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Identification of five new blast resistance genes in the highly blast-resistant rice variety IR64 using a QTL mapping strategy

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Abstract Rice progenies used for the construction of genetic maps permit exhaustive identification and characterization of resistance genes present in their parental cultivars. We inoculated a rice progeny derived from the cross IR64 × Azucena with different *Magnaporthe grisea* isolates that showed differential responses on the parental cultivars. By QTL mapping, nine unlinked loci conferring resistance to each isolate were identified and named *Pi-24*(*t*) to *Pi-32*(*t*). They could correspond to nine specific resistance genes. Five of these resistance loci (RLs) were mapped at chromosomal locations where no resistance gene was previously reported, defining new resistance genes. Using degenerate primers of the NBS (nucleotide binding site) motif found in many resistance genes, two resistance gene analogues (RGAs) IR86 and IR14 were identified and mapped closely to two blast RLs (resistance identified in this study, i.e. *Pi-29*(t) and *Pi-30*(t) respectively). These two RLs may correspond to the *Pi-11* and *Pi-a* blast resistance genes previously identified. Moreover, the *ir86* and *ir14* genes have been identified "in silico" on the *indica* rice cultivar 93-11, recently sequenced by Chinese researchers. Both genes encodes NBS-LRR-like proteins that are characteristics of plant-disease resistance genes.

Keywords Rice Blast · *Magnaporthe grisea* · Resistance genes · NBS-LRR · Genetic mapping

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Introduction

Rice blast caused by the fungal pathogen, *Magnaporthe grisea* Hebert (Barr), is one of the most devastating rice diseases. In this pathosystem, race-specific resistance is governed by the gene-for-gene relationship (Kiyosawa 1971; Silué et al. 1992). To-date, more than 40 major blast resistance genes have been mapped (reviewed in Imbe and Matsumoto 1985; Mackill and Bonman 1992; Tohme et al. 1993; Pan et al. 1996; Nagato and Yoshimura 1998; Tabien et al. 2000; Fukuoka and Okuno 2001). However, some of these genes could be identical or allelic, since very few allelism tests were performed. Kiyosawa (1984) described differential cultivars with one or two resistance genes named *Pi-*, followed by a different letter for each gene. Some of these genes have several alleles, such as loci *Pi-z* and *Pi-ta* with two alleles and *Pi-k* with five alleles. Some other resistance genes from tropical cultivars were introduced into isogenic lines using the susceptible cultivar Co39 as a recurrent parent (Imbe et al. 2000). Allelism tests performed on these isogenic lines showed that most of these genes were allelic or identical to known *Pi* genes (Inukai et al. 1994).

When testing the intensively studied Japanese differential cultivars with exotic isolates having a new avirulence gene, Imbe and Matsumoto (1985) discovered a new resistance gene, named *Pi-sh*. This showed that additional resistance genes could be detected even in well-characterised differential lines after exposure to a large set of isolates from diverse geographic or genetic origins. Many comparative inoculations of differential sets and resistant local cultivars were carried out, demon-

strating that almost all rice cultivars possess one, two or many resistance genes. In most cases, the resistance patterns of tested varieties differed from those of standard differentials such as the Japanese differentials. Such results indicated the presence of new resistance genes or combinations of genes in these cultivars. The characterization of these new genes was difficult to complete since it implies a long task. For each new rice cultivar, it requires comparison of its resistance pattern with those of reference cultivars having known resistance genes to a large set of differential isolates. Allelism tests must also be performed using progeny from crosses with standard differential cultivars having similar resistance patterns. This work is more tedious considering the increasing number of newly identified resistance genes. Therefore, the use of the molecular identification of resistance genes is of great interest.

During the last few years, many resistance genes have been cloned. A surprising result which has emerged is that a large number of resistance genes display a similar characteristic domain identified as a nucleotide binding site (NBS), often associated with a leucine rich repeat motif (LRR) (Meyers et al. 1999). The NBS motif possesses a highly conserved amino acid domain allowing primers to be designed for PCR amplification of potential resistance gene homologs. Using this strategy, many NBS homologs have been cloned in dicotyledous and monocotyledous plants such as potato (Leister et al. 1996), soybean (Kanazin et al. 1996; Yu et al. 1996), *Arabidopsis* (Aarts et al. 1998), barley (Leister et al. 1998) and rice (Mago et al. 1999). This strategy can be a powerful tool to facilitate the isolation of resistance genes that were previously mapped. Putative resistance gene fragments homologous to the NBS motif [resistance gene analogue (RGA) markers] can be rapidly identified by PCR and used as molecular markers on existing genetic maps. When co-localisation is observed between the RGA and a putative resistance locus, the RGA can be assigned to the resistance gene locus and used to further identify the resistance gene (Aarts et al. 1998; Collins et al. 1998; Leister et al. 1999; Shen et al. 1998).

