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ORIGINAL PAPER

A novel gene, *Pi40(t)*, linked to the DNA markers derived from NBS-LRR motifs confers broad spectrum of blast resistance in rice

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Abstract Rice blast disease caused by *Magnaporthe grisea* is a continuous threat to stable rice production worldwide. In a modernized agricultural system, the development of varieties with broad-spectrum and durable resistance to blast disease is essential for increased rice production and sustainability. In this study, a new gene is identified in the introgression line IR65482-4-136-2-2 that has inherited the resistance gene from an EE genome wild *Oryza* species, *O. australiensis* (Acc. 100882). Genetic and molecular analysis localized a major resistance gene, *Pi40(t)*, on the short arm of chromosome 6, where four blast resistance genes (*Piz, Piz-5, Piz-t*, and *Pi9*) were also identified, flanked by the markers S2539 and RM3330. Through *e*-Landing, 14 BAC/PAC clones within the 1.81-Mb equivalent virtual contig were identified on Rice Pseudomolecule3. Highly stringent primer sets designed for 6 NBS-LRR motifs located within PAC clone P0649C11 facilitated high-resolution mapping of the new resistance gene, *Pi40(t)*. Following association analysis and detailed haplotyping approaches, a DNA marker, 9871.T7E2b, was identified to be linked to the *Pi40(t)* gene at the 70 Kb chromosomal region, and differentiated the *Pi40(t)* gene from the LTH monogenic differential lines possessing genes *Piz, Piz-5, Piz-t*, and *Pi-9*. *Pi40(t)* was validated using the most virulent isolates of Korea as well as the Philippines, suggesting a broad spectrum for the resistance gene. Marker-assisted selection (MAS) and pathotyping of BC progenies having two japonica cultivar genetic backgrounds further supported the potential of the resistance gene in rice breeding. Our study based on new gene identification strategies provides insight into novel genetic resources for blast resistance as well as future studies on cloning and functional analysis of a blast resistance gene useful for rice improvement.

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Introduction

Rice (*Oryza sativa* L.) is the staple food for more than half of the world population and is considered as the lifeline of Asia, where it originally evolved (Khush 2004). However, several biotic and abiotic stresses threaten the sustainable production of rice. Rice blast disease caused by the fungus *Magnaporthe grisea* (Hebert) Barr (anamorph *Pyricularia grisea* Sacc.) is one of the most destructive diseases of rice worldwide. It has been estimated that, each year, the disease kills enough rice to feed 60 million people (Zeigler et al. 1994). An outbreak of blast can devastate rice fields, completely destroying crops in the most extreme cases (Ou 1985). Host–plant resistance based on the hypothesis of gene-for-gene interaction is the most economical and environmentally safe approach to control the disease (Jia et al.

2000). However, the pathogenicity of *M. grisea* isolates is highly variable and sometimes a small section of the virulent isolate spreads rapidly and overcomes resistance genes of rice cultivars (Fukuoka and Okuno 2001; Wang et al. 1994).

During the past decade, the genetics of blast resistance has been extensively studied and nearly 40 resistance genes have been identified. Most of the resistance 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; Gowda et al. 2006). Most of the resistance genes are from landrace, of indica subspecies. The only resistance gene, *Pi9*, has originated from a wild species, *O. minuta* (Liu et al. 2002). Five resistance genes, *Pib*, *Pita*, *Piz-5, Piz-t* and *Pi9*, have been cloned and sequenced (Bryan et al. 2000; Qu et al. 2006; Wang et al. 1999; Zhou et al. 2006). The gene *Pik-h* has been fine mapped (Sharma et al. 2005). A few resistance genes have been used for cultivar development, but these are often not durable. Moreover, most of the resistance genes are race-specific (Deng et al. 2006; Mackill and Bonman 1992). It is imperative to identify broad-spectrum blast resistance genes for effective protection against dynamic blast isolates of *M. grisea*.

The resistance genes *Pib*, *Pita*, *Piz-5, Piz-t* and *Pi9* produce NBS-LRR gene products that interact with the effecter gene of the pathogen and follow a gene-for-gene type of resistance (Bryan et al. 2000; Qu et al. 2006; Zhou et al. 2007). With the discovery of the complete sequence of the rice genome, it was found that NBS-LRR sequences are ubiquitous in the rice genome (http://rgp.dna.affrc.go.jp; http://www.genomics.org.cn). DNA markers within NBS-LRR motif sequences may provide additional links to look for new blast resistance genes.

The wild species of *Oryza* have rarely been used as sources for blast resistance except the only gene (*Pi9*) that was reported to have resistance to most of the South and Southeast Asian blast isolates (Lu et al. 2004). However, the *Pi9* gene as well as several genes on the *Piz* locus express a low level of resistance or susceptibility to the dynamic blast pathogen population in South Korea (Kim et al. 2004). It is necessary to look for novel resistance gene(s) for blast that can express a broad spectrum of resistance not only in Korea but also in South and Southeast Asia.

In this study, we have made an attempt to search for a new resistance gene from an EE genome wild *Oryza* species (*O. australiensis*) and fine-map the new gene using genome sequence information of Nipponbare through *e*-Landing on rice chromosome 6. We report here the differentiation of the new R gene from other known *Pi* genes using gene-marker association analysis of monogenic differential lines for blast resistance and by accuracy of pathogenicity assays using diverse isolates of *M. grisea*. Our results led us to the identification and fine mapping of the novel resistance gene *Pi40(t)* using new molecular approaches that will be useful for developing durable blast-resistant cultivars for rice improvement.

Materials and methods

Plant materials and DNA extraction

Five rice cultivars, 2 breeding lines, 12 blast monogenic differential lines (Tsunematsu et al. 2000) of japonica cultivar Lijiangxintuanheigu (LTH) background, and one wild *Oryza* species, *O. australiensis* (Acc. No. 100882), were used in this study (Table 1). Breeding line IR31917-45-3-2 is the progenitor of the introgression line IR65482-4-136-2-2 (Jena et al. 1991). Two blast-susceptible japonica cultivars, Jinbubyeo and Junambyeo, were used as the recurrent parents of the mapping population and recipient lines for R gene introgression. Two cultivars, Co39 and IR50, were used as susceptible checks for pathogenicity assays using Philippines isolates. Seeds of elite japonica cultivars Jinbubyeo and Junambyeo were obtained from the Genetics and Breeding Division of the National Institute of Crop Science (NICS), Rural Development Administration (RDA), Republic of Korea, and seeds of other lines were obtained from the Genetic Resources Center of the International Rice Research Institute (IRRI), Los Baños, Philippines.

Ninety-four F_2 and $F_{2:3}$ progenies from a cross between Jinbubyeo and IR65482-4-136-2-2 were used for genetic studies of blast resistance and developing markers tightly linked to the target locus for MAS applications. Resistant seedlings of 36 BC_2F_4 and 19 BC_3F_3 families from the crosses of Jinbubyeo × IR65482-4-136-2-2 and Junambyeo × IR65482-4-136-2-2, respectively, were used for MAS validity test of the new blast resistance gene, *Pi40(t)*. Total genomic DNA was extracted from young leaves according to Murray and Thompson (1980), with minor modifications.

