

Characterization of disease resistance gene homologues isolated from finger millet (*Eleusine coracana* L. Gaertn)

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Abstract Resistance gene homologues were isolated from finger millet (*Eleusine coracana* L.) using degenerate oligonucleotide primers designed to the conserved regions of the nucleotide binding site (NBS) of previously cloned plant disease resistance genes (R-genes) using polymerase chain reaction (PCR). Of the eleven primer combinations tested, only five showed amplification of resistance gene homologues in finger millet. BLAST search of cloned finger millet

DNA fragments showed strong homology to NBS-LRR-type R-genes of other crop species. Of the 107 clones sequenced, 41 showed homology to known R-genes, and are denoted as EcRGHs (*Eleusine coracana* resistance gene homologues), while 11 showed homology to pollen signalling proteins (PSiPs), and are denoted as EcPSiPs (*Eleusine coracana* pollen signalling proteins). The cloned EcRGH sequences were classified into four clusters, and EcPSiPs formed two separate clusters based on sequence homology at the amino acid level. The amino acid sequences of the cloned EcRGHs showed characteristic features of non-TIR-type R-genes, which have been identified in all the monocot species studied so far. Six EcRGHs-specific primers were designed based on the sequences obtained in finger millet; reverse transcription PCR was performed on the cDNA and revealed the expression of EcRGHs in finger millet. The ratio of non-synonymous to synonymous nucleotide substitution (dN/dS) in the NBS domains of finger millet RGHs varied from 0.3 to 0.7 for the different classes, which suggests a purifying selection, though the LRR region also needs to be considered to make predictions. This is the first report on RGHs in finger millet, which will serve as a valuable resource for finger millet improvement using molecular tools.

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Introduction

Finger millet (*Eleusine coracana* (L.) Gaertn), commonly known as Ragi (India), Bulu (Uganda), Wimbi (Swahili) and Tellebun (Sudan), is an important cereal crop for subsistence agriculture in dry areas of Eastern Africa, India and Sri Lanka. World finger millet production is about 4.5 million tonnes, of which 2.5 million tonnes are produced in Africa alone (ICRISAT and FAO 1996). India is one of the major cereal producing countries in the world and finger millet occupies a total area of about 1.5 million hectares with a production of 1.2 million tonnes (Anonymous 1995, 1996). Finger millet has an excellent nutritional value as its seeds contain 7–14% protein and are particularly rich in methionine, iron and calcium (Barbeau and Hilu 1993; Vadivoo et al. 1998). Even though finger millet is known to be one of the hardiest crops, it is affected by a number of diseases like blast, foot rot, smut, streak and mottling virus (Govindu et al. 1970). Blast of finger millet is a major disease caused by the fungus *Pyricularia grisea* (Cooke) Sacc. (formerly *Pyricularia oryzae* Cavara.), an anamorph of *Magnaporthe grisea* (Hulbert et al. 2001) Barr (Rossman et al. 1990) that causes blast disease in rice. It is a heterothallic, filamentous fungus pathogenic to almost 40 plant species in 30 genera of Poaceae including *Eleusine*. Blast caused by the fungus *Pyricularia grisea* is the most devastating disease affecting different aerial parts of the plant at all stages of its growth starting from seedling to grain formation. Yield loss due to blast may be around 28% (Vishwanath et al. 1986). In spite of a great deal of research on the pathogen and the disease, blast still remains a serious constraint to finger millet production in areas with conducive environments where the high-yielding cultivars become susceptible. Incorporation of genes for resistance in breeding populations, varieties and hybrids is the most effective means to manage diseases such as blast.

Plant disease resistance genes (R-genes) are important components of the genetic resistance mechanism in plants (Flor 1956) and have been studied in detail in many plants like *Arabidopsis*, tobacco, tomato, flax, rice and maize (Grant et al. 1995; Gassmann et al. 1999; Parker et al. 1997; Jones et al. 1994; Dixon et al. 1998; Whitham et al. 1994; Lawrence et al. 1995; Wang et al. 1999; Johal and

Briggs 1992). Resistance genes are present as gene clusters of different specificities in the plant genome and are either allelic in nature and/or closely linked. Novel techniques for deploying resistance to various biotic stresses, such as genetic transformation, molecular marker-aided selection and gene transfer from alien sources, have yet to make an impact on finger millet research.

Many biotechnological tools are being increasingly used to study the interaction between plants and potential pathogens at all stages of infection (Michelmore 1995) and considerable progress has been made in the identification and cloning of resistance genes using map-based cloning and transposon tagging (Foote et al. 1997; Seah et al. 1998). The availability of cloned disease resistance genes permits studies of resistance gene structure and function. The majority of the plant disease resistance genes cloned so far show conserved DNA sequences and amino acid domains irrespective of whether they confer resistance to viral, bacterial or fungal diseases. Sequence comparison of known R-genes shows remarkable similarities in general protein structure and conservation of specific domains that participate in protein–protein interaction and signal transduction (Sivaramakrishnan and Seetharama 2001; Baker et al. 1997; Gassmann et al. 1999). The similarity in the resistance gene sequences among the plant species, both at the nucleotide and amino acid levels, has made it possible to isolate such resistance gene homologues (RGHs) from any plant species of interest using polymerase chain reaction (PCR) with oligonucleotide primers designed to the conserved domains (Michelmore 1996). To isolate homologues of the known R-genes, the nucleotide binding site (NBS) of the NBS-LRR family of proteins is normally chosen based on the conserved motifs present (Bai et al. 2002; Cannon et al. 2008; Michelmore and Meyers 1998; Young 2000). This approach, known as the ‘candidate gene approach’, has been successfully used in a variety of plant species, including potato, soybean and lettuce, to isolate disease resistance gene homologues (Yu et al. 1996; Kanazin et al. 1996; Leister et al. 1996; Shen et al. 1998). The availability of rice genome sequence enabled the global characterization of NBS-leucine-rich repeat (NBS-LRR) genes, the largest class of plant disease resistance genes (Goff et al. 2002).