As new strategies for resistance genes are being tested (lineage exclusion, Zeigler et al. 1994), characterisation of the resistance gene diversity in rice is important. IR64 is one of the most cultivated rice variety in Asia and is highly resistant to blast disease under irrigated conditions (Bonman et al. 1989; Roumen et al. 1992). However if the blast resistance gene pyramid has been suggested to explain this, the genetics of resistance is not well known due, in particular, to its complex genealogic origin (Roumen et al. 1994). In this paper, by exploiting the genetic diversity of the rice blast fungus, we studied the number of major resistance genes present in IR64 and Azucena. A QTL (quantitative trait locus) detection approach was used in order to localize major or minor loci involved in the resistance. Several statistical methods were used to validate the results. Then, using the consensus primer of the NBS found in the R gene, we identified several RGAs from IR64 and tested whether these markers could be closely linked to the blast resistance genes described in this study.

Materials and methods

Rice cultivars and progeny

A progeny from the reference cross IR64 × Azucena, between an improved semi-dwarf indica (IR64) obtained by IRRI and an upland japonica from The Philippines (Azucena) was used. A population of 105 doubled-haploid lines (DH) was obtained by Guiderdoni et al. (1992) and used to map 200 molecular markers (Causse et al. 1994).

Rice cultivation

Rice plants (*Oryza sativa* L.) were grown in a greenhouse in trays of 40 × 29 × 7 cm filled with compost (7/8 Neuhaus compost no.9, 1/8 pouzzolane). Ten to 15 seeds of each DH line was sown in rows, in trays containing 14 lines each. Soil was kept moist with water and, once a week, with nutritive solution. Nitrogen fertilization with 8.6 g of nitrogen equivalent was done at 10, 3 and 1 day(s) before inoculation to increase susceptibility to blast.

Inoculation method

Inoculations with *M. grisea* Hebert (Barr) were performed 3 weeks after sowing either by injection or by spraying with conidial suspensions. For the spray method, 30 ml of a 50,000 conidia.ml^{-1} suspension with 0.5% gelatin were sprayed on each tray. Then rice plants were stored for 1 night in a controlled climatic chamber at 24 °C and 95% relative humidity. They were then transferred back to the greenhouse. For the injection method, plants were inoculated by injecting about 0.1 ml of a 25,000 conidia.ml^{-1} suspension with a syringe into the leaf sheaths. Two repetitions of parents and DH lines were grown and inoculated at different times. After 7 days, lesion types on rice leaves were observed and scored 1 (resistant) to 6 (susceptible) according to a standard reference scale (Silué et al. 1992). Progenies with scores between 1 and 3 were considered to be resistant and progenies with scores from 4 to 6 were considered to be susceptible. For QTL mapping, the exact score of each progeny (1 to 6) was used as a quantitative variable.

Isolates

Twenty nine rice blast isolates from 20 countries (including representatives from Latin America, Asia and Africa) were initially chosen from a collection of 1,500 field isolates from 55 countries, to maximize diversity. These isolates were screened on the parental lines (IR64 and Azucena) as well as 13 randomly chosen DH lines. Isolates showing clear differential reactions on parents and DH lines were then inoculated to the whole set of 105 available DH lines.

Molecular markers

Plant DNAs were isolated from lyophilized leaves using the CTAB method (Murray and Thompson 1980). A core map developed at IRRI (Huang et al. 1994) with RFLP, RAPD (random amplified polymorphic DNA) and isozyme markers was used. Marker density for regions of specific interest was increased using the following protocols: for RFLP markers, probes from the interspecific rice map (Causse et al. 1994) were used (kindly provided by Dr. S. McCouch, Cornell University, USA). Southern transfers,

hybridization and non-radioactive DNA labeling used for hybridization were done according to IRRI or CIMMYT protocols (Hoisington et al. 1994). For some probes, especially the RGA probes, radioactive labelling was performed using the Megaprime DNA labelling system (Amersham Life Science). Six restriction enzymes were used for the core map: *Dra*I, *Eco*RI, *Eco*RV, *Hin*dIII, *Sca*I, and *Xba*I. Fourteen additional enzymes were used to test for polymorphism with probes showing a monomorphic pattern. Several other RFLP markers were placed onto the map by different partners of the EGRAM (European Graminae Mapping Initiative) project. These markers mainly corresponded to rice, wheat, oats, barley, sorghum and sugar cane cDNAs with large-spectrum hybridization on the DNA of several grass species (Garsmeur et al., in preparation). For RAPD markers, PCR amplifications were carried out in 25 µl, as follows: 0.4 µM of primer, 0.02 U/µl of *Taq* polymerase (Appligene), 1 × buffer mix (Appligene), 150 µM of dNTP. Conditions were 95 °C – 5 min; 45 × (95 °C – 1 min; 35 °C – 1 min; 72 °C – 2 min); 72 °C – 6 min. PCR products were separated on 1.5% agarose gels and stained with ethidium bromide. STSs (sequence tagged sites) published by Inoue et al. (1994) were also used. These primers correspond to probes from the high density map of the Rice Genome Research Program – Japan (Kurata et al. 1994). PCR conditions were the same as in Inoue et al. (1994). When polymorphism between parents was not detected directly, amplification products were digested with restriction enzymes recognizing 4-bp sequences. Separation of PCR/digestion products were carried out on 2–3% agarose or 8% polyacrylamide gels and stained with ethidium bromide.