Blast inoculation

Three-week-old seedlings were inoculated with blast isolate suspension as described by Kim et al. (2004). The fungal spore suspension concentration was adjusted as 1.5×10^5 spores/ml. Seedlings were spray inoculated with 20 ml of spore suspension using an electric motor sprayer to ensure an even and uniform distribution of spores and were kept for 24 h in a dew growth chamber at 25°C in darkness and then transferred to a greenhouse with 12/12 h

Table 1 Plant materials used in the study

ID	Designation	R gene[a]	Remarks
1	IR31917-45-3-2	*Pib*	Recurrent parent of IR65482-4-136-2-2
2	IR65482-4-136-2-2	*Pib, Pita, Pi40(t)*	*O. australiensis* introgression line
3	Jinbubyeo	*Piz*	Korean elite japonica variety, recurrent parent
4	Junambyeo	Unknown	Korean elite japonica variety, recurrent parent
5	LTH	None	Japonica cultivar with no blast resistance gene
6	IRBLb-B	*Pib*	LTH monogenic line
7	IRBLz5-CA	*Piz-5*	LTH monogenic line
8	IRBLzt-T	*Piz-t*	LTH monogenic line
9	IRBLz-Fu	*Piz*	LTH monogenic line
10	IRBL9-w	*Pi9*	LTH monogenic line
11	IRBL3-CP4	*Pi3*	LTH monogenic line
12	IRBL5-M	*Pi5*	LTH monogenic line
13	IRBL1-CL	*Pi1*	LTH monogenic line
14	IRBLks-F5	*Pik-s*	LTH monogenic line
15	IRBLkp-K60	*Pik-p*	LTH monogenic line
16	IRBLta-K1	*Pita*	LTH monogenic line
17	IRBLta2-Re	*Pita2*	LTH monogenic line
18	Co39	*Pia*	Indica variety as check
19	IR50	Unknown	Indica variety as check
20	*O. australiensis*	Unknown	EE genome wild *Oryza* species (Acc. = 100882)

[a] R genes for lines ID1 and ID 2 were evaluated by using DNA markers reported as linked to seven blast resistance genes, *Pib, Pi9, Pi3, Pi5, Pi1, Pik*, and *Pita*. See Table 4 for the primer sets used. *LTH* Lijiangxintuanheigu

(day/night) photoperiod at 90% relative humidity for 7 days. Disease reactions were scored 7 days post inoculation. For Korean blast isolates, disease reaction was scored using a numerical system similar to that of Campbell et al. (2004). Several Philippine isolates were used to differentiate lines containing genes *Pi40(t)* and *Piz-t* (see below). Overall means scored from 15 to 25 representative seedlings per line were used for statistical analysis. Percent diseased leaf area (DLA) scorings (Asaga 1981) obtained from ten randomly selected seedlings were used for the Philippine blast isolates.

Preliminary screening of PCR-based DNA markers

From STS markers provided by the Rice Genome Research Program (http://rgp.dna.affrc.go.jp/E/publicdata/caps/index.html) and high-resolution rice linkage map with SSR markers (McCouch et al. 2002), DNA markers evenly spaced on rice chromosomes were preselected to detect polymorphism between the parental lines, Jinbubyeo and IR65482-4-136-2-2. Four restriction endonuleases (*Alu*I, *Hae*III, *Hin*fI, and *Rsa*I; NEB, Beverly, MA, USA) were used on monomorphic STS-PCR products to detect latent polymorphism. A total of 38 polymorphic markers were applied to the F_2 mapping population to conduct association analysis between marker genotypes of F_2 progenies and mean resistance levels of segregating $F_{2:3}$ progeny rows.

e-Landing on rice Pseudomolecule3 and Mirror Map construction

The concept of electronic chromosome landing (*e*-Landing) was adopted to determine the relative physical positions of DNA markers by projecting their primer sequences over a reference rice genome (Mirror Map). The primer sequences used the query sequences to localize on the rice Pseudomolecule3. The Sequence-BLAST menu at Gramene (http://www.gramene.org/Multi/blastview/BLA_SXIXXeafF) was used, where BLASTN and near-exact matches were the options for search tool and search sensitivity, respectively. When both primer sequences successfully recognized their physical locations to be annealed, the expected PCR product size was calculated to judge PCR products. Based on the defined physical locations, the BAC/PAC clones, including the primer annealing sites of the tested primers, were determined by using the Rice Pseudomolcule Information and Gene Search supported by TIGR (http://www.tigr.org/tdb/e2k1/osa1/pseudomolecules/). The determined cM position for each BAC/PAC clone was directly adopted from the TIGR database.

In addition to the 38 STS and SSR anchor markers, used for construction of a linkage map skeleton, 17 DNA markers previously reported tagging blast resistance genes as well as 10 STS markers tagging six NBS-LRRs on a PAC clone, P0649C11 (GenBank Acc. = AP005659), were used for *e*-Landing (see Table 4).

PCR amplification and the physical location of the *Pi40(t)* gene

Primers were synthesized by the Bioneer Company (Deajon, Korea). The DNA sequence data sets corresponding to the six NSB-LRRs on P0649C11 were used to design highly stringent primer sets. First, all possible oligonucleotide sequences were selected by using Primer3 (http://www.frodo.wi.mit.edu/primer3/) to have optimum melting temperature (*Tm*) of 65°C and 0.5–1.5 kb as their expected PCR products. Those oligonucleotides were then subjected to *e*-Landing on the rice Pseudomolecule3. PCR was performed in a total volume of 30 µl containing 10 ng of DNA template, 10 pmole of each primer, 1.5 mM of $MgCl_2$, 0.2 mM of dNTPs, and 1 U of *Taq* polymerase (Nurotics, Deajon, Korea). PCR began with one cycle at 95°C for 3 min, followed by 35 cycles at 95°C for 30 s, at 65°C for 30 s (see Table 4 for references), and at 72°C for 1 min, with a final extension at 72°C for 10 min (MJ Research PTC-100 thermocycler; Waltham, MA, USA). The primer annealing temperature for SSR markers was 55°C.

Detailed haplotypes of nine genotypes were evaluated for the intervening physical region of 1.81 Mb between markers S2539 and RM3330 on the chromosome 6 short arm to differentiate the *Pi40(t)* gene from the other blast resistance genes, and also to confirm the putative nesting place of *Pi40(t)* within the six NBS-LRRs on PAC clone P0649C11. Marker allele types at each of the 19 tested loci were determined based on the unique band sizes as well as banding patterns derived from cleaved STS-PCR products by six frequent cutters (*Alu*I, *Hae*III, *Hin*fI, *Rsa*I, *Taq*I, and *Tsp509*), where 4 µl of PCR product was digested by 2.5 U of each restriction endonuclease in a 20 µl reaction volume for 2 h following the manufacturer's instructions. Agarose gel (1.2%, 0.5× TBE, 150 V) and natural polyacrylamide gel (5% polyacrylamide, 0.5× TBE, 300 V) electrophoresis were used for STS-PCR and cleaved PCR products, respectively, and stained by ethidium bromide. Sequencing gel electrophoresis (5% polyacrylamide, 6 M urea, 1× TBE, 80 W) was used for SSR-PCR product separation and bands were visualized using Silver Sequence™ (Promega, Madison, WI, USA).

The F_2 mapping population as well as resistant BC progenies from the cross between Jinbubyeo and IR65482-4-136-2-2 were genotyped with informative markers to narrow down the putative locus of the blast resistance gene. The most tightly linked markers, 9871.T7E and 9871.T7E2b, were used for a MAS validity test on blast-resistant BC progenies. Marker 9871.T7E2b was used to detect the introgression test of an *O. australiensis* segment at the target locus. Some 50 ng of *O. australiensis* DNA template was used for the PCR amplification.

Statistical analyses

MAPMAKER/EXP 3.0 was used to analyze the linkage between molecular markers and the target gene (Lincoln et al. 1992). The marker intervals were calculated by using the Haldane mapping function (Haldane 1919). Chi-square analysis was used for segregation tests. PROC GLM of the SAS statistical package (SAS Institute 2000) was used to estimate the relative contribution of tested loci for blast resistance. For the *F* test of loci, markers having a *P* value less than 0.05 were declared as significant empirically. The percentage of phenotypic variation explained (R^2), additive genetic effects, and degrees of dominance were then estimated for the declared loci within the mapping population.

Results

Genetic evaluation of blast resistance

We have identified IR65482-4-136-2-2 as a new source of blast resistance by evaluating it with 15 recommended blast isolates of Korea; resistance was contributed by a single locus (Jeung et al. 2003). The details of the materials used, disease reaction and association analysis are given in Tables 1, 2 and 3. In order to find out whether blast resistance was contributed by any known gene(s), the corresponding gene homologues in IR65482-4-136-2-2 and its recurrent parent, IR31917-45-3-2, were tested by using tightly linked markers for seven blast resistance genes (*Pib, Pi9, Pi3, Pi5, Pi1, Pik*, and *Pita*; see Table 4 for the primer sets used). Both IR65482-4-136-2-2 and IR31917-45-3-2 did not share marker allele types with *Pi9, Pi3, Pi5, Pi1* and *Pik* (data not shown). DNA markers tagging the *Pib* and *Pita* genes amplified the same PCR products in terms of band size as well as band strength in IR65482-4-136-2-2, whereas IR31917-45-3-2 generated the same PCR product for the *Pib* locus only (Table 1). This result indicated that the *Pita* homologue in IR65482-4-136-2-2 might be integrated from the wild rice progenitor *O. australiensis*. However, due to susceptible reactions of the LTH monogenic differential lines with *Pib* and *Pita* genes to many Korean blast isolates (Table 2), we concluded that the resistance in IR65482-4-136-2-2 is unlikely to be contributed by either *Pib* or *Pita* genes.

