the BC_2F_1 populations. Thirty seedlings from each of the BC_2F_1 populations and 10–20 seedlings from each of the parents were evaluated for their reaction to PO6-6. BC_2F_1 progenies are either homozygous for the CRG4-2 recurrent parent allele (aa) or are heterozygotes (ab). The two classes were identified directly in the IR64-Sub1 × SHZ-2 cross from the PCR products. For the Swarna-Sub1 × SHZ-2 progeny, the heterozygotes were identified after cutting the PCR products with CEL1 enzyme to detect polymorphic strands.

Results

Mapping population construction

Segregation ratios of resistance phenotype and genotype in the F_2 populations were determined by testing for reaction to PO6-6 in two F_2 populations derived from crosses between TXZ-13 and two resistant varieties, SHZ-2 and BC-10. The F_2 populations segregated into 3R:1S (Table 4). The three genotypes in the F_2 families (RR, Rr and rr) fit a 1:2:1 segregation ratio (data not shown) using gene-specific markers CRG4-1 and CRG4-2. In the heterozygous F_3 families, the ratio of resistance and susceptible plants also followed a 3R:1S segregation pattern. These results indicated that a single dominant resistance locus is responsible for resistance to PO6-6 in SHZ-2 and BC-10.

Physical map construction

The *qBR9.1* locus was previously defined by the polymorphic SSR marker RM24022 (Liu et al. 2011). We constructed a physical map of this locus based on the reference sequence of cultivar Nipponbare (Fig. 1a).

Table 4 Chi square test for the segregation of resistance and susceptibility in F_2 populations inoculated with the rice blast isolate PO6-6

Cross	Total number of F_2 plants observed	Resistant plants	Susceptible plants	X^2	P^a value
SHZ-2/TXZ-13	343	261	82	0.2186	$0.90 > P > 0.80$
BC-10/TXZ-13	589	448	141	0.3530	$0.90 > P > 0.80$

[a] P value ≥ 0.05; indicates the goodness of fit to a 3R:1S ratio

To delimit the entire introgression region of *qBR9.1*, we identified two SSR markers, RM7364 and RM24031, that flanked the target region (Table 3). No polymorphism was found between SHZ-2 and TXZ-13, or between BC-10 and TXZ-13 using RM7364 and RM24031 (Fig. 1a). Based on annotation by Gramene, more than 49 predicted genes were located within the region flanked by RM7364 and RM24031 (Supplementary Table S1). Among the 49, two genes were predicted to encode NBS-LRR-containing proteins, one is in the LRR family and one is an accelerated cell death 6 (ACD 6) gene. To refine the *qBR9.1* locus, seven CRG markers were designed based on the predicted gene sequences (highlighted in Supplementary Table S1) of Nipponbare from TIGR 5.0. By PCR amplification, only five markers CRG3, CRG4 (including CRG4-1, CRG4-2, and CRG4-3), and CRG5 showed polymorphism between two pairs of parents, BC-10 versus TXZ-13, and SHZ-2 versus TXZ-13. CRG1 and CRG2 did not show polymorphism between parents. CRG2 showed SNPs between the parents as assayed by TILLING (Raghavan et al. 2007) but CRG1 did not. Two of the four polymorphic gene-specific markers, CRG4 (CRG4-1 and CRG4-2) and CRG5, and one SSR marker RM24022 were used for a recombination test in the two F_2 populations derived from SHZ-2 × TXZ-13 and BC-10 × TXZ-13. A total of six recombinants were recovered from the region defined by RM24022 and CRG5. These six recombinants were products of single-crossovers between three polymorphic markers. The recombinants together indicated that the essential region for resistance was at CRG4. The graphic genotypes and disease reaction of recombinants are shown in Fig. 1b. No recombinant was detected using CRG4-1 and CRG4-2 in the two F_2 populations. The data suggested that CRG4 cosegregates with the *qBR9.1* locus (Fig. 2). Together, the mapping data

