Efficient authentic fine mapping of the rice blast resistance gene *Pik-h* in the *Pik* cluster, using new *Pik-h*-differentiating isolates

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Abstract The *Pik-h* gene in rice confers resistance to several races of rice blast fungus (*Magnaporthe oryzae*), and has been classified as a member of the *Pik* cluster, one of the most resistance (*R*) gene-dense regions in the rice genome. However, the loss of a key mutant isolate has long made it difficult to differentiate *Pik-h* from other *Pik* group genes especially from *Pik-m*. We identified new natural isolates enabling the differentiation between *Pik-h* and *Pik-m* genes, and first confirmed the authenticity of the International Rice Research Institute (IRRI) "monogenic" line IRBLkh-K3, and then fine-mapped the *Pik-h* gene in the *Pik* cluster. Using 701 susceptible individuals among 3,060 siblings from a cross of IRBLkh-K3×CO39, the *Pik-h* region was delimited to 270 kb, the narrowest interval among the *Pik* group genes reported to date, in the cv. Nipponbare genome. Annotation of this genome region first revealed 6 NBS-LRR type *R*-gene analogs (RGAs), clustered within the central 120 kb, as possible counterparts of *Pik-h* and 6 other *Pik* group *R* genes. Interestingly, the *Pik-h* region and the cluster of RGAs were shown to be located 130 kb and 230 kb apart from *Xa4* and *Xa2* bacterial blight resistance genes, respectively, once classified as belonging to the *Pik* cluster. The closest recombination events were limited to the margins of the *Pik-h* region, and recombination was suppressed in the core interval with the RGA cluster. This fine-mapping, performed in a short time using an HEGS system, will facilitate utilization of the cluster's genetic resources and help to elucidate the mechanism of evolution of *R*-genes. The presence of natural isolates also confirmed that evolution of *Pik-h* corresponds to pathogen evolution.

Keywords *Pik -h* · Rice blast resistance · *Pik* resistance gene cluster · High resolution mapping · HEGS

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Introduction

Rice blast disease, caused by *Magnaporthe oryzae*, is one of the most serious diseases of rice, an important

staple crop for more than half the world's population. The use of disease resistance (*R*) genes is the most efficient and environmentally friendly way to control such diseases, and over 50 major rice blast *R* genes have been mapped to date (Hayashi 2005; Chen et al. 2006; Liu et al. 2005). However, the mechanisms of action of disease resistance genes and their race specificity are still unclear, although several hypotheses have been proposed (Jia et al. 2000; Orbach et al. 2000; Farman et al. 2002; Böhnert et al. 2004). Although isolation of the rice blast *R* genes has lagged behind efforts in *Arabidopsis* (Glazebrook et al. 1997; Meyers et al. 2003) and other crops, such as tomato and tobacco (Martin et al. 2003), 7 genes have recently been isolated (*Pib*; Wang et al. 1999, *Pita*; Bryan et al. 2000, *Pi9*; Qu et al. 2006, *Pid2*; Chen et al. 2006, *Pi2*; Zhou et al. 2006, *Pizt* and *Pi36*; Liu et al. 2007), 6 of which (*Pib*, *Pita*, *Pi9*, *Pi2*, and *Pizt*, *Pi36*) are of the nucleotide binding site-leucine-rich repeat (NBS-LRR) type, while *Pi2* encodes a receptor-like kinase. However, our understanding of the mechanism of initial recognition of pathogen signaling by *R*-genes is still far from complete. In addition, how the plant resistance genes could have evolved to recognize the highly mutable pathogens remains unclear.

More than 600 *R*-gene analogs of the NBS-LRR type were identified in the rice genome (Bai et al. 2002), and most of these are found in clusters. The rice *Pik* cluster is located near the telomeric end of the long arm of chromosome 11. This cluster includes the blast resistance genes *Pik*, *Pik-s*, *Pik-p*, *Pik-m*, *Pik-h* (Kiyosawa 1972; McCouch et al. 1994), *Pi44*(t) (Chen et al. 1999), and *Pi1* (Yu et al. 1996), and the bacterial blight resistance genes *Xa3* (Xiang et al. 2006), *Xa4* (Sun et al. 2004), *Xa22*(t) (Wang et al. 2003), and *Xa26* (Sun et al. 2004). This cluster represents one of the most highly concentrated regions of *R* genes in the rice genome (Inukai et al. 1994; Yu et al. 1996; Chen et al. 1999; Yang et al. 2003). Therefore, analyzing the genomic organization of this *Pik* cluster should facilitate effective utilization of these genetic resources and a better understanding of the molecular mechanism of *R* gene evolution. Recently, the *Pik*, *Pik-p*, and *Pik-m* genes were finely mapped (Hayashi et al. 2006). *Pik-m* was further finely mapped by Li et al. (2007).

The rice blast *R*-gene *Pik-h* was first reported by Kiyosawa and Murty (1969) from the test line K3

derived from a cross of an Indian cv. HR-22 (*indica*) and Japanese cv. Sasashigure. The *Pik-h* gene is expected to be allelic to *Pik*, because it is effective against a *Pik*-overcoming mutant isolate, Ken54-20-k^+, derived from the *Pik*-incompatible ken54-20, but other aspects of the resistance spectrum against Japanese and International Rice Research Institute (IRRI) standard races are the same as *Pik-m* (Hayashi 2005; Tsunematsu et al. 2000), which is also allelic to *Pik*. Later, *Pik-h* was shown to be present in a rather wide range of cultivars, including indica cvs. Tetep and Tadukan and the US cv. Dawn (Kiyosawa 1981). However, the key mutant blast isolate, Ken54-20-k^+, has been lost, making it difficult to differentiate *Pik-h*. A "monogenic" line, IRBLkh-K3, was constructed in IRRI by back-crossing from K3 with cv. LTH (Lijiangxintuanheigu), but differentiation of the *Pik* group monogenic lines with specific races has not been done (Tsunematsu et al. 2000). Although there have been some reports on examining the presence of *Pik-h* (Fjellstrom et al. 2004; Sharma et al. 2005), these reports included no descriptions of how *Pik-h* was differentiated from other *Pik* genes. It is necessary to settle this situation with some clear criteria for the gene. In addition, considering that *Pik-h* is found in various cvs., as described above, presence of some natural isolates corresponding to mutations in this gene was strongly suggested.

