Fine Mapping of a New Race-Specific Blast Resistance Gene, *Pi-hk2*, in *Japonica* Heikezijing from Taihu Region of China

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ABSTRACT

Heikezijing, a *japonica* rice landrace from the Taihu region of China, exhibited broad-spectrum resistance to more than 300 isolates of the blast pathogen (*Magnaporthe oryzae*). In our previous research, we fine mapped a broad-spectrum resistance gene, *Pi-hk1*, in chromosome 11. In this research, 2010-9(G1), one of the predominant races of blast in the Taihu Lake region of China, was inoculated into 162 recombinant inbred lines (RIL) and two parents, Heikezijing and Suyunuo, for mapping the resistance-blast quantitative trait loci (QTL). Three QTL (*Lsqt4-1*, *Lsqt9-1*, and *Lsqt11-1*) associated with lesion scores were detected on chromosomes 4, 9, and 11 and two QTL (*Lnqt11-1* and *Lnqt9-1*) associated with average lesion numbers were detected on chromosomes 1 and 9. The QTL *Lsqt9-1* conferring race-specific resistance to 2010-9(G1) at seedling

stages showed logarithm of the odds scores of 9.10 and phenotypic variance of 46.19% and might be a major QTL, named *Pi-hk2*. The line RIL84 with *Pi-hk2* derived from a cross between Heikezijing and Suyunuo was selected as *Pi-hk2* gene donor for developing fine mapping populations. According to the resistance evaluation of recombinants of three generations (BC₁F₂, BC₁F₃, and BC₁F₄), *Pi-hk2* was finally mapped to a 143-kb region between ILP-19 and RM24048, and 18 candidate genes were predicted, including genes that encode pleiotropic drug resistance protein 4 ($n = 2$), WRKY74 ($n = 1$), cytochrome b5-like heme/steroid-binding domain containing protein ($n = 1$), protein kinase ($n = 1$), and ankyrin repeat family protein ($n = 1$). These results provide essential information for cloning of *Pi-hk2* and its potential utility in breeding resistant rice cultivars by marker-assisted selection.

Rice blast, caused by the fungus *Magnaporthe oryzae*, is one of the most destructive diseases of rice. The annual loss of yield caused by rice blast was from 10 to 30%, which was enough for feeding approximately 60 million people (Skamnioti and Gurr 2009). The application of resistance (*R*) genes is considered as one of the most effective methods for minimizing crop losses caused by the disease (Fjellstrom et al. 2004). Developing rice cultivars with durable *R* genes to blast is the most economically and environmentally friendly management approach to control rice blast disease (Wang et al. 1994). However, resistant cultivars often remain effective for only a few years because the pathogen *M. oryzae* has sufficient genetic flexibility to eventually overcome them (Dean et al. 2005; Skamnioti and Gurr 2009). Thus, one strategy of pyramiding multiple *R* genes has been proposed as an alternative way for increasing the durability of resistance (Fjellstrom et al. 2004).

Over the past few years, approximately 100 rice *R* genes have been mapped (Sharma et al. 2012), and at least 24 of *R* genes have been cloned. Most of the *R* genes are dominant and only *pi21* and *pi55* are recessive (Fukuoka et al. 2009; He et al. 2012). Some *R* genes are clustered on chromosomes 6 and 11 (Chen et al. 2015), such as *Pi9*, *Pi2*, *Piz-t*, *Pigm(t)*, and *Pi50(t)* on chromosome 6 (Deng et al. 2006; Zhou et al. 2006; Zhu et al. 2012) and *Piks*, *Pikh*, *Pikm*, *Pikp*, *Pik*, *Pi1*, *Pi7(t)*, and *Pi44(t)*, located near the telomeric end of the long arm of chromosome 11 (Ashikawa et al. 2008; Campbell et al. 2004; Chen et al. 1999; Hua et al. 2012; Yuan et al. 2011; Zhai

