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Identification of three major *R* genes responsible for broad-spectrum blast resistance in an *indica* rice accession

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Abstract An *indica* rice accession HR4 was found to exhibit good resistance to rice blast in previous research. Inoculation with 116 different *M. oryzae* isolates revealed that HR4 has the broadest resistance spectrum of the six rice cultivars used in this study, which included two well characterized broad-spectrum resistance sources. To uncover the genetic mechanism of the broad-spectrum resistance in HR4, genetic analysis was carried out with three stable isolates. The results showed that a single dominant gene controlled its resistance to isolates GD93286 and GD00193, whereas two independent dominant genes were responsible for its resistance to isolate GD08T4. The resistance (*R*) gene in HR4 corresponding to isolate GD93286, named *Pi-h1(t)*, was found to reside in a region of ~235.9 kb on the long arm of chromosome 11, while the other two *R* genes identified with isolate GD08T4, named *Pi-h2(t)* and *Pi-h3(t)*, were linked to markers on chromosomes 1 and 12, respectively. The results indicated that the broad-spectrum resistance to rice blast in HR4 could be ascribed to multiple *R* genes. Identification of such multiple *R* genes will allow us to use markers more effectively for resistance improvement in rice breeding programs.

Keywords Rice blast · Inheritance of resistance · Resistance gene · Gene mapping

Wuming Xiao and Qiyun Yang have contributed equally to this work.

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Introduction

Rice (*Oryza sativa* L.) is the most important staple crop, feeding more than half of the world's population. Maintaining stable rice production is extremely important to feed the constantly growing human population (RoyChowdhury et al. 2012). However, rice production is challenged with many biotic and abiotic stresses. Among them, rice blast, caused by the filamentous ascomycete fungus *Magnaporthe oryzae*, is one of the most devastating and destructive diseases of rice worldwide (Couch and Kohn 2002). The blast fungus severely infects rice grown in areas where intensive rice cultivation is practised. The fungus causes lesions to all aerial parts of the plant, although

leaves and panicles are the most affected organs. Commonly, the disease reduces yield by 10–30 %, even up to 80 % under conditions favorable to the fungus (Skamnioti and Gurr 2009; Faivre-Rampant et al. 2011). Chemical control of the disease is effective, but it has economic and environmental costs. Growing resistant rice cultivars has generally been considered to be an effective and economical way of controlling the disease. However, many resistant cultivars are short-lived after deployment because of the high variation in *M. oryzae* population, due to a high level of genomic instability of the pathogen (Dean et al. 2005; Ballini et al. 2008). Therefore, blast resistance has become a major target of modern rice breeding programs.

Although many reports of durable or broad-spectrum resistance rice lines have been documented, such as Tetep (Ou 1985; Barman et al. 2004), Moroberekan (Ou 1985; Wang et al. 1994), IR36 (Wang et al. 1989), Sanhuangzhan 2 (Wu et al. 2004), Gumei 4 (Deng et al. 2006), Xiangzi 3150 (Huang et al. 2011), Er-Ba-Zhan (Zhu et al. 2012), Yuejingsimiao 2 (He et al. 2012), Tianjinyeshengdao (Wang et al. 2012), etc., the fact remains that understanding the genetic basis of resistance to blast in durable or broad-spectrum resistance rice lines is beneficial in developing resistant rice cultivars.

During the past decade, the genetics of blast resistance has been extensively studied. To date, over 80 blast resistance (*R*) genes have been identified, and are distributed on 11 rice chromosomes apart from chromosome 3 (Liu et al. 2010). So far, 22 have been cloned (*Pib*, *Pita*, *Pi9*, *Pi2*, *Piz-t*, *Pi-d2*, *Pi36*, *Pi37*, *Pikm*, *Pit*, *Pi5*, *Pid3*, *pi21*, *Pb1*, *Pish*, *Pik*, *Pikp*, *Pi54*, *Pia*, *NLS1*, *Pi25* and *Pi1*). Except for *Pi-d2* and *pi21*, most of them are nucleotide-binding site–leucine-rich repeat (NBS-LRR) genes (Wang et al. 1999; Bryan et al. 2000; Qu et al. 2006; Zhou et al. 2006; Chen et al. 2006; Liu et al. 2007; Lin et al. 2007; Ashikawa et al. 2008; Hayashi and Yoshida, 2009; Lee et al. 2009; Shang et al. 2009; Fukuoka et al. 2009; Hayashi et al. 2010; Takahashi et al. 2010; Zhai et al. 2011; Yuan et al. 2011; Sharma et al. 2005; Okuyama et al. 2011; Tang et al. 2011; Chen et al. 2011; Hua et al. 2012). *Pi-d2* encodes a serine/threonine-kinase membrane-spanning protein, while *pi21* encodes a protein with heavy-metal-binding and proline-rich domains (Chen et al. 2006; Fukuoka et al. 2009). Interestingly, resistance which requires pairs of NBS-LRR members was found in the case of *Pi5*, *Pia*, *Pik* and its alleles (*Pikm*, *Pikp* and *Pi1*) (Lee et al. 2009; Okuyama et al. 2011; Zhai et al. 2011; Ashikawa et al. 2008; Yuan et al. 2011; Hua et al. 2012).