We have screened 87 $F_{2:3}$ lines ($N = 94$) for blast resistance by using a specific Korean blast isolate, 01–01 (KJ105a). The frequency distribution of resistance levels of 87 $F_{2:3}$ segregating progenies exhibited an abnormal distribution pattern ($w = 0.8$; $P < 0.0001$, skewness = 1.45), with 52 plant-progenies completely resistant (0 score), 20 resistant (1–2 score), 6 moderately resistant (2–3 score), and 9 progenies completely susceptible (4–5 score). The

Table 2 Reactions of the new blast resistance gene donor line, IR65482-4-136-2-2, Jinbubyeo, and LTH mogenic lines to selected Korean and Philippine blast isolates

Isolate (race)	Parental lines[a]		LTH monogenic differential lines[a]											
	ID-2	ID-3	ID-6	ID-7	ID-8	ID-9	ID-10	ID-11	ID-12	ID-13	ID-14	ID-15	ID-16	ID-17
	DOR	RP1	*Pib*	*Piz-5*	*Piz-t*	*Piz*	*Pi9*	*Pi3*	*Pi5*	*Pi1*	*Pik-s*	*Pik-p*	*Pita*	*Pita2*
01-01(KJ105a)[b]	0	4	5	1	0	4	5	1	3	5	5	5	5	5
90-002(KI215)[b]	0	5	5	1	5	5	1	5	4	0	5	1	4	5
93-270(KJ103)[b]	1	1	4	0	1	0	0	4	1	1	4	4	2	1
97-134(KJ107)[b]	1	1	1	1	4	1	0	1	0	3	3	5	5	4
87-138(KJ-201)[b]	1	3	5	4	1	1	2	1	4	5	5	5	5	4
Ca89[c]	(0)	(0)	–	–	(70)	–	–	–	–	–	–	–	–	–
Ca23-49[c]	(0)	(0)	–	–	(0)	–	–	–	–	–	–	–	–	–
PO6-6[c]	(0)	(0)	–	–	(75)	–	–	–	–	–	–	–	–	–
M101-1-29-1[c]	(0)	(5)	–	–	(75)	–	–	–	–	–	–	–	–	–
M64-1-3-9[‡]	(0)	(0)	–	–	(0)	–	–	–	–	–	–	–	–	–

[a] The designations of each ID number are indicated in Table 1. DOR = resistant gene donor (IR65482-4-136-2-2), *RP1* Jinbubyeo

[b] Korean isolates. Reactions; *0* highly resistant, *1* resistant, *2* and *3* moderately resistant, *4* moderately susceptible, *5* highly susceptible

[c] Philippine isolates. Scores in parenthesis ( ) are % disease leaf area based on overall mean of 10 plants. *LTH* Lijiangxintuanheigu

resistance levels of Jinbubyeo and IR65482-4-136-2-2 were 4 and 0, respectively. The disease reaction of the $F_{2:3}$ segregating progenies indicated that blast resistance in IR65482-4-136-2-2 is controlled by major genetic factor(s), which could be easily localized on the rice chromosome following prompt DNA marker development.

Localization of the *Pi40(t)* gene on chromosome 6 via association analysis

Of the preselected 106 even-spacing STS and SSR markers, 38 markers (30 SSR and 8 STS) detected polymorphism between Jinbubyeo and IR65482-4-136-2-2. The mapping population was successively genotyped with those 38 markers using single-locus ANOVA to test the association between a marker locus and disease reaction phenotype of $F_{2:3}$ segregating progenies, two chromosomal regions were identified (App = 1 in Table 3). One genetic factor was located on the short arm of chromosome 6 distal to RM5963 and RM5745 (Fig. 1a) and the other was on the long arm of chromosome 9 tagged by RM0201, where the allele type of IR65482-4-136-2-2 and Jinbubyeo was favorable to increase blast resistance, respectively. Seven markers only for those two chromosomal regions were screened and eight additional informative markers (3 STS and 5 SSR) were applied for association analysis (App = 2 in Table 3). A region of chromosome 6 delimited by RM0527 and RM3330 (3.5 cM) was strongly associated with blast resistance, which explained 58.9–66.5% of the total phenotypic variation (Table 3). However, the other genetic factors localized on chromosome 9 identified by RM0215 were less effective ($R^2 = 0.137$).

The ANOVA confirmed that the blast resistance in the donor line (IR65482-4-136-2-2) was due to the gene on the short arm of chromosome 6, where at least four blast resistance genes (*Piz*, *Piz-t*, *Piz-5*, and *Pi9*) were previously reported (Conaway-Bormans et al. 2003; Hayashi et al. 2006; Jiang and Wang 2002; Liu et al. 2002). However, the monogenic differential lines with these resistance genes exhibited a differential reaction to the selected Korean blast isolates compared to line IR65482-4-136-2-2 (Table 2). This new blast resistance gene in IR65482-4-136-2-2 inherited from *O. australiensis* is designated as *Pi40(t)*.

Candidate BAC/PAC clone identification using *e*-Landing and mirror-map

Through *e*-Landing, we eventually constructed a "Mirror Map" for which the relative physical positions of the markers could be projected over a reference rice genome, Nipponbare (Table 4). The 1.81-Mb virtual contig composed of 14 overlapping BAC/PAC clones was identified as the intervening physical regions for the three markers, S2539, RM0527, and RM3330, that exhibited significant associations between markers and phenotype (Fig. 1). Simultaneously, the physical positions of the markers tightly linked to *Pi2* (*Piz-5*), *Piz*, *Piz-t*, and *Pi9* were also determined and placed on the Mirror Map (Fig. 1b, Table 4). A PAC clone, P0649C11 (GenBank Acc. = AP005659), containing 19 putative open reading frames (ORFs) was identified by surveying TIGR annotated gene content of each BAC/PAC clone in which 6 ORFs were annotated as NBS-LRR disease-resistance protein homologues (Fig. 1b, c). The other clones did not contain any ORFs having putative functions

Table 3 Summary of association analyses between DNA marker genotypes of F_2 progenies and mean resistance levels of segregating $F_{2:3}$ progeny lines

Locus[a]		Mirror Map[b]			Marker genotypes of F_2 progenies[c]				Single-locus ANOVA[d]			Genetic effects[e]	
Name	App	Ch	*e*%	cM%	A	H	B	χ^2	*F*	*P*	R^2	Add	DeD
S10372	1	6	2.6	2.9	19	55	20	2.3	4.8	0.010	0.103	−0.61	−0.23
RM0402	2	6	20.5	27.6	17	45	32	4.3	12.2	2.19E-05	0.225	−0.83	−0.06
RM5963	1	6	28.3	41.0	17	48	29	2.6	35.0	8.70E-12	0.455	−1.24	0.33
S2539	2	6	29.9	43.5	15	48	29	3.8	77.7	1.19E-19	0.655	−1.56	0.46
RG64-SAP	2	6	30.6	43.9	Affinity for B allele		Not estimated						
RM0527	2	6	31.6	45.3	17	46	31	3.6	83.4	1.10E-20	0.665	−1.51	0.46
9871.T7E	3	6	33.3	47.2	16	47	31	4.2	104.3	1.73E-23	0.713	−1.60	0.49
9871.T7E2b	3	6	33.3	47.2	16	47	31	4.2	104.3	1.73E-23	0.713	−1.60	0.49
9871.T9E3	3	6	33.4	47.2	Affinity for B allele		Not estimated						
9871.T10E4	3	6	33.4	47.2	16	47	31	4.2	104.3	1.73E-23	0.713	−1.60	0.49
9871.T14I1E2	3	6	33.5	47.2	16	47	31	4.2	104.3	1.73E-23	0.713	−1.60	0.49
RM3330	2	6	35.5	47.2	13	52	29	5.8	60.2	6.03E-17	0.589	−1.62	0.40
RM5745	1	6	40.0	52.2	12	54	28	6.7	46.1	3.02E-14	0.523	−1.58	0.35
RM0340	1	6	91.5	90.9	23	51	20	0.6	4.0	0.022	0.087	−0.53	0.36
RM0321	1	9	54.2	42.9	24	45	25	0.1	2.6	0.082			
RM0257	2	9	77.9	67.4	23	47	24	0.0	2.0	0.140			
S1057	2	9	82.6	77.1	21	53	20	1.2	2.2	0.121			
RM0201	1	9	87.4	83.4	23	52	19	1.1	3.4	0.040	0.074	0.47	-0.61
RM0215	2	9	91.9	89.0	21	51	22	0.5	6.7	0.002	0.137	0.56	-0.97
RM0205	1	9	98.7	100.0	21	56	17	3.2	0.3	0.709			