Recently, in the process of screening our Japanese rice blast isolate collection, we found two long sought key isolates that can differentiate *Pik-h* from the other *Pik* group genes. This race set will contribute greatly to differentiation between *Pik* group genes in various cvs. With these isolates, we confirmed the authenticity of the *Pik-h* gene in IRBLkh-K3. In addition, we confirmed that our fine-mapped gene using the cross CO39×IRBLkh-K3 and race V86010 to be *Pik-h*. This has localized *Pik-h* to a significantly narrower segment in the *Pik*-cluster compared to previous mapping reports, and verified the authenticity of *Pik-h* as a member of the *Pik* gene cluster. Mapping was performed efficiently using a high-efficiency genome scanning (HEGS) system involving high lane density polyacrylamide gel electrophoresis (Kawasaki and Murakami 2000). Here, we report the process of verification and fine mapping of the *Pik-h* gene.

Materials and methods

Plant materials and mapping populations

The original *Pik-h* line K3 (Kiyosawa and Murty 1969) was obtained from the gene bank of the National Institute of Agrobiological Sciences (NIAS). The *Pik-h* monogenic line IRBLkh-K3 (*indica*), derived from K3 and susceptible cv. CO39 (*Pik-h*: −, *Pi-a*: +, *indica*), was provided by IRRI, along with other monogenic lines IRBLk-Ka and IRBLkm-Ts. The 96 F_2 individuals of a CO39×IRBLkh-K3 cross were used for preliminary bulked segregant analysis (BSA). After inoculation as described below, 701 susceptible individuals were selected from 3,060 F_3 siblings of heterozygous F_2 individuals that were selected by segregation of sample progenies. Some of the susceptible (S) and resistant (R) homozygous lines from this cross were used to verify the authenticity of the mapped gene. DNA was extracted by a revised cetyl trimethyl ammonium bromide (CTAB) method for a large population (Xu et al. 2005), and then simple sequence repeat (SSR) and restriction fragment length polymorphism (RFLP)-derived sequence characterized amplified region (SCAR) analysis were performed with the HEGS system (Nihon-Eido, Tokyo, Japan), as described previously (Xu et al. 2005). Samples of approximately 5 µg of DNA were extracted from the leaves (1 g fresh weight) of the selected recombinants using a Kurabo NA2000 DNA isolation machine (Kurabo, Osaka, Japan) for further analysis.

Blast inoculation and disease evaluation

For fine-mapping, phenotypic analysis of F_2 and F_3 populations, *M. oryzae* isolate V86010 line, which overcomes minor *R*-genes against Japanese races in the CO39 background (Imbe et al. 2000), obtained from IRRI, was used for inoculation in an isolated greenhouse. The seedlings at the four- to five-leaf stage (about 10–14 days after sowing) were inoculated with a suspension of *M. oryzae* conidia ($2–3 \times 10^5$ spores/ml). The inoculated plants were then placed in darkness in a dew chamber (Koito, Tokyo, Japan) at 100% relative humidity for 24 h at 25°C, and subsequently transferred to a semi-temperature-controlled greenhouse, maintained at around 15–25°C. Disease symptoms of inoculated plants were recorded at 7–10 days after inoculation on a scale of 0–5 in accordance with the Japanese Ministry of Agriculture, Forestry, and Fisheries (MAFF) Microorganism Genetics Resources Manual Vol.18 (Hayashi 2005).

To characterize the authenticity of the *Pik-h* gene in IRBLkh-K3, in reference to K3, newly collected original Japanese isolates H05-56-1, H05-67-1, H02-20-1 from Ibaraki prefecture, and Kyu89-246 from Miyazaki, were used. For genotyping the *Pik-h* locus of the F_3 plants, F_4 progenies were inoculated with the new isolates under the same conditions as used for fine mapping. F_4 plants were used for phenotyping to avoid complication of the results by the presence of *Pia* as much as possible.

Development of SNP markers and physical mapping of the *Pik-h* locus

Single nucleotide polymorphism (SNP) markers were analyzed by sequencing according to the following method.

To find unique sequences, 6 bacterial artificial chromosome (BAC) clones of *Oryza sativa* L. cv. Nipponbare sequences in the International Rice Genome Sequencing Project (IRGSP) database (http://rgp.dna.affrc.go.jp/IRGSP/), within the region narrowed by the above fine mapping, were used for BLAST analysis (http://www.ncbi.nlm.nih.gov) against the complete rice genome sequence. Sequences found to be unique in the genome were used to design primers with the Primer3 software (http://fokker.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The uniqueness of each primer in the genome was confirmed again by BLAST analysis. The PCR products, showing a single band on 1% agarose gels, were directly sequenced using an ABI BigDye® Terminator v3.1 Cycle Sequencing Kit and ABI 3100 DNA sequencer (Applied Biosystems, Foster City, CA). In case in which normal PCR amplification with Takara Ex *Taq* polymerase (Takara Bio, Otsu, Shiga, Japan) was difficult, Phusion™ High-Fidelity DNA Polymerase (Finnzymes, Espoo, Finland) was used in accordance with the manufacturer's protocol.

The physical locations of RFLP-SCAR and SSR markers were checked in the Gramene marker database (http://www.gramene.org), in addition to BLAST searches as described above. These and SNP marker loci in Nipponbare were determined from the genome sequences and checked by graphical

genotyping with close recombinants. The location of the *Pik-h* gene was determined by phenotyping of the same recombinants, and a physical map around this region was constructed based on the Nipponbare genome database.

Results

Confirmation of *Pik-h* gene authenticity

In the *Pik* cluster blast resistance genes, the *Pik, Pik-s, Pik-p,* and *Pi1* have already been differentiated by Japanese and Philippine races from *Pik-h,* and only the differentiation of *Pik-h* and *Pik-m* has been difficult (Hayashi 2005; Tsunematsu et al. 2000).

With the newly collected blast isolates H05-56-1, H05-67-1, and H02-20-1 from Ibaraki Prefecture, and

Kyu89-246 from Miyazaki, the original line K3 and the IRRI *Pik-h, Pik-m,* and *Pik* monogenic lines IRBLKh-K3, IRBLkm-Ts, and IRBLk-Ka were tested for their resistance reactions (Table 1). The last *Pik* line was added as a reference. In addition, the background of these IRRI monogenic lines, LTH, was also tested. As shown in Table 1, the new isolates, H05-56-1 and H02-20-1, differentiated *Pik-h* in K3 from *Pik* and *Pik-m,* and confirmed the authenticity of *Pik-h* in IRBLKh-K3. It is notable that the resistance reaction of *Pik-h* to the new races is not strong (grade 2 in Table 1, with numerous small stopping lesions with brown fringes, without sporulation; Fig. 1) compared to other known races seen as grade 1 in Table 1. Interestingly, such mild reaction of *Pik-h* was also reported previously against the mutant isolate Ken54-20-k^+ (Kiyosawa and Murty 1969).