From our breeding programs, an *indica* rice accession named HR4 was found to endow universal resistance to panicle blast for over 10 consecutive cropping seasons in a natural blast nursery of Conghua (23.578N, 113.558E), Guangdong, China. The objectives of this study are: (1) to assess the resistance spectrum of HR4 in comparison to other wide-spectrum sources; (2) to determine the number of genes underlying the broad-spectrum resistance and (3) to map those genes to the rice genetic map. Understanding the genetic mechanism of HR4 will allow us to use markers more effectively to monitor the *R* genes in this wide-spectrum resistance source in future breeding practises.

Materials and methods

Blast inoculation and disease evaluation

We evaluated HR4 against 116 isolates of *M. oryzae* by individual inoculation, in comparison with Tetep, Kanto51, Yue-xiang-zhan (YXZ), Te-xian-zhan 13 (T13) and Li-jiang-xin-tuan-hei-gu (LTH) (Table 1). The isolates were highly diverse because they had been collected from different ecological areas of Guangdong Province over many years. Rice seedlings at the 3–4-leaf stage were spray-inoculated with *M. oryzae* spore suspensions (1.0×10^5 spores/ml) and then kept in darkness at 25–27 °C and over 90 % relative humidity for 24 h. The inoculated plants were subsequently kept under a 12/12-h (day/night) photoperiod at the same temperature and relative humidity for 6 days. Disease lesions on inoculated rice leaves were rated on a 0–9 scale, and the ratings were used to classify the seedlings into R (resistant 0–3) and S (susceptible 4–9) according to the description of IRRI (International Rice Research Institute 1996).

Genetic analysis

HR4, as male parent, was crossed with LTH, a universally susceptible *japonica* cultivar. F1 was selfed to produce the F2 population and backcrossed with LTH to produce the BC_1F_1 population. The F_1, F_2 and BC_1F_1 populations were inoculated with isolates

Table 1 Resistance reactions of HR4 and five other control cultivars to diverse isolates

