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Resistance gene analogues from rice: cloning, sequencing and mapping

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Abstract Degenerate oligonucleotide primers were designed on the basis of nucleotide-binding-site (NBS) motifs conserved between resistance genes of *Arabidopsis*, flax and tobacco and subsequently used as PCR primers to amplify resistance gene analogues (RGA) in rice. Primers amplified a major band of approximately 500 bp. Restriction analysis of the amplified product revealed that the band was made up of several different fragments. Many of these fragments were cloned. Sixty different cloned fragments were analysed and assigned to 14 categories based on Southern blot analysis. Fourteen clones, each representing one of the 14 categories of RGAs were mapped onto the rice genetic map using a Nipponbare (*japonica*) × 'Kasalath' (*indica*) mapping population consisting of 186 F_2 lines. Of the 14 clones representing each class 12 could be mapped onto five different chromosomes of rice with a major cluster of 8 RGAs on chromosome 11. Our results indicate that it is possible to use sequence homology from conserved motifs of known resistance genes to amplify candidate resistance genes from diverse plant taxa.

Key words Degenerate oligonucleotides · Disease resistance · Leucine-rich repeats (LRR) · Nucleotide-binding site (NBS) · Resistance gene analogues (RGA)

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The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers AF074886 to AF074899

Introduction

Genes conferring resistance to the major classes of plant pathogens, including bacteria, virus, fungi and nematodes, have been isolated from different plant species. Sequence analysis of the predicted proteins of these cloned disease resistance genes reveals that common motifs occur in resistance (R) genes of diverse origin and pathogen specificity. The structural motifs of various genes also suggest possible functions and cellular locations of resistance proteins. Based on common molecular features, the R-genes are classified into seven classes (Baker et al. 1997; Gebhardt 1997; Hammond-Kosack and Jones 1997).

Genes in class I encode cytoplasmic receptor-like proteins that contain a leucine-rich repeat (LRR) domain and a nucleotide-binding site (NBS). Genes included in this category are the *Arabidopsis RPS2*, *RPM1* and tomato *Prf* genes conferring resistance to strains of *Pseudomonas syringae* (Bent et al. 1994; Grant et al. 1995; Salmeron et al. 1996). The tomato *I2-C* gene conferring resistance to fungus *Fusarium oxysporum* (Ori et al. 1997), the tobacco *N* gene conferring resistance to tobacco mosaic virus, the rust resistance gene *L6* of flax and the *Arabidopsis RPP5* gene conferring resistance to downy mildew also belong to this category (Lawrence et al. 1995; Parker et al. 1997; Whitham et al. 1994). The gene sequence at the *Cre3* nematode resistance locus of wheat also belongs to NBS-LRR category of sequences (Lagudah et al. 1997).

Another class of resistance genes includes *Pto*, which confers resistance to the bacterial pathogen *P. syringae* pv. *tomato* (Martin et al. 1993). Though *Pto* does not possess any LRR or NBS, it is dependent on *Prf*, an NBS-LRR-containing protein, for its function. A third category, which includes the tomato *Cf-2* and *Cf-9* genes conferring resistance to the *Cladosporium fulvum* fungus and the sugar beet nematode resistance gene Hs^{pro-1} (Cai et al. 1997; Hammond-Kosack and Jones

1997), encodes a predicted extracytoplasmic protein containing LRRs. Resistance genes that form yet another class consist of a transmembrane receptor with an extracellular LRR domain and an intracellular serine-threonine kinase. The rice *Xa21* gene encodes this class of proteins (Song et al. 1995). The recently described bacterial blight resistance gene *Xa1* from rice, is however, very different from the *Xa21* protein and exhibits a NBS-LRR type of structural organization (Yoshimura et al. 1998).