Only DNA markers on chromosomes 6 and 9 are presented

[a] DNA markers were tested on 94 F_2 progenies. After conducting a first round of association analyses over well-defined anchor markers (application: App = 1), an additional 8 anchor markers were applied to narrow down the putative locations of blast-resistance genes on chromosomes 6 and 9 (App = 2). Six NBS-LRR ORF tagging polymorphic STS primer sets (see Fig. 4) were further applied to the F_2 mapping population (App = 3)

[b] Both percentage expressed physical (*e*%) and cM (cM%) positions are indicated (see Table 4)

[c] A and B are homozygous for the Jinbubyeo and IR65482-4-136-2-2 allele types and H indicates heterozygous progenies at the tested locus. Segregation-distorted loci are indicated with underlined χ^2 values for significant levels less than 0.05. The marker genotypes of RG64-SAP and 9871.T9E3 could not be evaluated because those primer sets had higher affinity to IR65482-4-136-2-2 allele types when the tested progenies were heterozygous

[d] Quantitatively acquired data sets for blast-resistance levels of each of 87 $F_{2:3}$ lines. For the *F*-test, markers having less than 0.05 for significance (*p*) were declared as significant empirically. The explainable phenotypic variation portion at the tested locus (R^2)

[e] Additive effect (Add), and degree of dominance (*DeD*) were then estimated at the declared loci: Add = (Bmean − Amean)/2 and DeD = Do/Add, where A and B are homozygous F_2 individuals for Jinbubyeo and IR65482-4-136-2-2, H is heterozygous individuals at the tested locus, and Do (dominant effect) = Hmean - (Bmean + Amean)

related to disease resistance. The PAC clone (P0649C11) was thus selected as the most putative physical region containing the *Pi40(t)* gene. The markers, S2539 and zt6057-Nip, were placed within any putative ORF having a putative function, peroxidase on P0038C05 (Gene ID = 3007.T00010) and similar to NBS-LRR disease resistance on P0649C11 (Gene ID = 9871.T00014). The other DNA markers were placed in the intergenic regions or partially shared with ORFs. Interestingly, the clone P0649C11 flanked by clones P0502B12 and B1197G05 was composed of many putative retrotransposon proteins (Fig. 1b).

DNA markers for *Pi40(t)* via haplotyping of the target region

Highly stringent primer sets for each NBS-LRR homologue were used to compare the marker allele types not only between IR65482-4-136-2-2 and IR31917-45-3-2 but also among the monogenic differential lines for *Piz-5*, *Piz-t*, *Piz*, and *Pi9* genes (Table 4; marker names having the Gene ID numbers '9871.T-'I or E' indicate the primer sequences derived from intron (I) and exon (E) regions, respectively). Two quality criteria were applied to ensure specific tagging

Table 4 List of locus-specific primer sets, their *e*-Landing-mediated physical positions on rice Pseudomolecule3, and corresponding BAC/PAC clones with their determined cM positions

Name	Primer sequences used for *e*-Landing[a]		Physical information[b]						Mirror Map[c]	
	Forward primer (5′→ 3′)	Reverse primer (5′→ 3′)	Ch	Start	Stop	*e*-PCR	*e*%	BAC/PAC	cM	cM%
Pib-1/2[d]	atcaactctgccacaaaatcc	cccatatcaccacttgttcccc	2	35,060,378	Rev	(600)	97.7	OJ1202_E07	154.1	97.6
S10372	ctccccgtattttgtaactg	tggtagcatttagggcatcc	6	811,663	812,889	1,227	2.6	P0541H01	3.6	2.9
RM0402	gagccatggaaagatgcatg	tcagctggcctatgacaatg	6	6,399,680	6,399,813	134	20.5	P0638H11	34.3	27.6
RM5963	cgaaaagtgggaagcaaatg	gcgtacccctagtggctgta	6	8,814,621	8,814,798	178	28.3	OJ1001_B06	51.0	41.0
R1679	atgcctatcagaaatggtc	atactctttacaggtggttga	6	8,956,750	8,958,589	1,840	28.7	P0528B02	53.5	43.0
MRG5836[d,e]	ttgttgtatacctcatcgac	agggtaagacgtttaacttg	6	9,308,941	9,309,108	168	29.9	P0038C05	54.1	43.5
S2539	ggactgagatggaatgtgct	gtagagtgatgacaaaatgacaa	6	9,325,104	9,327,007	1,904	29.9	P0038C05	54.1	43.5
RG64-SAP	gttgtttgagctctccaatgcctgttc	ctgcagtgcaatgtacggccagg	6	9,536,746	9,537,772	1,027	30.6	P0450D12	54.6	43.9
RM0527	ggctcgatctagaaaatccg	ttgcacaggttgcgatagag	6	9,862,291	9,862,523	233	31.6	OSJNBa0063H02	56.3	45.3
z4794-Nip[f]	tgaatgtgagaggttgactgtgg	cacgccacccttcaatggagact	6	10,022,552	10,022,691	140	32.1	OSJNBa0021H05	56.3	45.3
z4792-Nip[f]	tatattttggggcggaggtaggat	agtgttgtgcggcacgtgctttg	6	10,144,159	10,144,401	243	32.5	P0491D10	56.3	45.3
zt4792-Nip[f]	tatattttggggcggaggtaggat	agtgttgtgcggcacgtgctttg	6	10,144,159	10,144,401	243	32.5	P0491D10	56.3	45.3
9871.T5I1	gcctcacagccctttttggtc	tggatgatcttgtccggtgct	6	10,375,116	10,375,821	706	33.3	P0649C11	56.3	47.2
9871.T5E2	tgatgaggacttgggcagca	ttggcaccctcacacccttt	6	10,377,153	10,378,240	1,088	33.3	P0649C11	56.3	47.2
9871.T7E	ccatcccatctgaaaccatgc	cccccaggtcgtgataccttc	6	10,387,643	10,389,281	1,639	33.3	P0649C11	56.3	47.2
9871.T7E2b	caacaaacgggtcgacaaagg	cccccaggtcgtgataccttc	6	10,388,640	10,389,281	642	33.3	P0649C11	56.3	47.2
9871.T8I12	ccatgtagcgtaccatgacagca	agggaaggggattgggaattt	6	10,396,464	10,397,086	623	33.3	P0649C11	56.3	47.2
9871.T8E3	cgcggtaactgacagcaaagc	cgatcgtcaacgtccacagg	6	10,397,447	10,398,225	779	33.3	P0649C11	56.3	47.2
9871.T9I1	tctaggatggcaaaggcgtctc	gggagagggtcatagctcgaca	6	10,405,144	10,405,985	842	33.4	P0649C11	56.3	47.2
9871.T9E3	cgaatggtgggcctgttgtag	gcatggtttcagatgggatgg	6	10,407,213	10,408,138	926	33.4	P0649C11	56.3	47.2
9871.T10I2	atgggctcttacgcgaactcc	tggggatgtcgagagcaactc	6	10,421,300	10,421,926	627	33.4	P0649C11	56.3	47.2
9871.T10E4	atgggagcatgtcctgcaaaa	aataggggcgagctgtcgttc	6	10,425,195	10,426,296	1,102	33.4	P0649C11	56.3	47.2
z60510-Nip[f]	ggagttggtgcgacggtgccgttat	gcgcggaccggccagctagttgac	6	10,442,569	10,442,925	357	33.5	P0649C11	58.7	47.2
zt6057-Nip[f]	ggaagctcaaactaggaacgtgacga	actggaagtccctctatatgccc	6	10,445,390	10,445,678	289	33.5	P0649C11	58.7	47.2
9871.T14I1E2	gcccaaacacaagtggacaca	ccgatggcgatcctttagctt	6	10,445,409	10,446,373	965	33.5	P0649C11	58.7	47.2
9871.T14E23	tccagatccgcaatctcagga	acccgctcttgatggctgaat	6	10,446,381	10,447,525	1,145	33.5	P0649C11	58.7	47.2
z5765-Nip[f]	aatgtgaaattggatgagccggata	ttaccgatgttcgtcgctctcagg	6	10,553,791	10,554,147	357	33.8	B1197G05	58.7	47.2
pBA14[d]	tggtgcactcagaaagaa	gcagtgtcatcttgtctcc	6	10,902,552	Rev	(400)	34.9	P0036B02	58.7	47.2
RM3330	attattcccctcttccgctc	aagaaaccctcggattcctg	6	11,064,177	Rev	(145)	35.5	OSJNBa0055N24	58.7	47.2
RM5745	atgccaagtggacgatgtac	acatgtgggtagtgggatgg	6	12,493,013	12,493,215	203	40.0	P0578B12	64.9	52.2
RM0340	ggtaaatggacaatcctatggc	gacaaatataagggcagtgtgc	6	28,555,384	28,555,499	116	91.5	P0623A10	113.1	90.9
SSR140[d,e]	aaggtgtgaaacaagctagcaa	ttctaggggaggggtgtagaa	6		Fail	(115)	NA	NA	NA	NA
pB8[d]	cccaatctccaatgacccataac	ccggactaagtactggcttcgata	6		Fail	(500)	NA	NA	NA	NA