et al. 2011, 2014). Most *R* genes encode nucleotide-binding site leucine-rich repeat (NBS-LRR) proteins, except for *Pi-d2*, encoding a B-lectin receptor kinase (Chen et al. 2006) and resistance quantitative trait loci (QTL) gene *pi21*, encoding a proline-rich protein (Fukuoka et al. 2009). Among the 24 cloned genes, *Pi-ta* (Lee et al. 2009a), *Pi36* (Liu et al. 2007b) and *Pit* (Hayashi and Yoshida 2009) are reported as race-specific *R* genes. The race-specific recognition of *R* genes was usually determined by the conserved domains as the LRR region, the Toll/interleukin-1 receptor, and coiled-coil domains (Luck et al. 2000; Rairdan et al. 2008). In most cases, these conserved domains are involved in both pathogen detection and the activation of the downstream response (DeYoung and Innes 2006). *Pi-ta*, *Pi36*, and *Pit* trigger race-specific resistance through LRR domain recognition of the pathogen.

In our previous research, it was found that 'Heikezijing', a *japonica* rice landrace from the Taihu region of China, exhibited broad-spectrum resistance to at least 300 isolates. In total, 22 QTL conferring resistance to 17 isolates were identified in Heikezijing, and 5 QTL on chromosome 9 were effective against 13 isolates, with logarithm of the odds (LOD) scores of 2.65 to 14.57 and phenotypic variance of 7.43 to 68.61% (Shi et al. 2010; Wang et al. 2002). Main-effect locus *qt19-4-1* with the LOD score of 14.57 could explain 68.61% phenotypic variance against blast isolate JS02-95(ZE3). In this article, 2010-9(G1), one of the predominant isolates in the Taihu Lake region of China, was used to inoculate the mapping populations (recombinant inbred lines [RIL], BC₁F₁, BC₁F₂, BC₁F₃, and BC₁F₄) to fine map the race-specific *R* gene *Pi-hk2*.

MATERIALS AND METHODS

Plant materials and growth. In all, 162 RIL (F_{2:9}) derived from a cross between broad blast-resistant *japonica* landrace Heikezijing and blast-susceptible *japonica* landrace 'Suyunuo' were used to

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*The e-Xtra logo stands for "electronic extra" and indicates that one supplementary table is published online.

identify the resistant QTL to blast (*M. oryzae*). RIL84, selected from the RIL population and carrying few of the genomes from the resistance parent Heikezijing, was used as the target locus *Pi-hk2* donor to develop the fine-mapping population. The BC₁F₂ population with 1,560 plants, derived from a backcross of RIL84 and Suyunuo, was used for identifying recombinants in the target region with *Pi-hk2*.

Seed were sown in plastic trays (60 by 30 by 5 cm) with sieved garden soil, as described by Wang et al. (2002). In total, 28 lines and two parents (Heikezijing and Suyunuo as the resistant and susceptible controls, respectively) were sown in each tray, and 10 seeds/line were sown one den. Seedlings were grown in a greenhouse at 22 to 30°C with a light and dark cycle of 16 and 8 h, respectively, until they were at the four-leaf stage for disease evaluation and DNA preparation. For expression analysis of candidate genes, the leaves of Heikezijing seedlings inoculated by blast isolate 2010-9(G1) were sampled at 0, 4, 12, and 72 h after inoculation, frozen in liquid nitrogen immediately, and stored at -80°C.

Pathogen, inoculation, and disease evaluation. Isolate 2010-9 (G1), predominant in Jiangsu Province, China and provided by the Plant Protection Research Institute in Jiangsu Academy of Agricultural Sciences, was used for inoculating 162 F_{2:9} RIL and two parents. Seven isolates belonging to different races, including 2009-70-1 (A7), 2010-49-1 (B1), 2010-28 (C1), 2010-35-1 (D13), 2011-40 (E3), 2010-42-2 (F1), and 2010-9 (G1), were used to inoculate the *Pi-hk2* donor RIL84 and the parents Heikezijing and Suyunuo.

Rice seedlings (four-leaf stage) were put into inoculation chambers and inoculated with a 40-ml spore suspension (5×10^4 conidia/ml), as in the method described by Wang et al. (2002). Each line was inoculated in two independent experiments, with three replications in each experiment. After inoculation, the seedlings were kept in the dark at 26°C with relative humidity of 95% for 24 h, then transferred to a greenhouse with 25 to 28°C and 100% relative humidity, maintained by intermittently spraying water for 2 min every 3 h.