It has been observed that most of the above mentioned R-genes possess conserved amino acid motifs. These motifs consist of a nucleotide-binding-site domain and a hydrophobic domain (HD) with a consensus amino acid sequence Gly-Leu-Pro-Leu (GLPL), downstream of the NBS. By making use of conserved domains, various investigators have designed degenerate oligonucleotides for amplifying similar regions from the genomes of diverse plants species by the polymerase chain reaction (PCR) (Aarts et al. 1998; Collins et al. 1998; Feuillet et al. 1997; Kanazin et al. 1996; Leister et al. 1996, 1998; Shen et al. 1998; Speulman et al. 1998; Yu et al. 1996). Using a similar approach, we have PCR-amplified resistance gene analogues from rice with the aim of identifying clones that segregate with *Gm2*, a gall midge resistance gene, and other disease resistance genes.

We have previously mapped *Gm2* using restriction fragment length polymorphisms (RFLPs) and random amplified polymorphic DNAs (RAPDs) (Mohan et al. 1994; Nair et al. 1995). Our aim has been to isolate and clone the *Gm2* gene, a major dominant gene found in the indica variety 'Phalguna', which confers resistance to biotypes 1 and 2 of the pest. Though map-based cloning efforts are currently in progress in our laboratory, we are also interested in ascertaining if any RGAs isolated in this study cosegregate with *Gm2* locus and, if so, whether they could form a landmark for a map-based cloning strategy. Besides, if *Gm2* showed a structure similar to these genes, it could be isolated in this manner. Towards this goal, we have used primers based on the conserved motifs of previously isolated disease resistance genes to amplify similar regions from the resistant variety 'Phalguna'. The different RGAs were subsequently cloned, sequenced and mapped onto a rice genetic map. Analysis of sequence diversity was performed. Linkage of these RGAs with known disease resistance genes was also demonstrated, and map positions of these RGAs on the rice genetic map are described.

Materials and methods

Plant material

DNA from the 'indica' rice variety 'Phalguna' (resistant to gall midge biotype 1 and 2) was isolated as described earlier (Walbot 1988) and used as the template for all PCR amplifications.

Oligonucleotide primers and PCR amplification

PCR was performed in a total volume of 50 µl containing 10 m*M* TRIS-Cl (pH 8.8), 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 0.01% gelatin, 200 µ*M* of each of the four dNTPs, 50 pmol of each primer, 50 ng of rice genomic DNA and 2.5U *Taq* DNA polymerase (Stratagene, La Jolla, Calif.). Amplification of NBS-LRR domains was performed using the primers listed in Table 1a. Primer combinations were as

Table 1 **a** Oligonucleotides designed, based on the conserved peptide motifs NBS-LRR of *N*, *RPS2* and *L6*, for PCR amplification of RGAs from rice. **b** Primer combinations that amplified the 14 different categories of RGAs in the present study

a

Consensus motif	Primer designation	Sequence						
P-loop		G	G	V/I	G	K	T	T
	Sense							
	s1	GGT	GGG	GTT	GGG	AAG	ACA	ACG
	s2	GGI	GGI	GTI	GGI	AAI	ACI	AC
Hydrophobic domain (HD)		G	L	P	L	A/T	L	
	Antisense							
	as1	CCA	CGC	TAG	TGG	CAA	TCC	
	as2	IAA	IGC	IAG	IGG	IAA	ICC	
	as3	IAG	IGC	IAG	IGG	IAG	ICC	
	as4	ARI	GCT	ARI	GGI	ARI	CC	

b

Primer combination used	RGA clone number
s1 + as1	7
s2 + as2	12
s2 + as3	40, 47, 59
s2 + as4	1, 2, 8, 15, 16, 29, 33, 38, 42

follows: (I) primers s1 plus as1; (II) primers s2 plus as2; (III) primers s2 plus as3; (IV) primers s2 plus as4. Annealing temperatures were 55°C for set I and 45°C for sets II, III and IV. Cycling conditions consisted of a 3-min initial denaturation step at 94°C followed by 35 cycles at 94°C for 1 min, a 30-s annealing at the temperatures given above and a 30-s elongation at 72°C. Ten microliters of the reaction mix was loaded onto a 1.5% agarose gel to visualize the products.

