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Full Length Research Paper

Genome scanning for identification of resistance gene analogs (RGAs) in a highly durable blast resistance rice (*Oryza sativa* L.) cultivar, Moroberekan

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Disease resistance in plants is a desirable economic trait. Many disease resistance genes from various plants have been cloned so far. The gene products of some of these can be distinguished by the presence of an N terminal nucleotide binding site and a C-terminal stretch of leucine-rich repeats. Oligonucleotides already designed from sequence motifs conserved between resistance *N* genes of tobacco and *RPS2* of *Arabidopsis thaliana* were used as PCR primers (AS1/S2) to scan the rice blast disease resistant moroberekan genomic DNA. The primer pairs RL, CL and tobacco *N* gene which were used based on leucine-rich repeat regions of genes *RPS2* in *Arabidopsis*, *Cf9* of tomato against *Cladosporium fulvum* and *N* gene of tobacco confers resistance to the viral pathogen, tobacco mosaic virus respectively. The fragment amplified by the primer AS1/S2 was cloned and sequenced. The PCR products for the other three primers were sequenced directly. Homology search of the resultant nucleotide sequences and deduced amino acid sequences with the reported sequences available in public data bases of NCBI BLASTn and PSI blast indicated the presence of resistance protein-like gene in BRGA-1(blast resistant gene analogue-1), putative retro-elements and putative retro-transposons proteins in BRGA-2, mitochondrial DNA in BRGA-3 and NBS-LRR type resistance protein and NB-ARC domain containing expressed protein of *Oryza sativa* in BRGA-4.

Key words: Disease resistance, *Magnaporthe grisea,* leucine-rich repeats (LRR), Nucleotide-binding site (NBS), retrotransposon, rice blast disease, *Oryza sativa* L.

INTRODUCTION

Plants use a variety of strategies to defend themselves against microbial attack. One important defense mechanism is the plant's ability to recognize the presence of specific pathogens and initiate defense responses (Meaux and Olds, 2003). Plant genes for pathogen resistance can be used to engineer disease resistant crops (Leister et al., 1996). Pathogen recognition is mediated by resistance genes, most of which belong to an ancient family encoding proteins with nucleotide-binding site (NBS) and leucine-rich repeat (LRR) domains (Michelmore and Meyers, 1998; Young, 2000; Bai et al., 2002; Cannon et al., 2002). They control resistance to a wide variety of pathogens and pests including viruses, bacteria, fungi, nematodes and insects (Dangl and Jones, 2001). NBS-LRR genes are abundant in plant genomes, with approximately 150 described in the Colombia ecotype of *Arabidopsis* (Meyers et al., 2003) and many more estimated in the rice genome (Bai et al., 2002).

The success of a pathogen in infecting a host plant depends on how rapidly the plant recognizes the pathogen and activates appropriate defense reactions. If the pathogen carries an *Avr* (avirulence) gene whose product

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is specifically recognized by the product of the corresponding *R* (resistance) gene in the plant, resistance mechanisms are triggered rapidly resulting in disease resistance. If either the *Avr* or the *R* gene is absent, the pathogen is not recognized rapidly, the defense responses are activated slowly and disease ensues (Dangl and Jones, 2001).

The first class NBS-LRR genes of dicotyledonous plants can be subdivided into two distinct classes based on the structure of the N terminus of the protein, upstream of the NBS domain. The largest two classes contain nucleotide-binding domains (NBDs) and leucine-rich repeats (LRRs). The NBD is thought to be involved in signal transduction cascades through phosphorylation/dephosphorylation events with either ATP or GTP. The R-gene NBD domain has been expanded to include homology to eukaryotic cell death effectors (NBD-ARC) (Dangl and Jones, 2001).

Sequence-based markers, such as resistance gene analogues (RGA) that are based on the conserved sequences of leucine-rich repeats (LRRs) and nucleotide binding sites (NBSs), most likely lead to target genes for disease resistance. NBS-LRR super family became the premier pathogen surveillance system in cereal genomes, accounting for approximately 1% of the genes (Monosi et al., 2004). Sequences based on protein kinase (PK) motifs lead specifically to this class of genes which are involved in signal transduction processes in plants (Bent, 1996; Van der Linden et al., 2004). The mapping of RGA and PK markers on linkage maps has been used as a candidate gene approach to identify genes and pseudogenes with a possible role in the resistance mechanisms to various pathogens (Mago et al., 1999; Foolad et al., 2002; Quint et al., 2002; Mohler et al., 2002; Donald et al., 2002; Di Gaspero and Cipriani, 2003).

Complex clusters of R-genes are common in plant genomes. The isolation and characterization of more of these genes is important because they may provide clues about the complex mechanisms of resistance, the interactions involved in pathogen recognition and the evolution of the R-genes. Furthermore, cloned genes can be transferred to other species (Rommens et al., 1995; Thilmony et al., 1995) to study the resistance mechanism in a completely different genetic background.

The rice gene *Xa21* for resistance to the bacterium *Xanthomonas oryzae* corresponds to a receptor-like kinase protein in which a putative LRR domain, like that of *Cf9* in tomato is combined with a *Pto* type of serine/threonine kinase domain (Song et al., 1995). This study was carried out using the genomic DNA isolated from a cultivar, called moroberekan. Moroberekan is an upland japonica rice cultivar with apparently durable resistance that has been cultivated for many years in large areas of West Africa without losses to blast disease (Notteghem, 1985). Five resistance loci have been identified in African cultivar Moroberekan. Wang et al. (1994) located two major blast resistance loci, *Pi5(t)* and *Pi7(t)*, located on chromosomes 4 and 11 respectively, through restriction fragment length polymorphism (RFLP) analysis of the Moroberekan/CO39 recombinant inbred (RIL) population. Using this same RIL population, Naqvi and Chattoo (1995) and Naqvi et al. (1995) identified and mapped two more major resistance loci, *Pi10(t)* and *Pi157*, on chromosomes 5 and 12, respectively. Chen et al. (1999) mapped the blast resistance gene loci *Pi-44(t)* using bulk segregant AFLP analysis. Two dominant AFLP markers (AF_{348} and AF_{349}) linked to *Pi-44(t)* were identified. AF_{349} and AF_{348} were located at 3.3±1.5 and 11±3.5 cM from *Pi-44(t)*, respectively on chromosome 11.

Despite the large number of resistance genes (*R* genes) that have been identified and mapped and despite the fact the rice blast is a model pathosystem, only two blast resistance genes were cloned and characterized: *Pita* (Bryan et al., 2000; Jia et al., 2000) and *Pib* (Wang et al., 1999). A significant number of plant avirulence genes have been cloned, they encode small proteins secreted during infection and that are directly recognized by the corresponding resistance gene product. One exception to this model comes from the *Magnaporthe grisea* avirulence gene *ACE1* (Dioh et al., 2000; Bohnert et al., 2001). This gene encodes a large cytoplasmic enzyme that is unlikely to be recognized by the corresponding *R* gene product. Berruyer et al. (2003) identified the resistance gene corresponding to the cloned avirulence gene *ACE1* using pairs of isogenic strains of *M. grisea* differing only by their *ACE1* allele. Two RGAs, named IR86 and IR14, revealed a co-localization with two resistant loci's identified namely *Pi-29*(t) and *Pi-30*(t) which is mapped on chromosome 8 and 11, respectively by Sallaud et al. (2003) and both of this encoded NBS-LRR like proteins. They were designated as *ir86* and *ir14* genes as they have the characteristics of plant-disease resistance genes. Ramalingam et al. (2003) used candidate genes involved in both recognition resistance gene analogues (RGAs) and general plant defense response (DR) as molecular markers to test for association with resistance in rice to blast, bacterial blight (BB), sheath blight and brown planthopper (BPH). Most of the RGAs and DR genes detected a single locus with variable copy number and mapped on different chromosomes.

