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Isolation of Resistance Gene Candidates in Chilli and Use of Molecular Markers for Root Knot Nematode Resistance

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ABSTRACT

Chilli or hot pepper (*Capsicum annuum* L.), a major commercial vegetable crop of India is challenged with a number of pests and diseases and considerable economic damage, caused by root knot-nematode (RKN). Use of cultivars resistant to RKN would obviate the need for the use of pesticides. More than 40 resistant genes have been reported so far in various crops based on a candidate gene approach to address various biotic stresses. In the present study, isolation of these resistance gene candidates (RGCs) has been performed using a PCR-based approach involving degenerate primers designed based on the conserved nucleotide binding site (NBS) domains. Of the 41 clones sequenced, 9 NBS sequences with complete open reading frames (ORFs) had high similarity with the known resistance (*R*) genes, and are denoted as CaRGCs (*Capsicum annuum* resistant gene candidates). The presence of internal conserved motifs provided evidence that sequences isolated from chilli may belong to the NBS-LRR family. A cluster analysis based on the deduced amino acid sequence was carried out on chilli NBS-sequences, together with several analogous domains of known *R* genes, allowing chilli sequences to be classified into three major groups. The ratio of non-synonymous to synonymous nucleotide substitution (dN/dS) in the NBS domains of chilli RGCs ranged from 0.064 to 0.38 for the different classes, which suggests purifying selection. We report that the partial coding sequence CaRGC6, which shows similarity to a nematode resistant protein, could have a potential advantage in the development of a transgenic chilli provided the full length gene is isolated and characterized.

Keywords: degenerate primers, molecular markers, nucleotide binding site-leucine rich repeats (NBS-LRR), polymerase chain reaction, resistant gene (*R* gene)

INTRODUCTION

Chilli or hot pepper (*Capsicum annuum* L.) is one of the most lucrative cash crops and India is the largest producer in the world with a production of 13 lakh metric tonnes, grown in an area of 8 million ha in 2009 (FAOstat 2011). Besides imparting pungency and red color to food dishes, it is a rich source of vitamins and has medicinal properties (http://agroforestry.net/scps/). Root-knot nematodes (RKN) (*Meloidogyne* spp.) are a major pepper pest throughout the world (Di Vito *et al.* 1985; Chen *et al*. 2007) causing crop losses of about €80 billion per year (Blok *et al*. 2008; Djian-Caporalino *et al.* 2011). These are prevalent in open field and controlled conditions wherein they complete several generations within a year (Djian-Caporalino *et al.* 1999, 2011). They establish a permanent feeding site in the differentiation zone of the root by inducing nuclear division without cytogenesis in the host cells (Williamson and Gleason 2003; Wang *et al.* 2010) giving rise to large multinucleate cells called giant cells. This causes the formation of galls or root knots which is the typical feature of *Meloidogyne* spp. (Williamson and Hussey 1996; Vieira *et al.* 2011). These alternations severely affect the uptake of water and nutrients and interfere with the translocation of minerals and photosynthates in the host (Milligan *et al.* 1998; Vieira *et al.* 2011) resulting in wilted and stunted plants with significantly reduced yield levels. Besides, it makes the host plant more vulnerable to other soil-borne pathogens such as fungi and bacteria (Castagnone-Sereno *et al.* 1992). Environmental concerns and government regulations promote the use of non-chemical control measures. Cultivars resistant to this pest potentially render soil fumigant and toxic systemic nematicides unnecessary as they will be an efficient and durable control method (Djian-Caporalino *et al.* 1999). The use of resistant cultivars will obviate the need for using expensive and hazardous chemicals. Though conventional breeding methods are effective, they take longer durations to develop cultivars resistant or tolerant to nematodes. In this context molecular tools have major advantages of screening large number of samples in shortest possible time with high accuracy to breed for nematode resistance.