Cloning of PCR products

The major amplification fragment was gel-purified as described by Nair et al. (1995) and cloned into a plasmid vector pGEM-T (Promega, Madison, Wis.) following the manufacturer's instructions.

Grouping of RGAs based on Southern hybridization

Sixty clones were randomly picked and plasmid DNAs isolated by the alkaline lysis method of Sambrook et al. (1989). All the samples were run on a 1.5% agarose gel and Southern blotted (Mohan et al. 1994). Individual clones were categorized by Southern hybridization. Different clones were subsequently used as probes, and clones showing hybridization to the same probe were assigned to a category.

Sequencing of cloned inserts

At least one RGA from each category was completely sequenced by the dideoxy chain termination method using the Sequenase (version 2.0) kit (USB, Cleveland, Ohio). The primers T7 (5′-TAATACGACTCACTATAGGG) and pGEMT-R (5′-GCATCCAACGCGTTGGGAGC) were used for sequencing.

Mapping of RGAs

RFLP mapping of RGAs was done using 186 F_2 lines derived from an intraspecific cross between 'Nipponbare' × 'Kasalath'. For Southern analysis, 2 μg of rice DNA was digested with restriction enzymes, size-fractionated and blotted (Kurata et al. 1994). Hybridization was performed using an ECL System (Amersham, UK).

Sequence analysis and homology searches

Computer-aided similarity searches of DNA and amino acid sequences with the EMBL and Swiss Prot sequence database were carried out using the updated version of the BLAST algorithm (Altschul et al. 1990). Multiple sequence alignments of nucleic acid and amino acid sequences were carried out with CLUSTAL W provided in the MACVECTOR 6.0 suite of software (Oxford Molecular Group, UK).

Results

PCR amplification of RGAs

Using the different primer combinations mentioned in Table 1a, we were successful in amplifying a 500-bp band from genomic DNA of rice, as was expected from the distance between the sequence motifs in *N* and *RPS2* (Fig. 1). The 500-bp fragment was gel-purified and used for further analysis. Digestion of this product with various 4-bp-recognizing restriction enzymes (*Alu*I, *Hae*III, *Sau*3AI) resulted in many fragments, with the sum of the molecular weights of all the restriction fragments being much greater than the molecular weight of the original product, thus indicating the presence of a heterogeneous product. The PCR products were cloned, and 60 RGAs were randomly chosen and grouped into 14 categories based on cross hybridization under stringent conditions (data not shown). Table 1b shows the different primer combinations which amplified these RGAs.

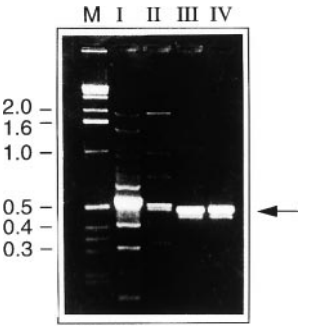

Fig. 1 PCR amplification products using rice DNA as template. Primer combinations are *I* (s1 + as1), *II* (s2 + as2), *III* (s2 + as3) and *IV* (s2 + as4), respectively. Primer sequences are listed in Table 1a. *M* 1-kb ladder. *Arrow* indicates the major bands which were cloned

Sequence analysis of rice PCR products

At least one clone from each of the 14 categories was sequenced. The 14 clones were further assigned to nine classes based on sequence comparison using CLUSTAL W analysis. RGA 29 was found to have an internal copy of the GLPL motif, the sequence of which was the basis for the design of the PCR primer in the antisense orientation. Several of the PCR-derived genomic fragments were homologous to each other. RGAs 1, 15, 16, 42, 47 and 59 shared between 86% and 94% identity, RGAs 8 and 38 were 73% identical, RGA 12 and 33 showed 54% identity. RGAs 2, 7, 29 and 40 did not show more than 50% identity with any of the other NBS-LRR sequences used in this study.