Qu et al. (2006) cloned rice blast resistance gene *Pi9* using a map-based cloning strategy. Analysis of selected *Pi9* deletion mutants and transformation of a 45-kb fragment from the BAC contig into the susceptible rice cultivar TP309 narrowed down *Pi9* to the candidate genes *Nbs2-Pi9* and *Nbs3-Pi9*. Disease evaluation of the transgenic lines carrying the individual candidate genes confirmed that *Nbs2-Pi9* is the *Pi9* gene. However, the molecular basis of *NBS2-Pi9* in broad-spectrum blast resistance remains to be elucidated. With the mentioned knowledge, already designed oligonucleotides sequences reported by Leister et al. (1996), Chen et al. (1998) and Whitham et al. (1996) were used as primers in PCR to

Table 1. Details of reported degenerate primers used to amplify putative rice resistance gene analogues (RGAs).

S/N	Primer	Sequence (5-3')	Resistance gene	Reference
1	AS1- F AS2 - R	GGTGGGGTTGGGAAGACAACG CCACGCTAGTGGCAATCC	*A. thaliana RPS2* gene (Leucine-Rich Repeat region) and tobacco N gene (Leucine-Rich Repeat region)	Leister et al. (1996)
2	RL-F RL-R	CGCAACCACTAGAGTAAC ACACTGGTCCATGATGAGGTT	*A. thaliana RPS2* gene (Leucine-Rich Repeat region)	Chen et al. (1998)
3	CL-F CL-R	TTTTCGTGTTCAACGACG TAACGTCTATCGACTTCT	Tomato *Cf9* gene (Leucine-Rich-Repeat region)	Chen et al. (1998)
4	N gene-F N gene-R	GCATCTTCTTCTTCTTCTTC GAGCCTTTGAGATTGGCCGC	Tobacco *N* gene (Nucleotide-Binding site / Leucine-Rich Repeat region)	Whitham et al. (1996)

amplify rice [moroberekan] genomic DNA. We obtained amplification products that may be suitable, that warrant a potential to be used as probes for the isolation of genes from rice and related plant species which confer resistance to different types of pathogens.

MATERIALS AND METHODS

Plant material and DNA isolation

Moroberekkan seedlings were obtained from Paddy Breeding Station, Coimbatore, India. Artificial screening for rice blast disease was conducted with standard testing conditions as reported by Sallaud et al. (2003) and scoring was done as per (SES, 2002). Fresh disease free seedlings were raised in pots. Leaf samples were collected from 15 days old seedlings were used for isolation and purification of total genomic DNA following the method of McCouch et al. (1988). The quantity of DNA present in the samples was determined using fluorometry. The quantity of DNA in the samples ranged from 700 to 2400 ng/µl in the undiluted DNA. After quantification, the DNA samples were diluted to 15 to 20 ng/µl for the isolation of putative RGAs through PCR analysis.

Primers used in the study

Four set of primers, already reported were used in this study to amplify putative rice resistance gene analogues (RGAs). The primers are (1) AS1/ AS2 (Leister et al., 1996); (2) RL (Chen et al., 1998); (3) CL (Chen et al., 1998); (4) tobacco N gene (Whitham et al., 1996). The details about primer sequences were given in Table 1.

Optimization of PCR conditions

Initially, the optimum annealing temperatures were determined for each primer pair using gradient PCR. Once optimized, the PCR reaction was performed in volumes of 20 µl containing 50 ng of genomic DNA, 0.2 µM each of forward and reverse primers, 50 µM of each dNTPs (M/s Bangalore Genei Pvt. Ltd., Bangalore) 1 X assay buffer (10 mM Tris-HCl, (pH 8.3) 50 mM KCl, 1.5 mM $MgCl_2$ and 0.5 units of *Taq* DNA polymerase (M/s Bangalore Genei Pvt. Ltd., Bangalore). PCR was performed on a *Bio-Rad* gradient thermal cycler with a PCR profile of 94°C for 5 min followed by 35 cycles of 1 min at 94°C, 1 min at different annealing temperatures optimized and 1 min at 72°C with a final extension of 15 min at 72°C.

After amplification, the PCR products were resolved in 1.2% agarose gel using 1 X TAE buffer, stained with ethidium bromide, visualized in gel documentation system (Alpha Imager ™1200, Alpha Innotech Corp., California, USA) and the results were documented. The sizes of the amplified fragments were estimated by comparison with 100 bp ladder (M/s Bangalore Genei Pvt. Ltd., Bangalore).

Gel elution of PCR products

The PCR amplified fragments were excised from the agarose gel and purified using a gel cleanup kit. For elution of DNA fragments, 1.2% low melting agarose was used, since gels from low melting agarose exhibit low background fluorescence when stained with ethidium bromide. The band corresponding to the desired DNA fragment was cut using a sterile scalpel. DNA was purified from sliced gel pieces using 'GenElute™ gel extraction kit' (SIGMA, St. Louis, USA). The final purified product was stored in -20°C.

Cloning and bacterial transformation

Cloning of the eluted fragments was carried out using the InsT/A clone™ PCR product cloning Kit (M/s MBI Fermentas Inc., USA), which is a convenient system for one-step cloning of PCR-amplified DNA fragments. Ligation of the purified DNA fragment was performed using pTZ57R/T vector as described in the supplier's manual. Vector (pTZ57R/T) and inserts were taken in 1:3 ratios. Ligation was performed in volumes of 30 µl containing 3 µl of plasmid vector pTZ57R/T DNA (0.165 µg, 0.18 pmol ends), 5 µl of eluted PCR fragment (approximately 0.54 pmol), 3 µl of 10 X ligation buffer, 3 µl PEG 4000 solution and 1 µl of T_4 DNA ligase (5 Units). The ligation mixture was kept for incubation overnight at 22°C.

Transformation was carried out using TransformAid™ bacterial transformation system with DH 5ά *Escherichia coli* culture. The transformed and incubated culture cells were finally plated on pre-

Table 2. Band size of fragments eluted from gel after amplification of moroberekan genomic DNA with four reported primers and the name designated.

S/N	Primer	Optimized annealing temperature (°C)	Eluted fragment size (bp)	Hypothetical name designated for the putative BRGA*
1	AS1 / AS2	56.1	~ 550	BRGA 1
2	RL F/R	53.7	~ 700	BRGA 2
3	CL F/R	51.9	~ 800	BRGA 3
4	Tobacco *N* gene	53.7	~ 500	BRGA 4

BRGA*, Blast resistant gene analogue.

warmed LB-agar plates (containing 100 µg ml^{-1} ampicillin, 100 µg ml^{-1} IPTG and 160 µg ml^{-1} X-gal) incubated overnight at 37°C. Putative transformed colonies were selected based on blue/white selection and streaked on LB-agar plates using a sterile Metaloop® and incubated overnight at 37°C. The white colonies were selected for colony PCR.

Clone analysis by PCR

Colony PCR was carried out for direct analysis of the positive transformants. Five putative transformant positive colonies were picked up and each colony was re-suspended in 20 µl of the PCR mixture. A negative control was included (with the non-transformed colony) along with the PCR. The reaction mixture was incubated for 5 min at 94°C to lyse the cells and inactivate the nucleases. PCR amplification was carried out with the same cocktail and PCR profile mentioned in Section 3.10.2, except positive colonies was used as a template DNA in each reaction, instead of the genomic DNA used earlier and the annealing temperature was fixed from the gradient PCR. The PCR products were analyzed in agarose gel electrophoresis.