Map construction

The genetic map of molecular markers and RGAs was computed using the multipoint functions performed by MapMaker/EXP v. 3.0 (Lander et al. 1987). The two-point LOD score threshold was set to 5, and r_{max} to 0.3. Ordering of markers was achieved using the 'order', 'try' and 'ripple' commands, which calculate likelihood ratios for the different possible multipoint orders. Conversion of recombination fractions into centimorgans (cM) was obtained with Kosambi's mapping function (Kosambi 1944). The final map was drawn using MapDisto v. 1.2 (http://www.mpl.ird.fr/mapdisto).

Cosegregation analysis between markers and resistance traits

A QTL detection approach was employed in order to localize loci with a major or minor effect on resistance. Several statistical methods were used. We first performed *F-tests* with the markers as the criteria using MapDisto v. 1.3. Quasi-simple interval mapping (Q-SIM) was then performed using Qgene v. 3.0.6u (Nelson 1997). Although it is based on an *F-test*, this analysis has similar properties to the simple interval mapping method based on the *LOD-score* test (Lander and Botstein 1989). When needed, composite interval mapping (CIM) was performed in order to resolve unclear QTL positions or to try to identify linked QTLs. CIM was computed using QTL Cartographer package v. 1.15c (Basten et al. 1994, 2001), with model 6 activated in the Zmapqtl module. Default parameters were used for choosing QTLs involved as cofactors and window size. QTL or major gene detection was also confirmed by the distribution-free Kruskall and Wallis test using MapQTL for Unix v.2.4 (Van Ooijen 1992). As a summary of results, we choose to present three statistical parameters for each detected QTL: the quasi-*LOD-score* (thereafter *LOD*) given by the Qgene software, and the *R-squared* (R^2) and additivity (*a*) given by MapDisto.

PCR amplification and cloning of RGAs

Rice cultivar IR64 was used for PCR-based isolation of resistance gene candidates. Genomic DNA was extracted from leaves following the procedure described by Edwards et al. (1991). Degenerated oligonucleotide primers LM637 and LM638 were synthesized according to Kanazin et al. (1996). PCR amplifications were performed in a total volume of 25 µl with 50 ng of genomic DNA and 1.25 Units of *Taq*Gold polymerase (Perking Elmer). DNA template was denatured at 94 °C for 10 min, followed by 35 cycles of 1 min at 64 °C, 30 s at 45 °C and 30 s at 72 °C. PCR products were separated on 1.5% agarose gels, isolated using the Geneclean II kit (BIO101). DNA fragments were cloned into the plasmid pGEMT- (Promega) following the manufacturer's instructions, and then transformed into *Escherichia coli* DH5α.

RGAs classification based on restriction analysis and sequencing

Positive clones were detected directly from colonies by PCR amplification using M13 universal and reverse primers. Clones were grouped by restriction enzymes recognizing 4-bp sequences (*Taq*I, *Hin*fI, *Rsa*I and *Alu*I). Typically, 3.5 µl of the PCR product was digested with ten units of restriction enzymes, and separated on 3% agarose gels. After classification, one member of each group was sequenced from both strands using T7 and SP6 primers by Genome express (Grenoble, France).

Results

Resistance evaluation

The inoculation of the 26 *M. grisea* isolates on parents and on the subset of 13 DH lines from the cross IR64 × Azucena revealed an important variability in DH-line resistance patterns. None of the isolates were virulent on both parents. Fourteen isolates were avirulent on both parents. Among them, seven were avirulent on all 13 DH lines, and seven were virulent on at least some of the DH lines. The 12 remaining isolates were avirulent on IR64 and virulent on Azucena. These 12 isolates were virulent to some DH lines and avirulent to others. All 12 isolates differed for their virulence to the subset of DH lines tested. These results strongly suggest the existence of a combination of avirulence genes in *M. grisea* isolates and of the corresponding resistance genes in the DH lines. Among the 12 isolates tested, six were kept for further studies since they showed clear susceptible reactions on at least about 25% of the DH lines (see Fig. 1). The observed resistant:susceptible ratios observed for these isolates could correspond to a genetic model involving one ore two major resistance genes and one or several QTLs with variable effects. These six isolates were inoculated on all the 105 available DH lines.