Many biotechnological tools are increasingly being used to characterize the interactions between plants and potential pathogens at all stages of interactions (Michelmore 1995). Although many genes are involved in these interactions, only few have been shown to determine genetic variation. Molecular markers allow the dissection of monogenic and quantitative resistance. Molecular studies are providing some insight into the evolution of new specificities within resistant genes and their manipulation to provide useful levels of resistance to the evolving pathogens.

Plant defense reactions to the disease are the result of an interaction between the resistant gene (*R*) in the plant and the avirulence gene (*avr*) in the pathogen (Ravensdale *et al.* 2011). Disease resistant genes were cloned from wide range of plant species either by map-based cloning (He 2010) or transposon tagging (Loebenstein 2009) from model plant systems or species with long history of genetic research. However, it is not feasible using these approaches to isolate *R* genes from a species with poor genetic information like chilli.

Research has shown that there is considerable structural similarity among resistant genes isolated from different

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plant species irrespective of type of invading pathogen. The *R* gene appears to be component of signal transduction pathway in the plant that elicits the defense response (Sivaramakrishnan and Seetharama 2001).

Most *R* gene isolates so far contain structural features nucleotide binding domain, leucine-rich repeats and serine/threonine protein kinase in different combinations (Staskawicz *et al.* 1995). This similarity among *R* genes has made it possible to isolate resistant gene candidates from any crop species using a candidate gene (CG) approach (Michelmore 1996; Kanazin *et al.* 1996; Leister *et al*. 1996; Shen *et al.* 1998; Reddy *et al.* 2010). The use of DNA markers will help to identify nematode resistant chilli germplasm for breeding purposes. Some of the elite varieties that are affected by nematode can be made resistant using marker-assisted back crossing.

This study was conducted with the objective of isolating and identifying *R* gene candidates from chilli which can be used against nematodes and also to assess the feasibility of using the molecular markers associated with nematode resistance from previous studies (Djian-Caporalino *et al*. 2007; Chen *et al*. 2007). Degenerate oligonucleotide primers designed to the conserved motifs in the nucleotide binding site 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, which will pave the way for the isolation and deployment of the full length gene through genetic transformation, thereby enhancing the genetic resistance to RKN. We report here the isolation and identification of such RGCs identified in chilli germplasm lines. Besides, an attempt was also made to identify the DNA markers associated with RKN resistance in chilli based on other reports.

Table 1 List of primers used in PCR to amplify chilli DNA.

Primer name	Primer sequence (5'- 3')
CG-S1	GGTGGGCTTGGGAAGACAAGC
CG-S2	GGI GGIGGIGGI AAI ACI AC
CG-AS1	CAACGCTAGTGGCAATCC
CG-AS3	IAG IGC IAG IGG IAG ICC
LM-637	A(A/G)IGCTA(A/G)IGGIA(A/G)ICC
LM-638	GGIGGIGTIGGIAAIACIAC
PLOOP-AA	CTACTACTACTAGAATTCGGNGTNGGNAAAACAAC
PLOOP-AC	CTACTACTACTAGAATTCGGNGTNGGNAAAACCAC
PLOOP-AG	CTACTACTACTAGAATTCGGNGTNGGNAAAACGAC
PLOOP-GA	CTACTACTACTAGAATTCGGNGTNGGNAAGACAC
GLPL-1	CTACTACTACTAGTCGACAGNGCNAGNGGNAGGCC
GLPL-2	CTACTACTACTAGTCGACAGNGCNAGNGGNAGACC
GLPL-4	CTACTACTACTAGTCGACAGNGCNAGNGGNAGCCC
GLPL-6	CTACTACTACTAGTCGACAANGCCAANGGCAATCC

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

Table 2 PCR amplified products from chillies genomic DNA, with different combinations of primers.

Primer combination	No. of clones obtained	No. of clones sequenced	No. of clones showing homology to known R-genes
S1/AS1	15	6	Nil
LM638/LM637	50	12	3
Ploop-GA/GLPL-6	25	14	6
Ploop-GA/GLPL-4	22	8	Nil
Ploop-AC/GLPL-4	4	2	Nil
Ploop-AA/GLPL-4	10	4	Nil
Total	126	41	9

6 degenerate primer combinations were used in isolation of CaRGCs

MATERIALS AND METHODS

Plant materials

Plant material for the present study was accessed from National Bureau of Plant Genetic Resources, Regional Station, Hyderabad, India. Material was initially phenotyped and the genotypes were selected based on the phenotype.

