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Identification and fine mapping of a resistance gene to *Magnaporthe oryzae* in a space-induced rice mutant

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Abstract Finding novel sources of resistance (R) to rice blast disease should facilitate breeding for improved resistance. The objectives of the present study were to evaluate reactions to blast and identify in a space-induced mutant an R gene to a representative isolate of rice blast pathogen. The mutant H4, its parent and twelve monogenic lines were evaluated for their responses to 35 isolates collected from Guangdong Province, China. H4 was found to be resistant to more isolates than its parent and the twelve monogenic lines, suggesting newly acquired resistance may be a function of one or more R genes. A representative isolate GD0193 was used to identify and map the R gene from H4. Genetic analysis revealed that resistance to the isolate GD0193 was controlled by a single dominant gene, designated *Pi46*(*t*). Linkage analysis using susceptible F2 individuals showed that *Pi46*(*t*) was mapped between the markers RM224 and RM27360 within 1.04 and 1.2 cM on the long arm of chromosome 11. Subsequently, *Pi46*(*t*) was delimited to an interval of approximately 183.7 kb flanked by the markers K67 and T94. These results provide essential information for the cloning of the *Pi46*(*t*) gene and will facilitate marker-assisted selection in rice breeding.

Keywords Fine mapping · Disease evaluation · Rice blast · Resistance (R) gene

Introduction

Blast, caused by the ascomycete fungus *Magnaporthe oryzae* (formerly known as *Magnaporthe grisea*, Couch and Kohn 2002), is one of the most devastating diseases of rice (*Oryza sativa* L.), and is responsible for significant yield losses under favorable environmental conditions worldwide. The use of resistance (R) genes in rice breeding is considered to be the most effective and economical strategy for blast control. However, many resistant varieties remain effective only for a few years after deployment, due to the emergence of new compatible pathogenic races (Hittalmani et al. 2000; Bonman et al. 1986). Therefore, it is necessary to pyramid R genes with different resistance spectra into a single elite cultivar in order to achieve durable resistance to this disease (Jeon et al. 2003).

The genetics of blast resistance in rice has been extensively studied. To date, more than 80 blast R

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genes or loci that confer resistance to different races of *M. grisea* have been documented (Ballini et al. 2008), most of which are distributed in clusters on chromosomes 6, 11 and 12. *Pi2*/*Piz*, *Pi8*, *Pi9*, *Pi13*, *Pi-d2*, *Pigm* and *Pi40* were mapped on chromosome 6 (Yu et al. 1991; Amante-Bordeos et al. 1992; Pan et al. 1998; Deng et al. 2006; Jeung et al. 2007); *Pi1*, *Pi7*, *Pi18*, *Pi38*, *Pi43*, *Pi44*, *PiCO39*, *Pif* and *Pik* on chromosome 11 (Wang et al. 1994; Chen et al.1999; Ahn et al. 2000; Hittalmani et al. 2000; Chauhan et al. 2002; Fjellstrom et al. 2004; Gowda et al. 2006; Luis et al. 2008; Lee et al. 2009a); and *Pita*, *Pi6*, *Pi12*, *Pi19*, *Pi20*, *Pi24*, *Pi39* and *Pi41* on chromosome 12 (Inukai et al. 1996; Hittalmani et al. 2000; Tsunematsu et al. 2000; Zhuang et al. 2002; Liu et al. 2007b; Yang et al. 2009). However, it is difficult to assess the actual number of R genes because allelism tests have not been easy to perform for R genes mapped in the same region of the genome due to unavailability of rice germplasm.

So far, more than ten major blast R genes have been cloned and characterized: *Pib*, *Pita*, *Pi9*, *Pi2*, *Piz-t*, *Pid2*, *Pi36*, *Pi37*, *Pikm*, *Pi5* and *Pid3* (Wang et al. 1999; Bryan et al. 2000; Qu et al. 2006; Zhou et al. 2006; Chen et al. 2006; Liu et al. 2007a; Lin et al. 2007; Ashikawa et al. 2008; Lee et al. 2009b; Shang et al. 2009). *Pid2* encodes a serine/threonine-kinase membrane-spanning protein, and the other genes belong to the NBS-LRR R gene family (Lee et al. 2009b). However, only one field blast R gene, *pi21*, has been cloned, which encodes a protein with heavy-metal-binding and proline-rich domains (Fukuoka et al. 2009). The cloned R genes have greatly advanced our understanding of the molecular mechanism of disease resistance in rice and should impact rice breeding for improved resistance to blast.