Disease reactions were evaluated 1 week after inoculation with the pathogen. A disease rating system, as described by Mackill and

Bonman (1992) and Silué et al. (1992), was modified to determine the phenotypes of each progeny of the mapping population. Lesion scores from 0 to 5 were based on lesion type and lesion size, as described by Shi et al. (2010). For data analysis, the seedlings with rating 0, 1, and 2 were considered to be resistant and those rated as 3, 4, and 5 were considered to be susceptible.

Marker development and identification. Plant DNA was extracted from the 3-week-old seedlings using the sodium dodecyl sulfate method (Pich and Schubert 1993). For fine mapping the *Pi-hk2* locus, three types of polymerase chain reaction (PCR)-based markers—simple-sequence repeat (SSR), intron length polymorphism (ILP), and insertion/deletion polymorphisms (InDel)—were developed in the target region with *Pi-hk2*. SSR markers were directly adopted from the International Rice Microsatellite Initiative (<http://www.gramene.org>) (McCouch et al. 2002), and the SSR detection procedures followed the methods described by Zhu et al. (2004). ILP markers were designed around intronic regions which showed higher polymorphism than exonic regions and were developed according to predicted exons by software tools GENSCAN (<http://genes.mit.edu/GENSCAN.html>) and softberry (<http://linux1.softberry.com/berry.phtml>) (Wei et al. 2005). For InDel markers, sequence comparisons were developed between ‘93-11’ and ‘Nipponbare’ in target regions by Pairwise BLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) (Wu et al. 2013). All primers of these new markers were designed by Primer Premier 5.0.

QTL mapping. QTL analysis was performed using a previously constructed molecular linkage map (Shi et al. 2010). A genetic map of 162 RIL F_{2:9} with a total distance of 2,457.16 centimorgans (cM) and an average distance of 17.68 cM between two markers was constructed by MapMaker/EXP v. 3.0, as previously reported (Lander et al. 1987; Shi et al. 2010). QTL analyses were performed using the modified algorithm inclusive composite interval mapping (Li and Ye 2007). The LOD threshold of 2.5 was used to detect QTL, as described by Lander and Kruglyak (1995). In this study, QTL detected by lesion number was labeled as *LnqtlA-B* and QTL detected by lesion score was labeled as *Lsqtla-B*,

Fig. 1. Distribution frequency of **A**, lesion scores and **B**, lesion number obtained on the progeny of 162 recombinant inbred lines (RIL) toward 2010-9(G1) isolate. X-axis is the trait of the lines and Y-axis is the number of lines with the same trait.

TABLE 1. Quantitative trait loci (QTL) for resistance to 2010-9(G1) detected based on lesion score and lesion number

Trait, QTL	Chromosome	Position ^a	Left marker	Right marker	LOD ^y	PVE (%) ^z	Add
Lesion score							
Lsqt14-1	4	63	RM6089	RM255	2.66	5.24	-0.52
Lsqt19-1	9	287	RM24097	RM24019	9.10	46.19	-1.62
Lsqt11-1	11	200	RM27088	RM27154	3.35	6.11	-0.58
Lesion number							
Lnqtl1-1	1	114	RM5853	RM1297	2.58	9.00	-0.63
Lnqtl9-1	9	26	RM24097	RM24019	5.94	14.10	-0.79

^a Position of peak logarithm of the odds (LOD) score.

^y Peak LOD score in the QTL.

^z Explained the contribution to the phenotypic variance.

as described by Shi et al. (2010), where *A* indicates the chromosome number and *B* represents the QTL.

Physical maps construction. In total, 1,560 BC₁F₂ individuals were used for constructing a genetic linkage map and susceptible individuals were used for mapping *Pi-hk2* by recessive class analysis methods. In all, 18 BC₁F₂ plants with a heterozygous region between RM24019 and RM24048 were used to generate BC₁F₃ (3,360) and 21 BC₁F₃ plants with a heterozygous region between RM24019 and RM24048 were used to generate BC₁F₄ (4,160). In total, 3,360 BC₁F₃ plants and 4,160 BC₁F₄ plants were used for genotyping and identifying recombinants between RM24019 and RM24048. The resistance phenotype of 13 recombinants obtained from BC₁F₂ (*n* = 4), BC₁F₃ (*n* = 4), and BC₁F₄ (*n* = 5) were used to fine map the *Pi-hk2*. The physical map of the target region with *Pi-hk2* can be subsequently constructed by BAC and PAC clones' sequences of Nipponbare in the International Rice Genome Sequencing Project (<http://rgp.dna.affrc.go.jp/IRGSP/>).