Isolation and restriction digestion of recombinant plasmid DNA

Highly pure plasmid DNA was obtained by means of ‘GenElute™ plasmid miniprep kit’ from SIGMA, St. Lois, USA). Double digestions of the plasmid DNA was performed with the restriction enzymes *Eco*R1and *Bam*H1 (M/s Fermentas, USA) according to the manufacturer’s instructions. About 0.1 to 1.0 µg of the plasmid DNA was restricted using 1 to 8 units of restriction endonucleases. After incubation at 37°C for 8 h, the restriction endonucleases were denatured by incubating the mixture at 60°C for 10 min. The digested DNA was analyzed by agarose gel electrophoresis.

Sequencing of the cloned and the eluted DNA

The recombinant plasmids for the primer (AS1/S2) were sequenced by submitting to custom sequencing services at M/s Bangalore Genei Pvt. Ltd., Bangalore and the purified PCR products for the primers (RL, CL, tobacco and N gene) to M/s Bioserve India Ltd., Hyderabad. The sequencing was done from one end of the vector with M13 primer using BigDye® Terminator v3.1 sequencing kit and analyzed on ABI PRISM® 377 genetic analyzer. Sequencing the purified products was done using the forward primer instead of M13 primer.

Sequence analysis

The nucleotide sequences obtained after sequencing from the M13 forward primer for AS1/S2 was analyzed to remove the vector backbone using VecScreen (Altshul et al., 1997). The contaminated region was cut using WINGENE (version 2.31). The forward nucleotide sequences obtained from the corresponding forward primers of RL, CL and tobacco N gene were used directly without employing VecScreen, since these sequences were obtained from the elutant. The amino acids were deduced from the nucleotide sequences in all possible frames using the translate option at the ORF finder of NCBI. The resulting amino acids were subjected to similarity search using the local alignment search algorithm, PSI BLAST. The scores of the alignment were computed with reference to block substitution matrix (BLOSUM). The result of the homology search is detailed as maximum identity for increased length of subject sequences, higher positive values and decreasing E-values. The lower the E-value, the more similar the sequences, in terms of homology to the previously reported sequences in the database.

DNA sequencing homology search was performed also using NCBI. Multiple alignments and similarity search of the peptide sequences were performed using the T-coffee sequence alignment software developed by Notredame et al. (2000).

RESULTS

Gene amplification

Four primer pairs AS1/S2 F/R (Forward / Reverse), RL F/R, CL F/R and tobacco *N* gene resulted in amplification of expected size DNA. The optimized annealing temperatures were used to amplify putative RGAs in rice. Primer combinations AS1/S2, RL F/R, CL F/R and tobacco *N* gene resulted in less intense multiple bands. For these primers, the expected band sizes (as per the previous reports) were eluted. Different size amplified bands were eluted (Table 2; Figure 1 a, b, c).

The DNA concentration was around 50 to 100 ng μl^{-1} for all the eluted fragments. The eluted fragment amplified by the primer AS1/S2 was ligated with the plasmid pTZ57RT and the ligated mixture was used for transformation. The purified PCR products for the other RGAs such as RL F/R, CL F/R and tobacco *N* gene were subjected direct automated sequencing.

Transformation of the ligated product in *E. coli*

E. coli (DH5α) was transformed with the ligated recombinant pTZ57R/T vectors carrying inserts. Colonies

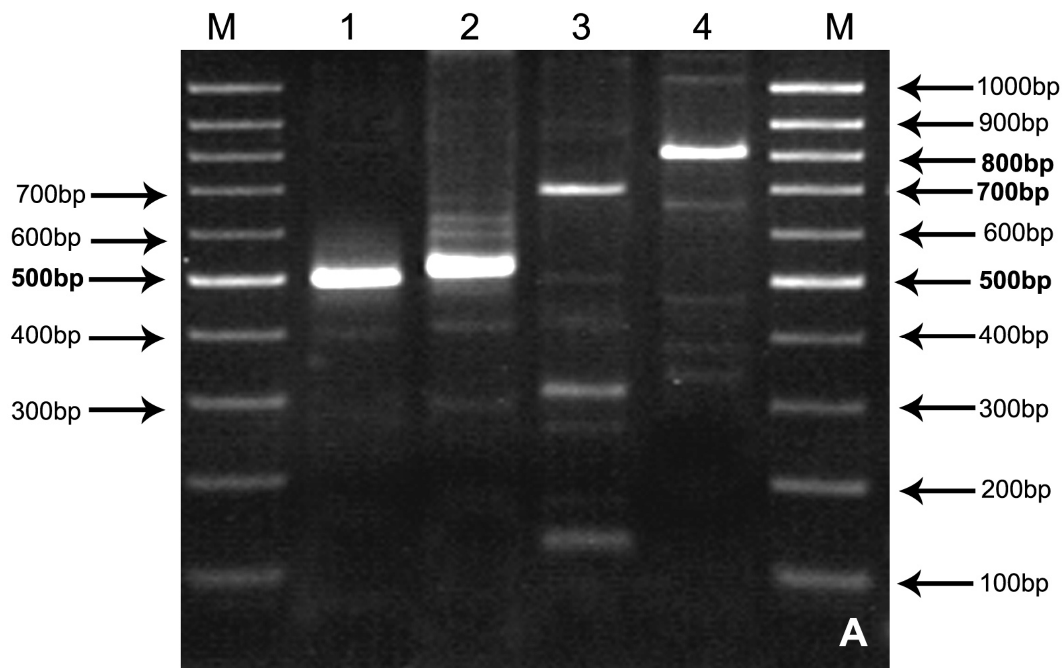

Figure 1. (A) Amplified PCR products (RGAs) of four primer sets in blast resistant rice genotype, Moroberekan. M - 100 bp ruler; lane 1: N; lane 2: AS1/S2; lane 3: RL; lane 4: CL. **(B)** eluted putative RGA fragments of AS1/S2 after electrophoresis. M - 100 bp ladder; lane 1,2: AS1/S2. **(C)** Eluted putative RGA fragments of N, RL and CL after electrophoresis. M - 100 bp ladder; lane 1: N; lane 2: RL; lane 3: CL.

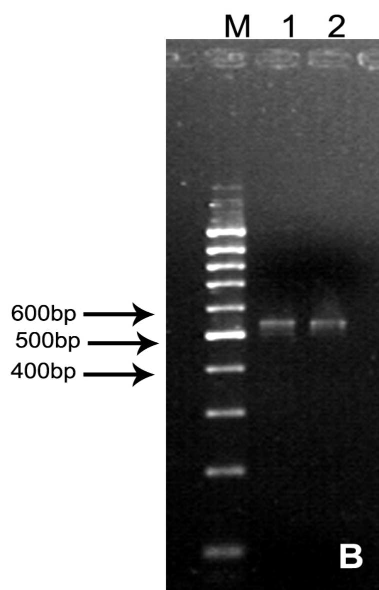

Figure 1. Contd..