As a first step in the identification and isolation of disease resistance genes from finger millet, the degenerate oligonucleotide primers designed to the conserved motifs in the NBS region were used to amplify DNA fragments. These DNA fragments were cloned, sequenced and screened for sequence homology with other known R-genes in the genome databases. We report here the identification and isolation of such RGs identified in cultivated finger millet. Identification, cloning and mapping of resistance genes corresponding to major pathogens would contribute significantly to improvement of finger millet and related crops.

Materials and methods

Amplification of NBS-like sequences using degenerate primers

A local popular high-yielding cultivar of finger millet developed by ANGRAU, UR762, was selected for the isolation of RGs from DNA. Genomic DNA was extracted from 5 g of leaf tissue from 2-week-old finger millet seedlings using a modified

cetyltrimethylammonium bromide (CTAB) method (Porebski et al. 1997). The degenerate primers used were designed based on the results of previous studies (Shen et al. 1998) so as to amplify DNA sequences spanning the most conserved domains of the NBS. In each pair, the forward primers were designed to anneal to the sequence encoding the *kinase-1a* or P-loop domain (Shen et al. 1998) and the reverse primers were designed in the antisense orientation corresponding to the coding sequences of an amino acid domain known as GLPLAL (Lawrence et al. 1995).

A total of 18 individual primers including forward and reverse primers were synthesized (Table 1) and tested in 11 possible combinations (Table 2) for the amplification of RGs from genomic DNA of finger millet cultivar UR762. PCR was performed with the different primer combinations in a 30 µl reaction volume containing 10 mM of Tris HCl (pH 8.3), 50 mM of KCl, 1.5 mM of MgCl₂, 0.2 µM of each forward and reverse primer, 100 µM of dNTPs, 1 U of Taq DNA polymerase (Invitrogen, USA) and about 50 ng of template DNA. PCRs were carried out in a PTC-100 thermal cycler (MJ Research, USA). Thermocycler parameters were: initial denaturation step at 95°C for 5 min, followed by 30 thermal cycles of

Table 1 List of primers used in PCR to amplify finger millet DNA

S. No.	Primer name	Primer sequence (5'→3')
1	CG-S1	GGTGGGCTTGGGAAGACAAGC
2	CG-S2	GGI GGI GGI GGI AAI ACI AC
3	CG-AS1	CAACGCTAGTGGCAATCC
4	CG-AS3	IAG IGC IAG IGG IAG ICC
5	LM-637	A(A/G)IGCTA(A/G)IGGIA(A/G)ICC
6	LM-638	GGIGGIGTIGGIAAIAACIAC
7	PLOOP-AA	CTACTACTACTAGAATTCGGNGTNGGNAACAAC
8	PLOOP-AC	CTACTACTACTAGAATTCGGNGTNGGNAACCAC
9	PLOOP-AG	CTACTACTACTAGAATTCGGNGTNGGNAACGAC
10	PLOOP-GA	CTACTACTACTAGAATTCGGNGTNGGNAAGACAC
11	GLPLAL-1	CTACTACTACTAGTCGACAGNGCNAGNGGNAGGCC
12	GLPLAL-2	CTACTACTACTAGTCGACAGNGCNAGNGGNAGACC
13	GLPLAL-4	CTACTACTACTAGTCGACAGNGCNAGNGGNAGCCC
14	GLPLAL-6	CTACTACTACTAGTCGACAANGCCAANGGCAATCC
15	RO1	GTG AGC CTT TAG TAC GAG
16	RO4	TAT GAG TGA TGA CCG ACC
17	RO1sgqip	GTG AGC CTT TAG TAC GAG TCN GGN CAR ATH CC
18	RO4hrdik1	TAT GAG TGA TGA CCG ACC YTT NAN RTC YCT RTG

Eighteen degenerate primers were used in the study designed by Leister et al. (1996), Shen et al. (1998), Kanazin et al. (1996)

Table 2 PCR amplified products from finger millet genomic DNA, with different combinations of primers

S. No.	Primer combination	No. of clones sequenced	No. of clones having ORF	No. of clones showing homology to known R-genes	Cluster no.
1	S1/AS1	9	03	03	A
2	S1/AS3	7	Nil	Nil	
3	S2/AS1	14	05	08	B,C,E
		13	06	06	A,F
4	S2/AS3	10	06	06	E
		14	08	12	D
5	LM638/LM637	12	03	05	E
		Nil		–	
6	RO1/RO4	–		–	
7	RO1sgqip/RO4 hrdik 1	7		Nil	
		7		Nil	
8	Ploop//GLPLAL4	–		–	
9	Ploop//GLPLALI	14	Nil	01	E
10	Ploop//GLPLAL6				
11	Ploop//GLPLAL2				
	Total	107	31	41	

11 degenerate primer combinations were used in isolation of EcRGHs

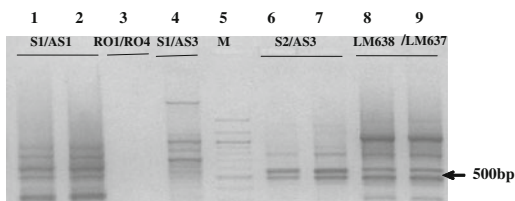
95°C for 1 min, 45/51°C for 1 min depending on the primers and 72°C for 1 min, with a final extension step of 10 min.