Table 1 Verification of the of the *Pik-h* genes in the original K3 and IRRI monogenic lines, and the mapping population, with the new *Pik-h*-differentiating blast isolates **H05-56-1** and **H02-20-1**

Rice lines	Rice blast isolates				Estimated genotypes
	H05-56-1	**H02-20-1**	H05-67-1	Kyu89-246	
K3	R(2)	R(2)	R(1)	R(1)	*Pik-h*
IRBLkh-K3(a)	R(2)	R(2)	R(1)	R(1)	*Pik-h*
IRBLkh-K3(b)	R(2)	R(2)	R(1)	R(1)	*Pik-h*
IRBLk-Ka	S(5)	S(5)	S(4)	R(1)	*Pik*
IRBLkm-Ts	S(5)	S(4)	R(1)	R(1)	*Pik-m*
LTH	S(5)	S(5)	S(5)	S(5)	+
RC-R Kh-101	R(1)	R(1)	R(1)	R(1)	*Pia, Pik-h*?
RC-R Kh-102	R(2)	R(2)	R(1)	R(1)	*Pik-h*
RC-R Kh-103	R(2)	R(2)	R(1)	R(1)	*Pik-h*
RC-S Kh-2	R(1)	R(1)	S(4)	S(3)	*Pia*
RC-S Kh-3	R/S(1/5)	R/S(1/4)	S(4)	S(4)	*Pia*/+
RC-S Kh-4	R/S(1/5)	R/S(1/5)	S(4)	S(4)	*Pia*/+

The standard lines and R/S-homozygotes from the mapping population of the CO39×IRBLkh-K3 were checked by their reactions. The estimated genotypes of the tested lines are indicated on the left

Rice disease symptoms are indicated in parentheses on the a scale from 0 to 5 as described in the MAFF manual (Hayashi 2005). The threshold for diagnosing R (resistant) or S (susceptible) was set between grades 2 and 3. R/S(1/5) indicates segregation of R(1) and S(5) individuals

The upper six are reference lines. K3: original *Pik-h* line. Two *Pik-h* IRRI monogenic-lines, IRBLkh-K3 (a) and (b), were kept independently by Hayashi and Kato. IRBLk-Ka and IRBLkm-Ts are IRRI monogenic-lines of *Pik* and *Pik-m,* respectively. LTH is the susceptible background cv. of these monogenic lines with no notable resistance genes

The new differential races H05-56-1 and H02-20-1 shown in bold letters were incompatible with some small lesions (grade 2) for *Pik-h* holding lines, but compatible (4–5) with *Pik* and *Pik-m* holding lines, such as LTH with no *R*-genes

The lower six lines are *Pik-h* +/− (R/S) homozygous lines of the mapping population from CO39×IRBLkh-K3. Their *Pik-h* (+/−) phenotypes (RC-R/S) have been diagnosed using the blast race V86010, which overcomes *Pia,* but not *Pik-h.* Therefore, by chance *Pia* comes into RC-R/S. Strong incompatibility (grade 1) of RC-R Kh-101 and RC-S lines against the two differential races was attributable to the presence of *Pia*
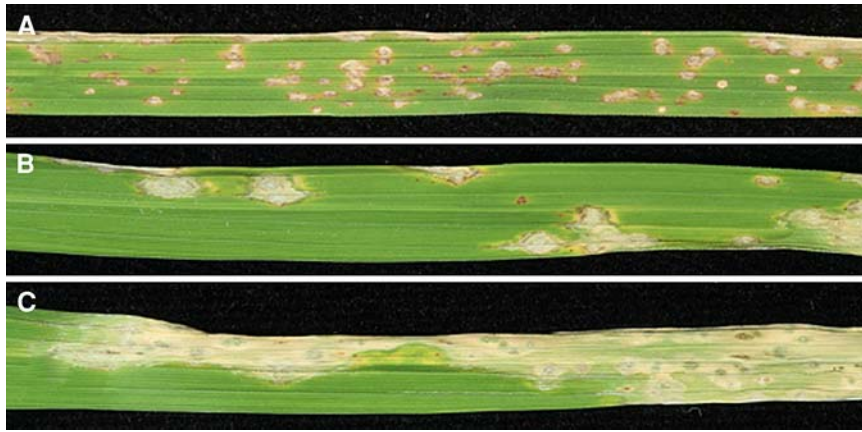
Fig. 1 Reaction of the *Pik* cluster blast resistance genes *Pik-h*, *Pik-m*, and *Pik* against the new differential blast race H05-56-1. For inoculation, a conidia suspension of $2–3 \times 10^5$/ml was sprayed onto all test plants at the same time, and after standing in a dew chamber for 24 h, the plants were moved to a greenhouse, and diagnosed on the 10th day after inoculation. (**a**) R-homozygous line RC-R Kh-102, from the cross of CO39×IRBLkh-K3 with only the *Pik-h* gene (Table 1). The resistance reaction of *Pik-h* varied from non-lesion type to this extent of small stopping-type lesions with browned fringes, but there were no progressive lesions or sporulation. (**b**) IRBLkm-Ts with *Pik-m*. (**c**) IRBLk-Ka with *Pik*. In (**b**) and (**c**), plants show susceptible reactions with sporulating progressive lesions, although sometime lesion density is lower than in (**a**). *Pik* seems more susceptible to this race than *Pik-m*

To confirm the authenticity of the F_2 and F_3 populations from IRBLkh-K3×CO39, used for subsequent fine mapping, some R and S homozygous lines of F_3 against V86010 from siblings were also checked for their reaction at their F_4 generation, to avoid complication with *Pia*, known to be present in CO39 (Tsunematsu et al. 2000), as much as possible. As shown in Table 1, the reactions of S homozygous siblings, RC-S Kh-2, 3, and 4, can be interpreted consistently as the absence of *Pik-h* and presence or segregation of *Pi-a*, and R homozygous lines RC-R Kh-101, 102, and 103 confirmed that the *R* gene of interest was *Pik-h*. The presence of *Pia* in RC-R Kh-101 and RC-S Kh-2, -3, -4 is by chance, and differentiated by stronger resistance reaction of grade 1 in Table 1, because the mapping isolate V86010 was selected to overcome *Pia* in CO39 (see Tsunematsu et al. 2000).