Using the resistance gene analogue sequences (GenBank accession numbers AF074886 to AF074899) to search the databases, we found significant sequence similarities not only to previously reported resistance genes *RPS2*, *RPP5* (from *Arabidopsis*) and *L6* (from flax), but also to resistance genes *Prf* and *I2C* of tomato and *Xanthomonas oryzae* resistance gene *Xa1* of rice. All these genes are members of the NBS-LRR class of plant resistance genes. Most of the clones also exhibited significant identity to resistance gene homologues

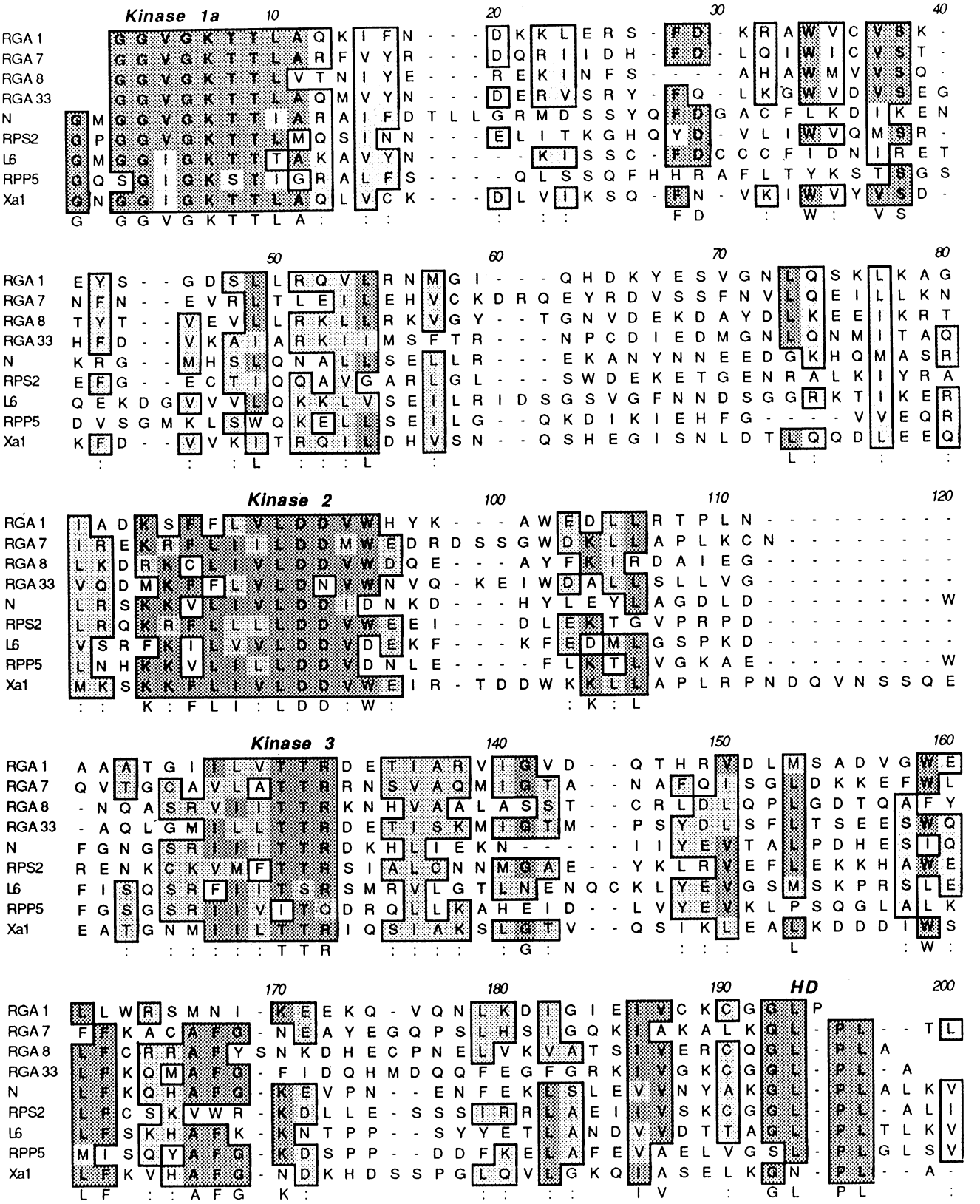

Fig. 2 Alignment of deduced amino acid sequences of 4 RGA classes of rice with that of other disease resistance genes of tobacco (*N*; accession no. U15605), flax (*L6*; accession no. U27081), *Arabidopsis* (*RPS2* & *RPP5*; accession nos. U14158, U97106) and rice (*Xa1*; accession no. AB002266) using CLUSTAL. The amino acid sequences shown are residues 216–392 of the *N* gene, 273–458 of *L6*, 182–355 of *RPS2*, 216–389 of *RPP5* and 326–508 of *Xa1*. The regions of homology are shown in *dark gray* (identity) and *light gray* (similarities). The conserved residues are indicated at the *bottom* as *letters* (identities) and as *:* (similarities)

isolated from *Arabidopsis* (Accession no. U97223-U97226), barley (Accession no. AF032679-AF032687), rice (Accession nos. Y09807-Y09812 and AF032688-AF032703), potato (Accession nos. U60069 and U60070) and soybean (Accession nos. U55803 and U55811). The homology of the RGA clones isolated in this study with these sequences varied from 52% to 99% amongst the different classes. For example, RGA 33 revealed homology varying from 99.2% with rice RGA (Accession nos. Y09811 and AF032696) to 53.6% with the *Arabidopsis* putative disease resistance gene analogue (Accession no. U97225), whereas