Cloning of PCR products

Amplified PCR products were separated by electrophoresis on 1.5% TBE agarose gels and stained with ethidium bromide for visualization (Fig. 1). The expected 500-bp size fragments corresponding to RGHs and other major fragments were excised from the gel and purified using Qiagen gel extraction kit (Qiagen, USA). Ligation reaction was set up in 10 µl

reaction volume with 50 ng of pGEM[®]-T Easy vector (Promega, USA) according to the manufacturer's protocol. Ligation was carried out at 14°C for 16 h.

Competent cells of *E. coli* DH10B strain were prepared using 10% glycerol (Sambrook et al. 1989) and bacterial transformation was effected by electroporation. Colony PCR was carried out for the confirmation of DNA inserts in the selected putative recombinant clones. PCR was performed in a total reaction volume of 30 µl with M13 universal forward and reverse primers. The PCR was carried out in a PTC-100[™] thermal cycler (MJ research[®], USA) and the parameters were: initial denaturation step of 95°C for 5 min followed by 30 thermal cycles of 95°C for 1 min, 55°C for 1 min and 72°C for 1 min, with a final extension step of 10 min. PCR products were verified for cloned inserts by resolving the DNA fragments on a 1.5% TAE agarose gel.



1, 2-S1/AS1; 3-RO1/RO4, 4-S1/AS3, 5-100bp ladder, 6, 7-S2/AS3, 8, 9-LM637/638.

Fig. 1 Agarose gel electrophoresis pattern of PCR amplified finger millet DNA. Lanes 1, 2 S1/AS1, 3 RO1/RO4, 4 S1/AS3, 5 100-bp ladder, 6, 7 S2/AS3, 8, 9 LM637/638. PCR was carried out using 11 degenerate primer combinations on finger millet DNA as the template. The amplified products were run on 1.5% agarose gel in TAE, stained with ethidium bromide and visualized on a UV-transilluminator

Sequencing and analysis

Initially, plasmids were isolated from five recombinant clones from each ligation event by alkaline lysis using the PEG precipitation method (Sambrook and Russell 2001) and were sequenced. BLAST searches (Altschul et al. 1997) were performed via the National Centre for Biotechnology Information (NCBI) web site.

Dot blot hybridization of putative RGH clones was carried out to select more clones for sequencing according to the method of Sambrook et al. (1989). Ninety-six clones were taken for dot blot analysis from each ligation event and EcRGH clones of the same ligation event were used as probes for hybridization in the dot blots. Additional clones were identified based on the intensity of hybridization in the dot blot and were sequenced. Homology search was done using BLAST in the GenBank database. *Eleusine coracana* resistance gene homologues (EcRGHs) sequences were aligned by ClustalW (Thompson et al. 1994) method using the MgAlign tool of DNASTAR software (Lasergene, USA). The reliability of phylogenetic tree topologies was tested by bootstrapping using the neighbour joining method of CLC Workbench3.1 software (www.clcbio.com). Amino acid sequences of NBS domains of three TIR-class R-genes (*L6* of flax, *N* gene of tobacco, *RPS4* of *Arabidopsis thaliana*) and three non-TIR R-genes (*RPS2*, *RPS5* and *RPP8* of *A. thaliana*) were included in the analysis. Seven rice R-genes and one pollen signalling protein (PSiP) gene were also included in the phylogenetic analysis for comparison.

Finger millet RGH-specific primers

Six RGH-specific primers were designed based on the RGHs isolated from finger millet following multiple alignments of RGHs using Primer3 software. Annealing temperatures were standardized using gradient PCR.

RT-PCR analysis

Total RNA was isolated from leaves of finger millet cultivar UR-762 using the RNeasy Plant Mini Kit (Qiagen, USA) method. Total RNA was treated with RNase-free DNase I (Promega, USA) to remove genomic DNA contamination in RNA preparations. For cDNA synthesis, 2 µg of DNA-free RNA was primed with oligo dT 23NV (New England Biolabs, USA) and the first strand cDNA was synthesized using 200 units of MMLV reverse transcriptase (New England Biolabs, USA). The second strand was synthesized using DNA Pol I, and the cDNA was purified and used for amplification with six finger millet RGH-specific primers. Control reactions were performed with RNA and cDNA using primers to

check the genomic DNA contamination. PCR amplified products were cloned into pGEM-T Easy vector system (Promega, USA) and sequence analysis was carried out as mentioned earlier.

GenBank submission

Cloned RGH sequences of finger millet were deposited in GenBank with the following accession numbers: EF408611–EF408639 and EU075219–EU075263. The expressed finger millet RGHs identified using six EcRGH-specific primers were also deposited in GenBank EST database (GH612831–GH612847).