The selected germplasm lines were screened for RKN resistance using the existing RKN population (*M. javanica*) at NBPGR, Hyderabad (Sarath Babu *et al.* 2011). Based on the phenotypic performance accessions viz, EC-391083 and EC-378688 were selected for DNA isolation for the identification of resistance gene candidates.

Isolation of plant DNA

Genomic DNA was extracted from young leaves using the method described by Fulton *et al.* (1995).

Amplification and cloning of NBS-like sequences from genomic DNA using the degenerate primers

A total of 14 individual primers including reverse and forward primers were synthesized (**Table 1**) and 20 combinations of degenerate primers were used to amplify the putative RGA from chilli (**Table 2**). PCR amplifications for cloning were performed in a 20-µl reaction volume containing 2 mM each of dNTP (Sigma Aldrich, St. Louis, USA), 10 ρmol of each primer (Bioserve, Hyderabad, India), 1 U of *Taq* polymerase (Jonaki Brit, Hyderabad, India) with the buffer solution supplied by the manufacturer, 1.5 mM $MgCl_2$ and 50 ng of template DNA. PCRs were carried out in a PTC-100 thermal cycler (MJ Research®, Foster City, CA, USA). Thermocycler parameters were: initial denaturation step was 95°C for 5 min, followed by 35 cycles of 95°C for 1 min, 45/50/51°C depending on the primers and 72°C for 1 min with the final extension step of 10 min. The amplified products were separated by electrophoresis on 1.2% (w/v) Tris-acetate-EDTA (TAE) agarose gel and stained with ethidium bromide for visualization (**Fig. 1**). The target PCR product (500 bp) was cleaned up by a gel extraction kit (Qiagen, Germantown, Maryland, USA). The ligation reaction was set up in 10-µl reaction volume with 50 ng of pGEM-T Easy vector (Promega, Madison, Wisconsin, USA) according to the manufacturer's protocol. Ligation was carried out at 14°C for 16 h. Competent *E. coli* DH10B cells were prepared using 10% glycerol (Sambrook *et al.* 1989) and bacterial transformation was performed by electroporation. The selected putative recombinant clones were confirmed by colony PCR using M13 universal primers (New England Biolabs Inc.). PCR was performed as follows: 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 and held at 4°C using a PTC-100 Thermal Cycler (MJ Research®). PCR products were verified for cloned inserts by resolving the DNA fragments on a 1.2% TAE agarose gel.

Sequence analysis of amplified products

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 (Bioserve, Hyderabad, India). These sequences were screened using the Vecscreen function of NCBI and BLAST searches (Altschul *et al.* 1997) were performed via the National Centre for Biotechnology Information (NCBI) web site. The sequence data were subjected to GenBank searches with BLASTN and BLASTP algorithms. Multiple alignment of amino acid sequences were performed using the CLUSTALX (Thompson *et al.* 1994) method using Megalign tool of DNASTAR software (Lasergene, USA). Phylogenetic analysis was performed by bootstrapping using the neighbour joining method of CLC Workbench 3.1 software (www.clcbio.com). Amino acid sequences of the following *R* genes (cloned from other species) were included in the phylogenetic analysis: N (U15605) from tobacco, L6 (U27081) from flax, RPS2 (U12860), RPS5 (AF074916) and RPP13 (AAF42831) from arabidopsis. I2 (AAU90295) from *Solanum demissum*, CaMi (DQ465824) from chilli. Searches for open reading frame was done using EXPASY translate tool.