Isolates	Tetep	Kanto51	LTH	HR4	YXZ	T13	Isolates	Tetep	Kanto51	LTH	HR4	YXZ	T13	Isolates	Tetep	Kanto51	LTH	HR4	YXZ	T13
GD006	R	R	S	R	R	–	GD05141	R	R	S	R	S	R	GD0860	R	R	S	R	S	S
GD0016	R	R	S	R	R	S	GD05166	R	R	S	R	R	R	GD08121	R	R	S	R	S	S
GD0021	R	R	S	R	R	–	GD05237	R	R	S	R	S	S	GD08159	R	R	S	R	S	R
GD0053	R	R	S	R	S	–	GD0602	R	R	S	R	R	S	GD08286	R	R	S	R	R	R
GD0090	R	R	S	R	R	R	GD0629	R	R	S	R	S	R	GD08472	R	R	S	R	S	R
GD00120	S	S	S	R	S	–	GD0630	R	R	S	R	R	R	GD08507	R	R	S	R	R	R
GD00164	R	R	S	R	R	S	GD0638	R	R	S	R	R	R	GD08682	R	R	S	R	R	R
GD00165	R	S	S	R	S	–	GD0689	R	R	S	R	S	R	GD08597	R	R	S	R	S	R
GD00193	R	R	S	R	S	S	GD06105	R	R	S	R	S	S	GD08679	R	R	S	R	S	S
GD0108	R	R	S	R	R	R	GD06128	R	R	S	R	R	R	GD08712	R	R	S	R	S	R
GD0118	R	R	S	R	S	R	GD06146	R	R	S	R	S	S	GD08753	R	R	S	R	S	S
GD0121	R	R	S	R	R	R	GD06164	R	R	S	R	S	S	GD08923	R	R	S	R	R	S
GD0140	R	R	S	R	S	R	GD0712	R	R	S	R	S	R	GD08937	R	R	S	R	S	S
GD0166	R	R	S	R	S	S	GD0717	R	R	S	R	S	S	GD08942	R	R	S	R	S	R
GD0174	R	R	S	R	S	S	GD0718	R	R	S	R	S	–	GD082011	R	R	S	R	S	S
GD0176	R	R	S	R	S	R	GD0732	R	R	S	R	S	–	GD08T3	R	S	S	R	R	R
GD01118	R	R	S	R	R	–	GD0751	R	R	S	R	R	–	GD08T4	R	S	S	R	R	R
GD01154	S	S	S	S	S	S	GD0761	R	R	S	R	S	–	GD08T6	R	S	S	R	R	S
GD01165	R	S	S	R	S	R	GD0773	R	R	S	R	R	–	GD08T28	R	S	S	R	R	R
GD01181	R	R	S	R	S	S	GD0786	R	R	S	R	S	–	GD08T29	R	S	S	R	R	S
GD01198	R	R	S	R	R	R	GD0792	R	R	S	R	S	–	GD09111	R	R	S	R	R	R
GD01216	R	R	S	R	S	–	GD07106	R	R	S	R	R	R	GD09126	R	R	S	R	S	S
GD0202	R	R	S	R	S	R	GD07107	R	R	S	R	R	R	GD09167	R	R	S	R	S	S
GD0252	R	R	S	R	S	S	GD07113	R	R	S	R	S	–	GD09318	R	R	S	R	S	S
GD0375	R	R	S	R	S	S	GD07116	R	S	S	R	S	S	GD09660	R	R	S	R	R	R
GD0494	R	R	S	R	S	S	GD07119	R	R	S	R	S	S	GD092004	R	R	S	R	S	S
GD0499	R	R	S	R	S	R	GD07126	R	R	S	R	S	R	GD93203	R	S	S	R	R	S
GD04121	R	S	S	R	S	S	GD07127	R	R	S	R	S	S	GD93286	R	S	S	R	S	S
GD04137	R	R	S	R	S	S	GD07136	R	R	S	R	R	R	GD9559	R	R	S	R	S	S
GD04147	R	R	S	R	S	R	GD07143	R	R	S	R	S	–	GD97230	R	R	S	R	R	R
GD04206	R	R	S	R	S	S	GD07153	R	R	S	R	S	S	GD97322	R	S	S	R	S	S
GD04218	R	R	S	R	S	S	GD07164	R	R	S	R	R	–	GD9844	R	R	S	R	S	S

Table 1 continued

Isolates	Tetep	Kanto51	LTH	HR4	YXZ	T13	Isolates	Tetep	Kanto51	LTH	HR4	YXZ	T13	Isolates	Tetep	Kanto51	LTH	HR4	YXZ	T13
GD0507	R	R	S	R	S	S	GD07175	R	R	S	R	S	–	GD9866	R	R	S	R	S	S
GD056A	R	R	S	R	S	R	GD0805	R	R	S	R	R	R	GD9884	R	R	S	R	R	R
GD0520	R	R	S	R	S	S	GD0809	R	R	S	R	R	R	GD98123	R	R	S	R	R	R
GD0525	R	R	S	R	S	S	GD0825	R	R	S	R	S	R	GD98164	R	R	S	R	R	R
GD0543	R	R	S	R	S	R	GD0830	R	R	S	R	S	R	GD98236	R	R	S	R	S	S
GD05118	R	R	S	R	R	R	GD0834	R	R	S	R	S	R	GD98288	R	R	S	R	R	R
GD05135	R	R	S	R	S	R	GD0844	R	R	S	R	R	R	RS (%)	98.3	88.8	0	99.1	35.3	47.9

R resistant, *S* susceptible

–, data not determined

RS resistance spectrum % = (no. of incompatible isolates/no. of total tested isolates) × 100 %

GD93286, GD00193 and GD08T4, respectively. All three isolates are stable, easy to culture, and avirulent to HR4 but virulent to LTH (Table 1). The number of R and S individuals was recorded for analysis of goodness of fit to Mendelian segregation by the Chi-squared test.

Construction of mapping population and linkage analysis

In this study, the F_2 individuals corresponding to isolate GD93286 were named group A, while corresponding to isolate GD08T4 were named group B. Ten of the most resistant and most susceptible individuals from group A were selected to form R and S DNA pools, respectively, for bulk segregant analysis (BSA) as described by Michelmore et al. (1991). The parental DNAs were initially screened via a panel of 252 microsatellite (SSR) primer pairs from IRMI (International Rice Microsatellite Initiative, http://www.gramene.org) with a uniform distribution on 12 chromosomes, and those which were informative were then tested on the pools, where this screen suggested possible linkage with susceptibility. All the S individuals in group A were then genotyped to confirm the linkage and also used as mapping population. Total DNA extraction, polymerase chain reaction (PCR) and electrophoresis were conducted according to Pan et al. (2003) with minor modifications. Likewise, the individuals linked with resistance to GD08T4 were screened and confirmed in group B in the same way.