Results

Of the 11 primer combinations used to amplify the finger millet template DNA, seven amplified a major band in the expected size range of ~500 bp or more, as reported in the literature for other plant species. Two primer combinations, RO1/RO4 and PLoop/GLPLAL4, did not give any amplification. A representative amplification pattern with different primer combinations is shown in Fig. 1. The recombinant clones were tested for the presence of DNA inserts by colony PCR, which showed size differences in the DNA amplicons. Five recombinant clones from each event were sequenced initially and homology search was carried out to find similarity with R-genes from other plant species. Further, dot blot hybridization was carried out on the recombinant clones using as probes the EcRGHs identified earlier by sequencing. A total of 107 recombinant clones were identified for sequencing based on the results of colony PCR and dot blot hybridization.

Of the seven primer combinations tested with finger millet genomic DNA, only five combinations, S1/AS1, S2/AS1, S2/AS3, LM638/LM637 and Ploop/GLPLAL, yielded amplification of RGHs. The BLAST search indicated that the isolated EcRGHs showed strong homology to NBS-LRR disease resistance protein homologues from *Oryza sativa*, *Saccharum officinarum*, *Pennisetum glaucum*, *Zizania latifolia*, *Ipomoea batatas*, *Zingiber cernuum*, *Lycopersicon hirsutum*, *Musa acuminata* and *Helianthus annuus* (Table 3). The other primer combinations showed DNA fragments of larger size on PCR amplification.

A total of 41 clones, excluding the two short sequences, were aligned by ClustalW into six clusters. Nucleotide sequences of greater than 65% similarity were considered to belong to the same cluster. The construction of a phylogenetic tree based on the nucleotide sequences also revealed six major branches that were considered to be separate clusters of EcRGHs as supported by high bootstrap values. Clones generated from the amplified products of S2/AS1 combination were very divergent and formed five clusters. A major cluster (E) was formed with the cloned RGHs amplified with S2/AS3, S2/AS1 and LM638/LM637 primer combinations that were about 500 bp in size, whereas the clones generated from the 600 bp fragment amplified with S2/AS3 formed a separate cluster (D). Two clusters B and F were formed with single clones. Clones generated from the amplified product with PLoop/GLPLAL primer combinations were included in cluster E (Table 2).

The nucleotide sequences of EcRGHs were translated and the amino acid sequences were aligned by the neighbour joining method (Fig. 2). The six clusters seen at the nucleotide level came down to four major clusters at the amino acid level, after eliminating the clones that did not show any open reading frame (ORF). Two clones from class C and one clone representing class B were eliminated as they did not show ORFs (cluster B and C were eliminated in Fig. 2 that were present in cluster analysis at nucleotide level). Of a total of 41 putative RGHs of finger millet, 31 RGHs showed uninterrupted ORFs after verification of translated amino acid sequences. The remaining RGHs had potential stop codons that resulted in distorted amino acid sequences upon translation; consequently these were unable to align with the other EcRGHs at the amino acid level.

Eleven clones from the Ploop/GLPLAL combination other than the RGH clones mentioned above showed homology to resistance genes from *Oryza sativa* and *Musa acuminata* in BLAST search but they did not have a non-TIR-NBS-LRR group characteristic or TIR characteristic consensus motifs. These 11 clones were aligned by ClustalW into two classes at the nucleotide level and upon translation only five out of the 11 sequences showed uninterrupted ORFs, which were divided into two classes based on 70% homology. These clones separated as different clusters when compared with EcRGHs and other RGHs from rice (Fig. 3).

Some of the recombinant clones (EcPSiPs) showed higher similarity with PSiPs in rice than RGHs and formed a separate cluster. EcRGH21 formed a cluster by itself both at the nucleotide and amino acid levels. The amino acid sequences of EcRGHs were further compared with the previously cloned plant disease resistance genes (Fig. 3). The EcRGHs formed separate groups with some similarity to other NBS-LRR genes from monocots but not from dicots. All the 31 NBS sequences contained the characteristic NBS motifs of *kinase-2* and *kinase-3a* of plant R-genes confirming that the clones characterized in the present study belong to the NBS-LRR gene superfamily. Amino acid sequences of the six clusters (A to F) have a tryptophan residue (W) at the end of the *kinase-2* motif, which is characteristic of the non-TIR sub-class of R-genes (Fig. 4). Logos were constructed based on the conserved residues in the *kinase-2* and *kinase-3a* motifs of the NBS domain of EcRGHs (Fig. 5a, b).

Amino acid sequences of each class were aligned and six oligonucleotide primers were designed to the conserved regions of the EcRGHs (Table 4). All the six EcRGH-specific primer combinations showed a DNA fragment in the size range of 400 bp (Fig. 6) when the cDNA from finger millet was used as a template. The amplicons were further verified by colony PCR and three clones from each of the six ligation events were sequenced and the sequence data was used for homology searches.

The ratios of non-synonymous to synonymous nucleotide substitution (dN/dS) were calculated for the four different EcRGH clusters and were considered to be indicators of evolutionary pressure on a class of genes. The values were found to be less than one (Table 5) in all the classes of EcRGHs.