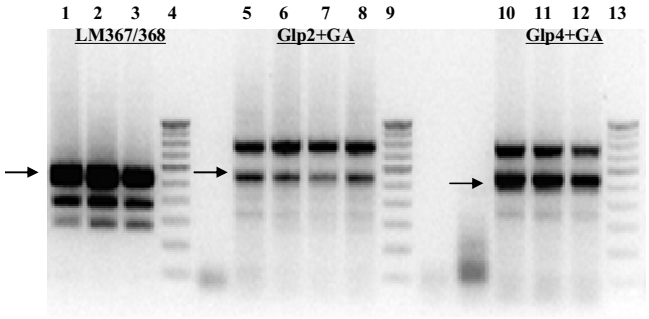


Fig. 1 Agarose gel electrophoresis pattern of PCR amplified chilli genomic DNA. PCR was carried out using 6 degenerate primer combinations on chilli DNA as the template. The amplified products were run on 1.5% agarose gel in TAE, stained with ethidium bromide and visualized on a UV-transilluminator. Arrow indicates the amplified band used for cloning RGCs. Lanes 1-3: chilli DNA amplicons with LM 367/368 primers, Lanes 4, 9, 13: 100-bp ladder, Lanes 5-7: Glp2+GA, Lanes 10-12: Glp4+GA.

GenBank submission

Cloned RGC sequences of chilli were deposited in GenBank with the following accession numbers: FJ605100-FJ605109.

RESULTS AND DISCUSSION

Seven out of the 20 oligonucleotide primer pairs produced a major band of the expected size (of 300-800 bp) according to the source species from which the primers were designed and other species reported in the literature (**Fig. 1**).

In each pair, forward primers were designed to anneal to the sequence encoding the kinase-1a or P-loop domain that is thought to interact with the γ-phosphate of ATP or GTP in some phosphorylation reactions. Reverse primers were designed in the antisense direction corresponding to the coding sequences of an amino acid domain, known as GLPL (Glycine Leucine Proline Leucine) or domain 5 region (Lawrence *et al.* 1995), which in the *Arabidopsis* RPS2 protein is postulated to reside in the *trans*-membrane region (LM-637).

The primers, listed (**Table 1**) amplified at least 3 bands of which one major band of approximately 500 bp was cloned. The remaining primer combinations were ignored because of the appearance of smears on the gel, which probably was due to the high level of degeneracy in the oligonucleotide sequences used. An additional band of 800 bp was also observed among the amplified products. However, it was not cloned as it was suspected to contain introns, as evident from studies on *Arabidopsis* (Aarts *et al.* 1998).

A total of 46 recombinant clones were identified based on colony PCR and sent for sequencing (Bioserve). Not surprisingly, the multi-sequence family of these sequences resulted in a pool of electromorphs of approximately the same size but different nucleotide sequences.

Relationships among the chilli *R* gene candidates

Nine out of the 46 sequences were unique with complete ORFs without stop codons and these were screened using vecscreen and analysed using the BlastX algorithm of NCBI for similarity search. CaRGC sequences showed similarity to the sequences from tobacco, populus, coffee, potato, cotton, chickpea, broad bean, sunflower, among others. All the sequences analysed showed similarity to the sequences with Toll/Interleukin receptors and none of them showed similarity to the sequences with a coiled coil (CC) domain. All the partial coding sequences were unique and the percent identity ranged from 9.9% between CaRGC 5 and 6 to 90.6% between CaRGCs 2 and 3. CaRGC6 showed the least identity among all the candidates studied indicating its uniqueness. Among the sequences, CaRGCs 1 and 9 showed 87% similarity and CaRGCs 5 and 8 had 83.7% similarity.

We searched for the sequences similar to CaRGC6 using the NCBI BlastX tool, as it was a unique sequence that did not show much similarity to any of the sequences from this study. Our search provided *Gro1* as the best hit for this sequence and reference into function of this gene indicated that this gene encodes for an NBS-LRR protein with resistance against the root cyst nematode *Globodera rostochinensis* race1 (Paal *et al.* 2004). However, these sequences were only 61% similar with a maximum score of 151 and an e-value of 3e-38. This again was interesting because the variation observed by the CaRGC sequence could have some important roles in the *R* gene-mediated signaling pathway, which needs to be investigated in future studies.