Resistance loci mapped in the IR64 × Azucena cross

The distribution frequency of the lesion scores obtained on the 105 DH lines with the six blast isolates are illustrated on Fig. 1. The score on the standard 1–6 scale of each DH line was used as a quantitative variable for QTL mapping on the IR64 × Azucena map previously developed. When considering resistance QTLs with a LOD score higher than 2.5, we detected nine resistance loci (RLs) mapped on 8 of the 12 rice chromosomes

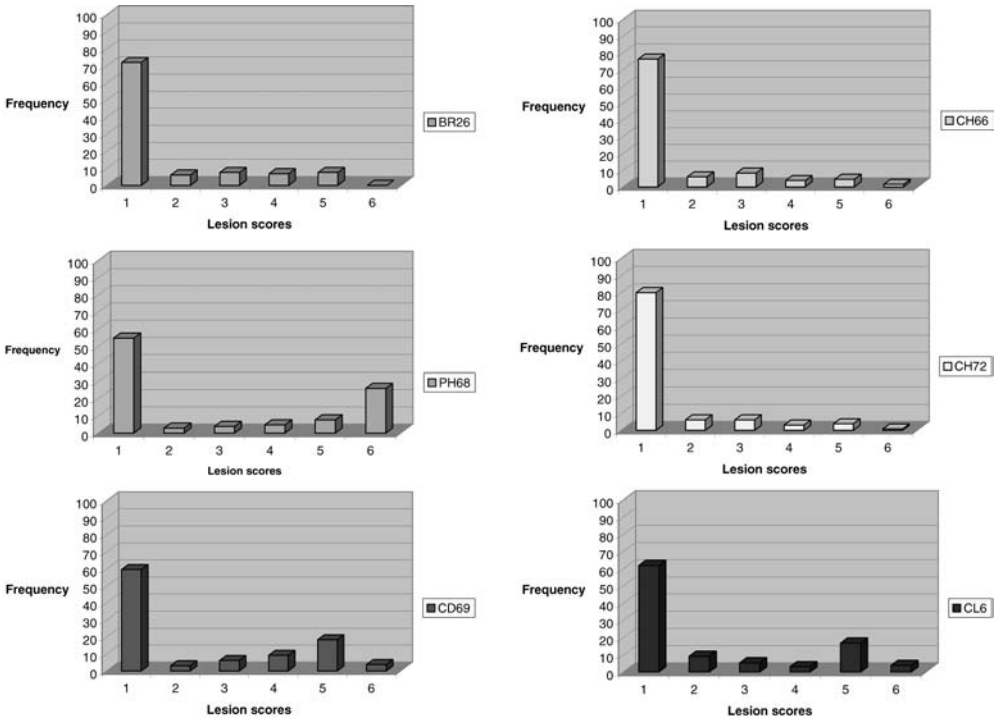

Fig. 1 Distribution frequency of the lesion scores obtained on the progeny of 105 DH lines (IR64 × Azucena cross) after inoculation with blast isolates BR26, PH68, CD69, CH66, CH72 and CL6. Lesion types were scored 1 (resistant) to 6 (susceptible) according to a standard reference scale (Silué et al. 1992). Frequency is calculated in percentage, indicating the ratio between the number of plants with the same lesion score on the total number of plants tested

(Table 1, Fig. 2). One isolate (CD69) revealed one RL, three isolates (BR26, CH72 and CL6) revealed two RLs and the last two (CH66 and PH68) revealed three RLs. Each RL was detected by one (six RLs), two (two RLs) or three isolates (one RL). None of these RLs were efficient against all the isolates, showing that all were isolate-specific and could be considered as putative specific resistance genes for blast. Six RLs were inherited from the indica cultivar IR64 and three RLs from the japonica cultivar Azucena. For CD69 and PH68, the results seem to fit well with the expected genetic model. The observed resistant:susceptible ratios were close to 1:1 and thus clearly showed the predominant effect of one major gene, which was evidenced by a very high *LOD score* and *Rsq* statistics (*LOD* = 19.43 and 17.64, *Rsq* = 0.54 and 0.47, respectively). For PH68, two additional QTLs with smaller effects were also found. For CH66 and CH72, the resistant:susceptible segregation is close to 3:1, indicating a possible action of two major genes. The results fit well to this model for CH72, as two loci were detected. For CH66, the accordance is less clear since three loci were evident. This shows that, at least for CH66, we cannot consider that the resistance segregation pattern is resulting in independant major genes. It could be considered as the result of the segregation of several QTLs, i.e. genes with less important individual effects on the trait. The observed *LOD* values, which reside between 2.33 and 4.28, and the *Rsq* values, which reside between 0.1 and 0.16, seem to support this model. For CL6 and BR26, the segregation is intermediate between a 1:1 and a 3:1 segregation. This seems to be in good accordance with the observed *LODs* and *Rsq*s, which indicate that these two traits are controlled by two genes, with one of a predominant effect.

Five RLs were mapped in chromosomal locations where no specific resistance gene to blast was previously described, leading to the identification of five new blast resistance genes. Following the current blast resistance gene nomenclature, they were named *Pi-24*(t) to *Pi-28*(t) (Table 1, Fig. 2).