Marker development and gene mapping

The preliminary locations of the *R* genes were determined through linkage analysis. Additional SSR markers distributed in the approximate region released by the IRMI were selected to screen polymorphism between the parents. Additionally, new InDel (insertion/deletion polymorphism) markers for HR4 and LTH were designed based on the InDels between the *japonica* (Nipponbare; http://www.rgp.dna.affrc.go.jp) and *indica* (9311; http://www.genomics.org.cn) genomes. Polymorphic markers were used to run the mapping population. Linkage analysis was performed with MAPMAKER/v3.0, and the Kosambi function was used to translate the recombination frequency into centiMorgans (cM) (Pan et al. 2003).

Table 2 Resistance reactions of the F_1, BC_1F_1 and F_2 populations to three isolates

Test isolates	No. of F_1 individuals		No. of BC_1F_1 individuals		χ^2 for 1:1	χ^2 for 3:1	No. of F_2 individuals		$\chi^2$for 3:1	χ^2 for 15:1
	R	S	R	S			R	S		
GD93286	13	0	18	14	0.37	–	1,756	623	1.73	–
GD00193	7	0	12	9	0.19	–	195	59	0.34	–
GD08TY4	16	0	24	7	–	0.01	1,417	85	–	0.79

R resistance, *S* susceptible; $\chi^2_{0.05} = 3.84$ ($df = 1$)

Construction of genetic map and physical map

For *Pi-h2(t)* and *Pi-h3(t)*, the genetic maps were constructed according to the linked markers with the *R* loci and public information. For *Pi-h1(t)*, the genetic map was also constructed according to linked markers at first, then the physical positions of the markers were determined based on the Nipponbare genome using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST). Subsequently, a physical map spanning the *R* gene locus was constructed, based on the contig map of Nipponbare (http://rgp.dna.affrc.go.jp/IRGSP/download.html). Finally, an integrated map including the genetic and physical maps spanning the locus was constructed.

Results

Assessment of resistance spectrum

To assess the resistance spectrum, we inoculated HR4 together with five other cultivars with 116 *M. oryzae* isolates collected from different ecological areas of Guangdong Province over many years. The inoculations showed that HR4 was resistant to 115 out of 116 isolates (99.1 %) and was only susceptible to isolate GD01154 (Table 1). Tetep and Kanto51, two well characterized wide-spectrum resistance sources, showed resistance to 98.3 and 88.8 % of isolates, respectively. Tetep was only susceptible to two isolates (GD00120 and GD01154). In contrast, YXZ and T13, the two very popular cultivars, showed a narrow resistance spectrum, with resistance to 35.3 and 47.9 % of isolates, respectively. LTH was absolutely compatible with all 116 isolates, demonstrating its universal susceptibility. The results confirmed that HR4 confers broad-spectrum resistance to *M. oryzae* isolates.

Resistance inheritance of HR4 to three isolates

Three stable *M. oryzae* isolates (GD93286, GD00193 and GD08T4) were used for genetic analysis of the blast resistance in HR4. All the F_1 plants were resistant to the three isolates, similar to HR4, indicating that the resistance in HR4 was dominant. The ratios of R:S in the BC_1F1 and F_2 populations to isolates GD93286 and GD00193 matched 1:1 and 3:1, respectively (Table 2). Therefore, single dominant genes in HR4, respectively, conferred resistance to these two isolates. However, phenotypes of resistance and susceptibility in the BC_1F1 and F_2 populations against isolate GD08T4 fitted the segregation ratios 3:1 and 15:1, respectively (Table 2). Hence, the resistance in HR4 to isolate GD08T4 was deduced to be controlled by two independent dominant genes and either gene can confer resistance to the isolate no matter whether it is homozygous or heterozygous.

Preliminary mapping of *R* gene to isolate GD93286

Among the 252 SSRs, 34 were null and 93 were polymorphic between the two parents, resulting in a polymorphic frequency of 42.7 %. After the R and S DNA pools in group A were analyzed using the polymorphic markers, RM224 and RM144 on the long arm of chromosome 11 were found to differentiate resistant bulk from susceptible bulk. Subsequently, we assayed all the 623 susceptible F_2 plants with the two SSR markers. The results showed that 12 recombinants were identified by RM224 toward the centromeric side, while 15 recombinants were detected by RM144 on the other side. This implied that the genetic distance of markers RM224 and RM144 from the *R* gene was 0.96 and 1.20 cM, respectively. Hence, the

R gene, tentatively named *Pi-h1(t)*, was preliminarily mapped in the region between RM224 and RM144.