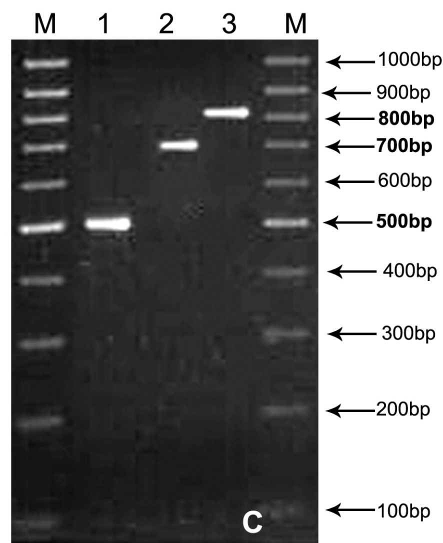

Figure 1. Contd..

emerged from 16 h of plating. Combinations of both blue and white colonies were obtained confirming successful transformation. White colonies were picked up and were grown in LB broth containing ampicillin.

Colony PCR and restriction analysis of transformants

Five recombinant colonies were screened for the presence of inserts by the colony PCR using the RGA AS1/S2. A negative control was included (with the non-transformed colony). Amplification of the expected size

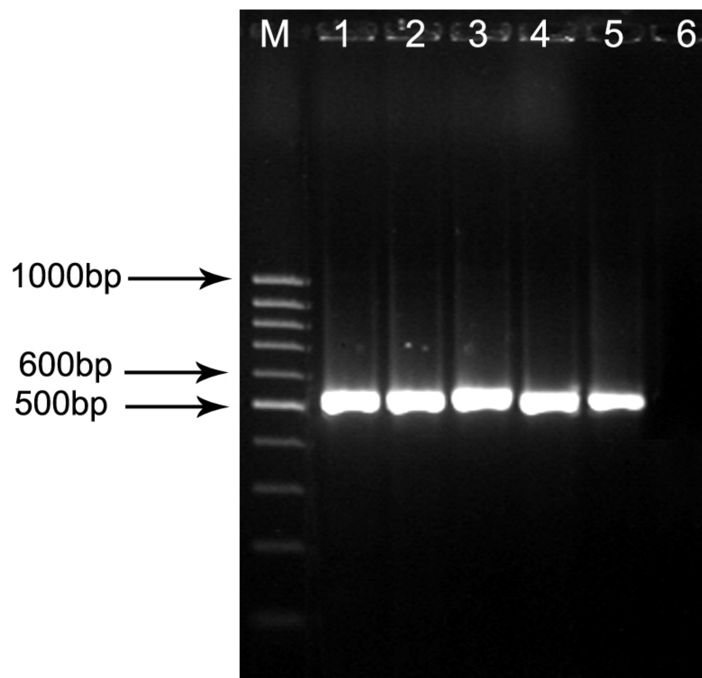

Figure 2. Colony PCR to check the presence of the insert for the primer AS1/S2. M: 100 bp ladder; LANE: 1 to 5 amplified PCR products of positive colonies; lane 6: negative control (non-transformed colony).

(~550 bp) was observed in all the five recombinant colonies except the negative control (Figure 2). The plasmids isolated from the recombinant clone were checked for the presence of insert by PCR. The recombinant plasmids were amplified by the gene specific primer AS1/S2. The recombinant clone exhibited specific amplification of expected size confirming the presence of the insert. Double digestion released the inserted DNA fragments from the recombinant plasmids. On resolving the digested mixture on a 1.2% agarose gel, the inserts of the expected size were released. This confirmed the insert in recombinant plasmids (Figure 3).

In silico analysis of the nucleotide sequences

Sequence analysis of BRGA-1 (AS1/S2)

The fragment cloned after PCR amplification of the rice genomic DNA of Moroberekan, by the primer combination AS1/S2 after sequencing resulted in a final size of 540 bp and was designated for convenience as BRGA-1. Homology search of BRGA-1 with the reported sequence available in public data bases of NCBI BLASTN 2.2.24+ (megablast) resulted in highly similar sequences with a total of 69 hits. Some of the highly similar sequences are listed in Table 3. BRGA-1 showed homology to resistance protein-like genes (RGAt1, RGAt2, sk110, etc.,) and some hypothetical proteins of *O. sativa* (*Indica* and *Japonica* cultivar group). Analysis of the nucleotide sequence with ORF finder of NCBI resulted in six frame translated protein sequences. The maximum size of amino acid sequence is from plus (2) frame with 68 amino acids and the start codon is from 161 to 367 bases with a length of 207 bases followed by 57 amino acids starting from 333 to 506 bases with a length of 174 bases. The translated sequence with 68 amino acids was searched with BLASTP 2.2.24+ of swissprot resulted in 6 blast hits. The sequence showed similarity with hypothetical proteins of *O. sativa Japonica* group and other unknown protein groups (Table 4). Multiple alignments of the translated peptide sequences of BRGA-1 were performed using the T-Coffee multiple sequence alignment (Notredame et al., 2000) and the results are presented in Figure 4.

Sequence analysis of BRGA-2

The purified PCR product of the rice genomic DNA by the primer combination RL F/R after sequencing resulted in a 702 bp, designated as BRGA-2. Homology search of BRGA-2 with the reported sequences public data bases of NCBI BLASTN 2.2.24+ (megablast) resulted in highly similar sequences with a total of 106 hits (Table 5). NCBI BLASTn (megablast) search revealed that BRGA-2 was homologous to the *O. sativa*-*Japonica* cultivar genomic DNA sequences. Analysis of the nucleotide sequence

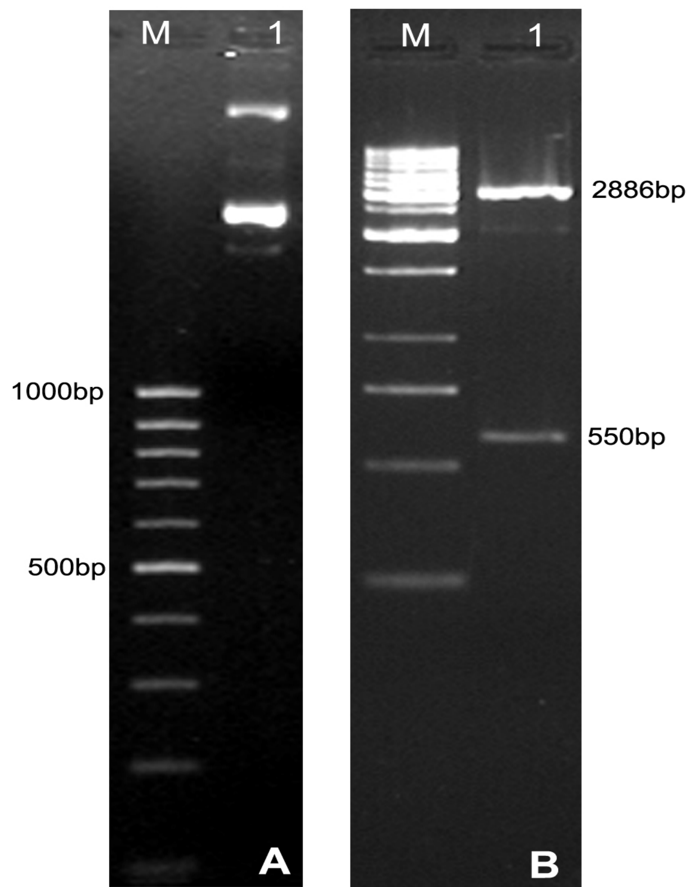

Figure 3. Restriction digestion of recombinant plasmid for checking the presence of the insert for the primer AS1/S2. (A) M: 100 bp ladder; lane 2, undigested recombinant plasmid; (B) M: 1 kb ladder; Lane 2, plasmid digested with *Eco*RI and *Bam*HI enzymes.

with ORF finder of NCBI resulted in six frame translated protein sequences. The maximum size of amino acid sequence is from plus (2) frame with 81 amino acids and the start codon is from 218 to 463 bases with a length of 246 bases followed by 45 amino acids starting from 564 to 701 bases with a length of 174 bases in minus (2) reading frame. The translated sequence with 81 amino acids was searched with BLASTP 2.2.24+ of swissprot resulted in 100 blast hits. BRGA-2 showed homology with putative retro-elements and putative retro-transposons proteins of the *O. sativa*-*Japonica* cultivars (Table 6). Multiple alignment of the deduced amino acid sequence of BRGA-2 with the related sequences is shown in Figure 5.