Discussion

Many classes of R-genes are present in plant species and arise from a combination of mainly three protein domains, namely nucleotide binding sites (NBS), leucine-rich repeats (LRR) and kinase. These together or separately form component(s) of a signal transduction pathway in plants to elicit the right kind of defence response to counteract invasion of the different pathogens. Among the different classes of R-genes reported so far, the majority belong to the

Table 3 BLASTX results of RGHS, cloned from Finger millet genomic DNA

EcRGH		BLASTX Result				
Primer combination	Clone	Cluster	Description of the matching protein in Genbank with high score	GenBank accession no.	Score (bits)	E value
S1/AS1	EcRGH10	A	<i>Saccharum</i> hybrid cultivar N11 NBS-LRR resistance protein (RGA761) gene, RGA761-R12 allele, partial cds	AY343981.1	203	5e-49
LM637/638	EcRGH12	A	<i>Hordeum vulgare</i> NBS-LRR type resistance protein (b9) gene, partial cds	AF032687.1	178	2e-4
	EcRGH2	E	<i>Oryza sativa</i> clone sk98 NBS-LRR-like gene, partial sequence	AY337914.1	64.4	2e-07
	EcRGH40	E	<i>Aegilops ventricosa</i> partial rae1 gene for resistance gene alike	AJ249943.1	351	3e-94
S2/AS1	EcRGH38	A	<i>Saccharum</i> hybrid cultivar N11 NBS-LRR resistance protein (RGA761) gene, RGA761-R12 allele, partial cds	AY343981.1	315	2e-83
	EcRGH22	A	<i>Pennisetum glaucum</i> isolate RGPM 226 disease resistance protein-like protein gene, partial sequence	AF513972.1	600	2e-168
PLOOP/GLPLAL	EcRGH1	E	<i>Pennisetum glaucum</i> isolate RGPM 229 disease resistance protein-like protein gene, partial sequence	AF513970.1	42.8	0.72
	EcRGH76	E	<i>Saccharum officinarum</i> clone RGA4-NCO376 NBS-LRR-like resistance protein gene, partial cds	EF059976.1	461	2e-127
S2/AS3	EcRGH68	E	<i>Saccharum officinarum</i> clone RGA4-NCO376 NBS-LRR-like resistance protein gene, partial cds	EF059976.1	457	2e-126

BLAST searches (Altschul et al. 1997) were performed via the National Centre for Biotechnology Information (NCBI) web site

Fig. 2 Phylogenetic analysis of EcRGHs at amino acid level. The tree was created by using CLC Workbench software based on the homology at amino acid level RGHS. Six classes at the nucleotide level reduced to four classes at the amino acid level

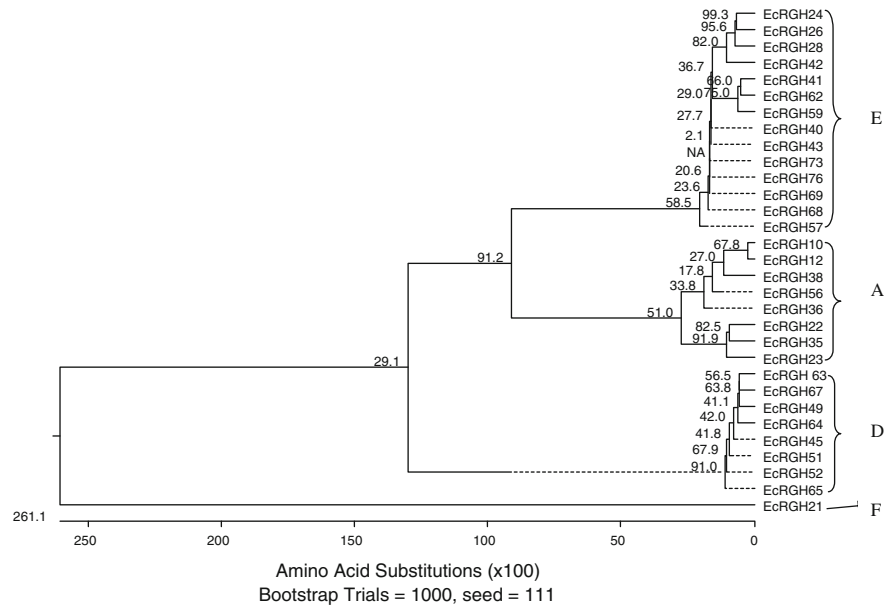
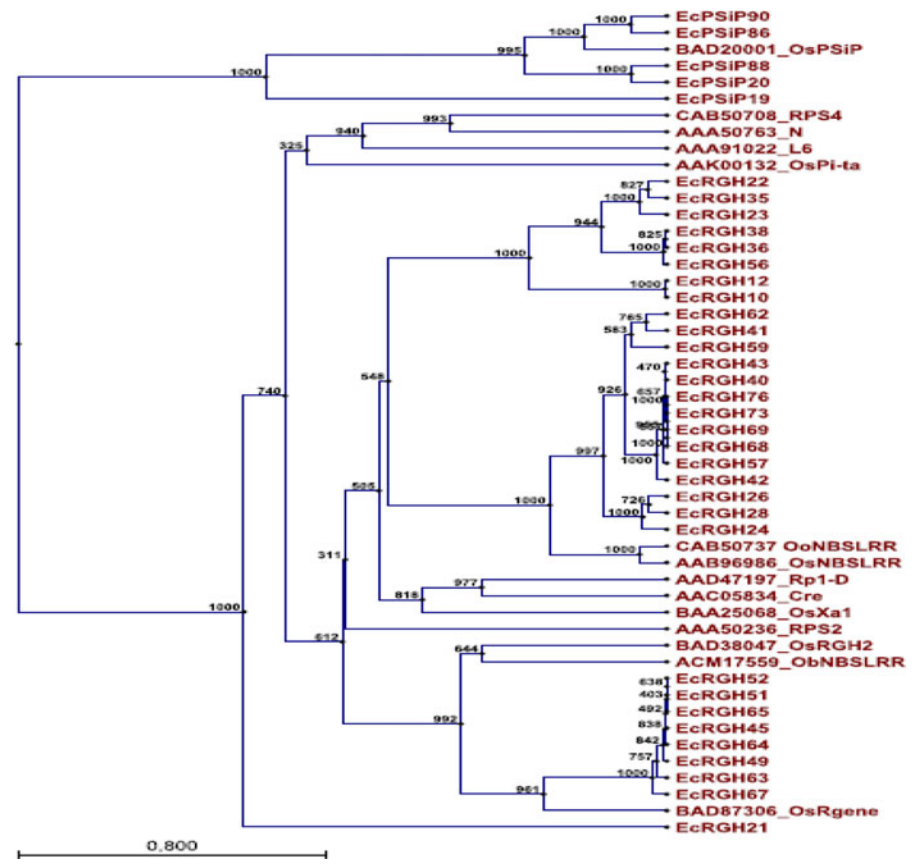