Relationships between the chilli NBS-like sequences and other plant disease *R* genes

Most of the RGC sequences amplified spanned the regions between the P-loop and GLPL motifs. The presence of the internal kinase 2 region (LIVCDB), excluding the P-loop and GLPL motifs (which were used to design the degenerate primers), confirms that these clones correspond to NBS sequences. The NBS domain is composed of several short amino-acid motifs that are highly conserved among family members and are interspersed among other largely divergent sequences (Meyers *et al*. 1999; Pan *et al*. 2000). The structure basically consists of 3 motifs, namely kinase-1a, kinase-2, and kinase 3a, and are distinct from protein kinases. The presence of the NBS, which is found in numerous ATP and GTP binding proteins suggest that although these *R* gene proteins do not possess intrinsic kinase activity, they could activate kinases or G proteins. NBS with its subdomains spans about 0.6 kb in many of the resistance genes. In addition, multiple alignment studies using the CaRGCs and the six most important *R*-gene peptide sequences showed that the similarity was especially high at three NBS motifs: P loop, kinase 2 and kinase 3A (**Fig. 2**).

After eliminating the RGC sequences with more than 95% amino acid similarity, a total of 9 candidates were isolated using the degenerate primers and aligned for phylogenetic analysis. Phylogenetic analysis grouped these 9 RGCs into 3 clades (**Fig. 3**). All the RGCs except for CaRGC1, CaRGC9 and CaRGC6 were grouped under clade 1 which showed similarity to the RPS2 family of *R* genes which also includes the *CaMi* gene ranging from 17 to 21.1%. The majority of RGCs from chilli were grouped with the CaMi gene than with other RGCs. CaRGC1 and CaRGC9 were grouped into clade 2 which showed 41 and 42.7% similarity to the tobacco *N* gene and 32.9 and 32.7% similarity to *L6*, respectively. Thus, RGCs with some differences in the NBS domain were also present in chilli.

The ratio of synonymous and non-synonymous substitutions was calculated for the three CaRGC clusters (**Table 3**). This ratio serves as an indicator of the evolutionary pressure exerted on a certain class of genes. The values were < 1, indicating purifying selection. A K_a/K_s ratio > 1 indicates diversifying selection and < 1 indicates purifying selection (Michelmore and Meyers 1998). Usually the NBS regions are fairly conserved across species as they are involved in signaling pathways inside plant cells. However, we cannot rule out the possibility that LRR domains which follow many of the NBS domains in *R* genes are generally under divergent selection (Michelmore and Meyers 1998).

Table 3 Rates of non-synonymous (dS) and synonymous (dN) nucleotide substitutions among chilli RGCs.

	Class A	Class B	Class C
dN/dS	0.064	0.383	0.211

dN/dS were calculated using the Nei and Gojobori (1986) method implemented in MEGA software.