Sequence analysis of BRGA-3

The purified PCR product of the rice genomic DNA by the primer combination CL F/R after sequencing resulted in a 772 bp fragment and designated as BRGA-3. Homology search of BRGA-3 with the reported sequences available in the public data bases gave 53 blast hits with NCBI BLASTn (somewhat similar sequences) search (Table 7). Similarity search with megablast option of NCBI resulted in a match of 2 highly similar blast hits. BRGA-3 showed homology with the mitochondrial DNA of *O. sativa* (*Japonica* cultivar-group), *Triticum aestivum* and with the genomic DNA of *O. sativa* (*Japonica* cultivar-group). Analysis of the nucleotide sequence with ORF finder of NCBI resulted in translated protein sequences in four frames namely +2, +3, -2 and -1 strands. The maximum size of amino acid sequence is from plus (2) frame with 94 amino acids and the start codon is from 44 to 328 bases with a length of 285 bases followed by 69 amino acids starting from 1 to 210 bases with a length of 210 bases in minus (2) reading frame. The translated peptide sequences with all possible frames were searched with BLASTP 2.2.24+ of swissprot and Pdb with no possible hits.

Table 3. Summary of homology search performed for BRGA-1 (AS1/AS2) sequence by NCBI BLASTN 2.2.24+ (Mega blast).

Accession number	Description	Maximum score	Query coverage (%)	E-value	Maximum identity (%)
AF406635.1	*Oryza sativa* (*indica* cultivar-group) clone RGAt1 resistance protein-like gene, partial sequence	878	92	0.0	98
AF406636.1	*Oryza sativa* (*indica* cultivar-group) clone RGAt2 resistance protein-like gene, partial sequence	865	92	0.0	98
AY970342.1	*Oryza sativa* (*indica* cultivar-group) clone sk110 resistance protein-like gene, partial sequence	822	86	0.0	98
AY970335.1	*Oryza sativa* (*indica* cultivar-group) clone sk60 resistance protein-like gene, partial sequence	800	86	0.0	97
AY970344.1	*Oryza sativa* (*indica* cultivar-group) clone sk59 resistance protein-like gene, partial sequence	699	82	0.0	95
AF406635.1	*Oryza sativa* (*indica* cultivar-group) clone RGAt1 resistance protein-like gene, partial sequence	878	92	0.0	98
AY970337.1	*Oryza sativa* (*indica* cultivar-group) clone sk63 resistance protein-like gene, partial sequence	641	92	1e-180	89
AC145127.1	*Oryza sativa Japonica* group chromosome 10 clone Pseudo10p0.0-10p4.4, complete sequence	638	97	1e-179	88
AC099400.2	*Oryza sativa Japonica* group chromosome 10 clone OSJNBa0096E22, complete sequence	638	97	1e-179	88
AY970338.1	*Oryza sativa* (*indica* cultivar-group) clone sk67 resistance protein-like gene, partial sequence	638	92	1e-179	89
AY970331.1	*Oryza sativa* (*indica* cultivar-group) clone sk55 resistance protein-like gene, partial sequence	636	92	5e-179	89

Table 4. Summary of homology search performed for BRGA-1 (AS1/AS2) peptide sequence by NCBI BLASTP 2.2.24+.

Accession number	Description	Maximum score	Query coverage (%)	E-value
ABA96756.1	Hypothetical protein LOC_Os12g11270 *[Oryza sativa* Japonica group]	39.3	32	0.15
ABA96644.1	Hypothetical protein LOC_Os12g09470 [*Oryza sativa Japonica* group]	38.5	32	0.33
BAD16988.1	Hypothetical protein [Oryza sativa *Japonica* group]	37.4	33	0.66
NP_001172365.1	Os01g0392800 [*Oryza sativa Japonica* group] >dbj|BAH91095.1| Os01g0392800 [*Oryza sativa Japonica* group]	35.0	33	3.5
AAK52573.1	Unknown protein [*Oryza sativa Japonica* group] >gb|AAP51836.1| hypothetical protein LOC_Os10g02200 [*Oryza sativa Japonica* group]	34.7	38	4.2
BAC99337.1	Hypothetical protein [*Oryza sativa Japonica* group] >dbj|BAD01175.1| Hypothetical protein [*Oryza sativa Japonica* group]	33.9	30	6.4

Sequence analysis of BRGA 4

The purified PCR product of the of the rice genomic DNA by the primer combination Tobacco *N* gene F/R after sequencing was a 492 bp sequence designated as BRGA-4. Homology search of BRGA-4 with the reported sequences available in the public data bases gave 110 blast hits with NCBI BLASTn (somewhat similar sequences) search. Analysis of the nucleotide sequence with ORF finder of NCBI resulted in translated protein sequences in a single frame (+1) strand. The maximum size of amino acid sequence is from plus (+1) frame with 56 amino acids and the start codon is from 295 to 465 bases with a length of 171 bases followed by 38 amino acids starting from 121 to 237 bases with a length of 117 bases in (+1) reading frame. NCBI BLASTn search revealed that BRGA-4 was homologous to the *O. sativa, Oryza rufipogon, Oryza meyeriana, Oryza officinalis* NBS-LRR-like protein and *O. sativa* clone RGA15 resistant protein like gene (Table 8). The translated protein sequences from BRGA-4 with 38 amino acids starting from 121 to 237 bases with a length of 117 bases in (+1) reading frame showed homology with NBS-LRR-like protein TM1 [*O. meyeriana*], NBS-LRR-like protein NR21

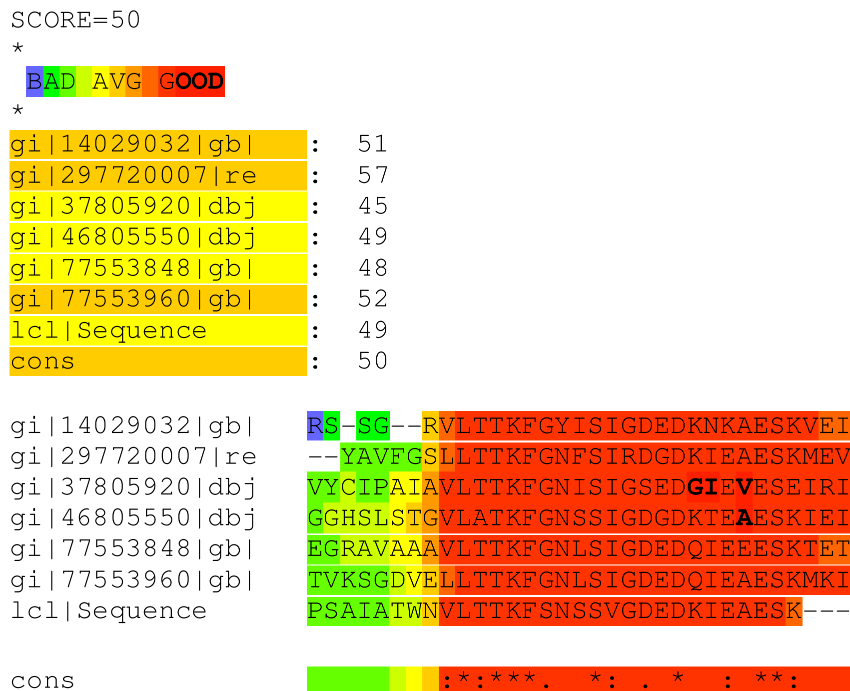

Figure 4. Multiple alignment of translated sequence of BRGA-1 (AS1/S2) with other related peptide sequences by T-COFEE version 8.93.