Four RLs were mapped in chromosomal locations where other specific resistance gene(s) to blast were located (Fig. 2). These may correspond to the specific resistance genes already described to these map positions or to different alleles. *Pi-29*(t), identified on chromosome 8, mapped closely to the *Pi-11*(t) (previously named Pi-Zh, Zhu et al. 1993) blast resistance gene. *Pi-30*(t), located on chromosome 11, mapped closely to the *Pi-a* blast resistance gene (Kiyosawa 1972; Goto et al. 1981). *Pi-31*(t), located on chromosome 12, mapped closely to the *Pi-6*(t), *Pi-157* and *Pi-ta*, (=*Pi-4*; allelic or closely linked to *Pi-ta*^{2}) blast resistance genes (Kiyosawa 1972; Mackill and Bonman 1992; McCouch et al. 1994; Naqvi and Chattoo 1996). *Pi-32*(t), located on chromosome 12, mapped closely to the *Pi-12*(t), *Pi-tq6* and *Pi-21*(t) blast resistance genes (Inukai et al. 1996; Tabien et al. 2000; Fukuoka et al. 2001).

RGAs isolation and mapping

Using degenerated primers LM637 and LM638, corresponding to the P-loop (GGVGK/NTT) and HD (GLPLT) motif (Kanazin et al. 1996) of the conserved NB-ARC domain, we amplified an expected 500-bp band from

Table 1 Summary of the loci identified for resistance to six blast isolates by QTL analysis in the present study. For each resistance locus, the closest marker on the genetic map is indicated. Three statistics are shown: *LOD* = maximum LOD score for the presence of a locus controlling the analysed trait estimated by the Simple Interval Mapping method; *Rsq* = percentage of variance explained by the locus, *a* = additivity for the locus. *a* > 0 indicates a favorable allele from IR64 parent, whereas *a* < 0 indicates a favorable allele from the Azucena parent

Chromosome	Closest marker	BR26	PH68	CD69	CH66	CH72	CL6	Resistance locus name	Possible identity
							LOD = 4.2		
1	K5						Rsq = 0.16 a = –0.69	*Pi-24* (t)	
		LOD[a] = 4.65			LOD = 2.70	LOD = 2.33			
2	RG520	Rsq[b] = 0.21 a[c] = 0.68			Rsq = 0.10 a = 0.47	Rsq = 0.10 a = 0.43		*Pi-25* (t)	
			LOD = 2.83						
5	RG313		Rsq = 0.11 a = –0.80					*Pi-26* (t)	
					LOD = 3.55				
6	Est-2				Rsq = 0.16 a = 0.56			*Pi-27* (t)	
8	RZ617 RGA-IR86						LOD = 6.30 Rsq = 0.22 a = 1.06	*Pi-29* (t)	*Pi-11* (t)
			LOD = 2.99						
10	RZ500		Rsq = 0.10 a = –0.73					*Pi-28* (t)	
11	OpZ11-f RGA-IR14				LOD = 4.28 Rsq = 0.12 a = 0.53	LOD = 3.24 Rsq = 0.10 a = 0.42		*Pi-30* (t)	*Pi-a*
			LOD = 17.64	LOD = 19.43					
12	O10-800		Rsq = 0.47	Rsq = 0.54				*Pi-31* (t)	*Pi-6* (t), *Pi-157*, *Pi-ta*, *Pi-ta2*
			a = 1.54	a = 1.61					
		LOD = 8.65							
12	AF6	Rsq = 0.29						*Pi-32* (t)	*Pi-12* (t), *Pi-tq6*, *Pi-21* (t)
		a = 0.90							

[a] *LOD* = SIM LOD (Qgene)
[b] *Rsq* = R-squared (MapDisto)
[c] *a* = additivity (MapDisto); *a* > 0, favourable allele from IR64; *a* < 0, favourable allele from Azucena

IR64 genomic DNA. One hundred clones were analyzed and grouped into 19 classes using 4-bp restriction enzymes. One member of each class was sequenced. Sequence comparison leads to the identification of only seven different RGA families having a partial putative ORF (open reading frame) with a significant homology to the NB-ARC domain of the resistance genes. This result indicated that seven putative RGAs had been identified (C. Sallaud, unpublished data). These RGAs were mapped to assess their genetic linkage to the RLs identified in this study. Using 105 DH lines of the IR64 × Azucena population, one member of each RGA family was successfully mapped. They were located on chromosomes 3, 7, 8 and 11. Two RGAs, named IR86 and IR14, revealed a co-localization with two RLs identified in this study.

RGA-IR86 was mapped on chromosome 8 between markers G104 and C225b. A RL [*Pi-29*(t)] identified in this study, and the blast resistance gene *Pi-11*(t) (Zhu et al. 1993), are also located at the same position. The IR86 nucleotide sequence of 501 bp shows 86% and 85% identity with two others RGAs, p8558-3 (NID: Y09812, Xue et al. 1998) and NBA3 (NID: AF159886, Zhou et al., unpublished) respectively. Using the blastN search on the new draft sequence of the rice (*O. sativa* ssp.*indica*) genome recently published by Yu et al. (2001), we identified a unique sequence with more than 98% nucleic acid identity with RGA-IR86. The sequence is located within contig10362. This contig is 8,554 bp in length, and contains an intron-less ORF of 3,111 bp starting at position 425 and ending at position 3,538 (Fig. 3a). In view of the high homology (98% nucleic acid identity) between RGA-IR86 and the ORF present within contig 10362, it is tempting to assume that this ORF represents an *ir86* gene; *ir86* encodes a putative protein of 1,037 amino acids having a nucleotide-binding site and a leucing-rich repeat domain that are characteristic of the NBS-LRR type of plant disease-resistance proteins (data not show). The