Candidate gene prediction and microarray data analysis. The sequences in the region of ILP-19 and RM24048 in BAC clone OJ1253E02 and PAC clone P0466E03 on chromosome 9 were downloaded from the RGP Web site. Open reading frames in the target region with *Pi-hk2* were predicted by GENSCAN (<http://genes.mit.edu>), FGENSH (<http://linux1.softberry.com/>), and Rice Genome Automated Annotation System (RiceGAAS) (<http://rgp.dna.affrc.go.jp>) software (Sakata et al. 2002).

The GENEVESTIGATOR (<http://genevestigator.com/gv/>) and Rice Oligonucleotide Array Database (<http://www.ricearray.org/>) were employed to analyze the expression of candidate genes based on 1,154 Affymetrix microarray datasets (<http://www.ricearray.org/>). The GENEVESTIGATOR was used to examine the expression patterns of candidate genes in all development stages and all organs.

The expression patterns of 18 candidate genes were detected by quantitative real-time PCR methods, as described by (Huang et al.

Fig. 3. *Lsqtl9-1* for resistance to blast detected based on lesion score. LOD = logarithm of the odds and red location was *Lsqtl9-1* position.

Fig. 2. Genetic locations of quantitative trait loci (QTL) for resistance to blast detected. Black diamonds are the QTL associated with lesion number and black stars are the QTL associated with lesion scores.

2008). The fold change in cDNA (target candidate gene) relative to the reference gene (18s-rRNA) was determined by the comparative cycle threshold method (Livak and Schmittgen 2001). Relative quantification relates the PCR signal of the target transcript in inoculated rice seedlings to that of uninoculated rice plants. Relative expression levels were calculated by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). All reactions were performed in triplicate. The primers for 18 candidate genes and 18s-rRNA in quantitative real-time PCR assay are shown in Supplementary Table S1.

RESULTS

Race-specific QTL *Lsqt19-1* was identified. Lesion scores and lesion numbers of 162 RIL ($F_{2,9}$) and two parents (Heikezijing and Suyunuo) inoculated with isolate 2010-9(G1) were evaluated. The two traits showed continuous frequency distributions among RIL populations (Fig. 1), indicating polygenic and quantitative resistance to the blast in resistant parent Heikezijing.

Five QTL were detected to be associated with lesion scores and lesion number, which were distributed on chromosomes 1, 4, 9, and

11 (Table 1; Fig. 2). Among them, *Lsqt14-1*, *Lsqt19-1*, and *Lsqt111-1*, associated with lesion scores, were distributed on chromosomes 4, 9, and 11, with LOD scores of 2.66, 9.10, and 3.35 and phenotypic variance of 5.24, 46.19, and 6.11%, respectively. The two QTL *Lnqt11-1* and *Lnqt19-1* associated with average lesion number were distributed on chromosomes 1 and 9, with LOD scores of 2.58 and 5.94 and phenotypic variance of 9.00 and 14.10%, respectively. The QTL *Lsqt19-1*, with LOD score of 9.10 and phenotypic variance 46.19%, was mapped between the SSR markers RM24019 and RM24097 on chromosome 9 (Fig. 3), which might be a major resistance QTL, and named as *Pi-hk2*.

The RIL populations were further inoculated by seven representative isolates: 2009-70-1(A7), 2010-49-1(B1), 2010-28(C1), 2010-35-1(D13), 2011-40(E3), 2010-42-2(F1), and 2010-9(G1). RIL84 conferring *Pi-hk2* showed resistance to 2010-9(G1) but significant susceptibility to the other six isolates (Table 2). This result indicated that *Pi-hk2* confers race-specific resistance to 2010-9(G1) at seedling stages.