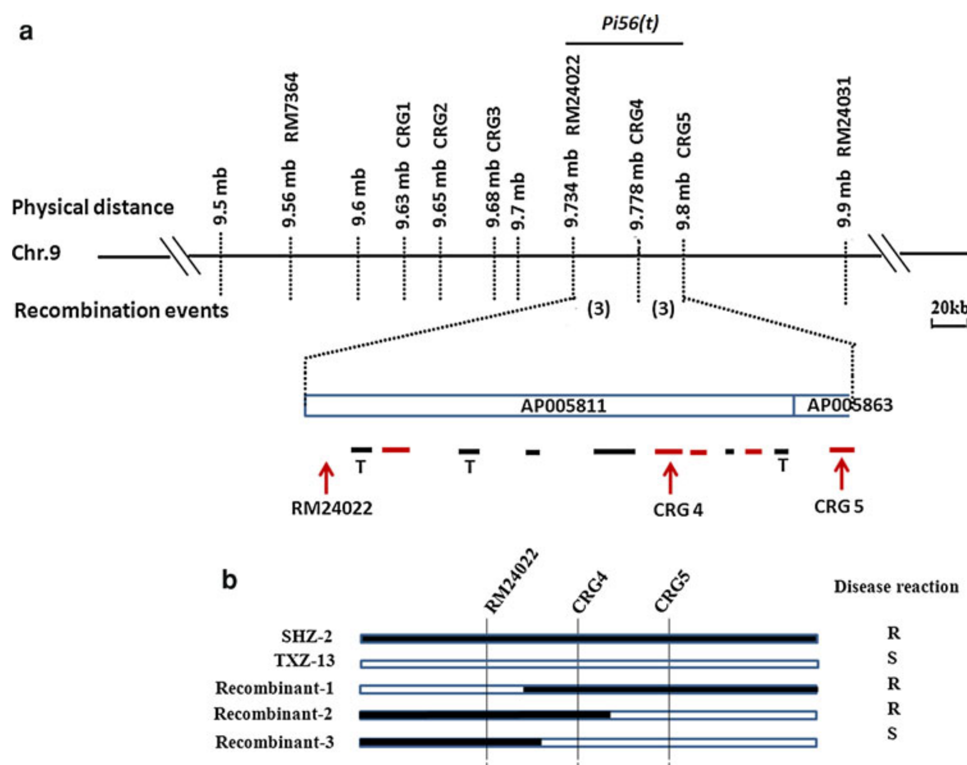

Fig. 1 **a** Physical maps of the *Pi56(t)* locus on chromosome 9 based on the Nipponbare sequence database from Gramene. Three SSR markers, RM7364, RM24022, and RM24031, and five gene-specific markers, CRG1, CRG2, CRG3, CRG4, and CRG5, identified in this study are localized on the physical map of BC-10 and SHZ-2 (*top*). The *numbers* below the markers are the physical distance from the top on the chromosome 9 based on the Nipponbare genome sequence from Gramene. The *numbers* below the map are the recombination events in the F_2 population (from BC-10 × TXZ-13). The details of the *Pi56(t)* region in the Nipponbare physical map are at the bottom with 11 predicted genes shown by *red* and *black bars* (listed in the Supplementary Table S1), among which three putative retrotransposon protein genes are indicated by T. There were five candidate resistance genes, CRG1, CRG2, CRG3, CRG4, and CRG5, located in the introgression region in BC-10 from SHZ-2. The *red arrows* point to the physical position of these markers. **b** Graphical genotypes of recombinants spanning the *Pi56(t)* region using SSR markers RM24022 and gene-specific markers CRG4 and CRG5. Six recombinants were detected in the region defined by the three polymorphic markers. Two of six recombinants (recombinant-1 genotype) were from crossover between RM24022 and CRG4. Three recombinants (recombinant-2) were from crossover between CRG4 and CRG5. One recombinant (recombinant-3) was also from a crossover between RM24022 and CRG but with a susceptible phenotype. These recombinants together indicated that the essential region for resistance centered at CRG4. The *black bar* is the introgression region from SHZ-2. The *white bar* is the TXZ-13 genome. *R* resistant, *S* susceptible (color figure online)

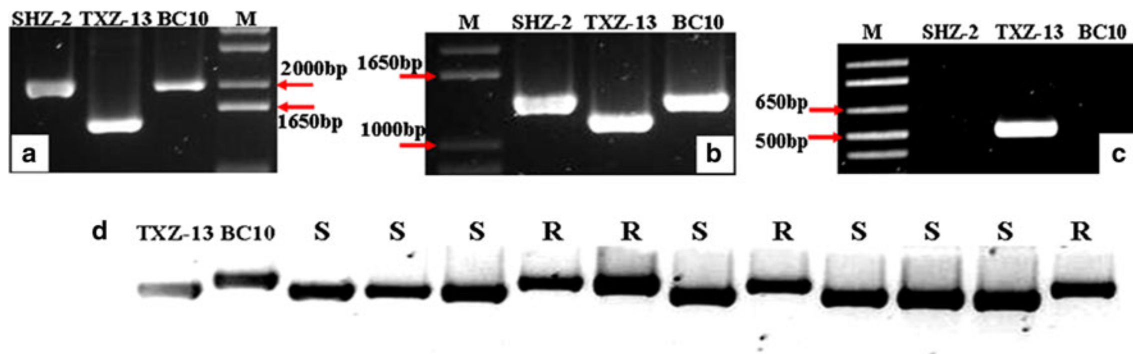

Fig. 2 PCR analysis using candidate gene-specific markers, CRG4-1, CRG4-2, and CRG4-3 which cosegregate with *Pi56(t)* resistance phenotype. **a** The differences of PCR products using CRG4-1 among SHZ-2, TXZ-13, and BC-10. **b** The differences of PCR products using CRG4-2 among SHZ-2, TXZ-13, and BC-10. **c** The differences of PCR products using CRG4-3 among SHZ-2, TXZ-13, and BC-10. **d** The cosegregation of CRG4-2 with *Pi56(t)* resistance in F_2 plants. '*M*' is the 1 kb plus DNA ladder. PCR products were detected after separation in a 1.2 % agarose gel and staining with 10 % SYBR Safe DNA gel stain (color figure online)

indicated that the *qBR9.1* locus is within an interval of ~69.1 kb on chromosome 9 flanked by SSR marker RM24022 and gene specific marker CRG5 (Fig. 1a).

Identification of the candidate gene for *qBR9.1*

Through segregation analysis in the two F_2 populations, *qBR9.1* was located between RM24022 and CRG5 and cosegregated with CRG4, including CRG4-1, CRG4-2 and CRG4-3 (Fig. 1a). In Nipponbare, the 69.1 kb region is covered by parts of two PAC clones, AP005811, and AP005863 and contains 11 predicted genes. Among these predicted genes, there is only one putative disease resistance gene with NBS-LRR domain (listed in Supplementary Table S1, highlighted in gray color). As a majority of blast resistance genes (*Pi*) are NBS-LRR family members, the CRG4 gene (Fig. 1a) was considered a strong candidate gene for the QTL *qBR9.1* and re-designated as *Pi56(t)*.