Preliminary mapping of *R* genes to isolate GD08T4 and constructing their genetic maps

Markers RM3359 and RM578 on the short arm of chromosome 1 were found to differentiate resistant bulk from susceptible bulk in group B, and they identified 20 and eight recombinants among the 85 susceptible F_2 plants, respectively, on the same side. Therefore, the *R* gene in HR4 on chromosome 1, tentatively named *Pi-h2(t)*, was located at genetic distances of ~4.7 and 11.7 cM away from markers RM578 and RM3359, respectively. The genetic location of *Pi-h2(t)* in relation to SSR markers is shown in Fig. 1. On the other hand, markers RM453, RM491 and RM179 on chromosome 12 were also found to differentiate resistant bulk from susceptible bulk in group B. RM453 and RM491 detected 21 and 15 recombinants, respectively, on the same side, while RM179 identified seven recombinants on the other side. The *R* gene in HR4 on chromosome 12, tentatively named *Pi-h3(t)*, was preliminarily mapped in the interval flanked by markers RM491 and RM179 at genetic distances of ~8.8 and 4.1 cM, respectively. The genetic location of *Pi-h3(t)* in relation to SSRs on chromosome 12 is shown in Fig. 1. Due to the small numbers of the susceptible F_2 plants to isolate GD08T4 and the lack of polymorphic SSR markers around the two *R* genes, we were not able to determine their precise locations in this study. This level of resolution, however, would be sufficient for marker-assisted selection (MAS) purposes.

Fine mapping of *Pi-h1(t)* and constructing its physical map

To narrow down the interval harboring *Pi-h1(t)*, eight SSRs (RM27298, RM27299, RM27318, RM27326, RM27334, RM3577, RM27360 and RM27363) within the interval between RM224 and RM144 were selected for polymorphic assay between the two parents. Additionally, we developed 19 new InDel markers by analyzing the equivalent homolog sequences between Nipponbare and 9311 available in the databases. Of the eight SSRs, three markers (RM27334, RM27360 and RM27363) were polymorphic. Of the 19 InDel markers, five were null and nine showed polymorphism between HR4 and LTH. The polymorphic SSR and InDel markers were used to screen the recombinants from RM224 and RM144 to narrow down the region encompassing the *Pi-h1(t)* locus.

As a result, markers K2, K3 and K5 detected seven, four and one recombinants derived from RM224,

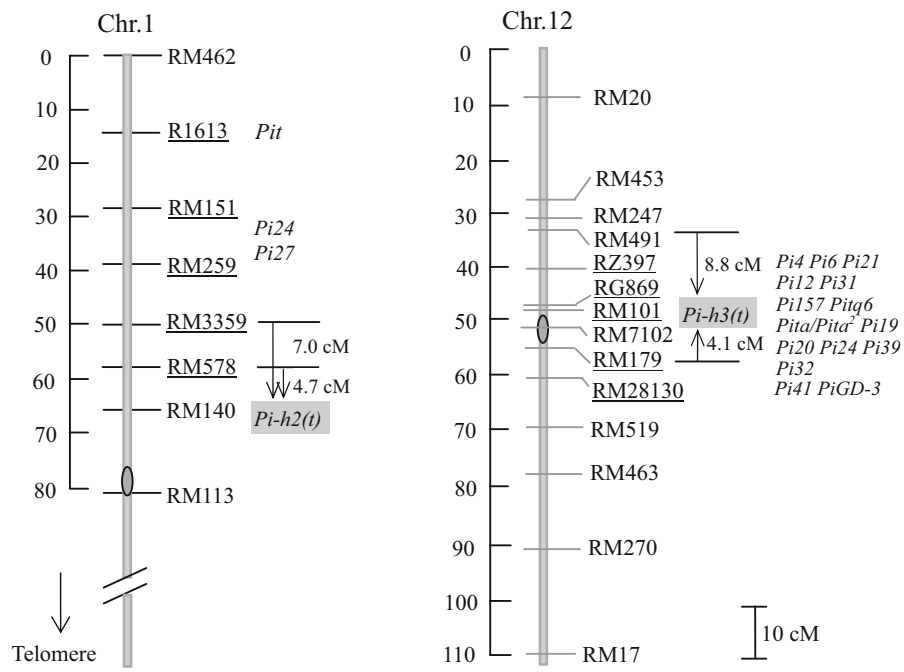

Fig. 1 Preliminary integrated linkage map of *Pi-h2(t)* and *Pi-h3(t)* and other blast resistance genes near them. *Note* the other blast resistance genes were integrated based on the reports by Ballini et al. (2008) and Yang et al. (2009). The *scale at the left* indicates genetic distance (cM). The resistance genes are anchored on the linkage map by compiling information on DNA markers linked to individual resistance genes. *Underlined* DNA markers are linked to the corresponding resistance genes