Table 5. Summary of homology search performed for BRGA-2 (RL F/R) nucleotide sequence by NCBI BLASTN 2.2.24+ (Mega Blast).

Accession number	Description	Maximum score	Query coverage (%)	E-value	Maximum identity (%)
AP003904.3	*Oryza sativa Japonica* group genomic DNA, chromosome 8, BAC clone:OJ1051_A08	479	54	1e-131	89
AP005388.2	*Oryza sativa Japonica* group genomic DNA, chromosome 8, BAC clone:OSJNBa0086F04	479	54	1e-131	89
AY360386.1	*Oryza sativa* (*Japonica* cultivar-group) chromosome 8 BAC OSJNBa0017M13, complete sequence	477	54	4e-131	89
AY360385.1	*Oryza sativa* (*Japonica* cultivar-group) chromosome 8 BAC OSJNBa0095C12, complete sequence	477	54	4e-131	89
AP005832.2	*Oryza sativa Japonica* group genomic DNA, chromosome 8, BAC clone:B1136D08	477	54	4e-131	89
AP004228.2	*Oryza sativa Japonica* group genomic DNA, chromosome 8, BAC clone:OJ1120_B07	477	54	4e-131	89
AC136150.4	*Oryza sativa Japonica* group chromosome 11 clone OSJNBa0023F12, complete sequence	473	54	5e-130	89
AP002968.2	*Oryza sativa Japonica* group genomic DNA, chromosome 1, PAC clone:P0416G11	473	54	5e-130	89
AP009092.1	*Oryza sativa Indica* group DNA, chromosome 8, BAC clone: K0253H11	468	54	2e-128	89
AC136521.2	*Oryza sativa* (*Japonica* cultivar-group) chromosome 5 BAC clone OSJNBa0034M22, complete sequence	468	54	2e-128	89
AC132490.2	*Oryza sativa Japonica* group chromosome 5 clone P0570A02, complete sequence	466	54	9e-128	89

Table 6. Summary of homology search performed for BRGA-2 (RL F/R) peptide sequence by NCBI BLASTP2.2.24+.

Accession number	Description	Maximum score	Query coverage (%)	E-value
AAM74416.1	Putative retro element [*Oryza sativa Japonica* group]	60.1	58	9e-08
ABB47077.1	Retro transposon protein, putative, unclassified [*Oryza sativa Japonica* group]	60.1	58	9e-08
ABB46599.1	Retro transposon protein, putative, unclassified [*Oryza sativa Japonica* group]	57.8	35	4e-07
AAM08509.1	Putative retroelement [*Oryza sativa Japonica* group]	57.4	35	6e-07
ABA96933.1	Retro transposon protein, putative, unclassified [*Oryza sativa Japonica* group]	57.4	39	7e-07
CAE04998.2	OSJNBb0093G06.6 [*Oryza sativa* (*Japonica* cultivar-group)]	56.6	76	9e-07
ABA94843.1	Retro transposon protein, putative, unclassified [*Oryza sativa Japonica* group]	56.2	37	1e-06
ABA91246.1	Retro transposon protein, putative, unclassified [*Oryza sativa Japonica* group]	56.2	37	1e-06
ABA93861.2	Retro transposon protein, putative, unclassified [*Oryza sativa Japonica* group]	55.8	35	2e-06
AAX95395.1	Reverse transcriptase putative [*Oryza sativa Japonica* group] >gb|ABA92966.2| retrotransposon protein, putative, unclassified [*Oryza sativa Japonica* group]	55.8	35	2e-06

[*O. rufipogon*], NBS-LRR-like protein [*O. sativa Japonica* group], NBS-LRR-like resistance protein [*O. sativa Indica* group] and NB-ARC domain containing protein expressed [*O. sativa Japonica* group] (Table 9). Multiple alignment of the deduced amino acid sequence of BRGA-4 with the related sequences by T-COFFEE multiple alignment software was shown (Figure 6).

DISCUSSION

The candidate gene approach might be an efficient way to establish the association between candidate genes and functionality. Several candidate gene markers were associated with qualitative and quantitative resistance for disease and an insect resistance. If these genes themselves are involved in resistance, they will be useful for marker-assisted selection breeding programs. However, to determine the generality of this approach, more candidate gene markers need to be placed on the map generated by populations segregating for the traits of interest. The publication of the draft rice genome sequences (Goff et al., 2002) has accelerated the identification of gene function using the candidate gene approach. The candidate genes share common sequence motifs, such as LRRs, NBSs and kinase domains, reflecting related functions in their roles in pathogen recognition (Anderson et al., 1997; Hulbert et al., 2001). These motifs shared the structural features that have been widely used to design degenerate oligo nucleotide primers to isolate R gene analogs (RGAs) by PCR amplification (Garcia-Mas et al., 2001).

In this study, four reported degenerate primers were used to amplify putative rice resistance gene analogues (RGAs) in the leaf blast resistance variety, Moroberekan and out of four fragments sequenced; three fragments were related by sequence to functional resistance genes.

For the sequence of BRGA-1, from the reported primer AS1/AS2, the amino acids were deduced from the nucleotide sequences in all possible frames using the ORF FINDER of NCBI. The sequence showed homology with hypothetical proteins of *O. sativa Japonica* group and other unknown protein groups. BLASTn search was done with the DNA sequence of 540 bp length as the query sequence for homology using two online programmes.

The significant alignment of the query sequence with negative E values indicated that the query sequence is having more than 95% homology with the *O. sativa,* (*Japonica* and *Indica* cultivar groups) having resistance protein-like genes. Since Moroberekan is a blast resistant, *Japonica* cultivar the homology search revealed that this cultivar possesses characteristic resistance enhancing proteins or gene(s) that might render durable resistance