Sequence and structure analysis of *Pi56(t)*

The sequence of the candidate gene CRG4 in Nipponbare (locus ID: LOC_Os09g16000 from Gramene) was downloaded from the TIGR 5.0 database. To identify the size and structure of the *Pi56(t)* gene in SHZ-2, one primer pair (F-cDNA) that amplified the entire coding region (Table 3) was designed for *Pi56(t).* The full-length coding region of *Pi56(t)* was amplified by RT-PCR in SHZ-2 and TXZ-13 and sequenced. Primer pairs CRG4-1, CRG4-2, and CRG4-3 were used to amplify the genomic sequence of *Pi56(t)* from SHZ-2 and BC-10. The sequence of *Pi56(t)* was determined by comparing the full-length coding sequence and three fragment DNA sequences including CRG4-1, CRG4-2, and CRG4-3 with the Nipponbare genomic DNA sequence.

The genomic sequence length of *Pi56(t)* in SHZ-2 is 3,810 bp (Supplementary Fig. S1) whereas the corresponding lengths of the *Pi56(t)* alleles are 3,045 bp in TXZ-13 and 3,172 bp in Nipponbare, (Supplementary Fig. S2). Sequence alignment among alleles of SHZ-2, TXZ-13, and Nipponbare revealed a 335 bp insertion in the first intron region of *Pi56(t)* in SHZ-2, a 106 bp insertion in allele of TXZ-13, and a 233 bp deletion in the fourth exon of the allele of *Pi56(t)* in TXZ-13 (Supplementary Fig. S3). In addition to the deletions, there are many sequence differences between *Pi56(t)* in SHZ-2 and the allele of *Pi56(t)* in TXZ-13. The *Pi56(t)* gene in SHZ-2 contains a 2,232 bp coding region, which is interrupted by three introns (1,249, 57 and 272 bp) (Fig. 3a).

Sequence analysis of *Pi56(t)*-encoded protein

Protein translation of the coding sequence of *Pi56(t)* revealed that the gene encodes a predicted 743-amino-acid polypeptide (Fig. 4) with a molecular weight of 84 kDa and a pI of 6.12. The N-terminal section contains three conserved motifs typical of NBS proteins (Fig. 4). The GLLGLGKT sequence (Fig. 4, positions 146–158) conforms to the kinase 1a (P loop) consensus, LIVLDGLL (positions 231–240), and CTRFIVTTS (positions 261–269) corresponding, respectively, to the kinase 2 and kinase 3a consensus motifs. At the C-terminal region of the protein is the LRR domain, which includes ten imperfect LRR repeats based on an IxxLxxLxxLxL consensus.

We compared the protein sequences of the *Pi56(t)* genes in SHZ-2, TXZ-13, and Nipponbare (Fig. 5). The allele of *Pi56(t)* in TXZ-13 encodes a protein of 537 amino acids without the LRR domain (Fig. 5). There are 34 amino acids differing from *Pi56(t)* in SHZ-2. The allele of *Pi56(t)* in Nipponbare also encodes a NBS-LRR protein of 743 amino acids but with only seven imperfect LRRs with 46 amino acids differing from *Pi56(t)* in SHZ-2. Of these differences,

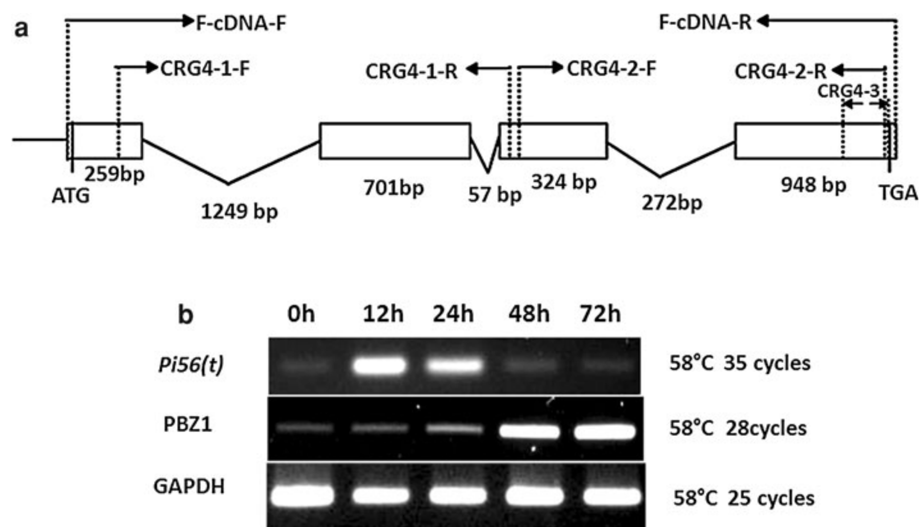

Fig. 3 The structure of *Pi56(t)* and its expression in infected BC-10 leaves after inoculation with PO6-6. **a** Structure of *Pi56(t)* and the position of the gene-specific primers used in the sequencing and RT-PCR analysis. Exons in the *Pi56(t)* gene are indicated by *horizontal lines* and *open squares*. Introns are indicated by *lines angled downward*. The positions of the initiation (ATG) and termination (TGA) codons are also indicated. **b** Analysis of expression pattern of *Pi56(t)* in BC-10 using semi-quantitative RT-PCR. Total RNA was isolated from BC-10 seedlings at 0, 12, 24, 48, and 72 h after inoculation with PO6-6. One microgram of total RNA was pretreated with RNase-free DNase I and subjected to reverse transcription. Semi-quantitative PCR with 35 cycles was performed using *Pi56(t)* specific primer CRG4-3. PBZ1 and GADPH are control genes