Fig. 3 Phylogenetic analyses of EcRGHs with EcPSiP and known rice R-genes at amino acid level. The tree was created by using the tree construction tool of CLC Workbench software. The algorithm used was UPGMA, and values indicated on the branches of the tree are the number of replicates taken in bootstrap analysis. Some sequences of known R-genes from rice were also included in the analysis for comparison



NBS-LRR class (Ayliffe and Lagudah 2004; Hulbert et al. 2001). The NBS domain has conserved amino acid sequences, which has given rise to the candidate

gene approach that uses the conserved motifs among the NBS-LRR class resistance genes in plant species for large-scale isolation of resistance gene analogues

Fig. 4 Alignment of EcRGHs proteins showing the conserved motifs like *kinase-2* and *kinase-3a* of the NBS domain of R-protein. All the amino acid sequences are aligned and the *kinase-2* and *kinase-3a* domains are marked using CLC Workbench software

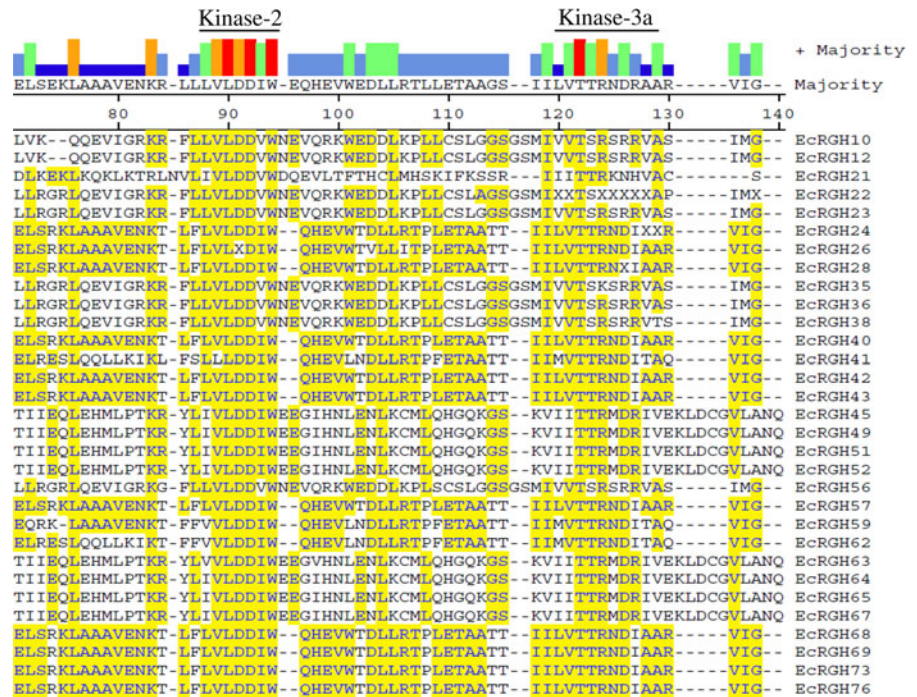


Fig. 5 **a** Logo of *kinase-2* motif of NBS domain of EcRGHs showing the highly conserved residues. **b** Logo of *kinase-3* motif of NBS domain of EcRGHs showing the highly conserved residues

from any plant species of interest using PCR with degenerate oligonucleotide primers (Shen et al. 1998; Madsen et al. 2003). The LRR region, on the other hand, is mostly involved in protein–protein interaction which is important in pathogen recognition by R-genes (Lehmann 2002).

The candidate gene approach was tried in one of the important coarse cereal crops, finger millet, in which little molecular work has been carried out so far. Sequence analysis indicated that the recombinant clones generated from one ligation event were not all similar but were different in terms of the actual nucleotide sequence and the length of the fragments amplified. The BLAST search indicated that cloned

Table 4 List of EcRGH-specific primers

S. no.	Primer name	Forward primer	Reverse primer
1	GSFM1	5'-gctaggagattacgacgatgac-3'	5'-atggtgttcactcagaaggac-3'
2	GSFM2	5'-gactaacaggagaatgctcatg-3'	5'-tgagtgaaggaagaactctctg-3'
3	GSFM3	5'-cttcagttgaagatgtggcactg-3'	5'-tgacaatagctctgccaatcacga-3'
4	GSFM4	5'-ggagatgggtagtggaaccagtg-3'	5'-gagccacacactcttagcga-3'
5	GSFM5	5'-tgacgacagaataaaggagca-3'	5'-ccatctcatcatcgggacatcaa-3'
6	GSFM6	5'-ttatcaggggagctgttga-3'	5'-cttagtcagaaggcaggtgaacg-3'