The typical resistance reaction of *Pik-h* on RC-R Kh-102 inoculated with the isolate H05-56-1 is shown in Fig. 1a. Although several small non-sporulating lesions appeared on the leaves, these were all of the stopping type with browned fringes, and did not extend as in the case of progressive lesions in the susceptible reaction, as shown in Fig. 1b and c. Other *Pik* group monogenic lines with *Pik-m* (IRBLkm-Ts: Fig. 1b) and *Pik* (IRBLk-Ka: Fig. 1c) developed severe progressive lesions with gray fringes and

sporulation with the same isolate. In *Pik-m*, the lesion density was lower than in the case of *Pik-h*. This lower density of susceptible lesions than that of the small lesions in resistant reactions is often observed in rice blast spray inoculation systems.

Segregation of *Pik-h* in the F_2 population

One hundred fifty-five F_2 plants derived from crosses of IRBLkh-K3×CO39 were examined for the presence or absence of *Pik-h* with the *M. oryzae* isolate V86010, which can overcome *Pia* and other minor *R* genes against Japanese isolates in CO39. The segregation of resistant and susceptible progeny was 117:38 in the F_2 populations, fitting a 3:1 ratio ($\chi^2 = 0.32$, $P > 0.05$) indicating that IRBLkh-K3 carries a single dominant *R* gene against the isolate V86010.

Fine mapping of the *Pik-h* locus

Forty-two DNA markers were selected from the RGP (http://rgp.dna.affrc.go.jp/) data (Kaji and Ogawa 1996; Hayasaka et al. 1996) in the region within 10 cM from *Pik* (110.1–120 cM) on Chromosome 11, and modified for PCR analysis. Twelve polymorphic markers were selected by preliminary BSA analysis using F_2 individuals (10 resistant and 10 susceptible

plants were selected to make pools). An example of BSA is presented in the side four lanes of Fig. 2. By screening 96 of the 155 F$_2$ individuals, the *Pik-h* region was narrowed to ∼2 cM flanked by the markers RM224 and Y6855RA (Figs. 2, 3). Then, 701 susceptible individuals were selected from among 3,060 F$_3$ hetero-siblings, and recombinants within the markers were selected with HEGS (Fig. 2). Among these, the 13 closest recombinants were selected between the flanking markers RM224 and Y6855RA, both linked to the *Pik-h* locus at 0.9 cM (Fig. 3).

Within this region, all the Nipponbare sequences obtained from IRSGP were segmented to sub-fragments of about 10 kb, and these were checked for their uniqueness in the genome by BLAST. From the unique regions, 90 PCR primers were designed using the Primer3 software and named such as Kh-(no.)F/R, depending on the optimal sequencing primers directions (Table 2). PCR was performed with these primers for IRBLkh-K3, and 19 primer pairs were found to amplify single bands. Among them, 8 bands properly positioned in the map were sequenced, and the SNPs between the parent cvs. were used as markers to determine the sites of close recombination events by sequencing with an ABI 3100 DNA sequencer.

In silico mapping of the *Pik-h* region and annotation of ORFs and RGAs

The above 8 SNP markers around the *Pik-h* region were located on a contig of 6 BAC clones of Nipponbare flanked by RM224 and Kh-38F (Fig. 3a) in reference to INE (http://rgp.dna.affrc.go.jp/E/giot/INE.html). Finally, the *Pik-h* region was delineated by the two closest flanking SNP markers, Kh-45F and Kh-A-3R on the north and south sides, respectively, with 5 and 2 recombination events on each side, spanning three BAC clones [OSJNBb0049B20 (131 kb), OSJNBa0047M04 (109 kb) and OSJNBa0036K13 (136 kb)]. According the complete sequence of Nipponbare chromosome 11 from IRGSP Release Build 4.0 Pseudomolecules of the Rice Genome, the physical distance between these two markers was estimated to be about 238 kb in length excluding a gap between OSJNBa0047M04 and OSJNBa0036K13. However, a gap-bridging BAC clone (110 kb; CHEF electrophoresis) was recently found, and by mapping of its end sequences and those of adjoining clones, the gap size was determined to be about 40 kb (RGP-team; Dr. T. Matsumoto, personal communication). Therefore, the *Pik-h* region size was delimited to about 270 kb, including the yet to be sequenced gap region. In comparison with other reports of *Pik* group genes, the *Pik-h* region was the narrowest and in the center of the previously reported regions (Fig. 3b).

The annotation of the expected ORFs in the region were determined by RiceGAAS (Rice Genome Automated Annotation System, http://ricegaas.dna.affrc.go.jp/: data not shown), and 54 ORFs were predicted; among these clones, 6 RGAs were found clustered near the center of the *Pik-h* region