Fig. 4 Deduced amino acid sequence of the *Pi56(t)* protein (743 amino acids). The NBS-LRR domain is between amino acids 122 and 399. The three conserved motifs forming the NBS region are underlined. In the NBS domain, the three *underlined sequences*, GLLGLGKT (positions 148–155), LIVLDGLL (positions 233–240), and CTRFIVTTS (positions 261–269) correspond to kinase 1a (P-loop), kinase 2, and kinase 3a, respectively. The C-terminal LRR region is shown separately from the rest of the sequence, which contains ten imperfect LRR repeats. The consensus is IxxLxxLxxLxL

1

MAGKATAFDVLRGLKRRVDRCRRQYAPWLPWKLDEALSVGVNPTLSLLLDMLLL
PPPAPVPGDELGTIKGALYELEDLLDDLDEHAGGIMMRNFKLHHQHTNPKHEKYK
EMAGFDEPTIIGRDTEKEDLKDLLSQSNPDDISILPIVGLLGLGKTSLARLVFEDNEE
GWDFDLRIWIHVDDNFDLEKFAVSIISEANKLMKGKFSHILNRSDCPSYLKFKDCIE
EILSSSSCLIVLDGLLYANEHWLPDLKYVLGKTKHKCTRFIVTTSSEEVAEVMQTIP
SYKLGGLSEDDCWTLFSEKAFGSRDATIHSWQTKIGKEINQDMETWLAAGNDEL
WELVERHSLEREVFSSFKQIYYDMSLMLKSCFLYLSVFPRGSDIDKDELIRQWIAL
DLINSNRHGTLPAVLHGEMFIEALVSISFLQIVNTSLVTKKKCKNPPPTILKVHSLVY
DFLRYIAAADIFTLDYAKSPNISVRNQPXRYAVLTNYSWQATMHEDLIAKAKAAKA
AIFRNCEATMPIADIFPILRYSRL 529

NBS

530

LDLSGCLFQELPAS
IGELKHLRFLNVSCFRITELPNE
MCCLRSLEYFDLSKTCIEVLPLFVGA
FDKLKYFNLHGCGKLKNLPRN
IGDLKRLEHLNLSCCPEIRELPSS
ISGLDELKLLNLSSCTKLELLPHQ
FGNLSRLEILELERCCNLQRLPES
FGGLSKLCSLSLASCSGLQRLPDC
IGELCSLEHLNISHAHLELPDS
LTKLQSLHIVNS 743

LRR

there is one amino acid located at kinase 1a (P-loop) and 14 amino acids located at the LRR domain (Fig. 5).

Liu et al. (2011) found that the expression pattern of *Pi56(t)* was the same in SHZ-2 and BC-10. *Pi56(t)* expressed at a very low level at the 0 time point and was induced by blast infection at 12 and 24 h after infection (HAI) and then reduced to a very low level at 48 HAI in SHZ-2 (Liu et al. 2011). An expression pattern similar to that for *Pi56(t)* described by Liu et al. (2011) was seen by RT-PCR in BC-10 (Fig. 3b). In contrast, *Pi56(t)* was constitutively expressed at a high level in TXZ-13 at all time points (Liu et al. 2011).