RGA 12, on the other hand, exhibited 97% and 82% homology to rice RGAs (Accession nos. AF032700 and AF032688) and 57% and 56% similarity with potato and the *Arabidopsis* disease resistance genes, respectively (Accession nos. U60069 and U97225). RGAs 2, 29 and 40 did not show significant homology (<50%) to any resistance genes or gene analogues reported so far. Interestingly, RGA 2 showed 73% identity to the rice 4-coumarate-CoA ligase gene in a FASTA search, while using BLAST it showed 59% identity to the *Toxoplasma gondii* chloroplast genome as the best homology and some rice heat-shock proteins. RGA 40 did not show homology to any sequence using the BLAST search, while a database search using FASTA showed homology to *Arabidopsis thaliana* chromosome 5 sequences. RGA 29 showed similarities to human and *Macaca mulata* sequences.

Of the 14 RGA sequences 4 (RGA 1, 7, 8, 33) could be translated into polypeptides uninterrupted by stop codons. The alignment of the deduced amino acid sequences of *RPS2*, *N*, *L6*, *Prf* and *Xa1* in the homologous regions is shown in Fig. 2. The various motifs of NBS, i.e. kinase-1a, kinase-2 and kinase-3, were observed in all the sequences. The hydrophobic region, represented by the GLPL domain, was also conserved in the 4 clones. In addition to the NBS region, several other amino acid residues were also conserved between most of the sequences. A homology search of the deduced protein sequences of the clones with the database revealed results similar to those based on nucleic acid sequences. All the sequences revealed considerable identity to resistance genes *RPS2*, *RPP5*, *Prf* and *Xa1* and R-gene homologues from *Arabidopsis*, barley, rice, potato and soybean. Considerable identity was also observed with regions of the *Arabidopsis* myosin proteins.