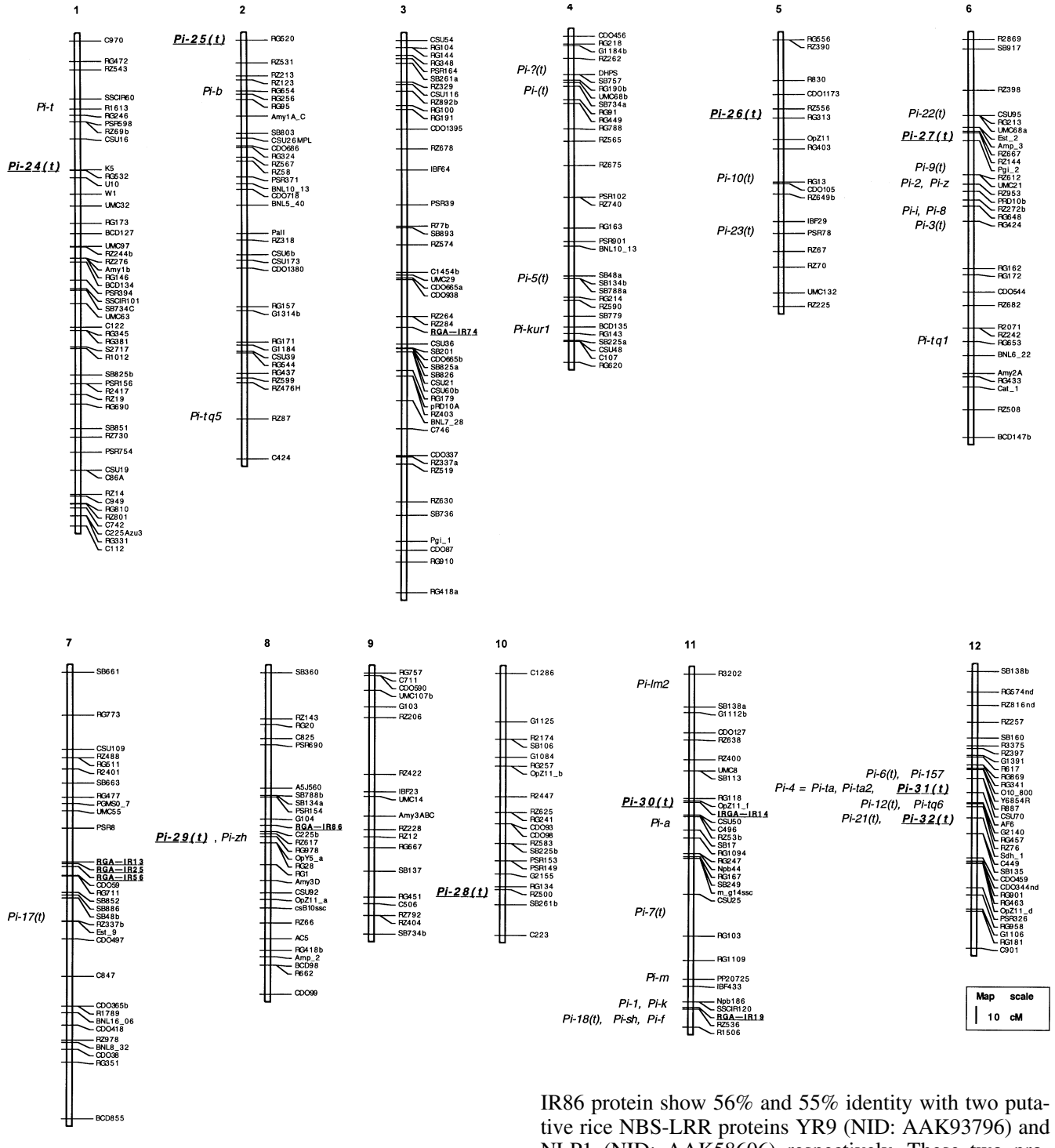

Fig. 2 Genetic localisation of several loci controling resistance to six blast strains in rice. The genetic map was obtained using a doubled-haploid population derived from anther culture of the F1 (IR64 × Azucena). The different types of markers were placed on the map by several laboratories (see text for details) and are indicated on the *right* of the linkage groups. The blast resistance loci which were identified either in the literature or in the present study (*in bold underlined*) are indicated on the *left* of the linkage groups. A tentative name was given to the resistance loci identified in the present study. Probes corresponding to the RGAs of the present study are *underlined* and are named as RGA-IRxx. Mapping function: Kosambi

IR86 protein show 56% and 55% identity with two putative rice NBS-LRR proteins YR9 (NID: AAK93796) and NLP1 (NID: AAK58606) respectively. These two proteins are deduced amino-acid sequences of cDNAs from seedling leaf (Yang et al., unpublished data) and root libraries (Zhou et al., unpublished data).