Many rice breeding institutions have developed diverse genetic resources, including near-isogenic lines, mapping and mutant populations induced by chemicals and irradiations, to identify genetic variations useful for trait improvement (Wu et al. 2005; Mei et al. 1998; Li et al. 2007a; Xiao et al. 2009). Among induced mutations, space opens a new opportunity for creating new plant germplasm. In this paper, we describe the creation of a new source of resistance to blast in a space-induced rice mutant H4 and the tagging of the corresponding R gene using molecular markers.

Materials and methods

Rice materials and *M. grisea* isolate

The control line, Zhonger Ruanzhan, is an elite *indica* rice variety with moderate-spectrum resistance to a number of tested blast isolates. It was released in 2001 as a conventional cultivar in Guangdong Province, China, and has been successfully planted on large scales. Fifty grams of dry seeds of Zhonger Ruanzhan had traveled to space in China's 18th recoverable satellite (orbit 63°, 200/350 km) for an 18-day flight in November 2003. The first to third generations of mutated offspring were inoculated with blast isolate GD0193, which is virulent to its parent at the seedling stage. The resistant individuals obtained were grown to produce advanced generations by self-pollination. Then the fourth generation was inoculated with 36 blast isolates to screen for desirable mutants, which were further planted in a natural blast nursery, Conghua, Guangdong, China, and tested for field resistance to various blast isolates. A desirable mutant named H4 that exhibited a high level of resistance to blast disease was obtained from the screening.

Magnaporthe grisea isolate GD0193, collected from Guangdong, China, is a highly stable race. When tested on 14 different monogenic lines, GD0193 can infect eight lines, including lines harboring *Pik*, *Pikp*, *Pi7*(*t*), *Pii*, *Piz*, *Pi3*, $Pita^2$, and *Pib*.

Blast resistance evaluation

To thoroughly investigate the resistance of the rice mutant H4, it, its parent line and twelve monogenic lines harboring different R genes (Tables 2 and 3) were tested for resistance to a total of 35 isolates collected from Guangdong, China. These monogenic lines were used as monogenic differential hosts to identify pathotypes and monitor variations of *M. grisea* races in the field.

Mapping population and blast phenotyping

For mapping the R gene in H4 to isolate GD0193, H4 was crossed with a highly susceptible variety B23 (*japonica*). F1 was selfed to produce the F2 population and backcrossed with B23 to produce the BC1F1 population. The F1, F2 and BC1F1 populations were

inoculated with GD0193 for genetic analysis. Two-week-old seedlings were spray-inoculated with spore suspension (1×10^5 spores/ml) and kept in a dew growth chamber for 24 h in darkness at 26°C. The plants were subsequently transferred into the semi-temperature-controlled greenhouse, which was maintained at around 22–30°C and 90% relative humidity. After 7 days, disease lesions on the inoculated rice leaves were scored on a scale of 0–9 according to the standard described by Marchetti et al. (1987), with ratings of 0–3 considered as resistant, and 4–9 as susceptible (S). The number of R and S individuals was counted and the data was subjected to chi-square analysis to test the goodness of fit to Mendelian ratios.

Linkage analysis and markers development

R and S DNA pools, representing 15 highly resistant and susceptible F2 individuals, were made for bulked segregant analysis (BSA; Zhu et al. 2004). DNA extraction, PCR and electrophoresis were conducted with minor modifications according to Chen et al. (2005). A total of 212 uniformly distributed simple sequence repeat (SSR) markers on 12 chromosomes, which were released by the IRMI (International Rice Microsatellite Initiative; http://www.gramene.org), were used for the polymorphism survey of the two parents. Polymorphic markers were used to screen R and S DNA pools to identify markers linked to the R gene. The markers differentiating R and S pools were used for linkage analysis of the susceptible F2 population (recessive class analysis; Pan et al. 2003). To finely map the R gene, new SSR markers were intentionally designed according to the reference genome sequence of cv. Nipponbare using the software SSRIT (http://www.gramene.org/microsat/ssrtool) and Primer Premier 5.0 (http://www.PremierBiosoft.com). In addition, new InDel (insertion/deletion polymorphism) markers for H4 and B23 were designed based on the InDels between the *japonica* (Nipponbare; http://www.rgp.dna.affrc.go.jp) and the *indica* (9311; http://www.genomics.org.cn) genomes. A total of 250 highly susceptible (recessive) F2 individuals were used for preliminary mapping of the R gene, with an additional 953 recessive F2 individuals for fine mapping. The recombination frequency between adjacent loci was estimated as $N_r/2N_T$ (N_r is the number of recombinants, and N_T is the overall population size; Pan et al. 2003; Yang et al. 2009). Subsequently the recombination frequency was transformed into centiMorgans (cM) according to the Kosambi function (Pan et al. 2003).