Fine mapping of *Pi-hk2*. In total, 62 plants (BC_1F_1) derived from backcrossing RIL84 and Suyunuo were inoculated with isolate 2010-9(G1) in the growth chamber; of these, 33 individuals were resistant to 2010-9(G1) and 29 were susceptible. The numbers of resistant and susceptible plants fit a 1:1 ratio ($\chi^2 = 0.25 < \chi^2_{0.05} = 3.84$), suggesting that the resistance of RIL84 to isolate 2010-9(G1) was controlled by a single dominant gene.

To fine map gene *Pi-hk2*, four additional markers (RM24048, RM24065, RM7390, and RM3912) between RM24019 and RM24097 were selected that showed polymorphisms between parents Heikezijing and Suyunuo. In all, 1,560 BC_1F_2 plants from 33 BC_1F_1 -resistant plants were genotyped by those markers, and 12, 8, 5, 16, and 8 recombinants were identified by RM24019, RM24048, RM24065, RM7390, and RM3912, respectively. Through phenotype assays of the recombinants, *Pi-hk2* was mapped in the region of RM24019 and RM24048 with a physical distance of 520 kb (Fig. 4A).

TABLE 2. Responses of Heikezijing, Suyunuo, and RIL84 to seven isolates^z

Isolate	Heikezijing	Suyunuo	RIL84
2009-70-1(A7)	0 (R)	2.5 (MS)	2.5 (MS)
2010-49-1(B1)	0 (R)	5 (S)	5 (S)
2010-28(C1)	0 (R)	5 (S)	5 (S)
2010-35-1(D13)	0 (R)	5 (S)	3 (S)
2011-40(E3)	0 (R)	4 (S)	5 (S)
2010-42-2(F1)	0 (R)	5 (S)	5 (S)
2010-9(G1)	0 (R)	5 (S)	0 (R)

^z R, MR, S, and MS represent resistant, moderately resistant, susceptible, and moderately susceptible, respectively.

Fig. 4. Genetic and physical maps of the region of the *Pi-hk2* locus. **A**, Fine linkage map generated by analyzing 1,560 BC_1F_2 segregating plants. Recombinant numbers are given between markers. **B**, Physical map of *Pi-hk2*, constructed from the Nipponbare genomic sequence. Numbers below the map are recombinant numbers. **C**, Counting map and candidate genes of the *Pi-hk2* locus. Short horizontal lines represent BAC/PAC clones of Nipponbare. These clones are anchored by the corresponding markers linked to the *Pi-hk2* locus. Arrows represent open reading frames predicted in the *Pi-hk2* region by RiceGAAS.

To evaluate their resistance phenotype, 13 recombinant plants identified between RM24019 and RM24048 were inoculated with 2010-9(G1). For further delimitation of the *Pi-hk2* locus, seven new markers were explored between RM24019 and RM24048 on the basis of sequences in a public database to determine the genotypes (Table 3). As a consequence of fine mapping, *Pi-hk2* was located in the region of ILP-19 and RM24048, which was 143 kb on physical map of the target region (Fig. 4B).

Candidate gene prediction and expression analysis. The target 143-kb region (10,021,585 to 10,166,709) conferring *Pi-hk2* in the Nipponbare sequence was covered by two BAC/PAC clones (OJ1253E02 and P0466E03), and 18 putative genes were predicted by GENSCAN, FGENSH, and RiceGAAS software (Fig. 4C). Among these 18 candidate genes (*P1* to *P18*), 6 putative genes (*P7*, *P8*, *P13*,

P14, *P16*, and *P17*) might encode functional proteins, including *P7* and *P8* encoding pleiotropic drug resistance protein 4, *P13* encoding WRKY74, *P14* encoding cytochrome b5-like heme/steroid-binding domain containing protein, *P16* encoding protein kinase, and *P17* encoding ankyrin repeat family protein. Five genes (*P1*, *P3*, *P12*, *P9*, and *P18*) might encode hypothetical proteins and seven genes (*P2*, *P4*, *P5*, *P6*, *P10*, *P11*, and *P15*) encode transposon/retrotransposon proteins (Table 4).