Distinct disease reaction patterns of *Pi56(t), Pi5*, and *Pi3(t)* to rice blast

The new major gene *Pi56(t)* was located in a cluster of resistance genes including two major blast resistance genes *Pi5* and *Pi3(t)* (Lee et al. 2009). To differentiate the three major genes, the disease reaction of two monogenic lines of *Pi5* and *Pi3(t)* and two pre-NILs of *Pi56(t)*, the donor parents of these genes, Moroberekan, SHZ-2 and BC-10, and susceptible control varieties (TXZ-13, CO39 and LTH) were tested using 20 differential standard isolates (Telebanco-Yanoria et al. 2008) under greenhouse conditions. Of

```
TXZ-13     MAGKATAFDVLRGLKRRVDRCRRQYAPWLPWKLDEALSVGVNPTLSLLLDMLLLPPPAPV 60
NBS-LRR    MAGKATAFDVLRGLKRRVDRCRRQYAPWLPWKLDEALSVGVNPTLSLLLDMLLLPPPAPV 60
SHZ-2      MAGKATAFDVLRGLKRRVDRCRRQYAPWLPWKLDEALSVGVNPTLSLLLDMLLLPPPAPV 60
                                          CC

TXZ-13     PGDELGTIKGALYELEDLLDDLDEHAGRNNDAEFKLHHQHANPQHEKYKEMVGVNGVTII 120
NBS-LRR    PGDELGTIKGALYELEDLLDDLDEHAGRNNDAEFKLHYQHANPQHEKYKEMVEVNGATII 120
SHZ-2      PGDELGTIKGALYELEDLLDDLDEHAGGIMMRNFKLHHQHTNPKHEKYKEMAGFDEPTII 120

TXZ-13     GRDTEKQYLKDLLSQSNPDDLSILPIVGLPGLGKTSLARLVFEDKEEGWDFDLRIWIHVD 180
NBS-LRR    GRDTEKQYLKDLLSQSNPDDLSILPIVGLPGLGKTSLARLVFEDKEEGWDFDLRIWIHVD 180
SHZ-2      GRDTEKEDLKDLLSQSNPDDISILPIVGLLGLGKTSLARLVFEDNEEGWDFDLRIWIHVD 180
                                  Kinase 1a(P-loop)

TXZ-13     DNFDLEKFAVCIISEANKLMKGKFSHILNRSDCPSYLKFKDCIEEILSSSSCLIVLDGLL 240
NBS-LRR    DNFDLEKFAVCIISEANKLMKGKFSHILNRSDCPSYLKFKDCIEEILSSSSCLIVLDGLL 240
SHZ-2      DNFDLEKFAVSIISEANKLMKGKFSHILNRSDCPSYLKFKDCIEEILSSSSCLIVLDGLL 240
                                                                Kinase 2

TXZ-13     YANEHWLPDLKYVLGETKHKCTRFIVTTSSEEVAQVMHTVPSYKLGGLSEDDCWTLFSEK 300
NBS-LRR    YANEHWLPDLKYVLGETKHKCTRFIVTTSSEEVAQVMHTVPSYKLGGLSEDDCWTLFSEK 300
SHZ-2      YANEHWLPDLKYVLGKTKHKCTRFIVTTSSEEVAEVMQTIPSYKLGGLSEDDCWTLFSEK 300
                              Kinase 3a

TXZ-13     AFGSRDATIHSWQTKIGKAINQDMETWLAAGNDELWELVERHSLEREVFSSFKQIYYDMS 360
NBS-LRR    AFGSRDATIHSWQTKIGKAINQDMETWLAAGNDELWELVERHSLEREVFSSFKQIYYDMS 360
SHZ-2      AFGSRDATIHSWQTKIGKEINQDMETWLAAGNDELWELVERHSLEREVFSSFKQIYYDMS 360

TXZ-13     LMLKSCFLYLSVFPRGSDIDKDELIRQWIALDLINSSRHGTLPAFLHGEMFIEALVSRSF 420
NBS-LRR    LMLKSCFLYLSVFPRGSDIDKDELIRQWIALDLINSSRHGTLPAFLHGEMFIEALVSRSF 420
SHZ-2      LMLKSCFLYLSVFPRGSDIDKDELIRQWIALDLINSNRHGTLPAVLHGEMFIEALVSISF 420

TXZ-13     LQIVNTSSVTEKKCKNPPPTILKVHSLVYDFLRYIAADDIFTLDYAKSPNISVRNQPFRY 480
NBS-LRR    LQIVNTSLVTEKKCKNPPPTILKVHGLVYDFLRYIAADDIFTLDYAKSPNISVRNQPFRY 480
SHZ-2      LQIVNTSLVTKKKCKNPPPTILKVHSLVYDFLRYIAAADIFTLDYAKSPNISVRNQPXRY 480
                                                               LRR domain
TXZ-13     AVLTNYSWQAKMHEDFIAKAKAAKAAIFRNCEATMPTADIFPILRYSRTQELA------- 533
NBS-LRR    AVLTNYSWQAKMHEDFIAKAKAAKAAIFRNCEATMPIADIFPILRYSRLLDLSGCLFQEL 540
SHZ-2      AVLTNYSWQATMHEDLIAKAKAAKAAIFRNCEATMPIADIFPILRYSRLLDLSGCLFQEL 540
           LRR domain
TXZ-13     ------PKHW-------------------------------------------------- 537
NBS-LRR    PTSIGELKHLRYLNVSFFRITELPNEMCCLRSLEYLDLSKTCIEVLPLFVGAFDKLKYFN 600
SHZ-2      PASIGELKHLRFLNVSCFRITELPNEMCCLRSLEYFDLSKTCIEVLPLFVGAFDKLKYFN 600
                                   LRR domain
TXZ-13     ------------------------------------------------------------
NBS-LRR    LHGCGKLKNLPQNIGDLKRLEHLSLSCCPEIRELPSSISGLDELKLLNLSSCTKLELLPH 660
SHZ-2      LHGCGKLKNLPRNIGDLKRLEHLNLSCCPEIRELPSSISGLDELKLLNLSSCTKLELLPH 660
           LRR domain
TXZ-13     ------------------------------------------------------------
NBS-LRR    QFGNLSCLESLEMAGCCSLQRLPESFGGLSKLCSLSLASCSSLQRLPDYIGELCSLEYLN 720
SHZ-2      QFGNLSRLEILELERCCNLQRLPESFGGLSKLCSLSLASCSGLQRLPDCIGELCSLEHLN 720

TXZ-13     -----------------------
NBS-LRR    ISHAHLELPDSLTKLQSLHIVNS 743
SHZ-2      ISHAHLELPDSLTKLQSLHIVNS 743
```