Table 4 continued

Name	Primer sequences used for *e*-Landing[a]		Physical information[b]						Mirror Map[c]	
	Forward primer (5′→ 3′)	Reverse primer (5′→ 3′)	Ch	Start	Stop	*e*-PCR	*e*%	BAC/PAC	cM	cM%
JJ81[d]	tcatcaaactcagttaaact	agcgaaaatcatttatcaca	9		Fail	(500)	NA	NA	NA	NA
JJ113[d]	ctcttggtgatctttgttac	ggatgatgtgatctgcagag	9		Fail	(500)	NA	NA	NA	NA
YL100/102[d]	caatgccgagtgtgcaaagg	tcaggttgaagatgcatagc	12	10,606,438	10,606,840	403	38.6	OJ1103_B10	50.4	46.1
YL155/87[d]	agcaggttataagctaggcc	Ctaccaacaagttcatcaaa	12	10,607,647	10,608,689	1,043	38.6	OJ1103_B10	50.4	46.1

[a] The primer sequences were used as the query to localize on Rice Pseudomolecule3

[b] When both primer sequences successfully recognized their physical locations to be annealed, the expected PCR product size (*e*-PCR: 'Stop' – 'Start' + 1 bp) was used to judge PCR products. For both cases of *e*-Landing failure (Fail) or single primer annealing (For or Rev), the expected PCR product sizes, which were previously reported, are indicated within parentheses in the '*e*-PCR' column. When only one primer sequence displayed clear annealing (For: forward primer only, Rev: reverse primer only), the defined physical location is placed in the 'Start' column to calculate the percentage expressed relative physical positions, '*e*%', compared to the total physical lengths of each chromosome. The determined physical regions followed by *e*-Landing were used to match corresponding BAC/PAC clones depending on the 'Rice Pseudomolcule Information and Gene Search' supported by TIGR

[c] The determined cM positions for each BAC/PAC clone were adopted directly from the TIGR database, and were estimated using the marker sequences obtained from the Cornell RiceGenes Database and the Japanese Rice Genome Program. To more easily compare cM positions from various mapping populations having different total lengths, the percentage expressed relative genetic position, 'cM%', was also calculated

[d] Previously reported *Pi*-gene-specific markers for *Pib, Pi9, Pi3, Pi5, Pil, Pik*, and *Pita*

[e] MRG5836 is synonymous for RM6836, but SSR140 is not synonymous for RM8140 as described in the Gramene database (http://www.gramene.org/db/markers/)

[f] The primer sets are composed of Nipponbare-specific forward and reverse primers, and Zenith (*Piz*)- or Toride 1(*Piz-t*)-specific primers were not used

of the NBS-LRR homologues during PCR amplification: (1) high annealing temperature and (2) singleton amplification. Twelve primer sets were synthesized and all successfully generated the expected target bands without serious false positives (Table 4; Fig. 1c, 2a).

Detailed haplotypes of IR65482-4-136-2-2, IR31917-45-3-2, and the monogenic differential lines with *Piz-5*, *Piz-t*, *Piz*, and *Pi9* genes for the 1.81-Mb physical segment flanked by S2539 and RM3330 were determined by using unique allele types derived from 19 DNA markers (Table 5; Fig. 2b). Identical haplotypes as well as pathotyping results between Jinbubyeo (ID-3) and IRBLz-Fu (ID-9) suggested that Jinbubyeo has the *Piz* gene in addition to the minor genetic factor identified on the long arm of chromosome 9 (Tables 2, 4, 5). This was also confirmed by checking 'Fukunishiki' as one of the progenitor lines of Jinbubyeo. The haplotype of IR31917-45-3-2 (ID-1) was fairly distinct from that of IR65482-4-136-2-2 (ID-2), and indicated integration of a 1.81-Mb DNA segment from *O. australiensis* (Table 5). The F_2 mapping population was genotyped by using five NBS-LRR derived markers to conduct single-locus ANOVA by using the pathotyping results (Table 3). The homozygous and heterozygous alleles of IR65482-4-136-2-2 could not be distinguished at the 9871.T9E3 locus, and the genotype data at the locus were not collected (data not shown). Association analysis confirmed that the physical region including six NBS-LRR homologues was the most putative location of *Pi40(t)* ($F = 104.3$, $R^2 = 0.713$), which was likely to be an additive gene (degree of dominance = 0.49). However, the genetic intervals among the four DNA markers could not be estimated due to the small size of the mapping population ($N = 94$).

Fine mapping of the *Pi40(t)* gene

The marker allele types of the blast R genes (*Piz-5*, *Piz-t*, *Piz*, and *Pi9*) were compared to infer their putative precise locations (Table 5). The locus 9,871.T8E3 was considered as one of the putative locations for the *Piz-5* (ID-7) and *Piz-t* (ID-8) genes because the inferred allele type of those two monogenic lines was different from that of LTH (ID-5). Under the assumption that the allele types of *Pi40(t)* could not be shared with any other R genes (*Piz-5*, *Piz-t*, *Piz*, or *Pi9*), due to their uniqueness in terms of gene source and reaction to a range of blast isolates tested (Tables 1, 2), the probable ORFs for the *Piz* gene could be one of three: 9871.T7, 9871.T10, or 9871.T14. Similarly, the possible ORFs for *Pi9* (ID-10) could be either 9871.T7 or 9871.T14 (see Table 5). The ORF 9871.T5 could be excluded from consideration, because all tested genotypes showed a similar banding pattern even after applying six endonucleases to the 1,794-bp region tagged by 9871.T5I1 and 9871.T5E2 (see Table 5).