Physical mapping of the R gene

To construct a physical map, primer sequences of linked markers were matched with the reference sequence of cv. Nipponbare by using BLASTN (http://www.ncbi.nlm.nih.gov/BLAST). The corresponding bacterial artificial chromosome (BAC) clones of cv. Nipponbare were downloaded from NCBI, and sequence alignments were conducted using Pairwise BLAST (http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html). Subsequently, a contig map spanning the R locus was established using the matched clones, and the physical map was constructed by the composite sequence derived from the contig map (Deng et al. 2006; Liu et al. 2007b).

Results

Resistance characterization of H4

To evaluate the potential usefulness of mutant H4 for resistance improvement in Guangdong, China, H4, its parent, and twelve monogenic lines harboring R genes on different chromosomes, were tested with blast resistance spectrum to 35 isolates collected from Guangdong, China. The results, that H4 conferred resistance to all the 35 isolates, indicated that H4 governed a broader blast resistance spectrum than its parent and the 12 monogenic lines (Table 1). It proved that the mutant H4 should be an excellent resistance source for improving blast resistance in rice. The results also showed that H4, its parent and the twelve monogenic lines exhibited different resistance responses to part of the 35 isolates (Table 2), implicating different resistance mechanisms in these lines.

Inheritance of resistance to isolate GD0193 in H4

All the 12 F1 plants from the cross between H4 and B23 exhibited high resistance to GD0193, indicating dominant resistance in H4. In total 5,345 F2 and 43 BC1F1 individuals derived from the cross mentioned

Table 1 Resistance spectra of H4, Zhonger Ruanzhan (ZE) and 12 monogenic lines

Lines	H4	ZE	IRBL3	IRBL4	IRBL6	IRBL7	IRBL8	IRBL9	IRBL10	IRBL16	IRBL18	IRBL22	F98-7	F128-1
Gene	*Pi46(t)* and others	Unkown	*Pii*	*Piks*	*Pik*	*Pikp*	*Pikh*	*Piz*	*Piz5*	*Pish*	*Pi1*	*Pi9*	*Pikm*	*Pita*^{2}
Resistance spectrum (%)	100	57.1	8.6	2.9	54.3	51.4	71.4	25.7	88.6	28.6	94.3	60.0	8.6	65.7

Resistance spectrum % = (no. of incompatible isolates/no. of total tested isolates) × 100%; the 12 monogenic lines were provided by the Institute of Plant Protection, Chinese Academy of Agricultural Sciences

above were inoculated with isolate GD0193. Segregation of resistant (R) and susceptible (S) progenies fitted a 3:1 ratio in the F2 population (4,037R:1,308S, $\chi^2 = 0.77$, $P > 0.25$) and an 1:1 pattern in the BC1F1 population (24R:19S, $\chi^2 = 0.38$, $P > 0.45$). This suggested that a single dominant gene in H4 conferred its resistance to the isolate.

Preliminary mapping of the R gene

A total of 212 SSR markers distributed on 12 chromosomes were deployed to identify the markers linked with the R gene. Two polymorphic markers, RM224 and RM27360, on the long arm of chromosome 11 were associated with the R gene. To confirm the reliability of linkage between the two markers and the R gene, we assayed the genotypes of 250 highly susceptible F2 plants using the two markers. Five recombinants were detected at the RM224 locus towards the centromeric side, and six different recombinants at the RM27360 locus towards the telomeric side. Hence, the R gene, tentatively named *Pi46*(*t*), was preliminarily located in the region flanked by markers RM224 and RM27360.