Fig. 5 Comparison of the deduced amino acid sequence of *Pi56(t)* in SHZ-2, TXZ-13, and Nipponbare by CLUSTAL W (1.83) multiple sequence alignment. NBS-LRR refers to the allelic amino acid sequence of *Pi56(t)* in Nipponbare. Hyphens indicate sequence gaps. The numbers in the *right column* indicate the position of the rightmost amino acid. The differences of amino acid sequence are highlighted in *red* and *green* among varieties. LRR domain is highlighted in *gray*. *Horizontal line* under the sequences indicates CC domain. *Horizontal line* with *brown* highlight indicates Kinase domain

the 20 isolates, the pre-NILs of *Pi56(t)* (lines of BC_4F_5-10-19 and BC_4F_5-10-4) showed complete resistance to 16 isolates (PO6-6, 43, M39-1-3-8-1, CA41, M64-1-3-9-1, M39-1-3-8-1, M36-1-3-10-1, JMB8401, IK81-25, IK81-3, BN111, V850196, JMB840610, BN209, M101-1-2-9-1, B90002, and C923-49) and moderate resistance to three isolates (M39-1-2-21-2, V850256, and V86010); and only showed susceptibility to one isolate CA89 (Table 2). In contrast, the monogenic lines of *Pi5* (lines of IRBL5-M) showed complete resistance to eight isolates (PO6-6, M39-1-3-8-1, M39-1-2-21-2, M36-1-3-10-1, JMB8401, IK81-25, BN111, and V850196), moderate resistance to one isolate (43), and susceptibility to 11 isolates (CA89, CA41, M64-1-3-9-1, IK81-3, V850256, V86010, JMB840610, BN209, M101-1-2-9-1, B90002, and C923-49) (Table 2). The monogenic lines of *Pi3(t)* (lines of IRBL3-CP4) also showed a different spectrum of resistance from *Pi5* and *Pi56(t)* (Table 2). These results showed that *Pi56(t)* has a pattern of resistance distinct from those of *Pi3(t)* and *Pi5*. With the set of pathogen isolates available for assay, we

cannot rule out the possibility that the recurrent parent TXZ-13 may have additional resistance genes that could affect the disease reaction of *Pi56(t)*. Nonetheless, the results are consistent with the proposition that *Pi56(t)* is a new blast resistance gene located within the cluster of resistance genes on chromosome 9.

Polymorphism of newly developed markers for *Pi56(t)*

To ensure that the markers developed for *Pi56(t)* are useful for breeding programs, the polymorphism of the newly developed markers was tested with 19 selected varieties from Asia and Africa. Of these 19 varieties, 13 were considered popular varieties or as donor parents in breeding programs. Polymorphic markers differentiating *Pi56(t)* from the corresponding alleles in these varieties will be useful in marker-aided selection (MAS) programs. CRG4-1 showed good polymorphism among most of the 19 varieties except HYB30 and TDK-Sub1. On the other hand, CRG4-2 only showed polymorphism among some varieties (Table 5; Supplementary Figs. S4 and S5). CRG4-3 showed good polymorphism among most of the varieties except TDK-Sub1. The reaction of those varieties to isolate PO6-6 indicated that the varieties with the SHZ-2 specific-marker CRG4-1 showed strong resistance to PO6-6 except TDK-Sub1, which showed moderate resistance to PO6-6. However, the varieties without CRG4-1 showed susceptibility or moderate resistance to PO6-6. The CRG4-2 marker was not associated with resistance phenotype to isolate PO6-6 among the 19 varieties. The varieties with the same CRG4-3 marker as SHZ-2 also showed resistance to PO6-6, or moderate resistance to PO6-6, such as TDK-Sub1 (Table 5; Supplementary Figs. S4 and S5). These results suggested that the newly developed markers CRG4-1 and CRG4-3 are diagnostic of blast resistance conferred by *Pi56(t)* and can be used for MAS breeding programs.

Marker validation in two breeding populations

Cosegregation of blast reaction phenotypes and CRG4-2 alleles was assayed in two BC_2F_1 families of SHZ-2 (resistant to PO6-6) and recurrent parents IR64-Sub1 and Swarna-Sub1 (both susceptible to PO6-6). The backcross families were developed by selecting for the SHZ-2 CRG4-2 allele. By assigning "r" for the recurrent parent allele and "R" for the SHZ-2 allele, IR64-Sub1 × SHZ-2 BC_2F_1 progeny segregated 13(rr):17(Rr) for the CRG4-2 marker while Swarna-Sub1 × SHZ-2 BC_2F_1 progenies segregated 18(rr): 7(Rr). In both populations, the heterozygous progenies (Rr) carrying the SHZ-2 allele were resistant. All IR64-Sub1 × SHZ-2 rr progenies were susceptible. The assay validated the usefulness of the CRG4-2 marker for MAS. It also confirms the dominance of *Pi56(t)* in different genetic backgrounds.

Table 5 The polymorphism of *Pi56(t)*-specific markers relative to 19 selected varieties using PCR method

Variety	Geographical coverage/adapted environment	Reaction to PO6-6	*Pi56(t)*-specific marker		
			CRG4-1	CRG4-2	CRG4-3
SHZ-2	South China (donor of *Pi56(t)*)	R	+	+	+
BC-10	South China	R	M	M	M
TXZ-13	South China	S	P	P	P
IR64-Sub1	South and SE Asia, Africa	S	P	P	P
Swarna	India	S	P	M	P
Samba Mahsuri-Sub1	India	S	P	M	P
Ciherang-Sub1	Indonesia	S	P	P	P
HYB30	Korea	R	M	M	P
BR11-Sub1	Bangladesh	S	P	M	P
TDK-Sub1	Laos/Cambodia	MR	M	M	M
WITA-4	Africa	S	P	P	P
FARO44	Africa	S	P	P	P
NERICA-L-19	Africa	S	P	P	P
NERICA-L-49	Africa	S	P	P	P
IR64	South and SE Asia, Africa	S	P	P	P
BC-116	South China	S	P	P	P
LTH	South China	S	P	P	P
CO39	India	S	P	P	P
Nipponbare	Japan	MR	P	P	P

P polymorphic relative to SHZ-2 allele, *M* mono-morphic relative to SHZ-2, + contains the marker, *MR* moderate resistance to PO6-6