Gene-specific primers were designed taking into consideration conserved regions of EcRGHs isolated

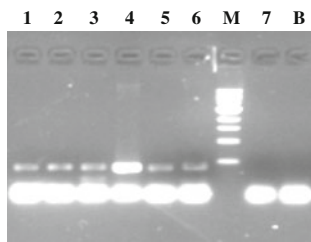


Fig. 6 Agarose gel electrophoresis pattern of finger millet DNA. RGHs amplified by RT-PCR with EcRGH-specific primers. Lanes 1–6 DNA fragments amplified with 6 different RGH-specific primers, lane M 1-kb DNA ladder, lane 7 RNA used as control template (for no DNA contamination), lane B no DNA (blank). PCR was carried out using gene-specific primers and amplified product was run on 1.5% agarose gel in TAE, stained with ethidium bromide and visualized on a UV transilluminator. Control reactions were performed with RNA and cDNA using primers to check the genomic DNA contamination

Table 5 Rates of non-synonymous (dN) to synonymous (dS) nucleotide substitutions among EcRGHs

Class 1	Class 2	Class 3	Class 4
0.693	0.537	0.306	0.709

dN/dS was calculated for each class using the Nei and Gojobori method implemented in MEGA software

putative RGHs of finger millet showed homology to NBS-LRR-type disease resistance protein homologues from some of the monocots and also to other plant species like tomato, ginger and sunflower, suggesting the highly conserved nature of NBS-LRR domains in different crop species.

The percentage identity between clusters was less than 50%, and within each cluster there was more than 70% identity. Such phylogenetic grouping was further supported by high bootstrap values. Other approaches used to classify RGH sequences include cross-hybridization, but the results vary with the stringency of post-hybridization washes (Shen et al. 1998) and this approach was not used to classify EcRGHs. The use of degenerate forward primer with normal reverse primer can be used to amplify very divergent RGHs from different plant species, as has been demonstrated with peanut and finger millet in our laboratory (Srinivas Reddy 2008, unpublished data). The RGHs amplified with S2/AS3 and LM637/LM638 primers showed maximum homology with each other and were considered to belong to the same cluster, which could be due to the primer sequence

similarity. Although many of the sequences showed high levels of similarity, a few nucleotide differences that were present might have functional importance. For example, an NBS region in potato associated with virus resistance line had a difference of only 12 nucleotides from the homologous regions found in all of the susceptible lines (Sorri et al. 1999).

It is well established that the protein-level search and comparisons showed more homology with the NBS-LRR region of many RGHs than at the nucleotide level. Thus, pair-wise comparison at the amino acid level is more reliable than at the nucleotide level for establishing the identity of RGHs (Totad et al. 2005). Hence, nucleotide sequences were translated into polypeptides and the comparison of amino acid sequences of EcRGHs showed similarity with NBS region of well-characterized R-genes of rice, *RPS2*, *RPS5* and *RPP8* of *A. thaliana*. Five of the recombinant clones (EcPSiPs) had higher homology with PSiP (Moutinho et al. 2001) of rice and lacked the *kinase-2* and *kinase-3a* motifs characteristic of NBS type of R-genes. These were eliminated from cluster analysis of NBS sequences at the amino acid level.

The highly conserved *kinase-2* and *kinase-3a* motifs were seen in all the EcRGHs isolated from finger millet. The NBS-LRR genes are usually grouped into two different subfamilies (Meyers et al. 1999; Pan et al. 2000); subfamily I contains the TIR element (Toll-interleukin-1 receptor-like domain) and has been found only in dicots, whereas the subfamily II which lacks the TIR domain (called non-TIR) was found in both monocots and dicots. The presence of a tryptophan (W) residue in the *kinase-2* (FXXXXW) motif of the NBS domain in all the EcRGHs cloned indicates that these belong to the non-TIR-NBS-LRR subclass of R-genes as reported for most monocots (Meyers et al. 1999). The last amino acid residue of the *kinase-2* domain can be used to predict with 95% accuracy whether an R-gene belongs to the TIR-NBS-LRR class or the non-TIR-NBS-LRR family; conservation of tryptophan (W) at this location is tightly linked to the non-TIR class of R-genes (*RPS2*, *RPS5* and *RPP8*) of *A. thaliana*, whereas conservation of aspartic acid residue (D) or its uncharged derivative asparagine (N) is characteristic of the TIR class of R-genes (*N* and *L6*) (Meyers et al. 1999; Pan et al. 2000; Jeong et al. 2001; Penuela et al. 2002). In the TIR-NBS-LRR group there is a

characteristic consensus motif FXXXXF and a highly conserved glycine that is present between *kinase-1a* and *kinase-2* motifs, whereas the non-TIR-NBS-LRR group contains the consensus sequence FXXXXW (Pan et al. 2000).