Fine mapping of *Pi46*(*t*)

To finely locate the *Pi46*(*t*) locus, we detected an additional 953 recessive F2 individuals with markers RM224 and RM27360. Some susceptible F2 plants died soon after disease evaluation, and thus their DNA could not be extracted. In total, 25 recombinants were identified by RM224 and 29 by RM27360, which suggested that they are linked to the R gene within 1.04 and 1.2 cM, respectively. Chromosome walking of the gene was conducted from both loci with the 54 recombinants.

To find additional markers associated with *Pi46*(*t*), 16 SSR markers in the target region were adopted from IRMI for further linkage analysis. Only one marker, RM27358, locating in the same BAC clone with RM27360, exhibited polymorphism between H4 and B23, which detected 23 recombinants derived from RM27360. Additionally, 16 SSR markers and 27 InDel markers between RM224 and RM27358 were developed according to sequence information of the *japonica* Nipponbare and the *indica* 9311 genomes. Among them, five SSR markers and seven Indel markers were found to display polymorphism

Table 2 Phenotypes of H4, Zhonger Ruanzhan (ZE), and twelve monogenic lines to eight isolates of *M. grisea* collected from Guangdong, China

Lines	Gene	Different isolates of *M. grisea* collected from Guangdong							
		GD0176	GD3286	GD0193	GD8236	GD595	GD1165	GD07126	GD0830
H4	*Pi46*(*t*) and others	R[a]	R	R	R	R	R	R	R
ZE	Unkown	S	S	S	S	R	R	R	R
IRBL3	*Pii*	S	S	S	R	S	S	S	S
IRBL4	*Piks*	S	S	S	S	S	S	S	S
IRBL6	*Pik*	R	S	S	R	R	S	S	S
IRBL7	*Pikp*	R	S	S	R	R	S	S	S
IRBL8	*Pikh*	R	R	S	R	R	R	S	R
IRBL9	*Piz*	S	S	S	S	S	S	S	S
IRBL10	*Piz5*	R	R	S	R	S	R	R	R
IRBL16	*Pish*	S	S	S	S	S	S	S	R
IRBL18	*Pi1*	R	S	R	R	R	S	S	R
IRBL22	*Pi9*	R	S	R	R	S	S	S	R
F98-7	*Pikm*	S	S	S	S	S	S	S	R
F128-1	*Pita*^{2}	S	S	S	S	S	R	R	R

[a] *R* resistant, *S* susceptible

The 12 monogenic lines were provided by the Institute of Plant Protection, Chinese Academy of Agricultural Sciences

between H4 and B23 (Table 3). For further linkage analysis, all these 12 polymorphic markers were used to screen the recombinants detected by RM224 and RM27358. On the centromeric side, 15, 11, 2, 1, 1 and 1 recombinants derived from RM224 were identified with markers T26, T9, K107, K53, K134 and K67, suggesting that genetic distances away from the R gene were 0.62, 0.46, 0.08, 0.04, 0.04 and 0.04 cM (Fig. 1a), respectively. On the opposite side, 9, 2, 2 and 1 recombinants derived from RM27358 were detected by markers T124, K46, K47 and T94, which meant that genetic distances away from the R gene were 0.38, 0.08, 0.08, and 0.04 cM (Fig. 1a), respectively. No recombinants were identified at the K22, O151 and T103 loci, indicating that these markers co-segregated with the *Pi46*(*t*) locus (Fig. 1a). Thus, the *Pi46*(*t*) gene was delimited in a region flanked by K67 and T94, and the genetic region spanning the R gene locus was about 0.08 cM in length (Fig. 1a).

Physical mapping of the *Pi46*(*t*) locus in silico

To physically map the *Pi46*(*t*) locus, all the anchor markers used in the chromosome walking to the locus were located on the respective BAC clones of reference cv. Nipponbare by BLASTN analysis, and their physical positions were determined. The identified BAC clones were aligned as a contig map covering the locus by using Pairwise BLAST analysis (Fig. 1b). Subsequently, a physical map covering the *Pi46*(*t*) locus was constructed based on the two overlapping clones flanked by the two closest markers K67 and T94 (Fig. 1c). According to this map, the *Pi46*(*t*) locus was physically located in an interval flanked by K67 and T94, which covered ~183.7 kb in length based on the reference sequence.