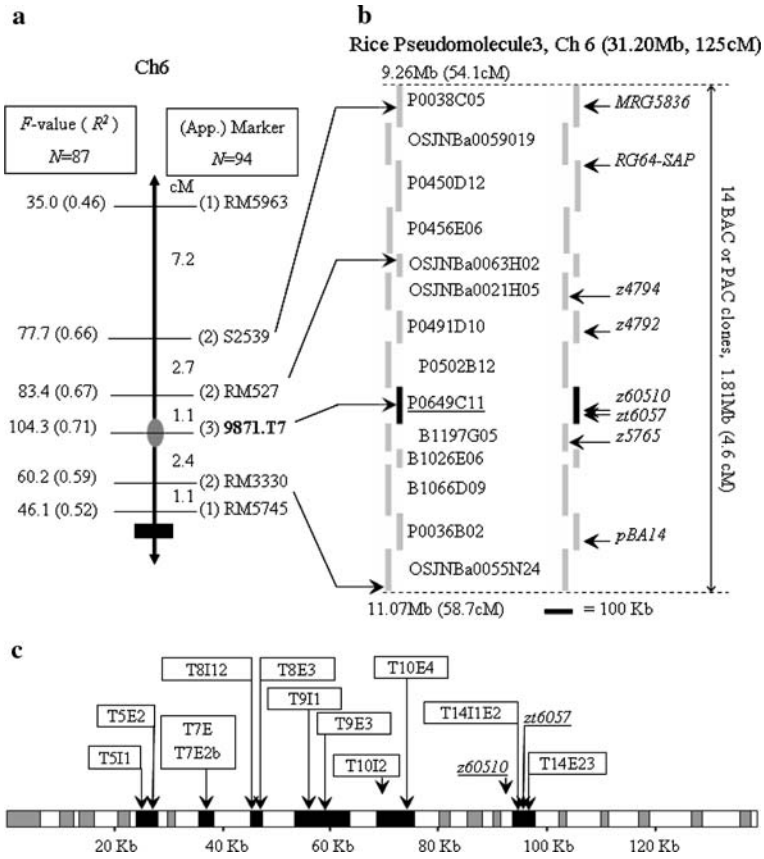

Fig. 1 **a** Linkage map skeleton of the chromosome 6 short arm with six DNA markers used to narrow down the putative location of the R-gene. Markers were applied based on F-statistics from single-locus ANOVA results (*App.* application, see also Tables 3, 4). The genetic intervals are shown in centiMorgan (*cM*). The *black rectangle* indicates the centromere region. The PCR primer set for marker 9871.T7 (in *boldface*) tagging the resistance gene that originated from Nipponbare PAC clone P0649C11 was determined as the putative location of the *Pi40(t)* gene based on comparative haplotype analyses. **b** *e*-Landing-mediated high-resolution map of the *Pi40(t)* locus and determined physical positions of specific markers for *Pi2* (*Piz-5*), *Piz*, *Piz-t*, and *Pi9* reported earlier. *e*-Landing of three DNA markers, used for second application (S2539, RM527, RM3330; App. = 2 in **a**), on Rice Pseudomolecule3 delimited the corresponding 1.81 Mb virtual contig composed of 14 BAC or PAC clones. The physical positions of previously reported markers (*italics*) on the virtual contig were also evaluated via *e*-Landing except for SSR140 and pB8. **c** PAC clone P0649C11 (138,870 bp, GenBank Acc. AP005659), containing 6 NBS-LRR disease resistance protein homologues (*black rectangles*) out of 19 putative ORFs predicted by TIGR. Of the ten previously reported primer sets for *Pi2* (*Piz-5*), *Piz*, *Piz-t*, and *Pi9* loci (**b** and Table 4), applied to *e*-Landing procedures, only two SNP markers for the *Piz* loci, z60510 and zt6057 (*italics*), could be placed on the P0649C11 clone

The number of marker allele types resolved by 12 specific primer sets varied from 1 (9871.T5I1 and 9871.T5E2) to 6 (9871.T7E, and 9871.T14E23) (Table 5). The locus 9871.T7E2b expressed seven marker allele types by which all tested genotypes could be differentiated except between IR65482-4-136-2-2 and IRBLzt-T (Table 5, Fig. 3a). We concluded that the 9871.T7 locus could be the most putative location for the *Pi40(t)* gene.

Evidence of *O. australiensis* chromosome segment integration

The STS marker 9871.T7E2b amplified the predicted size of 642 bp band for both IR65482-4-136-2-2 and *O. australiensis*. The cleaved banding patterns by *Hin*fI and *Tsp509*I supported the integration of *O. australiensis* DNA segment(s) into IR65482-4-136-2-2, which had occurred near the 9871.T7 locus (Fig. 3b).

MAS validity test and cross-evaluation of blast resistance level

The linkage between the *Pi40(t)* gene and PCR markers 9871.T7E and 9871.T7E2b was validated as complete linked marker by genotyping resistant BC_2F_4 or BC_3F_3 progenies in the genetic backgrounds of Jinbubyeo and Junambyeo. Blast reaction with the isolate 01–01 showed segregation of 36 and 19 lines as resistant out of 95 and 68 lines tested in the genetic background of Jinbubyeo and Junambyeo, respectively. Some 115 (Jinbubyeo BCs) and 77 (Junambyeo BCs) resistant individuals (scored as 0–2) randomly selected for amplification of 9871.T7E (data not

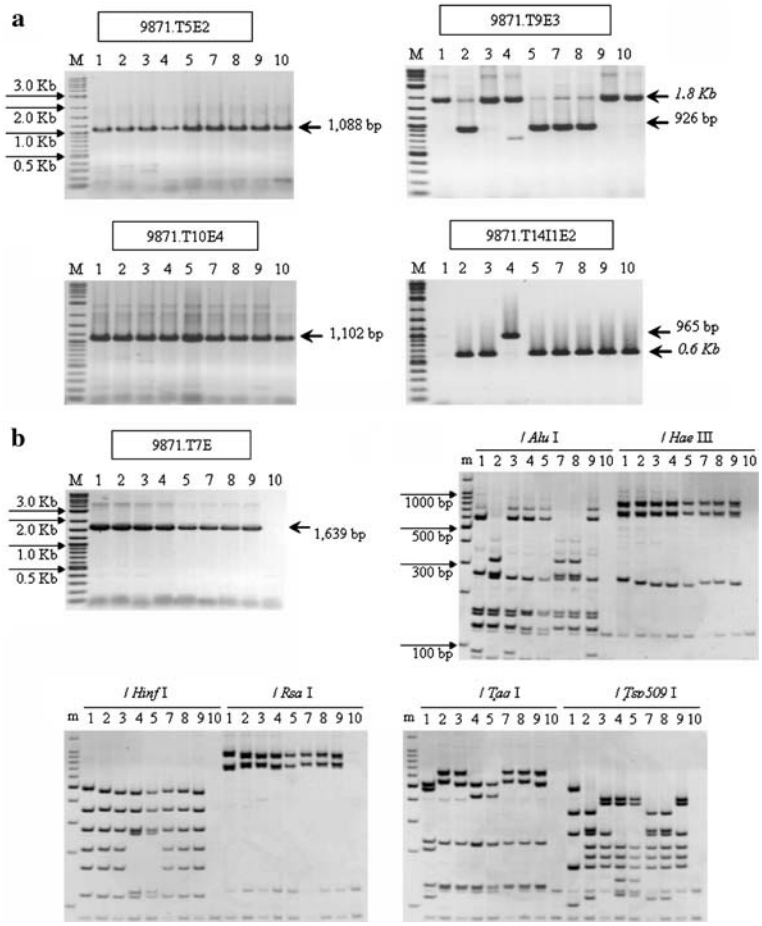

Fig. 2 PCR analysis using NBS-LRR tagging primer sets and differentiation of allele types by restriction endonucleases. **a** Confirmation of expected banding patterns using designed primer sets as detected by *e*-Landing and *e*-PCR (Table 4). Note that 9871.T9E3 detected insertions (1.8 kb in *italics*) on ID-1, 3, 4, 9, and 10 (IR31971-45-3-2, Jinbubyeo, Junambyeo, IRBLz-Fu, and IRBL9-w, respectively), whereas ID-2, 5, 7, and 8 (IR65482-4-136-2-2, LTH, IRBLz5-CA, and IRBL-zt-T, respectively) generated the exact PCR product (926 bp) as expected. However, the primer 9871.T14I1E2 generated the expected PCR product (965 bp) only in Junambyeo (ID-4), IR31917-45-3-2 (ID-1) generated a null allele, and the others showed a deleted allele type (0.6 kb). **b** Detection of latent polymorphism at the tagged locus using six restriction endonucleases. Expected band size of 1,639 bp was identified by the primer 9871.T7E in all tested genotypes except for ID-10 (IRBL9-w). The banding patterns generated by *Hae*III and *Rsa*I cleavages were considered as the same pattern. *Alu*I cleavage, for example, generated three unique banding patterns (pattern I = ID-1, 3, 9; pattern II = ID-2, 7, 8; pattern III = ID-4, 5). However, two, three, and four unique banding patterns were identified by *Hin*fI, *Taq*I, and *Tsp509* I enzymes, respectively. The primer 9871.T7E (null at ID-10) within tested nine genotypes further discriminated two allele types into a total of five different allele types (Type A = ID-1; Type B = ID-2, 7, 8; Type C = ID-3; Type D = ID-4, 5; Type E = ID-9). *Line IDs* are listed in Table 1. Agarose gel was used for gel images having the DNA step marker lane 'M' (2-Log DNA ladder; NEB), and a natural polyacrylamide gel was used for those having 'm' (100 bp DNA ladder; Promega). In both cases, ethidium bromide staining was used for visualization

shown) and 9871.T7E2b (Fig. 4) loci did not exhibit any homozygous susceptible marker allele type of Junambyeo. This result confirmed that the *Pi40(t)* gene was the essential genetic component for resistance against blast infection.