Fig. 2 An example of HEGS/SSR (RM224) screening of recombinants around the blast resistance gene *Pik-h*, from susceptible F$_3$ individuals of CO39×IRBLkh-K3. Right side four lanes show an example of bulked segregant analysis with the F$_2$ population. M: ΦX174 DNA-*Hae*III marker; C: CO39; K: IRBLkh-K3; BS: susceptible bulk; BR: resistant bulk of F$_2$, including heterozygotes. The arrows R and S indicate resistant and susceptible type bands, respectively
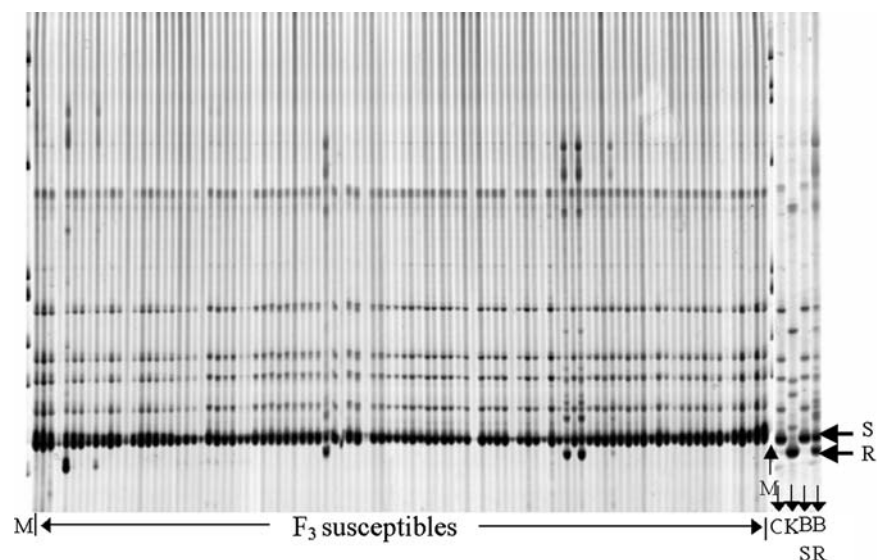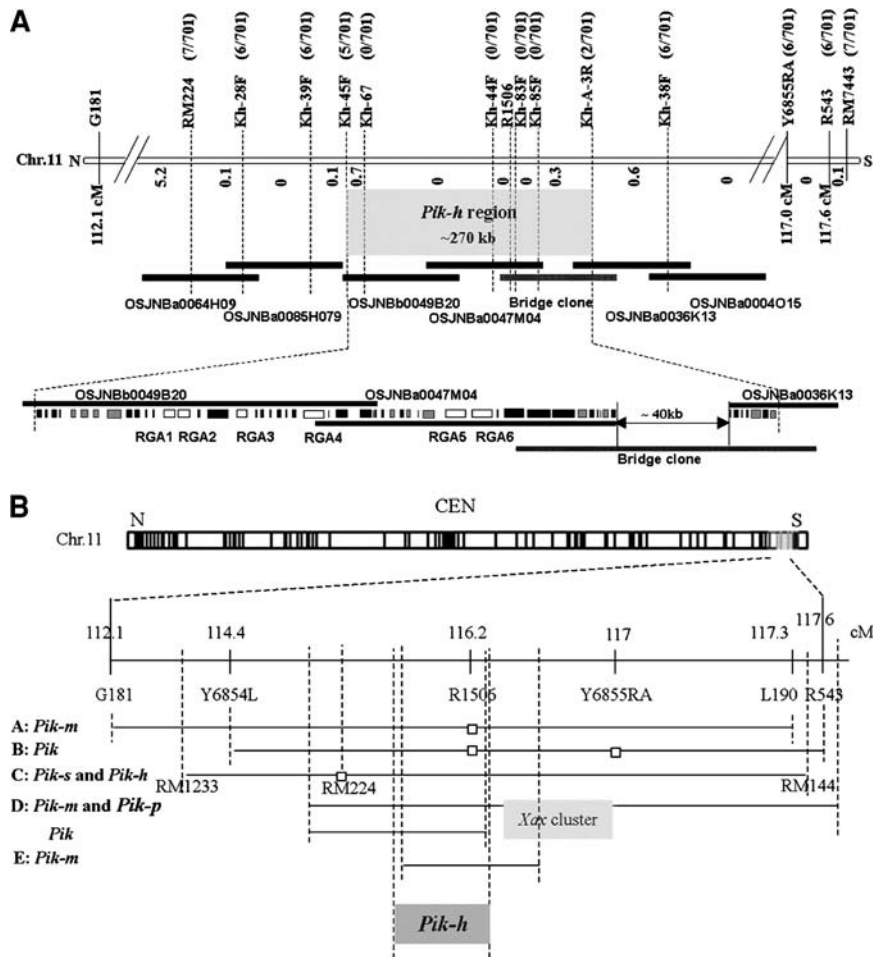
Fig. 3 Genetic and physical maps around the *Pik-h* region at the end of the long arm of chromosome 11, based on Nipponbare BACs (**a**), and comparison with the previous reports of fine-mapping of *Pik* cluster genes (**b**). (**a**) Marker locations on the chromosome and BAC clones, with recombination data in parentheses. The cumulative numbers of recombinants of markers to the R-gene per tested individual are shown at the top in parentheses. Genetic distances between the markers and the *Pik-h* gene region are indicated under the chromosome in Kosambi units. The details of the *Pik-h* region in the Nipponbare physical map are shown on the bottom with 54 predicted ORFs. There is a still unsequenced gap of 40 kb near the right end of the region, although a bridge clone

covering the gap was recently found. There are six resistance gene analogs (RGAs); RGA1 to 6, as candidate counterparts of the real *Pik-h*, and possibly also of the other *Pik*-group R-genes. There are also 11 transposon-related ORFs indicated by gray boxes. (**b**) Previously reported *Pik*-group genes regions. A: *Pik-m* (Kaji and Ogawa 1996); B: *Pik* (Hayasaka et al. 1996); C: *Pik-s* and *Pik-h* (Fjellstrom et al. 2004). D: *Pik-m*, *Pik-p*, and *Pik* (Hayashi et al. 2006); E: *Pik-m* (Li et al. 2007). Comparison was performed using the Nipponbare genome database with cM addresses. All the blast resistance gene regions overlap as *Pik* clusters, while recently cloned bacterial blight resistance genes *Xa4* and *Xa26*, (hatched rectangle on the right) were outside of the *Pik-h* region

(Fig. 3a). All the RGAs were of the nucleotide binding site leucine rich repeat (NBS-LRR) type, ranging in size from 1.6–3.4 kb. The cluster of 6 RGAs was 230 kb apart from the recently cloned *Xa4* and *Xa26* (Fig. 3b). The presence of 6 RGAs as a cluster in the center of not only *Pik-h* region but also of all *Pik* group gene regions is of special

interest, because this suggests that some of the RGAs may be counterparts of not only *Pik-h*, but also of other *Pik* group genes.