respectively, on the centromeric side, while markers RM27363, RM27360, K18, RM27334, K16 and K15 identified 14, 11, five, three, two and two recombinants derived from RM144, respectively, on the telomeric side. No recombinants were identified at the K7, K12 and K13 loci, indicating that these markers are co-segregating with the *Pi-h1(t)* gene (Fig. 2). Therefore, the *Pi-h1(t)* locus was finally delimited to the interval flanked by K5 and K15.

Table 3 lists all the linked markers with *Pi-h1(t)*. The tightly linked markers were located on the respective BAC/PAC clones of reference cv. Nipponbare by BLASTN analysis, and their physical positions were determined. The identified BAC/PAC clones were aligned as a contig map, and a physical map covering the *Pi-h1(t)* locus was subsequently constructed (Fig. 2). Finally, the *Pi-h1(t)* locus was localized to a 235.9-kb interval bounded by K5 and K15, which covers two BAC clones and a gap. The equivalent sequence of Nipponbare is annotated to contain 11 candidate genes, including NBS-LRR (nucleotide-binding site and leucine-rich repeat) and PK (protein kinase) type resistance gene analogs (RGAs), where *Pikm*, *Pikp*, *Pik* and *Pi1* reside (Ashikawa et al. 2008; Yuan et al. 2011; Zhai et al. 2011; Ashikawa et al. 2012; Hua et al. 2012).

Discussion

The present research by inoculation assay demonstrated that the *indica* rice accessions HR4 and Tetep conferred broad-spectrum resistance, reaching 99.1 and 98.3 %, respectively. Tetep is well-known for its durable and broad resistance to rice blast; it is also widely used in breeding programs worldwide for developing high-yielding blast-resistant cultivars (Ou 1985; Barman et al. 2004; Sharma et al. 2010). Tetep's broader-spectrum resistance than HR4 in this study does not mean that the resistance of HR4 to rice blast is better than Tetep, because the isolates used in this study were collected only from the ecosystem of Guangdong Province, where HR4 was identified. Therefore, the isolates seemed favorable to HR4. In fact, the resistance-spectrum of HR4 was only broader than that of Tetep by 0.8 %, or just one more isolate than Tetep. It is possible that the resistance spectrum of HR4 and Tetep would be different if tested by isolates from distinct ecosystems. YXZ and T13, two ever-popular *indica* cultivars, were widely cultivated in Guangdong Province 10 years ago. The decline of their blast resistance proved that many resistant varieties remain effective only for a few years after deployment (Hittalmani et al. 2000; Xiao et al. 2012),