The expression profiles of these candidate genes in various rice tissues were investigated based on microarray data deposited in the GENEVESTIGATOR. The expression of 12 candidate genes could be detected in rice culm (stem), sheath, root, panicle, leaf, pollen, inflorescence, node, embryo, and endosperm (Fig. 5). The expression of *P1*, *P3*, *P5*, *P11*, *P16*, *P17*, and *P18* showed higher expression

TABLE 3. Information on polymerase chain reaction-based markers used for mapping of *Pi-hk2*

Marker name	Marker type ^x	Primer sequence(5'–3')	Product size (bp) expected	Temp (°C) ^y	Gel ^z
RM24019	SSR	F:GGACAAGATGATGACACTCTGTACC R:TGCTTCTTAGTTCTCAGCCACACC	84	55	A
RM24030	SSR	F:GTATGCCATATCCAACGACAATCC R:GTGTGATTCTGATTGGCTGAAAGG	82	55	A
ID-3	InDel	F:CGGTCAATACATCATCTCCTTCCTC R:CTGCCATGACGTTCCAAGC	714	54	C
ILP-7	ILP	F:CCCAAATTCCAGTCACCGCCGTCCG R:CGCGCGATAAATGGTCGCCGCGCAGC	179	59	A
ID-5	InDel	F:TCTCAAGCATTACGATGACAGCCTT R:AGGGCTGGGAACATTGGTATGGTTT	1,245	60	C
RM24032	SSR	F:ATTCAACATGGGCTGCCTGTCC R:GCTGCAGTCTGAATGGTGGTACG	239	52	B
ID-9	InDel	F:AATCTACTTTCAGATGGTTTGGTCC R:GCTAGTCTTAACGACATACTTGACG	867	55	C
ILP-19	ILP	F:GACGACGGGGAGGCGGGATC R:AGGAGGGCCGCCAAGCAC	151	52	A
RM24048	SSR	F:CTAGCTTCGTCAAATCACGTAGG R:GGATAGATGGATGGATGGATGG	158	55	A
RM24065	SSR	F:TGCAGAGACAAGATAGGAATGC R:CATGCATGTCAATGTGATCG	145	52	A
RM7390	SSR	F:TGAGAGCTCGTAGGAAGTGTC R:CAGAGTCAGCAATCGCTAAGG	244	52	B
RM3912	SSR	F:CACTCAGATTGGCCGATCC R:GCTGATCCAGATCTACCTGACACC	263	58	B
RM24097	SSR	F:GTCCTTCGTCCACGGCAACTACG R:TACGCCGAGGTACAGGTCAAGC	82	55	A

^x SSR = simple-sequence repeat (SSR), InDel = insertion/deletion polymorphisms, and ILP = intron length polymorphism.

^y Annealing temperature. All of the polymerase chain reactions (PCR) were conducted as below: after preheating for 5 min at 95°C, 32 PCR cycles (40 s at 95°C, 40 s at the corresponding temperature described above, and 40 s to 2 min at 72°C), followed by 10 min at 72°C.

^z A = 8% acrylamide, B = 6% acrylamide, and C = 2% agarose.

TABLE 4. Candidate genes at the *Pi-hk2* region in Heikezijing

Number	Annotated genes	Prediction of protein function ^y	N ^z
1	LOC_Os09g16390	Expressed protein	171
2	LOC_Os09g16400	TP, putative, CACTA, En/Spm subclass, expressed	336
3	LOC_Os09g16410	Expressed protein	149
4	LOC_Os09g16420	RP, putative, Ty3-gypsy subclass, expressed	197
5	LOC_Os09g16430	RP, putative, Ty3-gypsy subclass, expressed	1,262
6	LOC_Os09g16440	TP, putative, unclassified, expressed	722
7	LOC_Os09g16449	Pleiotropic drug resistance protein 4, putative, expressed	479
8	LOC_Os09g16458	Pleiotropic drug resistance protein 4, putative, expressed	423
9	LOC_Os09g16470	Hypothetical protein	68
10	LOC_Os09g16480	RP, putative, centromere-specific	168
11	LOC_Os09g16490	RP, putative, unclassified	209
12	LOC_Os09g16500	Expressed protein	107
13	LOC_Os09g16510	WRKY74, expressed	362
14	LOC_Os09g16520	Cytochrome b5-like heme/steroid-binding domain containing protein, expressed protein	119
15	LOC_Os09g16530	TP, putative, unclassified, expressed	1,586
16	LOC_Os09g16540	Protein kinase, putative, expressed	724
17	LOC_Os09g16550	Ankyrin repeat family protein, putative, expressed	688
18	LOC_Os09g16560	hypothetical protein	160

^y TP = transposon protein and RP = retrotransposon protein.