In plant genomes, NBS-LRR-type genes are abundant, with approximately 150 described in the Columbia ecotype of *Arabidopsis* (Meyers et al. 2003), more than 500 in the rice genome (Bai et al. 2002; Goff et al. 2002; Monosi et al. 2004; Yang et al. 2006) and only about 50% in the sorghum genome in comparison to those present in rice (Paterson et al. 2009). The NBS-LRR genes of rice are very similar to the non-Toll-interleukin-1 receptor homologue region (non-TIR) class R-genes of *Arabidopsis* but none showed similarity to the TIR class genes. In monocots, non-TIR-NBS types of R-genes are the major class and the present work also indicated that a large number of R-gene homologues present in the finger millet genome belong to the major non-TIR-NBS-LRR class of R-genes. Prior to this report, no RGHS of finger millet were available in the literature or in GenBank. Consequently, the present study represents the first report on RGHS in finger millet.

The EcPSiPs have neither the FXXXXF characteristic of TIR-NBS-LRR nor the FXXXXW characteristic of the non-TIR-NBS-LRR group. PSiP is a signalling protein specific to pollen tissue and is involved in the control of polarity. The perturbation of its function with ODNs (oligodeoxynucleotides) suggests its fundamental role in the maintenance of transduction mechanisms operating in pollen tubes. The analysis of a PSiP amino acid sequence indicated the presence of a NBS motif A (also known as P-loop) for binding ATP or GTP, and LRRs predicting a role in signalling pathways. LRRs have been suggested to function downstream as mediators of protein–protein interactions in signal transduction pathways (Suzuki et al. 1990). The sequence resemblance of PSiP to disease resistance proteins evokes the possibility that in plants AC (adenylyl cyclase) enzymes might be disguised under the broad spectra of large gene families, like the R-gene family. Plant disease-resistance genes like the *RPS2* gene contain leucine zipper, P-loop domains and LRRs, an interaction domain similar to that of yeast AC (Bent et al. 1994). The intimacy between AC and stress signalling proteins in plants has been suggested (Bolwell

1992), and the cellular responses driven by pathogens elicit a response similar to the one operating in pollen tubes.

Expression studies of RGHS in a particular tissue are not yet studied in detail; there are also very few reports on R-gene expression (Aswati Nair and Thomas 2007; Hammond-Kosack and Jones 1997; Hulbert et al. 2001; Yoshimura et al. 1998; Wang et al. 1999; Fourmann et al. 2001; Rivikin et al. 1999; Vincente and King 2001). Elucidation of finger millet RGHS expression was carried using primers representative of each class of RGHS. These RGHS have potential to be used as probes for further R-gene isolation studies.

Rates of non-synonymous to synonymous nucleotide substitution quantify selection pressure by comparing the rate of substitution at silent sites (d_S , K_A), which are presumed natural, to the rate of substitutions at non-silent sites (d_N , K_S), which possibly experience selection (Rocha et al. 2006; Mustonen and Lassig 2007). A ratio of K_A/K_S greater than one indicates diversifying selection and less than one indicates purifying selection (Michelmore and Meyers 1998). Lower K_A/K_S values in the TIR than the non-TIR sequences in various plant species and in *A. thaliana* were observed, suggesting greater functional constraints in the TIR subfamily (Cannon et al. 2008). The NBS region is predicted to be the signalling component that activates plant defences and has been found to be highly conserved across different plant species. Therefore, it is expected that there are few non-synonymous to synonymous changes in this region of R-genes in finger millet. The K_A/K_S ratio of finger millet RGHS was found to be less than one, suggesting purifying selection. However, the LRR region of these genes is predicted to interact (directly or indirectly) with the pathogen controlling specificity and is the most divergent region of this class of resistance genes. Hence, it is important to identify and sequence the LRR regions of the isolated RGHS, and only then the predictions made on this class of R-genes on the basis of NBS region can be further validated. We agree with the conclusions drawn by Michelmore and Meyers (1998) while studying the NBS-LRR gene superfamily in various species, that it is under purifying selection whereas the LRR domain is under divergent selection, as indicated by the high K_A/K_S ratio. Further, the slow evolution of R-genes versus the

faster evolution of avirulence genes of pathogens leading to new races needs to be considered for durable resistance in crop plants.

A number of resistance genes to rice blast like *Pi-ta*, *Pi36* and *Pi37* have an NBS-LRR domain that recognizes the fungal pathogen, *M. grisea*, in a race-specific manner (Bryan et al. 2000; Lin et al. 2007). Similarly, the *Pi2* gene is a member of a gene cluster comprising nine gene members (named *Nbs1-Pi2* to *Nbs9-Pi2*) and encodes a protein with NBS and LRR domains that confer (Zhou et al. 2006) resistance to rice blast pathogen. Though a genetic linkage map is available for finger millet (Dida et al. 2007), it is far from saturation, and the inclusion of more markers in the map will make it possible to draw comparisons between rice and finger millet blast in terms of the identification of the resistance genes and their significance.

Efforts are already underway to map the RGs for use in marker-assisted selection and also for the isolation of full-length R-genes. NBS profiling is another method of isolating more RGs from finger millet and this has been applied in other crop species (van der Linden et al. 2004). The availability of cloned disease resistance genes will permit studies of resistance gene structure, function and evolution. The molecular cloning of disease resistance genes will uncover new ways to deploy these genes against many of the biotic constraints, which will have a major impact in agriculture. Information on more R-gene sequences is necessary to delineate more structural domains which are the basis for the search for RGs in any crop plant (Totad et al. 2005). Sequencing of more plant genomes will make it possible to assess the evolutionary significance of the NBS-LRR class of resistance genes in host–pathogen interaction at the molecular level.

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