RFLP mapping of RGAs

One representative of each of all 14 different categories of resistance gene analogues were used as RFLP probes

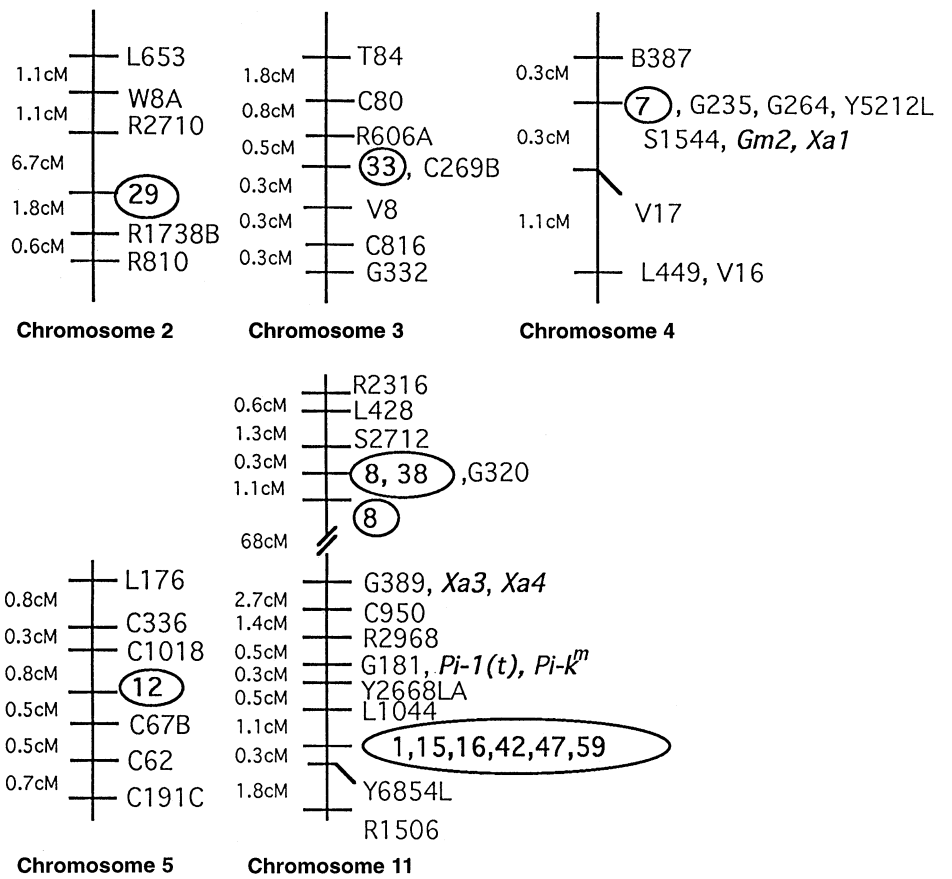

Fig. 3 A portion of the RFLP linkage map of the five chromosomes onto which the different RGAs isolated in this study have been mapped. Markers are indicated to the *right* of the *vertical lines* (Harushima et al. 1998). *Numbers* in *circles* are RGAs (this study). *Slanted bars* on *chromosome 11* represent a break to show two different portions of the same chromosome. Values to the *left* of each chromosome represent map distances in centiMorgans (cM) as shown in Harushima et al. (1998). Map not to scale

for mapping onto a genetic map of rice in order to ascertain whether any of the clones cosegregated with previously mapped disease resistance genes. Of the 14 clones 12 could be mapped to a total of seven different loci on 5 of the 12 linkage groups. Two RGAs (nos. 2 and 40) could not be mapped because of a lack of polymorphism between the parents. Four of the RGAs (12, 29, 33 and 38) behaved like single-copy sequences during RFLP analysis. Many of the RGAs (1, 15, 16, 42, 47, 59) showed similar RFLP patterns, suggesting that they constitute different members of the same multigene families. RGAs with about 90% sequence identity between themselves showed similar RFLP patterns.