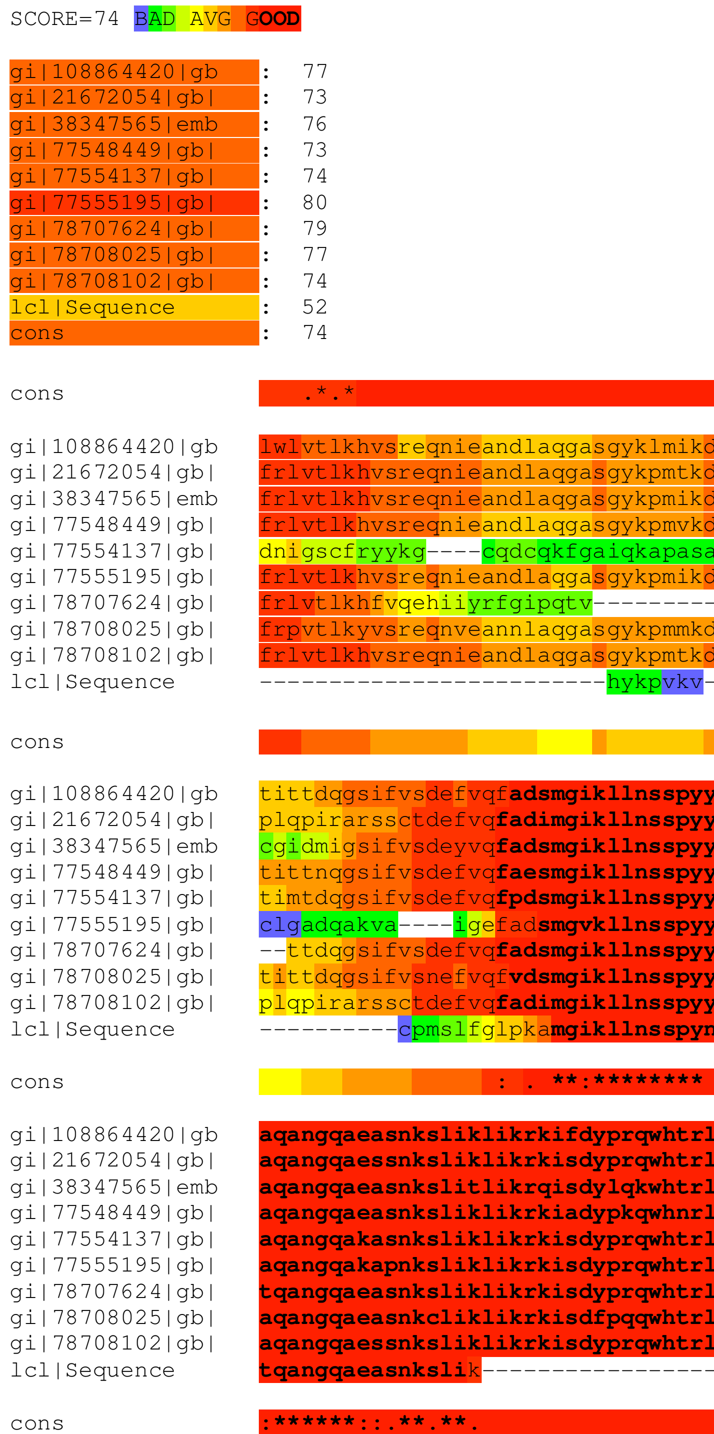

Figure 5. Multiple alignment of translated sequence of BRGA-2 (RL F/R) with other related peptide sequences by T-COFEE version 8.93

for both biotic and abiotic stresses as results were not specific indicating the gene or protein for disease resistance alone.

Deduced amino acid sequence of BRGA-2, shared homology with unclassified putative retrotransposon proteins, polyproteins and retroelements of *O. sativa* (*Japonica* cultivar group). Putative retroelements and polyproteins have been shown to co-localize locally with disease resistance genes in *Arabidopsis* and poplar (Lescot et al., 2004) and in apricot (Soriano et al., 2005). Retro transposon *Tos3* has been reported to be linked with leaf blast resistance gene *Piz* at 0.4 cM and

Table 7. Summary of homology search performed for BRGA-3 (CL F/R) nucleotide sequence by NCBI BLASTN 2.2.24+ (blastn).

Accession number	Description	Maximum score	Query coverage (%)	E-value	Maximum identity (%)
AP006757.2	*Oryza sativa Japonica* group genomic DNA, chromosome 1, PAC clone:P0551C06	648	79	0.0	83
AP003791.4	*Oryza sativa Japonica* group genomic DNA, chromosome 1, BAC clone:B1065G12	648	79	0.0	83
AP011077.1	*Oryza sativa Indica* group mitochondrial DNA, complete genome, cultivar: lead rice	506	62	1e-139	83
AP011076.1	*Oryza* rufipogon mitochondrial DNA, complete genome	506	62	1e-139	83
BA000029.3	*Oryza sativa Japonica* group mitochondrial DNA, complete genome	506	62	1e-139	83
DQ167400.1	*Oryza sativa* (*Japonica* cultivar-group) cultivar Nipponbare mitochondrion, complete genome	506	62	1e-139	83
DQ167807.1	*Oryza sativa* (*Japonica* cultivar-group) isolate PA64S mitochondrion, complete genome	506	62	1e-139	83
DQ167399.1	*Oryza sativa* (*Indica* cultivar-group) isolate 93-11 mitochondrion, complete genome	506	62	1e-139	83
AY187000.1	*Oryza sativa* (*Indica* cultivar-group) mitochondrial RAPD molecular marker PWH-17 sequence	497	62	5e-137	83
AC136501.2	*Oryza sativa Japonica* group chromosome 11 clone OSJNBb0044M15, complete sequence	170	21	1e-38	83
EU365401.1	Bambusa oldhamii mitochondrion, complete genome	127	33	1e-25	80

Table 8. Summary of homology search performed for BRGA-4 (N gene F/R) nucleotide sequence by NCBI BLASTN 2.2.24+.

Accession	Description	Maximum score	Query coverage (%)	E-value	Maximum identity (%)
AY337922.1	*Oryza sativa* clone sk77 NBS-LRR-like gene, partial sequence	219	58	1e-53	78
AY337914.1	*Oryza sativa* clone sk98 NBS-LRR-like gene, partial sequence	219	58	1e-53	78
AY337912.1	*Oryza sativa* clone sk95 NBS-LRR-like gene, partial sequence	219	58	1e-53	78
AY337908.1	*Oryza sativa* clone sk91 NBS-LRR-like gene, partial sequence	219	58	1e-53	78
AY043283.1	*Oryza sativa* subsp. japonica NBS-LRR-like protein (YR9) mRNA, complete cds	219	58	1e-53	78
AF220735.1	*Oryza sativa* NBS-LRR-like protein (YR10) gene	219	58	1e-53	78
EU293163.1	*Oryza* rufipogon clone 19 NBS-LRR resistance-like protein gene, partial cds	214	58	6e-52	78
AY518220.1	*Oryza sativa* (*Indica* cultivar-group) NBS-LRR-like protein A (NL-A), NBS-LRR-like protein B (NL-B), NBS-LRR-like protein C (NL-C), and NBS-LRR-like protein D (NL-D) genes, complete cds	214	58	6e-52	78
AY337867.1	*Oryza sativa* clone sk11 NBS-LRR-like gene, partial sequence	214	58	6e-52	78
AF220745.1	*Oryza sativa* NBS-LRR-like protein (YR23) gene, partial cds	214	58	6e-52	78
AF220743.1	*Oryza sativa* NBS-LRR-like protein (YR21) gene, partial cds	214	58	6e-52	78
AF220734.1	*Oryza sativa* NBS-LRR-like protein (YR9) gene, partial cds	214	58	6e-52	78

Table 9. Summary of homology search performed for BRGA-4 (N gene F/R) peptide sequence by NCBI BLASTP2.2.24+.