Discussion

Numerous laboratories in different rice-growing countries have been trying to induce blast resistance by chemical treatment or radiation (Wu et al. 2005; Zhang et al. 2003), which is the routine method of generating genetic variability for breeding research and genetic studies. Space travel can also induce mutations (Mei et al. 1998; Li et al. 2007b; Xiao et al. 2009). Among the complex factors in space, including high-energy particles, cosmic rays, microgravity, super-high vacuum, magnetic fields etc., all kinds of high-energy particle radiation are considered to be

Table 3 PCR-based markers linked to *Pih4*(*t*) locus

Markers	Primer sequence (5′–3′)[c]	Genomic position (bp)[d]	Annealing temperature °C)[e]	Expected size (bp)
RM224	F: ATCGATCGATCTTCACGAGG	26796469–26796487	56	122
	R: TGCTATAAAAGGCATTCGGG	26796590–26796571		
RM27360	F: CATGTTGCGTGTTTGTATACCACTC	28135116–28135140	57	248
	R: GCCGCTGGTGAGTCGTAATGG	28135363–28135343		
RM27358	F: ACGACAAGAAGACCGACTACTCC	28105787–28105809	56	291
	R: ATGCGTTTGAACAGCTATCAGG	28106077–28106056		
T26[a]	F: ATTCTTCCTCCCTTCCGCTTGC	26898253–26898232	57	192
	R: CGTGCCAGTCCAATCAACA	26898062–26898080		
T9[a]	F: CGACAAATCAATAGGGCAACA	26948794–26948814	54	96
	R: AAAGGAGCCACGCACGGT	26948889–26948872		
K107[b]	F: ACATCAATGGCTACAACT	27136149–27136166	53	183/188
	R: TGCTAACGGTGCTGGTAT	27136336–27136319		
K53[b]	F: TTCCATCCCGCCCAACCT	27493029–27493046	55	128/137
	R: ACCAGCCTTTCTCCGCCAC	27493150–27493132		
K134[b]	F: GATGGCGAGATGGTTGTC	27539755–27539772	55	180/192
	R: GCCTTTGAGATAGGGATTGC	27539934–27539915		
K67[b]	F: ATGGTCCCAACGGTGCTT	27595148–27595165	54	126/134
	R: ATTCCCGTTGGTGACATT	27595282–27595265		
T124[a]	F: TGAATGGACGGTTGGATAG	28060957–28060975	55	201
	R: TTTAGGCTTGAGAATGGATG	28061157–28061138		
K46[b]	F: AATGGGGTTGAGTTGGAG	27846001–27846018	55	241/248
	R: GCAGCTTTGGGGAAATAC	27846241–27846224		
K47[b]	F: CCGTGAAACTGTTGAAAG	27830159–27830176	55	80/91
	R: TCTGTCTGGACCTATCCC	27830238–27830221		
T94[a]	F: GGGAGTGAGCCGAACAGA	27778861–27778844	58	108
	R: TGGTGGACTGCCAACTTCT	27778754–27778772		
K22[b]	F: CCATTGCTCTTGCTCGTC	27600993–27600976	54	129/132
	R: TCACTTCAGCGTCGTCAT	27600865–27600882		
O151[b]	F: CCAGCCGAACAGAGCAAG	27617176–27617159	58	204/226
	R: AGCAACAGCAGCGACACC	27616973–27616990		
T103[a]	F: TGGTGGCAAGAAGGCTGTA	27747635–27747617	57	158
	R: CGTGTCCGCTCTGCTGTT	27747478–27747495		

[a] SSR marker

[b] InDel marker

[c] *F* forward, *R* reverse

[d] Genomic position of each marker was determined by using BLASTN based on the reference sequence of rice cv. Nipponbare

[e] All PCR reactions were conducted following the procedure: 4 min at 94°C, 35 PCR cycles (30 s at 94°C, 35 s at the corresponding temperature given above and 35 s at 72°C), and a final extension step of 5 min at 72°C. After PCR, the products were detected on 8% acrylamide gel

the major factor that induces changes in organisms (Badhwar 1997; Wei et al. 2006; Li et al. 2007b). In 1987, China began to use recoverable satellites to conduct space mutation breeding for crops. Carrying crop seeds on recoverable satellites to create novel germplasm has become an important part of mutation breeding in China (Wei et al. 2006). So far, many commercially novel varieties with high yield and