RGA-IR14 was mapped on chromosome 11, between markers OpZ11-f and CSU50. OpZ11-f and IR14 are the markers closest to the RL *Pi-30*(t) identified in this study. The *Pi-a* blast resistance gene was previously mapped in this position (Kiyosawa 1972; Goto et al. 1981). The IR14 nucleotide sequence exhibits more than 99% homology with two putative RGAs identified as

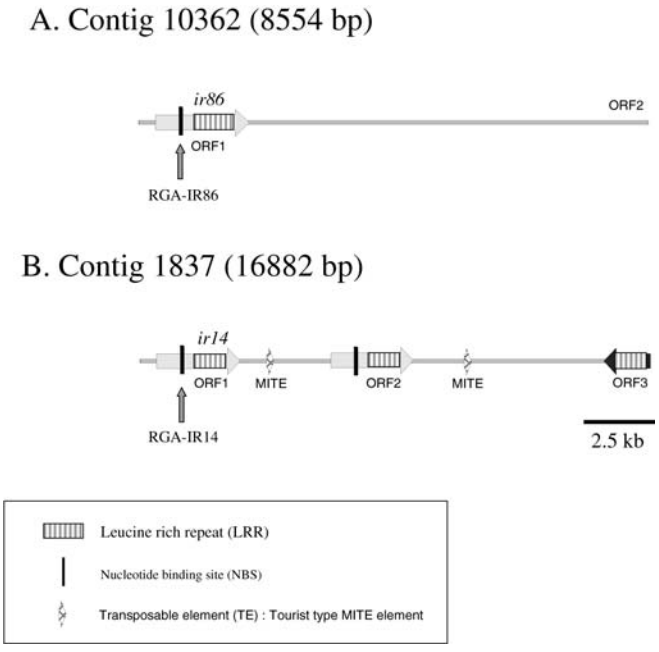

Fig. 3 Schematic representation showing putative ORFs encoding for NBS-LRR-like proteins. Contig sequences are nucleic acid sequences from the *O. sativa* ssp. *indica* cultivar 93-11 genome. **A** Contig 10362 with a putative ORF of 1,037 amino acids coding for NBS-LRR-like proteins. **B** Contig 1837 with three putative ORFs coding for NBS-LRR-like proteins. Three base insertions or deletions at positions 2,826, 6,801 and 7,651 have been introduced manually to obtain ORF1 and ORF2 encoding NBS-LRR-like proteins of 912 and 907 amino acids, respectively. Position of the RGA sequence homologous to the contig is indicated with a *black vertical arrow*. *Horizontal black shaded boxes* indicate the leucine-rich repeat protein motif (LRR), the *vertical black bar* indicates nucleotide binding-site domains (NBS), *horizontal shaded arrows* indicate tourist MITE-type transposable elements

RGA8 (NID: AF074889, Mago et al. 1999) and h359-1 (NID: Y09807, Xue et al. 1998). RGA 8 was isolated from the *indica* rice variety Phalguna and was mapped at the same position on chromosome 11. Using the blastN search on the draft sequence of the Chinese *indica* rice genome (cultivar 93-11), we found only one sequence with high homology to IR14 (97% nucleic acid identity). This homology obtained with a different cultivar suggests that it corresponds to the *ir14* gene. This sequence belongs to contig1837 which is 16,882 bp in length. This contig contains two putative ORFs (ORF1 and ORF2) encoding NBS-LRR-like proteins of 912 and 907 amino acids respectively (Fig. 3b). Both proteins share a high degree of homology (70% identity), and a significant homology with *RPR1* (64% identity), a NBS-LRR-like protein as well as to other NBS-LRR proteins (data not shown). *Rpr1* is located within another contig of the *indica* rice genome. One and two frameshift mutations caused by nucleotide insertions or deletions in ORF1 and ORF2 respectively have to be manually corrected in the sequence at positions 2,826, and 6,801 and 7,651, to obtain the two putative ORFs. We do not know if these insertions or deletions correspond to sequencing errors, or if they leads to inactive alleles, or pseudogenes. A third, although partial ORF (ORF3) encoding a putative protein having a homology to LRR proteins is located at the end of the contig in an opposite orientation.

Discussion and conclusion

This study showed that genetically fixed progeny like doubled-haploid lines, constitute a very useful tool for the characterization of resistance alleles originating from two parental lines. Such permanent populations permit the exploitation of the genetic diversity of the pathogen to detect specific resistance genes. When more than one resistance allele is combined in the segregating population, the QTL mapping method can be more powerful than Mendelian analysis to map these genes, even if the resistance observed in the population is not quantitative (Fig. 2).

We inoculated a limited set of 13 DH lines from the cross IR64 × Azucena with 26 *M. grisea* isolates. Among them, each of the 12 isolates giving a polymorphic reaction between the two cross parents gave a different resistance pattern on the set of DH lines. This complex pattern reflects the different combinations of at least nine segregating resistance loci (RLs).