Discussion

Pi56(t) is a new broad-spectrum R gene conferring resistance to rice blast

In this study, we mapped a new major QTL, *qBR9.1*, with broad-spectrum resistance to rice blast (Liu et al. 2011) into an interval of 69.1 kb on chromosome 9 using F_2 populations. We combined recombinational genetics and sequence annotation analysis to narrow down the region of *qBR9.1* and identified the candidate gene within this region. First, *qBR9.1* was identified by the SSR marker RM24022 on chromosome 9 in BC-10, a BC_3F_2 line derived from SHZ-2. Then, flanking

SSR markers, RM7364 and RM24031, were chosen to delimit the introgression region of *qBR9.1*. Second, seven resistance gene-specific markers, CRG1, CRG2, CRG3, CRG4 (CRG4-1, CRG4-2, and CRG4-3), and CRG5, were developed to complete the chromosome walk to the target region based on gene annotation. Through the cosegregation analysis, CRG4 (including CRG4-1, CRG4-2, and CRG4-3) was found to be the anchor gene-specific marker that did not recombine with *qBR9.1*. CRG4 corresponded to a putative disease resistance gene encoding an NBS-LRR protein, a common type of R proteins in plants (Liu et al. 2005; Bai et al. 2002; Hulbert et al. 2001; Monosi et al. 2004). The gene structure and annotation suggest that CRG4 is the most likely candidate gene for *qBR9.1*. Furthermore, because CRG4 showed complete cosegregation with *qBR9.1,* we concluded that CRG4 is the blast resistance gene in SHZ-2, tentatively designated as *Pi56(t).*

The *Pi56(t)* locus is located in a region of chromosome 9 with known blast resistance genes including *Pi15(t)* and *Pi5/Pi3/Pii* locus (Lin et al. 2004; Jeon et al. 2003; Yi et al. 2004; Lee et al. 2009). To differentiate these resistance genes, an integrated physical map of these resistance genes was constructed by analysis of positions of the *Pi15(t)*-linked markers and *Pi5/Pi3/Pii*-linked markers on the physical map of *Pi56(t)* (Fig. 6). In this map, the *Pi56(t)* locus was defined by RM24022 and CRG5, which were located from 9,734,211 to 9,803,312 bp on chromosome 9; *Pi15(t)* locus is located at the genomic positions 9,589,508–9,634,143 bp on chromosome 9; the *Pi5/Pi3/Pii* locus is flanked by C1454 and S04G04, which are located at the genomic positions 9,630,841–9,799,145 bp on chromosome 9. Thus, the *Pi56(t)* locus is different from *Pi15(t)* locus. An overlap exists between the *Pi56(t)* and *Pi5/Pi3/Pii* locus positions in a ~65 kb interval flanked by RM24022 and S04G03, but their cosegregation markers are different (Fig. 6). *Pi5/Pi3* was tightly linked to C1454 and S04G04 (Jeon et al. 2003), whereas *Pi56(t)* is tightly linked to CRG4, the candidate gene marker identified in this study. Lee et al. (2009) found that *Pi5*-mediated resistance required the presence of both *Pi5-1* and *Pi5-2*. As noted, LOC_Os09g15840 (9,672,999–9,673,694 bp) was annotated as *Pi5-1* and LOC_Os09g15850 (9,680,879–9,688,737 bp) was annotated as *Pi5-3* (Lee et al. 2009). According to the Fig. 2 of Lee et al. 2009, *Pi5-2* was located less than 30 kb from *Pi5-3*. Hence by inference, *Pi5-2* is located about 57 kb from *Pi56(t)* (Fig. 6).

To functionally differentiate the three major resistance genes, we used a set of differential isolates that recognize *Pi3(t)*, *Pi5*, and *Pi56(t)*. The disease reaction revealed that each of the major resistance genes has different resistance spectrum to 20 standard isolates (Table 2).

Comparing the protein sequences of *Pi56(t)*, *Pi5-1*, and *Pi5-2*, we found that *Pi56(t)* protein sequences are different from those of *Pi5-2* and *Pi5-1* (Supplementary Fig. S6). Taking together the physical position, disease reaction pattern, and the protein sequences of *Pi56(t), Pi5-1,* and *Pi5-2,* we inferred that *Pi56(t)* is different from the adjacent *Pi5-1*, *Pi5-2*, and *Pi3(t)*.