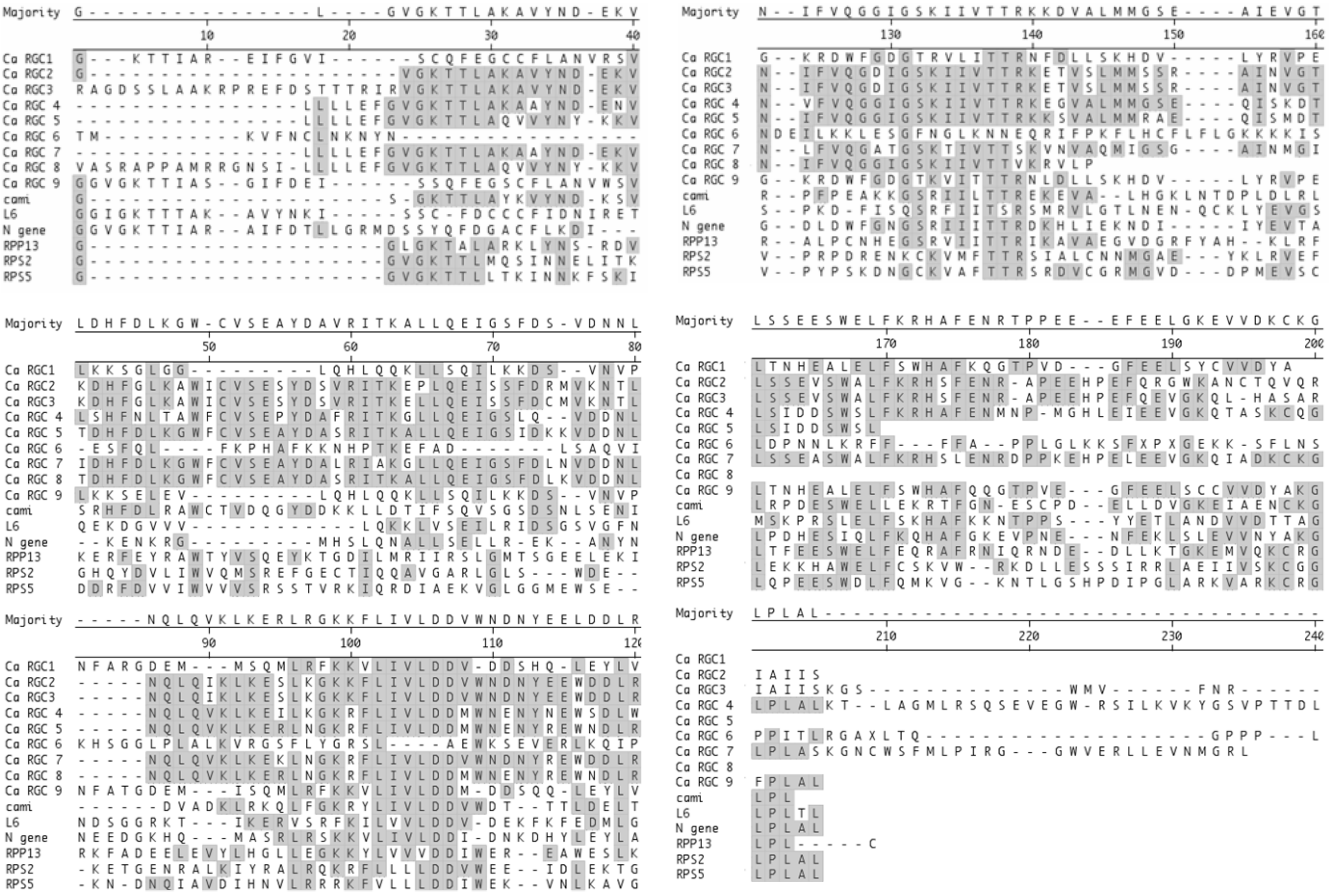
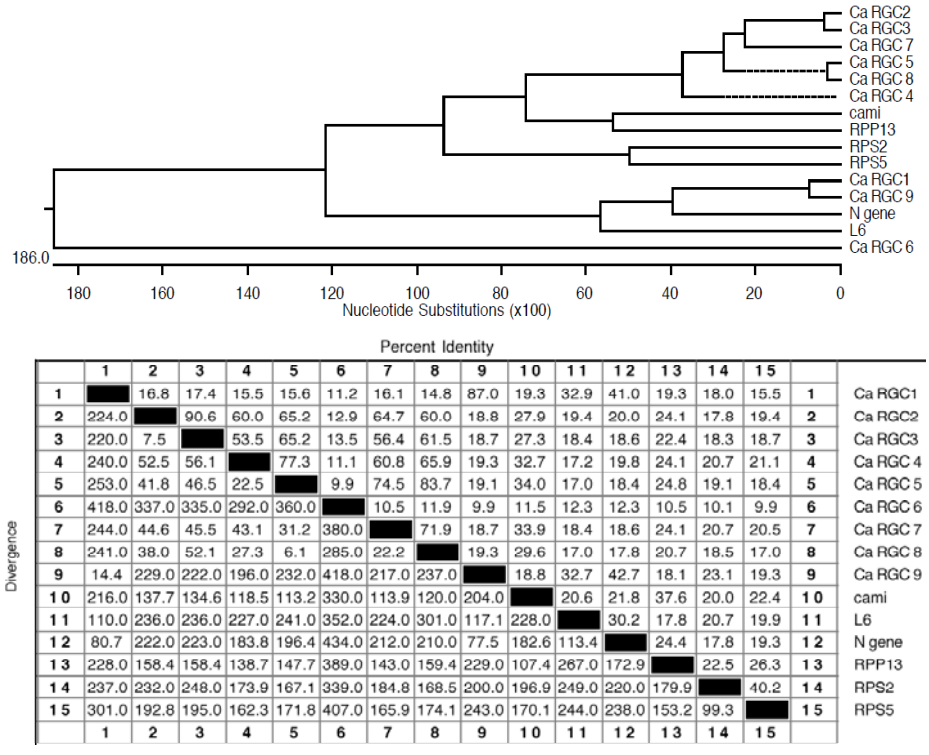