Five out of the nine RLs identified in this study mapped in chromosomal regions where no specific resistance gene to blast has been previously described. Thus, these five loci can be considered to be new resistance genes to the blast fungus. This is a significant number when compared with the 40 rice resistance genes to blast already described. This is all the more surprising since only six blast isolates and two rice cultivars were used in this study. These data suggest that the number of resistance genes yet to be discovered remains large.

For the four RLs that mapped in chromosomal regions where specific resistance genes were already described, allelism tests between reference cultivars with known resistance genes and some DH lines having only one RL will be necessary to demonstrate whether these RLs are new resistance gene alleles. As more DH lines are produced and more markers are mapped, a more accurate localisation of the existing resistance genes will be possible. Moreover, as more avirulence genes will be identified, the use of isolates with only one avirulence gene will be another powerful tool to simplify the identification of RLs.

The presence of six RLs in IR64 is unexpected, since most rice cultivars seem to have one or two known resistance genes. Such an unplanned pyramiding of resistance genes has only been found in the rice cultivar Hama Asahi (Kiyosawa et al. 1991). The origin of the six RLs from IR64 is not known since the genealogy of this cultivar is very complex and involves different resistant parental cultivars such as Tetep, Tadukan, Peta, Taichung Native 1, CP-SLO, Chow Sung, BPI 76, NM S4, PTB 18, TKM6, GP 15, MUDGO, 17-1LT, W1263, Dee-geo-woo-gen and *Oryza nivara*. Progeny of such crosses were tested in IRBN nurseries (with susceptible spread-

ers and a natural local population of *M. grisea*). This resistance screening is likely to have led to the selection of progeny that had accumulated different resistance genes. IR64 is resistant to *M. grisea* in many countries under irrigated conditions. In Asia, its resistance in the field is considered to be good, even in areas where virulent isolates on IR64 are present.

The NBS motif belongs to a larger domain family, the NB-ARC domain, which is shared by proteins involved in the regulation of cell death in animals and resistance genes in plants is often associated with the LRR domain (known as NBS-LRR-like proteins). More than 70% of the approximately 40 resistance genes that have been cloned up to now, possess a NB-ARC domain. Moreover, recent genome sequence data reveal that the NBS-LRR class represents as much as 1% of the Arabidopsis genome. In this paper, we have shown that with a limited set of degenerate primers homologous to the NB-ARC domain, two out of seven distinct RGAs were mapped very close to the blast resistance genes. Moreover, another RGA identified in this study, IR19, is located at the end of chromosome 11 where multiple blast resistance genes have been identified (*Pi-1*, *Pi-k*, *Pi-18*, *Pi-sh*, *Pi-f*). These results confirmed other studies in various plants such as Arabidopsis, Rice, Maize, Lettuce and Potato (Leister et al. 1996, 1999; Botella et al. 1997; Aarts et al. 1998; Collins et al. 1998; Shen et al. 1998), showing that RGAs are localized where known resistance genes had been previously mapped. These examples show the utility of the candidate gene approach to facilitate the cloning of resistance genes. The genome of the *japonica* rice cultivar *Nipponbare* is being sequenced by an international rice consortium and 50% of the sequence is already available. More recently, a draft sequence of the *indica* cultivar 93-11, which covers 97% of the genome, has been published by Chinese researchers (Yu et al. 2001). By data mining on rice genome sequences, the number of NBS-LRR genes have been estimated to be more than 500. It is feasible to design specific primers that will allow the identification of RGAs alleles in different rice varieties. With the rice physical map being available for the *Nipponbare* cultivar, it will be easier to co-localize the putative resistance gene, with the RL, found by QTL analysis. In this paper, we demonstrate that this strategy could be promising. By taking advantage of the available rice genome data, (http://btn.genomics.org.cn/rice), we have identified "in silico" two genes corresponding to two RGA markers (IR14 and IR86) which co-localized with blast disease RLs. Both genes encode a putative NBS-LRR-like protein. One of them (*ir14*) is highly homologous with *rpr1* which has been identified by differential display in a screen to find genes induced by probenazole, a chemical inducer of acquired resistance to *M. grisea* (Sakamoto et al. 1999). On the same contig, we also identified two putative NBS-LRR-like proteins, one of which is highly homologous to *ir14*, suggesting gene duplication. Interestingly, *rpr1*, which shows significant homology to *ir14*, mapped on chromosome 11 close to RGA-IR14. This seems to indicate that a resistance gene cluster is present in this region. This has not been previously found. By screening an IR64 BAC library with RGA-IR14 and RGA-IR86 probes we have identified several BAC clones (data not shown) that will be used to confirm whether *rpr1* belong to this cluster, and the precise physical organization of this resistance gene cluster.

We have presented here a consensus map for most blast resistance genes identified to-date (Fig. 2). While co-localization with a gene candidate is effective, the work is far from complete. The next step is to use them as molecular markers in resistance gene segregation analysis. The same strategy is being used to identify QTLs using rice ESTs with defense-response gene-like sequences (Wang et al. 2001). Finally, mutagenesis and/or complementation analysis will be necessary for the final proof of the resistance gene function. This work is in progress for the putative resistance genes, *ir14* and *ir86*, found in this study.

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