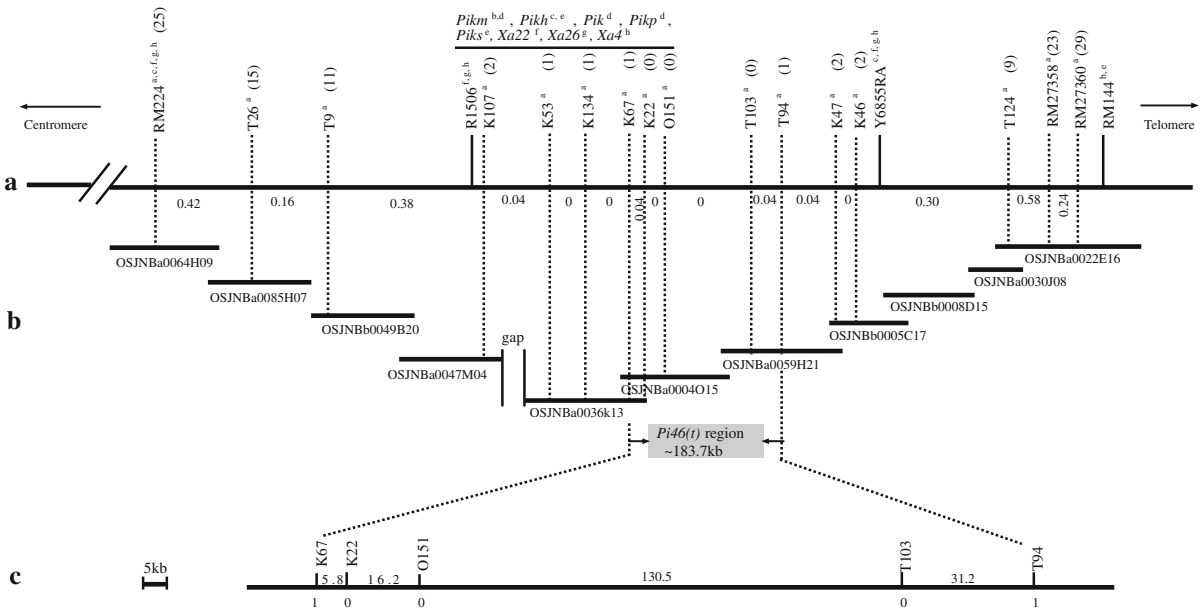

Fig. 1 **a** An integrated map of nine resistance genes including the *Pi46*(*t*) gene on rice chromosome 11. The positions of the resistance genes and markers were integrated from the following articles: *a* this study, *b* Li et al. 2007a, *c* Xu et al. 2008, *d* Hayashi et al. 2006, *e* Fjellstrom et al. 2004, *f* Wang et al. 2003, *g* Yang et al. 2003, *h* Sun et al. 2003. The *numbers below* the map are relative genetic distances in cM and the corresponding markers are listed *above* the map. The *numbers in parentheses* after the markers are the numbers of recombinants detected between the corresponding markers and the *Pi46*(*t*) gene. **b** A contig map spanning the *Pi46*(*t*) locus. The *short horizontal lines* represent BAC clones of cv. Nipponbare released by IRGSP (International Rice Genome Sequencing Project; http://www.rgp.dna.affrc.go.jp) and are anchored by the corresponding markers linked to the *Pi46*(*t*) locus. The *vertical dotted lines* denote the positions of each marker. **c** A physical map of the *Pi46*(*t*) locus between the markers K67 and T94. The *numbers above* the map are estimated relative physical distances in kb based on the reference sequence, and the *numbers below* the map are the numbers of recombinants detected between the corresponding markers and the *Pi46*(*t*) gene

good quality have been released, and a large quantity of new breeding lines with good traits have been created from various crop seeds after space mutation, which shows a promising application for this technique in crop breeding (Cyranoski 2001; Wei et al. 2006; Cheng et al.2007; Xiao et al. 2009). In this study, the control line, Zhonger Ruanzhan, was resistant to 20 of the 35 tested isolates, whereas the mutant H4 was resistant to all 35 isolates. Obviously, H4 gained a broader resistance spectrum, indicating that H4 obtained new resistance not carried by the control line. The resistance gained was probably due to one or more resistant genes, which could be attributed to genetic mutagenesis occurring in the seeds while in the retrievable satellite.