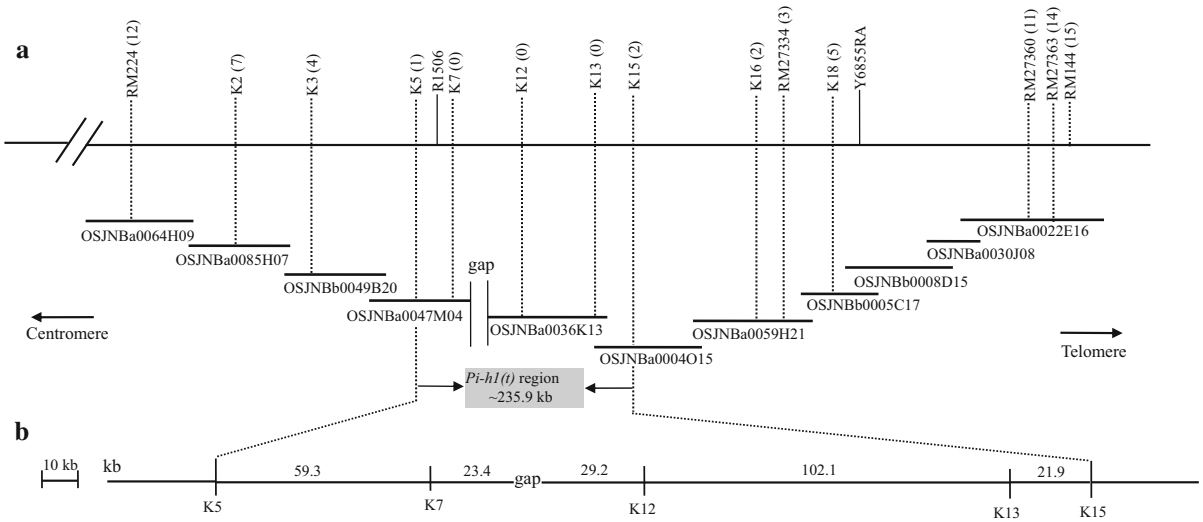

Fig. 2 Physical map of the *Pi-h1(t)* locus on rice chromosome 11. *Note* the two *long black horizontal lines* represent the rice chromosome. The 11 overlapping cv. Nipponbare BAC clones in **a** are indicated by *short horizontal lines*. The positions of markers on corresponding clones are shown by *vertical dotted lines*. The *numbers in parentheses* after the markers in **a** are the numbers of recombinants detected between the corresponding markers and the gene. The *numbers between markers* in **b** indicate the corresponding physical distance

Table 3 Molecular markers used to map the *R* genes in HR4 in this research

Marker	Forward primer sequence (5′–3′)	Reverse primer sequence (5′–3′)	Types	Annealing temperature (°C)	Product size (bp)
RM3359	CTCCGATTACCACCAATCAGG	TCTAATCCACTCCGCTAATCACC	SSR	56	183
RM578	AGATATACACGGCAATCCGATCC	AGATATACACGGCAATCCGATCC	SSR	56	287
RM453	GCTCGCATCTCTCTCCCTTATCG	GTTGTAGTTGATCCTCCCGTTGC	SSR	56	161
RM491	CACATGATGCGTAGCGAGTTGC	TTATGCCTCTCCCTTCCCAATTCC	SSR	56	270
RM179	CCCCATTA GTCCACTCCACCACC	CCAATCAGCCTCATGCCTCCCC	SSR	57	190
RM224	ATCGATCGATCTTCACGAGG	TGCTATAAAAGGCATTCGGG	SSR	57	122
K2	ATTCTTCCTCCCTTCCGCTTGC	CGTGCCAGTCCAATCAACA	SSR	57	192
K3	CGACAAATCAATAGGGCAACA	AAAGGAGCCACGCACGGT	SSR	54	96
K5	GATGACAGACCGTTGAGC	TACCTTCACCCTTTCCAT	InDel	56	197/206
K7	ACATCAATGGCTACAACT	TGCTAACGGTGCTGGTAT	InDel	53	183/188
K12	TTCCATCCCGCCCAACCT	ACCAGCCTTTCTCCGCCAC	InDel	55	128/137
K13	ATGGTCCCAACGGTGCTT	ATTCCCGTTGGTGACATT	InDel	54	126/134
K15	CCAGCCGAACAGAGCAAG	AGCAACAGCAGCGACACC	InDel	58	204/226
K16	TGGTGGCAAGAAGGCTGTA	CGTGTCCGCTCTGCTGTT	InDel	57	158/162
K18	AATGGGGTTGAGTTGGAG	GCAGCTTTGGGGAAATAC	InDel	55	241/248
RM27334	CAACCCTCCTATTCATTTAGCC	TATCCTTATCTATCCCGTCTCTCC	SSR	56	256
RM27360	CATGTTGCGTGTTTGTATACCACTC	GCCGCTGGTGAGTCGTAATGG	SSR	57	248
RM27363	ACTGCGTCCTCGTCACCTTCTGC	CACTCCTCCGCCTTCTTGTACGG	SSR	56	190
RM144	CATGTTGTGCTTGTCCTACTGC	AGCTAGAGGAGATCAGATGGTAGTGC	SSR	57	245

whereas they both showed good resistance to blast when they were released in the late 1990s. It reminds us of the continuously evolving process to develop blast-resistant varieties and the importance of mining durable or broad-spectrum resistance rice lines.