Mapping data obtained by co-segregation analysis of these RGAs with RFLP markers in the 186 F_2 lines derived from a cross between 'Nipponbare' × 'Kasalath' revealed that most of the RGAs detected single loci. Six RGAs (1, 15, 16, 42, 47 and 59) mapped to a single locus on chromosome 11. RGA 8 mapped to two positions on chromosome 11 which were 1.1 cM apart. RGAs 7, 12, 29, 33 and 38 detected single loci on chromosomes 4, 5, 2, 3 and 11, respectively. Notably, RGAs 12, 29, 38 and the RGA cluster consisting of RGAs 1, 15, 16, 42, 47, 59 on chromosome 11 mapped to new locations on the rice genome previously not covered by any other RFLP markers in this mapping population. The map positions of the resistance gene analogues are shown in Fig. 3.

Several of the RGAs were found to be linked to known resistance genes. Of the 14 RGAs 8 mapped to rice chromosome 11. Six RGA sequences (1, 15, 16, 42, 47, 59) showed segregation with bacterial blight resistance genes, *Xa3* and *Xa4*, and several blast resistance genes such as *Pi-1*(*t*) and *Pi-k*^{m} . RGA 7, which mapped to chromosome 4, showed cosegregation with markers G235 and G264 and close linkage to V17. These markers had previously been shown to be tightly linked to the *Gm2* gene (Rajyashri et al. 1998) and *Xa1* (Yoshimura et al. 1996). Upon hybridization with the Nipponbare YAC library, RGA 7 identified the same YAC as the *Gm2* flanking markers, indicating a tight physical linkage (data not included).

Discussion

We have been able to identify at least 9 categories of resistance gene analogues from rice based on homology to the NBS region of various disease resistance genes such as *N*, *RPS2*, *RPP5* and *L6*. A simple PCR-based strategy was employed to isolate these RGAs with the aim of isolating a RGA clone cosegregating with the gall midge resistance gene *Gm2* and also to other disease resistance loci in rice. NBS-containing genes have been previously cloned from *Arabidopsis*, lettuce, maize, potato, rice and soybean using a similar strategy, and a number of these have also been mapped onto their respective genetic maps (Aarts et al. 1998; Collins et al. 1998; Kanazin et al. 1996; Leister et al. 1996, 1998; Shen et al. 1998; Speulman et al. 1998; Yu et al. 1996). The high sequence homology of the rice amplification products with NBS motifs of *N*, *RPS2*, *RPP5*, *L6*, *Prf* and resistance gene homologues of *Arabidopsis*, barley, potato, rice and soybean indicates that this strategy could be useful for isolating resistance genes.

The cosegregation of the NBS-containing DNA sequences with known disease resistance loci as shown in earlier studies has also been demonstrated in the present investigation. In potato and soybean some of these genes were present in clusters, while others mapped to several chromosomal locations (Kanazin et al. 1996; Leister et al. 1996; Yu et al. 1996). Also, in *Arabidopsis* and maize, map positions of various RGAs could be correlated to several disease resistance loci (Aarts et al. 1998; Collins et al. 1998; Shen et al. 1998; Speulman et al. 1998).

In the present study 12 different RGAs were mapped to seven loci on 5 of the 12 linkage groups in rice. Similar results were observed in the case of soybean where the different RGAs mapped to 8 of 26 linkage groups (Kanazin et al. 1996). Seven RGAs cosegregated with previously mapped resistance genes. Six of the RGA sequences (1, 15, 16, 42, 47, 59) showed segregation with *Xanthomonas* resistance genes *Xa3* and *Xa4* (Xinghua et al. 1996) and blast resistance genes *Pi-1(t)* and *Pi-k*^{m} (Kaji and Ogawa 1996; McCouch et al. 1995). RGA 7 mapped in close proximity to the gall midge resistance gene *Gm2*-flanking markers (Rajyashri et al. 1998) and bacterial blight resistance gene *Xa1* on chromosome 4 (Yoshimura et al. 1996) and also hybridized to the same YAC on which *Gm2* is presumably located (Rajyashri et al. 1998). r4, an RGA isolated in another study, has also been shown to map to the same region (Leister et al. 1998). Interestingly, the 5 chromosomes onto which the RGAs isolated in this study have been mapped also harbour other resistance genes such as *Pi-b* on chromosome 2 and a quantitative trait locus (QTL) for blast resistance on chromosome 3 (McCouch et al. 1995), rice tungro spherical virus (RTSV) and green leafhopper resistance on chromosome 4 (Sebastian et al. 1996), *xa-5* on chromosome 5 (Blair and McCouch 1997) and *Pi-7(t)* and *Xa-21* on chromosome 11 (McCouch et al. 1995; Ronald et al. 1992). It would be useful to determine the exact map position of the resistance genes in relation to the RGAs, however this is not possible due to the fact that different mapping populations were used to map these resistance genes. Interesting details should emerge once the Cornell rice map (Causse et al. 1994) and Rice Genome Group map (Harushima et al. 1998) are integrated.