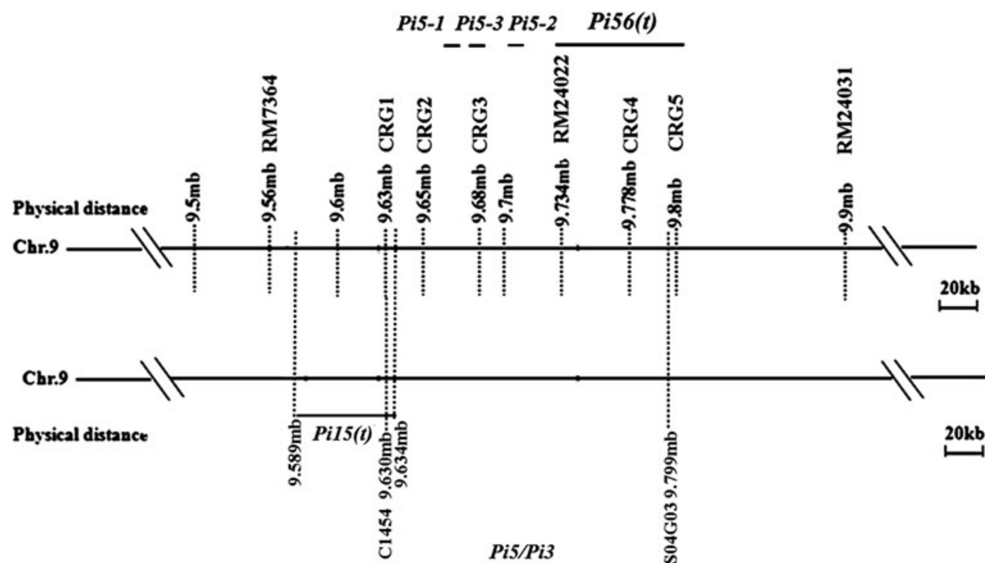

Fig. 6 Integrated physical mapping including the three rice blast resistance genes *Pi15* (Lin et al. 2007)*, Pi5/Pi3* (Jeon et al. 2003; Lee et al. 2009; Lin et al.2007), and *Pi56(t)* on chromosome 9 based on the Nipponbare sequence database from Gramene. The *upper horizontal bar* is SHZ-2 genome. The *lower horizontal bar* is Nipponbare genome. The position of the resistance genes and markers was integrated on the basis of the data from the following sources: **a** Lin et al. 2007; **b** Jeon et al. 2003; **c** Lee et al. 2009; **d** This study. The markers used in this study and their physical positions are located on the top

The structure of *Pi56(t)* and its protein sequence

Based on the deduced amino acid sequence, *Pi56(t)* belongs to the NBS-LRR class of resistance genes. In contrast to the NBS-LRR genes from dicot plants, the NBS-LRR genes from cereals often have introns in the NBS region (Bai et al. 2002; Qu et al. 2006). Similar to *Pib* (Wang et al. 1999), *Pi-ta* (Bryan et al. 2000), and *Pi9* (Qu et al. 2006), *Pi56(t)* has three introns (1,249, 57 and 272 bp) in its NBS region. Whether this unique structure in the *Pi56(t)* gene has any biological significance to its broad resistance spectrum will need further study.

The LRR region of the rice NBS-LRR genes contains approximately 15 % leucine residues and varies considerably in size and sequence (Bai et al. 2002). The LRR occupies almost the entire C-terminal region of the proteins (Meyers et al. 2003), as in the case of *Pib*, *Pita*, *Pi9*, *Pi36*, and *Pi37*, but the repeats are mostly imperfect, with only a few conforming to any consensus sequence (Bai et al. 2002; Lin et al. 2007). The LRR domain of the *Pi56(t)* protein contains ten imperfect repeats which is similar to the *Pib* protein and extends to the end of the C-terminal. The protein of the corresponding *Pi56(t)* allele in TXZ-13 does not have the LRR domain. In Nipponbare, there are only seven imperfect LRR in the allele of *Pi56(t)* with 14 amino acids difference. Nipponbare confers partial resistance to the blast isolate PO6-6 (data not shown). LRR domains are typically thought to be the major determinant of specificity in R genes, hence often a high level of polymorphism exists between alleles in these domains (Hulbert et al. 2001). The LRR sequences of different *Pi56(t)* alleles proteins are consistent with this hypothesis. Further research is needed to understand if the sequences at the C-terminal play any role in the determination of the specificity with respect to particular pathogen isolates.

The development of molecular markers for *Pi56(t)*

The traditional phenotypic screening methods to evaluate resistance to *M. oryzae* are time-consuming, labor-intensive, and require specialized facilities. With high-resolution genetic and physical mapping strategies, molecular markers associated with desired agronomic traits, including disease resistance, can be deployed in breeding (Fjellstrom et al. 2004, 2006; Yi et al. 2004). A number of PCR-based markers have also been developed for several blast resistance genes, such as *Pi2* (Hittalmani et al. 1995), *Piz, Pib, Pi-k*^{h}, and *Pi-k*^{s} (Fjellstrom et al. 2004, 2006), *Pita* (Jia et al. 2002), *Pi5* (Yi et al. 2004). These DNA markers offer an efficient and rapid means to select for the presence of multiple blast resistance genes. However, because of sequence similarity and clustering of *Pi* genes, for a marker to be effective it must be highly specific. In this study, we have developed three PCR-based DNA markers for *Pi56(t)*, including CRG4-1, CRG4-2, and CRG4-3, which cosegregated with the *Pi56(t)* resistance phenotype. CRG4-1 and CRG4-3 showed polymorphism among most of the 19 selected varieties (Table 5, Supplementary Figs. S4 and S5) using a simple PCR method. Varieties such as SHZ-2 with the marker CRG4-1 showed strong resistance to *M. oryzae* PO6-6 with one exception (TDK-Sub1) that only showed moderate resistance. Further study will determine if this is due to SNP differences in the *Pi56(t)* alleles of SHZ-2 and TDK-sub1 or if it is due to the genetic background. However, all of the varieties lacking the marker CRG4-1 were susceptible or moderately resistant to PO6-6. As a simple PCR marker, CRG4-2 only showed polymorphism among some varieties and was not associated with resistance. However, CRG4-2 can be used for discriminating resistance and susceptibility successfully using PCR and TILLING methods. Thus, by combining CRG4-1, CRG4-2, and CRG4-3, the tools are available for introduction of *Pi56(t)* from SHZ-2 into a range of popular rice varieties, such as IR64-Sub1, Swarna-Sub1, and HYB 30.

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