^z Number of amino acids.

levels in pollen. *P7* and *P8* were found to show a higher expression level in sheath; *P12* in endosperm and pollen; *P13* in inflorescence, leaf, and root; and *P14* in panicle and culm (stem).

Real-time reverse-transcription PCR was performed to examine the expression pattern of candidate genes in Heikezijing seedlings induced by 2010-9 (G1). The results showed that *P11*, *P13*, and *P18* were dramatically more highly expressed in Heikezijing, and were induced by inoculation. Among those candidate genes, the expression of *P13* was the highest; the other candidate genes were expressed relatively less either with or without inoculation (Fig. 6). Genes *P1*, *P3*, *P4*, *P5*, *P6*, *P9*, and *P15* were not expressed before or after inoculation. According the structure and expression of candidate genes, two genes (*P13* and *P18*) might be the candidates of gene *Pi-hk2*.

DISCUSSION

It was reported that there were some broad-spectrum or durably resistant cultivars such as ‘Moroberekan’, ‘Suweon’, ‘Teqing’, ‘Sanhuangzhan 2’, ‘GuMei 2’, and ‘XZ3150’ (Ma et al. 2015). Several *R* genes have been identified from Moroberekan (Jeon et al. 2003), ‘Suweon 365’ (Ahn et al. 2000), Teqing (Tabien et al. 2000), and Sanhuangzhan 2 (Liu et al. 2004). Heikezijing, one *japonica* rice landrace from the Taihu region of China, exhibited broad-spectrum resistance to at least 300 isolates belonging to seven Chinese blast races (Shi et al. 2010; Wang et al. 2002). In our previous research, a broad-spectrum *R* gene, *Pi-hk1*, has been identified on chromosome 11 of Heikezijing. In this article, we fine mapped race-specific *R* gene *Pi-hk2* on chromosome 9.

Race-specific resistance closely follows the classical gene-for-gene relationship. The isolation and subsequent characterization of *R* genes will help to unravel the molecular mechanisms underlying the interaction between host and pathogen. To date, five pairs of avirulence (*Avr*) and *R* genes (*AvrPita* versus *Pi-ta*, *Avr-Pik* versus *Pik*, *AvrPiz-t* versus *Piz-t*, *Avr-Pia* versus *Pia*, and *Avr1-CO39* versus *Pi-CO39*) have been intensively studied in rice blast

resistance (Cesari et al. 2013; Jia et al. 2000; Kanzaki et al. 2012; Li et al. 2009a, b; Zhai et al. 2014), and only *Pi-ta* was reported as a race-specific *R* gene (Lee et al. 2009a). In this research, seven isolates belonging to races 2011-40(E3), 2009-70-1(A7), 2010-49-1(B1), 2010-28(C1), 2010-42-2(F1), 2010-35-1(D13), and 2010-9(G1) were inoculated in RIL populations, and it was found that only blast isolate 2010-9(G1) could be controlled by *Pi-hk2*. This suggested that *Pi-hk2* might confer race-specific resistance to predominant isolate 2010-9 (G1) in Jiangsu Province, China.

Gene *Pi-hk2* conferring resistance to the blast in landrace Heikezijing was mapped into an interval of 143 kb between ILP-19 and RM24048 on chromosome 9. It has been reported that three blast *R* genes (*Pi5/Pi3/Pi-i*, *Pi-15*, and *Pi56*) are located near this region (Liu et al. 2013b). In order to distinguish *Pi-hk2* from them, an integrated physical map with these *R* genes and linked markers were constructed base on the Nipponbare sequence (Fig. 7). Three linked markers (C1473, RM240044, and CRG5) were used to amplify the genomic DNA of Heikezijing and Suyunuo. No PCR product was detected by C1473 and RM24044 in Heikezijing and Suyunuo, and no fragment difference was detected by CRG5 in Heikezijing and Suyunuo. Otherwise, the whole gene sequences of *Pi56*, *Pi5-1*, and *Pi5-2* genes were amplified from Heikezijing and Suyunuo, and the sequence alignments showed no differences of these three genes’ sequences between Heikezijing and Suyunuo. In this map, the *Pi-hk2* locus defined by ILP-19 and RM24048 was different from the loci of the other three genes. All of these results suggested that *Pi-hk2* might be a new blast *R* gene.