Two RGAs, 2 and 40, could not be mapped due to lack of polymorphism. The very fact that most of the clones show polymorphism between the parents indicates that significant polymorphism exists around the region of the RGAs in rice. Of the 14 RGA sequences

characterized in this study 8 mapped onto chromosome 11, which is known to contain a large number of disease resistance genes (Causse et al. 1994). Clustering of R-genes at a single locus is well-documented (Kanazin et al. 1996; Leister et al. 1996, 1998; Yu et al. 1996). Clusters of disease resistance and defence response genes are known to exist in the *Arabidopsis* genome (Botella et al. 1997). It has been proposed that the clustering of R-genes is due to tandem duplication followed by unequal crossing over (Hulbert 1998).

The conservation of features such as NBS and LRR motifs among a large proportion of cloned R-genes has suggested that conserved sequences can be used to find additional resistance genes. This strategy will be beneficial in placing tightly linked loci to known resistance genes and possibly in identifying candidate resistance genes. Of the 12 RGAs that could be mapped in the present study, 3 RGAs and a cluster consisting of 6 RGAs mapped to regions not previously covered by any RFLP markers, thereby increasing the marker coverage of the genetic map of rice. It may be borne in mind that one may not be able to clone all the possible members of the multigene family. Besides, due to the lack of polymorphism between the parents some members of RGAs may not be mapped. Also, Southern hybridization revealed a number of bands of which only some of the major bands were mapped. This could lead to a biased mapping of the loci as NBS, LRR motifs could be found in other regions of the genome and these could show signals in the Southern analysis.

In addition, there could be a number of sequences that share the common NBS motifs that may not be a disease resistance gene at all. These may be relics of once functional disease resistance genes or may assume a function in the evolutionary future due to recombination in disease resistance clusters (Hulbert 1998). In this regard, it is interesting to see that 6 RGAs that cluster together on chromosome 11 have sequence identity amongst themselves varying from 86% to 94% (data not shown), these could form an ideal region for the origin of a new RGA driven by the mechanism of inter- and intragenic crossovers following mispairing (Hulbert 1998).

Pairwise comparisions of the derived amino acid sequences of the 4 RGAs (1, 7, 8, 33) which could be translated, among themselves and with resistance genes from other plants, revealed amino acid identities ranging from 15% to 41% and amino acid similarities ranging from 44% to 57% (data not shown). The homology between them was mainly restricted to the different motifs of NBS (as seen in Fig. 2), which formed the basis of isolation of these RGAs, thereby indicating sequence diversity between the various resistance genes and resistance gene analogues. The fact that these RGAs can be translated and that 2 of them (RGAs 1 and 7) are tightly linked to known resistance genes (Fig. 3) suggests that they may be involved in disease resistance. However, in order to establish the presence of a functional gene, we need to isolate the corresponding cDNA clones and use them to isolate the complete genes. We are presently in the process of isolating full-length cDNA clones using the various categories of putative resistance genes from rice (RGAs) that we have isolated in this study.

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