The IR65482-4-136-2-2 genotype was also evaluated for Philippine blast isolates along with the check lines, including IR31917-45-3-2 and IRBLzt-T. None of the tested isolates was compatible with IR65482-4-136-2-2, but IR31917-45-3-2 and IRBLzt-T were susceptible to several isolates (Table 2; Fig. 3c). Our results suggested that the *Pi40(t)* gene indeed has a broad spectrum of blast resistance as well as a different genetic value from that of *Piz-t*.

Discussion

Developing broad-spectrum durable resistance to blast pathogen is the primary objective of most rice breeding programs worldwide. However, to date, the available R genes are mostly race-specific and short-lived. The availability of sequence information of Nipponbare, novel genetic resources of alien introgression lines, and new strategies accelerating the fine mapping of R genes provides new ways to develop broad-spectrum blast resistance in rice.

Qualitatively interpreted disease reaction results are often used to infer genotypes of segregating progenies at

Table 5 Determined detailed haplotypes of nine genotypes on the near-centromeric region of chromosome 6

Locus	Physical information		Line discrimination[a]		ID-1	ID-2	ID-3	ID-4	ID-5	ID-7	ID-8	ID-9	ID-10
	Start (bp)	Interval (kb)	Method	Type	RPD	DOR	RP1	RP2	LTH	*Piz-5*	*Piz-t*	*Piz*	*Pi9*
S2539	9,325,104	–	CAPs	2	A	B	A	B	A	C	B	A	B
RG64-SAP	9,536,746	211.64	CAPs	2	A	B	A	C	A	B	C	A	B
RM0527	9,862,291	325.55	SSR	4	A	B	C	B	A	B	D	C	D
z4794-Nip	10,022,552	160.26	CAPs	2	A	A	A	B	B	A	A	A	A
9871.T5I1	10,375,116	352.57	CAPs	1	a	a	A	a	A	A	A	A	a
9871.T5E2	10,377,153	2.04	CAPs	1	A	A	A	A	A	A	A	A	A
9871.T7E	10,387,643	10.49	CAPs	6	A	B	C	D	D	B	B	E	–
9871.T7E2b	10,388,640	1.00	CAPs	6	a	B	C	D	D	E	B	F	f
9871.T8I12	10,396,464	7.83	CAPs	2	–	A	A	–	A	a	A	a	–
9871.T8E3	10,397,447	0.98	CAPs	4	A	B	C	A	C	D	B	C	C
9871.T9I1	10,405,144	7.70	CAPs	2	A	A	B	A	A	A	A	B	a
9871.T9E3	10,407,213	2.07	STS	2	A	B	A	A	B	B	B	A	A
9871.T10I2	10,421,300	14.09	STS	2	A	A	A	A	B	A	A	A	A
9871.T10E4	10,425,195	3.90	CAPs	2	A	A	B	A	A	A	A	B	A
zt6057-Nip	10,445,390	20.20	STS	3	–	A	B	A	A	A	A	B	B
9871.T14I1E2	10,445,409	0.02	CAPs	4	–	A	B	C	B	A	A	B	B
9871.T14E23	10,446,381	0.97	CAPs	6	–	A	B	C	D	A	A	B	E
pBA14	10,902,552	456.17	CAPs	3	A	A	A	–	B	–	–	–	B
RM3330	11,064,177	161.63	SSR	5	A	A	B	C	B	A	D	B	E

The designations for each ID number appear in Table 1

[a] *CAPs* The digested STS-PCR products by six restriction enzymes (*Alu*I, *Hae*III, *Hin*fI, *Rsa*I, *Taq*I, *Tsp509* I) were used to detect latent SNPs, and overall allele types were determined by summing up all banding patterns. The Null allele type (−) was considered as one unique allele along with other allele types. Small letters indicate that the amount of amplified PCR product from the breeding line was less than that of other

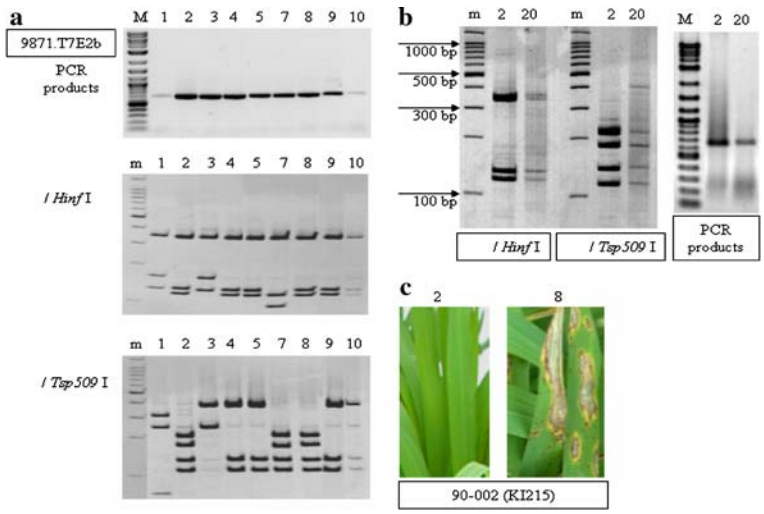

Fig. 3 Confirmation of the novelty of the *Pi40(t)* gene. Line IDs, electrophoresis conditions, and DNA step markers are the same as in Fig. 2. **a** The primer, 9871.T7E2b, amplified a 642-bp fragment in all tested materials. ID-1 and ID-10 (IR31917-45-3-2 and IRBL9-w) were differentiated as a different allele type from another eight genotypes due to their weak band intensity. Combined interpretation over the banding patterns from the *Hin*fI and *Tsp509* I digested PCR products discriminated all LTH monogenic lines (ID-5, 7, 8, 9, 10 for LTH, *Piz-5*, *Piz-t*, *Piz*, *Pi9*, respectively) and also the blast resistance gene donor line (ID-2) from its recurrent parent (ID-1) and the two Korean japonica varieties used as recurrent parents of this study (ID-3 and ID-4). Note that the allele type of IR65482-4-136-2-2 could not be differentiated from that of IRBLzt-T (ID-8). **b** Comparisons on the PCR banding patterns from *Hin*fI and *Tsp509* I cleavages between the introgression line (ID-2) and *O. australiensis* (ID-20) indicate alleles different from IR31917-45-3-2 (**a**; ID-1) that originated from *O. australiensis*. **c** Highly susceptible reactions of IRBLzt-T (ID-8) to a selected Korean isolate, 90-002 (KI215), suggested that *Pi40(t)* is a new blast resistance gene having a broad spectrum of resistance against rice blast (see also Table 1)

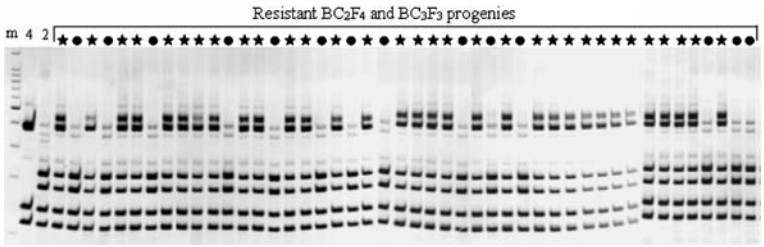

Fig. 4 MAS validity tests of blast-resistant BC progenies in a susceptible japonica cultivar genetic background. Partial gel image of detected marker genotypes on the 9871.T7E2b locus among 38 randomly selected BC_3F_3 and 39 BC_2F_4 progenies from a cross between Junambyeo (ID-4) and IR65482-4-136-2-2 (ID-2). An endonuclease, *Tsp509* I, was used to detect polymorphism between parental lines. Progenies marked with a *closed circle* are homozygous for the IR65482-4-136-2-2 allele and a *star* indicates heterozygous progenies. Note that none of the resistant BC progenies was homozygous for the Junmanbyeo allele. 'm' = 100 bp DNA ladder (Promega). A natural polyacrylamide gel was used for electrophoresis, followed by ethidium bromide staining