This study finely located the *Pi46*(*t*) gene coincidently in a region near the telomere of the long arm of chromosome 11. How was the new resistance generated? Analysis of R-like genes in the whole rice genome revealed that most of the R-like genes (24.98%) reside on chromosome 11 (Zhou et al. 2004; The Rice Chromosomes 11 and 12 Sequencing Consortia 2005). In particular, the long arm of rice chromosome 11 contains almost twice the number of R-like genes compared to the short arm, and large gene clusters (35 and 37 members) are present between regions of 80–90 cM (between 20,001,952 and 22,632,644 bp) and 110–119 cM (between 26,213,144 and 28,180,239 bp), respectively (The Rice Chromosomes 11 and 12 Sequencing Consortia 2005). Our results showed that the *Pi46*(*t*) gene is located in the cluster spanning the region of 110–119 cM on the long arm of chromosome 11, one of the most highly concentrated regions of R-like genes in the rice genome (Chen et al. 1999; Yang et al. 2003; Xu et al. 2008). It is possible that the space environment may cause changes in the genome such as insertion, deletion and recombination. The changes in clusters rich in R-like genes may activate existing R genes or give rise to new R genes. We found high polymorphisms between the parent and the

space-induced mutants on rice chromosome 11 (Xiao et al. 2009), implicating potential hotspots for space mutation in this chromosome. Analysis of reference sequences of cv. Nipponbare indicated that the target region flanked by markers K67 and T94 is rich in repetitive sequences (data not shown). The repetitive sequences might provide a mechanism for unequal crossover, resulting in recombinant events between R-like homologues, and consequently new R genes. Some reports have proved that the region covering the *Pi46*(*t*) gene is of hyper-variability and high divergence (Xu et al. 2008; Ashikawa et al. 2008).

Our results showed that the ratios of physical to genetic distances were not uniform across the telomeric region of rice chromosome 11 harboring the *Pi46*(*t*) locus, which was also reported by other researchers (Sun et al. 2003; Wang et al. 2003; Yang et al. 2003; Li et al. 2007a; Xu et al. 2008). There are cross-cold and cross-hot regions in the rice genome. Only a few recombination events occurred in the interval between K107 and K46, indicating that this region is a typical cross-cold region. The regions with more recombination events were flanked by marker pairs T9–K107 and K46–T124. The two outside regions, RM224–T9 and T124–RM27360, displayed the most frequent recombination events and are a typical cross-hot region. Overall, the cross-cold and cross-hot regions found in this study are consistent with other researchers' results (Li et al. 2007a; Xu et al. 2008). The three co-segregation markers in this study spanned a region of ~146.7 kb, suggesting that recombination events were highly suppressed in the core interval covering the R locus. Suppression of recombination had also been observed in other R-gene-containing regions, such as *Pita*^{2} (Nakamura et al. 1997), *Pi-CO39* (Chauhan et al. 2002), *Pi5* (Jeon et al. 2003) and *Pi37* (Chen et al. 2005) loci.

In addition to the broader resistance spectrum of H4, the differential reactions between H4 and the monogenic lines to the tested 35 isolates indicated that the R gene(s) in H4 differs from those in the monogenic genes. There might be more than one R gene in H4 contributing to its broad resistance spectrum to blast isolates. The R gene *Pi46*(*t*) in H4 was finely mapped in a region near the telomere of the long arm of chromosome 11, which also harbors other blast R genes including *Pik*, *Pikh*, *Pikm*, *Pikp*, *Piks*, *Pish*, *Pi1*, *Pif*, *Pilm2* and *Pi43*(*t*) (Lee et al. 2009a). A series of test crosses will be needed to determine allelic relations of *Pi46*(*t*) with other known blast R genes. The equivalent sequence in cv. Nipponbare of the ~183.7 kb region was subjected to gene annotation by RiceGAAS (http://www.ricegaas.dna.affrc.go.jp), and 13 candidate genes, including NBS-LRR (nucleotide-binding site and leucine-rich repeat) and PK (protein kinase) type resistance gene analogs (RGAs), were identified (data not shown). In fact, we had performed RT-PCR analysis and found that a candidate gene is expressed constitutively from 0 to 48 h after inoculation, with a slight increase from 8 to 24 h (data not shown). Whether this candidate is *Pi46*(*t*) is currently being tested. The markers developed in this study could be used in the local rice resistance breeding program by marker-assisted selection.

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