Although there were 7 recombination events in this region, all occurred in the marginal regions near the flanking markers (Fig. 3a). The four markers, Kh-67, Kh-44F, Kh-83F, and Kh-85F, within about

Table 2 PCR primer sequences of the markers near *Pik-h*. Primer name's F/R indicates their optimal direction for sequencing the amplicons

Primer	Type	Forward			Reverse	
Kh-28F	SNP	5′	ctccgaaatcttcagctcactt	3′	5′ tactgaatgccaactggtatcg	3′
Kh-38F	SNP	5′	acccattttgcagtttctgact	3′	5′ gtaaaaggacaagccaatcagg	3′
Kh-39F	SNP	5′	tcaattcccacttctccaag	3′	5′ tgaatcctataccgccaaag	3′
Kh-44F[a]	SNP	5′	gtttcagcattcacaacgag	3′	5′ catgatgtggttgttttccc	3′
Kh-45F	SNP	5′	ttcatccttttcaggcaacc	3′	5′ aacgtacacgcacaccatgt	3′
Kh-67	Dominant	5′	gatggcggcgtacatattct	3′	5′ tcatcagatggcattgcttc	3′
Kh-83F	SNP	5′	tggacaagttcgtggtggta	3′	5′ aagccatcttctttgcctga	3′
Kh-85F	SNP	5′	gtcatgagcgacttggtgaa	3′	5′ ttgcgagtcatcttttgtcg	3′
Kh-A-3R	SNP	5′	acgcagcacctctagctacc	3′	5′ gtcaagtagctcccgtcgtc	3′
Tetep-3[b]	Dominant	5′	agatcagcggtggaaaattc	3′	5′ cactcacagcaatgcctcat	3′
R543	RFLP-SCAR	5′	ttgcaactgctaagatgcct	3′	5′ atcacttcatctgtggatac	3′
RM224	SSR	5′	tgctataaaaggcattcggg	3′	5′ atcgatcgatcttcacgagg	3′
RM7443	SSR	5′	tgctgcgtgttactttggtg	3′	5′ aacccttcatcaggctacgc	3′
Y6855RA	RFLP-SCAR	5′	ctgcagattacggagaccaa	3′	5′ tcgacatatgggaccgatca	3′

Their map locations are indicated in Fig. 3

[a] Amplified only with Phusion DNA polymerase

[b] Used only for PCR comparison of the "*Pik-h* ORF" between Tetep and IRBLkh-K3

200 kb of core region showed no recombination with the *Pik-h* gene in these F₃ siblings (Fig. 3a).

Discussion

There have been several genetic studies of the rice blast resistance genes in the *Pik* region. This region is on the near-telomeric end of the long arm of chromosome 11, between RFLP markers G181 and R543, closely linked with RM224 and R1506 (Kaji and Ogawa, 1996; Hayasaka et al. 1996; Yu et al. 1996; Fjellstrom et al. 2004; Hayashi et al. 2006; Li et al. 2007). The *Pik-h* gene was identified in the experimental line K3, which was derived from a cross of Indian cv. HR-22 and Japanese cv. Sasashigure (Kiyosawa and Murty 1969), by suppressing the isolate Ken54-20-k^+, which has a mutation in *Avr-Pik* and was separated from a lesion developed in the incompatible Ken54-20 inoculation against lines with the *Pik* resistance gene. Therefore, *Pik-h* is expected to be allelic to *Pik*, and indeed there was no recombination between the resistances against the wild-type isolate Ken54-20 and mutant isolate Ken54-20-k^+, *i.e.*, between *Pik* and *Pik-h* (Kiyosawa and Murty 1969). Further, the resistance spectra of

the *Pik* group genes are identical for the 12 IRRI isolates used for IRBL *Pi* monogenic line development (Tsunematsu et al. 2000) except for a slight difference in *Pi1*. The resistance spectra of *Pik*, *Pik-m*, and *Pik-h* group genes are also very similar to the Japanese standard differentiating set of 31 blast races (Hayashi 2005).

Later, *Pik-h* was found to be widely distributed in several cvs. as in Indian Charnak, Vietnamese Te-tep, Philippine Tadukan, Russian Roshia 33, American Dawn (Kiyosawa 1981), and Japanese cvs., such as Fuji120, Mutsunishiki, and Chugoku 31 (Kiyosawa 1978). Thus, *Pik-h* was found more frequently than *Pik-p* or *Pik-m* with each single cv. known at that time. However, the detailed analysis of *Pik-h* has been severely hindered due to the loss of the key differentiating mutant isolate Ken54-20-k^+. For example, although Fjellstrom et al. (2004) reported that *Pik-h* is rather widely distributed in American rice cvs., his paper lacks a critical description of the race system differentiating *Pik* cluster genes, except the easily discernable *Pik-s*. With the conventional USA (Dr. Y. Jia, personal communication) and Philippine (Tsunematsu et al. 2000) race set, it is not possible to discriminate *Pik-h* from some other closely related *Pik* group genes, such as *Pik-m*.

Therefore, our new identification of natural *Pik-h*-differentiating isolates will facilitate utilization of *Pik* group genes in breeding, and analysis of evolution of the *R* genes in the *Pik* cluster, and also that of the *AvrPik* group genes in blast fungus, and of their recognition mechanism.

In the present study, for genetic analysis of *Pik-h*, we used the monogenic line IRBLkh-K3 with the LTH (Lijiangxintuanheigu) genetic background (Tsunematsu et al. 2000). The original report on IRBL monogenic lines (Tsunematsu et al. 2000) did not differentiate the *Pik* group lines with race reactions, probably because of the lack of appropriate differentiating races, and in confidence regarding the donor lines. However, it became necessary to confirm the authenticity of the *Pik-h* gene in IRBLkh-K3 and siblings of its cross with CO39 to refute the claim of "*Pik-h* cloning" by Sharma et al. (2005). As shown in Table 1, we have verified that the gene conferring resistance against V86010 in the cross was actually *Pik-h*.

We have also verified that the *Pik-h* gene maps both genetically and physically to the same region as the *Pik* gene cluster with higher resolution than in any previous report (Fig. 3a, b), confirming the association of *Pik-h* with the cluster. Use of the HEGS system greatly facilitated the fine mapping, also in the SSR marker system, while hitherto HEGS has mainly been used with AFLP marker systems (Hori et al. 2003). One marker analysis of 768 susceptible siblings was performed by overnight electrophoresis of 800 lanes with 8 set gel plates each with 100 lanes by a single researcher.

Pik-h gene was mapped within an interval of about 270 kb, including a gap of 40 kb that has yet to be sequenced, which was bridged by a recently found clone, based on the sequences of cv. Nipponbare. Within 54 predicted ORFs (RGP Rice Genome Annotation Database: http://ricegaas.dna.affrc.go.jp/) in this region, six were RGAs with the NBS-LRR structure. We suppose that some of these may be counterparts of the real *Pik* group genes, including *Pik-h*, although the genome structure around this region will be very different between *japonica* and *indica*, as described in the following section. Therefore, this report may facilitate cloning of other *Pik* cluster genes. Especially, *Pik-s* is found in several domestic and modern *japonica* lines although it is absent in Nipponbare. This indicates that the sequence differences between the Nipponbare

RGA and the *Pik-s* gene are rather small, and it is possible that one of the RGAs identified here may be the counterpart. Therefore, we are currently also attempting to amplify the *Pik-s* gene using the sequences of the 6 RGAs shown in Fig. 3a.