Fig. 2 Alignment of CaRGCs proteins showing the conserved motifs like kinase-2 and kinase-3a of the NBS domain of R protein. All the amino acid sequences are aligned and the kinase-2 and kinase-3a domains are marked using CLC Workbench software.



	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15		
1		16.8	17.4	15.5	15.6	11.2	16.1	14.8	87.0	19.3	32.9	41.0	19.3	18.0	15.5	1	Ca RGC1
2	224.0		90.6	60.0	65.2	12.9	64.7	60.0	18.8	27.9	19.4	20.0	24.1	17.8	19.4	2	Ca RGC2
3	220.0	7.5		53.5	65.2	13.5	56.4	61.5	18.7	27.3	18.4	18.6	22.4	18.3	18.7	3	Ca RGC3
4	240.0	52.5	56.1		77.3	11.1	60.8	65.9	19.3	32.7	17.2	19.8	24.1	20.7	21.1	4	Ca RGC 4
5	253.0	41.8	46.5	22.5		9.9	74.5	83.7	19.1	34.0	17.0	18.4	24.8	19.1	18.4	5	Ca RGC 5
6	418.0	337.0	335.0	292.0	360.0		10.5	11.9	9.9	11.5	12.3	12.3	10.5	10.1	9.9	6	Ca RGC 6
7	244.0	44.6	45.5	43.1	31.2	380.0		71.9	18.7	33.9	18.4	18.6	24.1	20.7	20.5	7	Ca RGC 7
8	241.0	38.0	52.1	27.3	6.1	285.0	22.2		19.3	29.6	17.0	17.8	20.7	18.5	17.0	8	Ca RGC 8
9	14.4	229.0	222.0	196.0	232.0	418.0	217.0	237.0		18.8	32.7	42.7	18.1	23.1	19.3	9	Ca RGC 9
10	216.0	137.7	134.6	118.5	113.2	330.0	113.9	120.0	204.0		20.6	21.8	37.6	20.0	22.4	10	cami
11	110.0	236.0	236.0	227.0	241.0	352.0	224.0	301.0	117.1	228.0		30.2	17.8	20.7	19.9	11	L6
12	80.7	222.0	223.0	183.8	196.4	434.0	212.0	210.0	77.5	182.6	113.4		24.4	17.8	19.3	12	N gene
13	228.0	158.4	158.4	138.7	147.7	389.0	143.0	159.4	229.0	107.4	267.0	172.9		22.5	26.3	13	RPP13
14	237.0	232.0	248.0	173.9	167.1	339.0	184.8	168.5	200.0	196.9	249.0	220.0	179.9		40.2	14	RPS2
15	301.0	192.8	195.0	162.3	171.8	407.0	165.9	174.1	243.0	170.1	244.0	238.0	153.2	99.3		15	RPS5
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15		

Fig. 3 Phylogenetic analysis of CaRGCs at nucleotide level. The tree was created by using CLC Workbench software based on the homology at nucleotide level of RGCs.