Tetep was reported to carry at least four *R* genes in its genome, *Pi-4*^{a} (*Pita*) on chromosome 12 (Inukai et al. 1994; Hittalmani et al. 2000), *Pi-tp(t)* on chromosome 1 (Barman et al. 2004), and *Pi54* (*Pi-k*^{h}) and *Pi1* on chromosome 11 (Sharma et al. 2005, 2010; Hua et al. 2012). Coincidently, the *R* genes in HR4 were also located on these three chromosomes. However, fine-scale mapping results showed that the *R* gene *Pi-h1(t)* in this study was located in the opposite side of *Pi54* but in the same region where *Pi1* resides (Sharma et al. 2005, 2010; Hua et al. 2012). The results indicated that *Pi-h1(t)* is different from *Pi54* but allelic or linked with *Pi1*. Meanwhile, *Pi-h2(t)* in this study and *Pi-tp(t)* are also located in different regions on chromosome 1, because RM578 is ~18.9 Mbp away from RM246, the marker linked with *Pi-tp(t)* (Barman et al. 2004; www.gramene.org). The interval spanning *Pi-h3(t)* in HR4 overlaps with that of *Pita* (Hittalmani et al. 2000), meaning that they may be allelic or linked. The broader resistance spectrum of HR4 than Tetep in this study suggests that there should be other *R* gene(s) in HR4 which have not been identified. Nevertheless, HR4 and Tetep share a common ground, which is that their broad-spectrum resistance results from the combination of multiple *R* genes harbored in their genomes. Kanto51 (*japonica*), one of the differential hosts used in China to identify pathotypes and monitor variations of *M. oryzae* races in the field, was reported to carry a major *R* gene, *Pik*, on the long arm of chromosome 11 (Ashikawa et al. 2012). In this study, *Pi-h1(t)* mapped to the same region where *Pik* resided. This suggests that *Pi-h1(t)* could be one of the alleles of *Pik*, like *Pikm*, *Pikp*, *Pik* and *Pi1*, because the genome analysis of resistant cultivars, including Tsuyuake, K60, Kusabue, Kanto51 and C101LAC, reveals only two adjacent NBS-LRR candidates, which confer resistance as a *R* gene by interaction, in the genomic region of respective cultivars (Ashikawa et al. 2008; Yuan et al. 2011; Zhai et al. 2011; Ashikawa et al. 2012; Hua et al. 2012). However, wide diversity in the genomic region was observed in different cultivars, which means that the flexible nature of the genomic region facilitates the generation of new *R* specificities during the process of evolution (Ashikawa et al. 2010, 2012; Costanzo and Jia 2010). Except for *Pik*, no other *R* gene(s) is reported in Kanto51, which may account for the narrower resistance spectrum of Kanto51 than HR4.

Virtually, to ascertain the relationship of *Pi-h1(t)* to *Pik*, *Pikm*, *Pikp* and *Pikh*, we have amplified the equivalent sequences in HR4 using primers which were designed according to the mRNA of *Pikm-1* and *Pikm-2*. The results of amplicon sequencing demonstrated that *Pi-h1(t)* is an allele of *Pik*, and it also includes two members *Pi-h1(t)-1* and *Pi-h1(t)-2*. BLAST results showed that the coding sequence of *Pi-h1(t)* was highly similar to those of *Pikp* and *Pikh*. The levels of sequence identity between *Pi-h1(t)-1*/*Pikp-1*, *Pi-h1(t)-1*/*Pik1*, *Pi-h1(t)-1*/*Pikm1* and *Pi-h1(t)-1*/*Pikh-1* reach 99.9, 97.5, 97.4 and 99.9 %, respectively (Supplementary Fig. A and Table A), while the levels of sequence identity between *Pi-h1(t)-2*/*Pikp-2*, *Pi-h1(t)-2*/*Pik2*, *Pi-h1(t)-2*/*Pikm2* and *Pi-h1(t)-2*/*Pikh-2* are 100.0, 99.9, 99.9 and 100.0 %, respectively (Supplementary Fig. B and Table B). Likewise, the cDNA sequence of *Pi-h3(t)* on chromosome 12 was verified to be the same as that of *Pita* from Katy but different from that of Yashiro Mochi (Supplementary Fig. C and Table C). As for *Pi-h2(t)* on chromosome 1, it may be a novel *R* gene, as no *R* genes have been reported in the region.

The successful identification of three *R* genes in the present study was attributed to the two isolates, GD93286 and GD08T4, which showed different pathogenicity. We did not select the two isolates at random to construct mapping populations and map the *R* gene(s). Our former research identified *Pi-h1(t)* on chromosome 11 with isolate GD93286, and introgressed it into several elite but highly susceptible restoring lines via MAS. However, the restoring lines improved only with *Pi-h1(t)* were always compatible with some isolates, including GD08T4 etc., when subjected to inoculation assay (Supplementary Tab. D). The results remind us that there exists more than one *R* gene in HR4. The distinct resistant genetic patterns of HR4 to isolates GD93286 and GD08T4 in the present study illustrated the rationale for using the two isolates, which resulted in the mapping of three different *R* genes. Genetic analysis indicated that a dominant major *R* gene was responsible for the resistance of HR4 to isolate GD00193. The *R* gene was proved to be *Pi-h1(t)* on chromosome 11 in other research

(unpublished data). There is no doubt that understanding the genetic mechanism in HR4 will allow us to use this wide spectrum resistance source more effectively with MAS in breeding programs.

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