Although we have 4 co-segregation markers within this region, it was still not easy to localize the *R* gene candidate further by simply enlarging the size of the mapping population, because recombination in 200 kb around the target region is highly suppressed, and seven recombination events occurred only in the region near the flanking markers (Fig. 3a). Therefore, construction of a new BAC library from a *Pik-h*-containing resistant cv. will be needed for cloning of *Pik-h*. Indeed, no data are available on the regions in the *indica* Kasalath (http://rgp.dna.affrc.go.jp/E/publicdata/kasalathendmap/index.html) or 9311 (Rice Genome Database, http://rise.genomics.org.cn/rice/index2.jsp) genome, corresponding to the Nipponbare BAC OSJNBb0049B20 to OSJNBa0036K13 in Fig. 3a. Although Chinese *indica* cv. Minghui 63, which contains *Xa26*(t), has a BAC contig that includes Y6855RA and R1506, slightly overlapping with our *Pik-h* region (Sun et al. 2004) with R1506, their rather short contig of Minghui 63 (around 100 kb) corresponds to a very long (more than 0.5 Mb) region in the Nipponbare map (Fig. 3). This absence of genome data in *indica* cvs. is likely due to the hypervariability of the genome in this region, as often seen around several resistance genes. Clustering of resistance genes other than *Pik* genes, such as the *Xa* gene group, has been reported in this region. However, we speculate that these *Xa* resistance genes, such as *Xa3*, *Xa4*, and *Xa26*, are not completely allelic to the *Pik*-group rice blast genes. As the resistance spectra of the *Pik*-group genes are so close that the epitopes recognized by them, i.e., *Avr* gene products of the blast races, are thought to be very similar, and will be different from the bacterial epitopes recognized by *Xa* gene products. Indeed, the *Pik-h* region and the cluster of RGAs were shown to be located 130 kb and 230 kb apart from *Xa4* and *Xa26*, respectively (Fig. 3b). However, the markers developed in the present study will be useful for research and breeding to introduce other resistance genes in this region.

Considering the character of the *Pik-h* gene and the region discussed above, the claim of Sharma et al. (2005) about "cloning of the *Pik-h* gene from Tetep" is very doubtful. First, they presented no race

characterization verifying their gene's authenticity. Second, their "locus" is located more than 10 cM (JRGP RFLP 2000 Map) apart from the *Pik* cluster. Third, our PCR comparison of their "*Pik-h* ORF" between Tetep and IRBLkh-K3 revealed 5.5% deviation at the amino acid level (data not shown), a difference that is too large to consider their "gene" to also be functional in IRBLkh-K3. Fourth, the possibility that their "gene" is another *R*-gene is also small because their annotated "LRR and NBS sequences" are only 20 to 30 amino acids in length, which is one order shorter than the normal *R*-genes. Thirty amino acids can scarcely constitute a single unit of an LR "repeat." Such structures will not be able to function as a ligand recognizing domain or a nucleotide binding site. Finally, no concrete evidence of complementation was presented at the 4th International Rice Blast Conference in Oct. 2007, in Changsha, China, to which they submitted an abstract.

A BAC library of a cultivar containing the *Pik-h* is being constructed to develop a real contig around the gene, and to finally clone the gene and elucidate the genomic character of this highly *R*-gene clustered region.

References

Bai J, Pennill LA, Ning J, Lee SW, Ramalingam J, Webb CR, Zhao B, Sun Q, Nelson JC, Leach JE, Hulbert SH (2002) Diversity in nucleotide binding site-leucine-rich repeat genes in cereals. Genome Res 12:1871–1884

Böhnert HU, Fudal I, Dioh W, Tharreau D, Notteghem JL, Lebrun MH (2004) A putative polyketide synthase/peptide synthetase from *Magnaporthe grisea* signals pathogen attack to resistant rice. Plant Cell 16:2499–2513

Bryan GT, Wu KS, Farrall L, Jia Y, Hershey HP, McAdams SA, Faulk KN, Donaldson GK, Tarchini R, Valent B (2000) A single amino acid difference distinguishes resistant and susceptible alleles of the rice blast resistance gene *Pi-ta*. Plant Cell 12:2033–2045

Chen DH, Dela Vina M, Inukai T, Mackill DJ, Ronald PC, Nelson RJ (1999) Molecular mapping of the blast

resistance genes, *Pi44(t)*, derived from a durably resistant rice cultivar. Theor Appl Genet 98:1046–1053

Chen XW, Shang JJ, Chen DX, Lei CL, Zou Y, Zhai WX, Liu GZ, Xu JH, Ling ZZ, Cao G, Ma BT, Wang YP, Zhao XF, Li SG, Zhu LH (2006) A B-lectin receptor kinase gene conferring rice blast resistance. Plant J 46:794–804

Farman ML, Eto Y, Nakao T, Tosa Y, Nakayashiki H, Mayama S, Leong SA (2002) Analysis of the structure of the *AVR1-CO39* avirulence locus in virulent rice-infecting isolates of *Magnaporthe grisea*. Mol Plant-Microbe Interact 15:6–16

Fjellstrom R, Conaway-Bormans CA, McClung AM, Marchetti MA, Shank AR, Park WD (2004) Development of DNA markers suitable for marker assisted selection of three Pi genes conferring resistance to multiple *Pyricularia grisea* pathotypes. Crop Sci 44:1790–1798

Glazebrook J, Rogers EE, Ausubel FM (1997) Use of *Arabidopsis* for genetic dissection of plant defense responses. Annu Rev Genet 31:547–569

Hayasaka H, Miyao A, Yano M, Matsunaga K, Sasaki T (1996) RFLP mapping of a rice blast resistance gene *Pik*. Breed Sci 46(Suppl 2):68

Hayashi N (2005) MAFF Microorganism genetic resources manual. Vol 18. Rice blast fungus. Natl Inst of Agrobiological Sciences

Hayashi K, Yoshida H, Ashikawa I (2006) Development of PCR-based allele-specific and InDel marker sets for nine rice blast resistance genes. Theor Appl Genet 113(2):251–260