Search for markers associated with nematode resistance

The selected resistant and susceptible parents were screened using the RAPD markers E8, F16, U4 and G11. These four markers reportedly co-segregated with nematode resistance in a study of screening 800 RAPD markers (Djian-Caporalino *et al*. 2001). Among these four RAPD markers, G11 co-segregated with the nematode resistant accessions (**Fig. 4**), indicating its potential to be used in nematode resistant

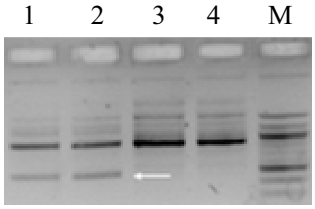


Fig. 4 Polymorphism observed in the bulks with RAPD-G11 primer. PCR was carried out the RAPD primer G-11 on chilli DNA as the template. The amplified products were run on 1.5% agarose gel in TAE, stained with ethidium bromide and visualized on a UV-transilluminator. Arrow indicates the amplified band that differentiates nematode susceptible from resistant bulks of chilli. Lanes 1 and 2: Resistant bulk (RB); Lanes 3 and 4: Susceptible bulk; Lane 5: 100-bp ladder.

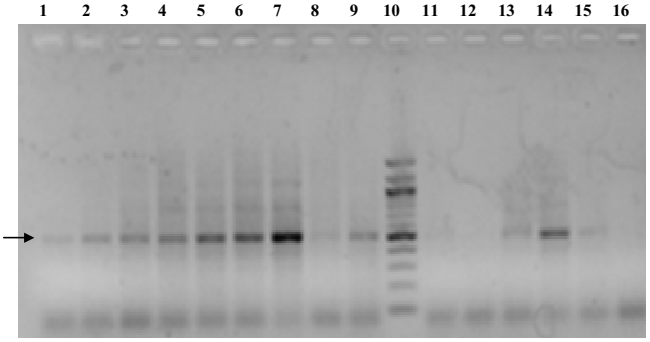


Fig. 5 Polymorphism observed in the chilli accessions with gene-specific primers DP1 and DP2 spanning kinase 1a and hydrophobic domain of *CaMi* gene. PCR was carried out the gene-specific primes DP-1 and DP-2 on chilli DNA as the template. The amplified products were run on 1.5% agarose gel in TAE, stained with ethidium bromide and visualized on a UV-transilluminator. Arrow indicates the amplified band that differentiates nematode susceptible from resistant bulks of chilli. Lane 1: SD-6159; Lane 2: SD-3577; Lane 3: EC-402113; Lane 4: EC-405253; Lane 5: SD-6188; Lane 6: EC-391087; Lane 6: EC-391087; Lane 7: EC-378688; Lane 8: PSR 7075; Lane 9: EC 391083; Lane 10: 100 bp ladder; Lane: 11: EC 339043; Lane 12: EC 402105; Lane 13: PBC 204; Lane 14: EC 389238; Lane 15: EC 399538; Lane 16: Control.

breeding programs.

We also performed degenerate PCR amplification of the resistant and susceptible accessions using DP1 and DP2 primers (Chen *et al*. 2007). These primers were used to isolate a full length resistant gene in chilli resistant accession PR 205. We observed a consistent segregation pattern of this degenerate primer combination with nematode resistance (**Fig. 5**). Dense coverage of the parental material with more molecular markers for the resistant and susceptible bulks from a mapping population would result in the identification of new race-specific genes in the chilli genome. An alternative to this approach would be to bulk the resistant versus susceptible chilli parents and utilize an association mapping approach to discover the co-segregating molecular markers.

To summarize the above study, an integrated approach of "candidate genes" and genetic mapping for RKN resistance would aid in the genetic improvement of chilli. Besides, it also serves as an excellent tool for plant breeders working towards RKN resistance. Full length gene isolation using the CaRGC6 sequence and its subsequent characterization would help to uncover the role of this sequence in the resistance against RKN in pepper.

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