There were two main references about disease rating systems. Mackill and Bonman (1992) showed that 0-to-5 scales were decided by lesion type and lesion size. Silué et al. (1992) showed a picture with different lesion type, lesion size, and lesion number in a 1-to-6 scale. In our manuscript, we referred these two articles and made a modification as described as Shi et al. (2010). In our scoring system, scores from 0 to 5 were based on lesion type and lesion size.

More than 70 *R* genes from different plant species have been cloned and characterized, and most of them encode NBS-LRR proteins (Liu et al. 2007a). Among 24 rice blast *R* genes, 22 genes encode NBS-LRR proteins, except for *Pi-d2* encoding a B-lectin receptor kinase (Chen et al. 2006) and *pi21* encoding a proline-rich protein that includes a putative heavy metal-binding domain and putative protein–protein interaction motifs (Fukuoka et al. 2009). In all, 18 putative genes were predicted in the target 143-kb region conferring *Pi-hk2* in the Nipponbare sequence. Among these

Fig. 5. Tissue-specific expression profiles of candidate genes based on microarray data from the GENEVESTIGATOR (<https://genevestigator.com/gv/>).

Fig. 6. Real-time polymerase chain reaction analysis for the expression patterns of candidate genes in Heikezijing induced by inoculation isolate 2010-9(G1); 18S-rRNA was used as an internal control. Data represent means and standard errors of three replicates.

Fig. 7. Integrated physical mapping of four resistance genes (*Pi15*, *Pi5/Pi3/Pi-i*, *Pi56*, and *Pi-hk2*) which were mapped on chromosome 9. Positions of the resistance genes and markers were integrated on the basis of the data from the following references: *Pi15* (Lin et al. 2007); *Pi5/Pi3/Pi-i* (Jeon et al. 2003; Lee et al. 2009b; Yi et al. 2004); *Pi56* (Liu et al. 2013b), and *Pi-hk2* (this article). Markers used in this study and their physical positions are located on the top of the chromosome.

candidate genes, seven genes (*P2*, *P4*, *P5*, *P6*, *P10*, *P11*, and *P15*) encode transposon/retrotransposon proteins and only six genes (*P7*, *P8*, *P13*, *P14*, *P16*, and *P17*) encoded for function proteins. *P7* and *P8* encoded pleiotropic drug resistance protein 4. However, the sequence similarity of *P7* and *P8* was low, and they may not have generated by duplication. *P13* encodes a WRKY transcription factor WRKY74, with conserved motif WRKYGQK at its N terminus. WRKY transcription factors generally act as transcriptional regulators through W boxes, cis-elements that are enriched in the promoters of several defense-related genes. It binds specifically to the DNA sequence motif (T)(T)TGAC(C/T), which is known as the W box. *P14* encoded cytochrome b5-like heme/steroid-binding domain containing protein. The genome of *Arabidopsis thaliana* has 15 proteins with a cytochrome b5-like heme/steroid binding domain, and 1 of them acted as a regulator positively controlling LR formation, and functions independently of the ARF7/19-mediated auxin signaling pathway (Ikeyama et al. 2010). *P16* encoded protein kinase, which played a role in a multitude of cellular processes, including division, proliferation, apoptosis, and differentiation. *P17* encoded ankyrin repeat family protein, which is one of the most common protein-protein interaction motifs in nature. Li and Wang (2013) reported that, among the 13 rice WRKY genes, 11 genes are involved in the interaction of rice with *M. oryzae*, which causes blast diseases not only in rice but also in barley. Compared with other candidate genes, *P11*, *P13*, and *P18* were expressed higher based on the result of real-time PCR analysis in Heikezijing induced by inoculation with 2010-9(G1). According to these results, it appeared that *P13* and *P18* were more possibly related to rice blast resistance and might be the candidate genes of *Pi-hk2*. Among these predicted genes, none of these genes encoded NBS-LRR protein, and our future work will focus on fine mapping and identifying the gene *Pi-hk2*.

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