Hori K, Kobayashi T, Shimizu A, Sato K, Takeda K, Kawasaki S (2003) Efficient construction of high-density linkage map and its application to QTL analysis in barley. Theor Appl Genet 107(5):806–813

Imbe T, Tsunematsu H, Kato H, Khush GS (2000) Genetic analysis of blast resistance in IR varieties and resistant breeding strategy. In: Tharreau D, Lebrun MH, Talbot NJ, Notteghem JL (eds) Advances in rice blast research. Kluwer Academic Publishers, pp 34–44

Inukai T, Nelson RJ, Zeigler RS, Sarkarung S, Mackill DJ, Bonnan JM, Takamure I, Kinoshita T (1994) Allelism of blast resistance genes in near isogenic lines of rice. Phytopathology 84:1278–1283

Jia Y, McAdams SA, Bryan GT, Hershey HP, Valent B (2000) Direct interaction of resistance gene and avirulence gene products confers rice blast resistance. EMBO J 19:4004–4014

Kaji R, Ogawa T (1996) RFLP mapping of a blast resistance gene, *Pik-m*, in rice. Breed Sci 46(Suppl 1):70

Kawasaki S, Murakami Y (2000) Genome Analysis of *Lotus japonicus*. J Plant Res 113:497–506

Kiyosawa S (1972) Genetics of blast resistance. Rice breeding IRRI. Manila, Philippines. pp 203–225

Kiyosawa S (1978) Identification of blast-resistance genes in some rice varieties. Jpn J Breed 28:287–296

Kiyosawa S (1981) Gene analysis for blast resistance. Oryza 18:196–203

Kiyosawa S, Murty VVS (1969) The inheritance of blast-resistance in *india* rice variety, HR-22. Jpn J Breed 19:269–276

Li L, Wang L, Jing J, Li Z, Lin F, Pan QH (2007) The *Pik^m* gene, conferring stable resistance to isolates of *Magnaporthe*

oryzae, was finely mapped in a crossover-cold region on rice chromosome 11. Mol Breed 20:179–188

Liu X, Wang L, Chen S, Lin F, Pan Q (2005) Genetic and physical mapping of *Pi36 (t)*, a novel rice blast resistance gene located on rice chromosome 8. Mol Genet Genomics 274:394–401

Liu X, Lin F, Wang L, Pan Q (2007) The *in silico* map-based cloning of *Pi36*, a rice coiled-coil nucleotide-binding site leucine-rich repeat gene that confers race-specific resistance to the blast fungus. Genetics 176:2541–2549

Martin GB, Bogdanove AJ, Sessa G (2003) Understanding the functions of plant disease resistance proteins. Annu Rev Plant Biol 54:23–61

McCouch SR, Nelson RJ, Tohme J, Zeigler RS (1994) Mapping of blast resistance genes in rice. In: Rice blast disease. In: Zeigler RS, Leong SA, Teng PS (eds) CABI and IRRI, Wallingford, Oxon, UK, pp 167–186

Meyers BC, Kozik A, Griego A, Kuang HH, Michelmore RW (2003) Genome-wide analysis of NBS-LRR-encoding genes in *Arabidopsis*. Plant Cell 15:809–834

Orbach MJ, Farrall L, Sweigard JA, Chumley FG, Valent B (2000) A telomeric avirulence gene determines efficacy for rice blast resistance gene *Pi-ta*. Plant Cell 12:2019–2032

Qu S, Liu G, Zhou B, Bellizzi M, Zeng L, Dai L, Han B, Wang GL (2006) The broad-spectrum blast resistance gene *Pi9* encodes a nucleotide-binding site-leucine-rich repeat protein and is a member of a multigene family in rice. Genetics 172:1901–1914

Sharma TR, Madhav MS, Singh BK, Shanker P, Jana TK, Dalal V, Pandit A, Singh A, Gaikwad K, Upreti HC, Singh NK (2005) High-resolution mapping, cloning and molecular characterization of the *Pik-h* gene of rice, which confers resistance to *Magnaporthe grisea*. Mol Genet Genomics 274:569–578

Sun XL, Cao YL, Yang ZF, Xu CG, Li XH, Wang SP, Zhang QF (2004) *Xa26*, a gene conferring resistance to *Xanthomonas oryzae* pv *oryzae* in rice, encodes an LRR receptor kinase-like protein. Plant J 37(4):517–527

Tsunematsu H, Yanoria MJT, Ebron LA, Hayashi N, Ando I, Kato H, Imbe T, Khush GS (2000) Development of monogenic lines of rice for rice blast resistance. Breed Sci 50:229–234

Wang ZX, Yano M, Yamanouchi U, Iwamoto M, Monna L, Hayasaka H, Katayose Y, Sasaki T (1999) The *Pib* gene for rice blast resistance belongs to the nucleotide binding and leucine-rich repeat class of plant disease resistance genes. Plant J 19:55–64

Wang C, Tan M, Xu X, Wen G, Zhang D, Lin X (2003) Localizing the bacterial blight resistance gene, *Xa22(t)*, to a 100-kilobase bacterial artificial chromosom. Phytopathology 93:1258–1262

Xiang Y, Cao YL, Xu CG, Li XH, Wang SP (2006) *Xa3*, conferring resistance for rice bacterial blight and encoding a receptor kinase-like protein, is the same as Xa26. Theor Appl Genet 113:1347–1135

Xu X, Kawasaki S, Fujimura T, Wang CT (2005) A protocol for high-throughput extraction of DNA from rice leaves. Plant Mol Biol Rep 27:291–295

Yang Z, Sun X, Wang S, Zhang Q (2003) Genetic and physical mapping of a new gene for bacterial blight resistance in rice. Theor Appl Genet 106:1467–1472

Yu Z, Mackill DJ, Bonman JM, McCouch S, Guiderdoni E, Notteghem JL, Tanksley SD (1996) Molecular mapping of genes for resistance to rice blast (*Pyricularia grisea* Sacc). Theor Appl Genet 93:859–863

Zhou B, Qu SH, Liu GF, Dolan M, Sakai H, Lu GD, Bellizzi M, Wang GL (2006) The eight amino acid differences within three leucine-rich repeats between *Pi2* and *Piz-t* resistance proteins determine the resistance specificity to *Magnaporthe grisea*. Mol Plant-Microbe Interact 19: 1216–1228