the resistance locus. However, qualitatively expressed data may cause serious bias per se, due to phenotypic mis-scoring as well as environment fluctuations during evaluation (Tabien et al. 2002). Therefore, incorporation of the resistance genes using DNA markers of a high-density linkage map would be challengeable, especially because of the ambiguous judgment on heterozygous progenies (Jena et al. 2006). To overcome these practical problems, we made a quantitative interpretation of the disease reaction data, since it is less sensitive to even modest numbers of phenotypic mis-scores. Based on the principles of linkage disequilibrium (Remington et al. 2001), and observed abnormality of frequency distribution of resistance levels from $F_{2:3}$ segregating progenies, only 2–5 anchor markers representing each chromosome were initially tested to find the chromosomal location of an effective genetic factor. A locus, contributed by Jinbubyeo, was localized on the subterminal region of chromosome 9, where a QTL was reported in Lemont and Teqing recombinant inbred lines (Tabien et al. 2002). The presence of the *Pi40(t)* gene on chromosome 6 was indicated even by markers tagging telomere regions due to its strong allele substitution effect. In spite of the small population size ($N = 87$) for association analysis, with selective markers for the putative chromosomal regions, plotting of F values from single-locus ANOVA provided the unbiased locations of effective loci as well as estimated genetic effects. Our study demonstrated for the first time that merging of quantitative data (pathogenicity) and a restricted amount of qualitative (marker alleles) data via association analysis would be one of the most practical approaches not only to map unknown resistance genes but also to evaluate their informative genetics such as breeding value and degree of dominance.

We adopted here the concept of *e*-Landing (Jena et al. 2006), by which all anchor markers were preplaced on a physical map constructed *in silico* based on the contig map of BAC/PAC clones of Nipponbare supported by TIGR. Our *e*-Landing approach offered the substitution of previously reported DNA markers with well-evaluated pools of primer sets of STS and SSR anchor markers to test the possibility of known blast genes that might be involved in the broad-spectrum resistance of IR65482-4-136-2-2. After we identified the 1.81-Mb virtual contig as the most putative location of the *Pi40(t)* gene, it was realized that at least four blast resistance genes, *Piz-5*(*Pi2*), *Piz*, *Piz-t*, and *Pi9*, were previously placed on the same chromosomal location. However, the determined marker intervals are informative only within the mapping population used to differentiate marker positions.

To avoid the time-consuming efforts of tedious genotyping of a large number of segregating progenies, we proposed here a new concept of "Mirror-Map". We searched the corresponding physical regions on the Nipponbare genome for DNA markers of each blast resistant gene, based on an assumption that there may be a high level of synteny relationships among the rice lines used for *Piz-5*, *Piz*, *Piz-t* and *Pi9* tagging DNA markers as well as those used in our study. By projecting markers over the Nipponbare reference genome, their physical and genetic intervals were successfully elucidated. We could identify a PAC clone, P0649C11, that includes six NBS-LRR disease resistance protein homologues as the most likely nested place of five rice blast resistance genes, including *Pi40(t)*. The haplotyping results suggest that the ORF orders among the recurrent parents, the donor line for resistance, and monogenic differential lines are collinear and that there is a high degree of synteny at the micro level. Recent studies on *Pi2*, *Pi9*, *Piz*, and *Piz-t* genes also confirmed that the putative physical regions for these blast resistance genes contained a cluster of the NBS-LRR gene homologues (Hayashi et al. 2006; Qu et al. 2006; Zhou et al. 2007).

The Nipponbare genome harbors about 600 NBS-LRR motifs and these are considered as candidate resistance genes (Monosi et al. 2004; Zhou et al. 2004). The structural features and resistance specificity to avirulence proteins of NBS-LRR have been investigated (Jia et al. 2000; Martin et al 2003; Qu et al. 2006; Zhou et al. 2006). Clusters of resistance genes have been identified in diverse plant

species (Song et al. 1995; Sun et al. 2004; Qu et al. 2006; Wei et al. 2002), where NBS-LRR genes are the most prevalent class out of six distinct resistance gene classes. Clustering of genes suggest that the tandem repeats of resistance gene homologues at a locus may provide a variety of opportunities for plants to evolve new specificities of resistance when the corresponding avirulence gene in the pathogen has mutated (Dean et al. 2005). Although six NBS-LRR disease resistance protein homologues were identified within the PAC clone P0649C11, they expanded up to only 70-kb physical regions, which corresponded to a 0.1-cM genetic interval based on the Mirror Map (Fig. 1c; Table 4). In our study, the 70-kb physical region was dissected by 12 primer sets and haplotypes for nine genotypes, including monogenic differential lines for *Piz-5*, *Piz-t*, *Piz*, and *Pi9* were determined (Table 5). The most likely locus/loci for each blast resistance gene on chromosome 6 could be successfully assigned. The reliability of our approach is also supported posteriori by standard map-based cloning of the *Pi9* gene (Qu et al. 2006). The *Pi9* gene was putatively localized to the genomic region containing *Nbs2–Pi9*, which is highly collinear to the 9871.T7 locus. In our study, 9871.T7 and 9871.T14 loci are predicted as the most putative ORFs for the *Pi9* gene. It is noteworthy to mention that comparative analysis of determined haplotypes for LTH monogenic differential lines could make it possible to develop a PCR primer set for the 9871.T7E2b marker, by which all LTH monogenic differential lines were discriminated against each other.

Comparative analysis on determined haplotypes for the 1.81-Mb physical intervals suggested that micro-colinearity was highly conserved across all tested genotypes, even for IR65482-4-136-2-2 and IRBL9-w. IR65482-4-136-2-2 carrying *Pi40(t)* gene was developed as an introgression line through an interspecific cross between IR31917-45-3-2 and *O. australiensis* (Jena et al. 1991). The *Pi9* gene, which was also introduced into *O. sativa* from a wild species, *O. minuta* (Liu et al. 2002). *O. minuta* and *O. australiensis* have two distinct genome types as well as genome sizes (Wing et al. 2005); therefore, the alien DNA segments, including *Pi40(t)* and *Pi9* genes, might have precisely recognized their destination within the *O. sativa* genome during interspecific crosses. A recent study strongly suggested that the physical positions of alien DNA fragment integration might be dependent on the host genome structure (Wang et al. 2005). Our previous studies on another *O. australiensis* introgression line, IR65482-7-216-1-2, having the *Bph18(t)* gene also supported the hypothesis (Jena et al. 2006). We have detected an interesting genomic feature nearby the alien DNA integration for blast resistance. The flanking BAC/PAC clones of Nipponbare were the clones composed of many putative retroelements—P0502B12 and B1197G05 for the *Pi40(t)* and *Pi9* genes.

The possible mechanism of alien gene introgression into *O. sativa* is by restricted reciprocal recombination as detected in this study and as reported earlier (Jena et al. 1992, 2006). However, our study indicates that the marker polymorphism-based judgment on integration events may lead to under- and overestimations on the total number of integration events and their fragment sizes, especially at the physical regions maintaining a high level of synteny between *O. sativa* and its wild relatives. An integration test for the 70-kb physical regions, including the *Pi40(t)* gene, revealed polymorphism between IR65482-4-136-2-2 and IR31917-45-3-2 at seven loci out of 13 loci tested (Table 5). Without two double crossovers within the 7.7-kb (between 9871.T8E3 and 9871.T9I1) and 18-kb (between 9871.T10I2 and zt6057-Nip) intervals, the monomorphic regions could not be interpreted as *negative-for-integration events*. Therefore, the actual size of the *O. australiensis* DNA segment in IR65482-4-136-2-2 around the *Pi40(t)* gene might be at least 1.1 Mb, which was flanked by S2539 and 9871.T14E23. The comparative genomics program of OMAP will provide comprehensive information not only on the genome structure in the region but also on the detailed sequence variation among the NBS-LRR family members (Wing et al. 2005).

In our study, advanced breeding lines derived from BC progenies were used to validate the markers 9871.T7E and 9871.T7E2b as markers completely associated with the *Pi40(t)* gene. They have passed meioses independently 3 or 4 times and rapidly regressed into the unique haplotypes of their recurrent parental lines. The occurrence of resistant BC progenies from two genetic backgrounds with resistance-specific marker alleles in either the homozygous or heterozygous state suggests that the breeding value of the *Pi40(t)* gene estimated by using progenies from Jinbubyeo × IR65482-4-136-2-2 could be stably maintained in other susceptible japonica cultivars.

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