Accession number	Description	Maximum score	Query coverage (%)	E-value
AAO39130.1	NBS-LRR-like protein TM1 [*Oryza meyeriana*]	40.2	73	0.10
AAO39132.1	NBS-LRR-like protein TM7 [*Oryza meyeriana*]	40.2	73	0.10
AAO39125.1	NBS-LRR-like protein NM10 [*Oryza meyeriana*]	39.4	73	0.17
AAO39133.1	NBS-LRR-like protein TM27 [Oryza meyeriana]	38.2	73	0.34
NP_001176707.1	Os11g0673600 [Oryza sativa Japonica Group] >dbj|BAH95435.1| Os11g0673600 [*Oryza sativa Japonica* group]	37.8	73	0.45
EAZ40863.1	hypothetical protein OsJ_25344 [*Oryza sativa Japonica* group]	37.8	73	0.45
AAR99709.1	NBS-LRR-like protein C [*Oryza sativa Indica* group]	37.8	73	0.45
AAO39124.1	NBS-LRR-like protein NR21 [Oryza rufipogon]	37.8	73	0.45
AAO39119.1	NBS-LRR-like protein NR9 [Oryza rufipogon]	37.8	73	0.45
AAF43654.1	NBS-LRR-like protein [*Oryza sativa Japonica* GROUP] >gb|ABX76052.1| NBS-LRR resistance-like protein [Oryza rufipogon]	37.8	73	0.45
AAF43664.1	NBS-LRR-like protein [*Oryza sativa Japonica* group]	37.8	73	0.45
AAK93796.1	NBS-LRR-like protein [*Oryza sativa Japonica* group]	37.8	73	0.45
AAF43662.1	NBS-LRR-like protein [*Oryza sativa Japonica* group]	37.8	73	0.45
AAF43651.1	NBS-LRR-like protein [*Oryza sativa Japonica* group]	37.8	73	0.45
AAF43649.1	NBS-LRR-like protein [*Oryza sativa Japonica* group]	37.8	73	0.45
AAF43653.1	NBS-LRR-like protein [*Oryza sativa Japonica* group]	37.8	73	0.45
AAM69515.1	NBS-LRR-like resistance protein [*Oryza sativa Indica* group]	37.8	73	0.45
AAO37922.1	resistance protein [*Oryza sativa Indica* group]	37.8	73	0.45
AAB96994.1	NBS-LRR type resistance protein [*Oryza sativa Japonica* group]	37.8	73	0.45
AAM69500.1	NBS-LRR-like resistance protein [*Oryza sativa Indica* group]	37.8	73	0.45

retrotransposon *BARE-1,* linked 0.28 cM from the *Mla* locus of multigene family that confers resistance to the powdery mildew of barley (Wei et al., 2002; Kalendar et al., 2000). The consequence of insertion of retrotransposon [*Retrofit* (*copia*)] in *Xa 21D* gene (receptor like kinase) leads to a truncated protein with partial resistance activity in rice; the predicted protein encodes a new class of LRR resistance gene products lacking a trans-membrane protein and kinase domain (Song et al., 1995).

Our deduced amino acid sequence of BRGA-3, showed no homology in the SwissProt database search. Hence, BLASTn of the NCBI search was done which resulted in 31 hits on the query sequence. The significant alignment of the query sequence with the negative E values indicated that the query sequence is having homology with the sequences from mitochondrial DNA of *O. sativa, Japonica* and *Indica* cultivar groups.

The deduced amino acid sequence of BRGA-4 shared homology with NBS-LRR type resistance protein. NCBI BLASTn search of BRGA-4 shared homology with the NBS-LRR like gene from both *Indica* and *Japonica* cultivar groups of *O. sativa.* Since Moroberekan is a highly resistant variety for leaf blast, it is evident from the results obtained that it contains the resistance gene with conserved sequences of NBS-LRR domains.

Earlier reports indicated that the major blast resistance genes consisted of NBS-LRR domains for disease resistance. The putative *Pi-k*^{h} gene cloned from Tetep was 1.5 kbp in size with a single ORF and belongs to the nucleotide binding site-leucine rich repeat (NBS-LRR) class of disease resistance genes (Sharma et al., 2005). *Pi-ta* is a member of the putative cytoplasmic NBS-receptor class of *R* genes. The resistance gene *Pi-ta* encoded a highly interrupted and relatively rudimentary leucine-rich repeat (LRR) motif, compared with that seen in other members of this class (Bryan et al., 2000).

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

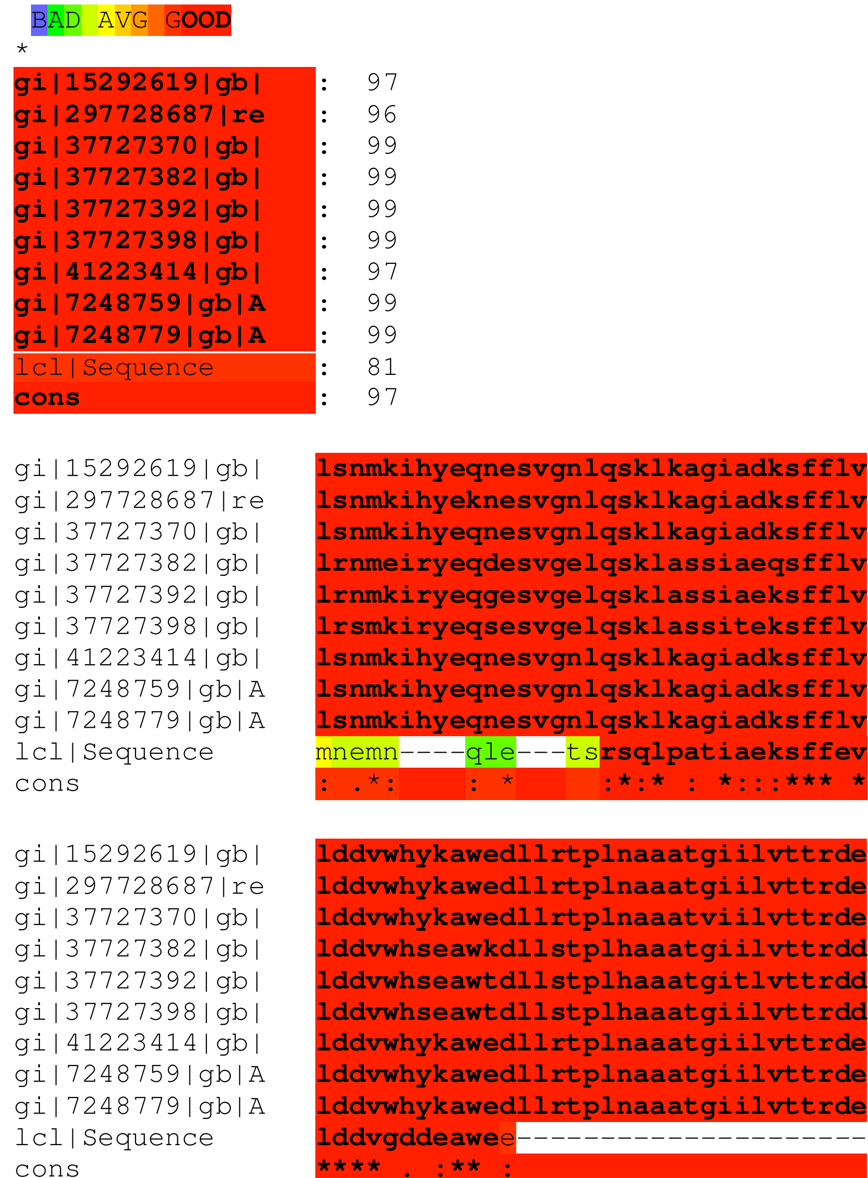

Figure 6. Multiple alignment of translated sequence of BRGA- 4 (N-gene) peptide sequences by T-COFEE version 8.93.

class represents as much as 1% of the genome in *Arabidopsis* (Sallaud et al., 2003).

Sallaud et al. (2003) demonstrated using degenerate primers of the NBS motif found in many resistance genes, two resistance gene analogues (RGAs) IR86 and IR14 that were identified and mapped closely to two blast resistance loci, *Pi-29*(t) and *Pi-30*(t), respectively. These two RLs might 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. Both genes encoded NBS-LRR-like proteins that are characteristics of plant-disease resistance genes.

Conclusion

In conclusion, identification of resistant gene analogues in durable genotypes, and confirming them with functional studies will be helpful to combat diseases to greater

extent, where they can be cloned into respective cultivars which possess desirable traits like yield.

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