Short Communication

IDENTIFICATION AND CHARACTERIZATION OF NBS-LRR CLASS RESISTANCE GENE ANALOGS IN MOTHBEAN (VIGNA ACONITIFOLIA)

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SUMMARY

Commercial moth bean (Vigna aconitifolia) cultivars are highly susceptible to bacterial, viral and fungal pathogens. The identification of resistance gene analogs holds great promise for development of resistant cultivars. The major known family of plant resistance genes encodes proteins with nucleotide-binding site (NBS) and C-terminal leucine-rich repeat (LRR) domains. Three degenerate primers designed on identified resistance genes (R-genes) were used in combinations to identify resistance gene analogs in the moth bean cv. FMM-96. These primers amplified products with the expected size of 450-600 bp. When their predicted amino acid sequences were compared to each other and to those of known R-genes, a considerable sequence similarity was found. The identification of conserved domains, such as kinase-1a, kinase-2 and hydrophobic motif confirmed that the sequences belong to the NBS-LRR class gene family.

Key words: Vigna aconitifolia, multiple sequence alignment, cloning.

Control of plant pathogens, whichever they are, is predominantly based on a combination of farming practices, chemical treatments and the use of disease-resistant cultivars obtained by breeding.

Molecular marker technologies have located and mapped resistance genes in many plant species, facilitating their genetic analysis and transfer to their commercial cultivars (Palomino et al., 2006). These genes share striking structural similarities, suggesting that certain signallling events are common to all or most plant defense systems. Currently, more than 50 resistance (R) genes have been cloned and characterized from both monocotyledonous and dicotyledonous plants through map-based cloning, transposon tagging and genome homology analysis (Joshi et al., 2010).

On the basis of conserved motifs, R-genes have been classified into five classes (Hammond-Kosack and Jones, 1997). Class I is represented by the maize HM1 gene, which encodes a reductase that deactivates HC toxin of the fungus Cochliobolus carbonum. Class II is represented by the majority of functionally identified R-genes (RPS2, RPM1, N, L6, etc.), which encode cytoplasmic receptor-like proteins that comprise a leucine-rich repeat (LRR) domain and nucleotide-binding site (NBS). Class III includes the Pto gene from tomato, which does not have an NBS-LRR domain but encodes a protein with a serine-threonine protein kinase domain. Class IV includes the Xa21 gene of rice, which encodes an extra-cytoplasmic LRR domain and an intracellular serine-threonine kinase domain, whereas class V represents the Cf genes of tomato that encode transmembrane receptors with an extracellular LRR domain and an intracellular serine-threonine kinase.

These conserved domains in genes represent opportunities for designing simple PCR-based strategies with degenerate primers for the amplification and isolation of related sequences in other plant species (Kanazin et al., 1996). The structural specificity has made it possible to isolate potential resistance gene analogs (RGAs) by homology-based techniques in diverse plant species such as soybean (Kanazin et al., 1996; He et al., 2003), maize (Collins et al., 1998), lettuce (Shen et al., 1998), rice (Mago et al., 1999), common bean (Rivkin et al., 1999), citrus (Deng et al., 2000), wheat (Lacock et al., 2003), sorghum (Totad et al., 2005), and ginger (Nair and Thomas, 2007). The resistance gene analogs were also used as molecular markers for tagging disease resistance loci in different hosts, e.g. Arabidopsis (Aarts et al., 1998), rice (Ilag et al., 2000) and tomato (Zhang et al., 2002).

With the increase of R gene sequences deposited in databases, homologous analysis has become a useful approach to unravel R genes (Michelmore, 2000). Although the overall sequence homology among different R genes is limited, short stretches of peptide sequences such as

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kinase-1α (MGVGKTT) and domain 2 (GLPLAL) in the NBS-LRR class are highly conserved. For example, such motifs in the NBS domain occur across different plant species, making it possible to isolate R gene analogues (RGAs) from different crops using degenerate PCR (Yu et al., 1996; Pan et al., 2000; Tian et al., 2004; Wan et al., 2010; Zhang et al., 2011; Mutlu et al., 2006).

Mothbean [Vigna aconitifolia (Jacq.) Marechal, family Leguminoseae], is a major commercial crop in the semi-arid regions of Rajasthan (India). It fixes atmospheric nitrogen and is a source of lysine and leucine amino acids and certain vitamins or precursors, carotene in particular (Kumar, 2002). This plant, however, is infected by a number of fungi, among which Fusarium moniliforme, Alternaria alternata, Colletotrichum truncatum, Curvularia lunata, Gibberella fujikuroi and Myrothecium roridum (Singh and Srivastava, 1989).

In the present study, three pairs of degenerate oligonucleotide primers based on the NBS domain of resistance genes were used to identify new resistance gene analogs from the V. aconitifolia-resistant cv. FMN-96 by PCR analysis. Further, from the sequencing data, the diversity of the NBS-LRR class of RGAs obtained from mothbean was investigated by comparison with known resistance gene sequences.

Genomic DNA was isolated from V. aconitifolia using the method of Doyle and Doyle (1990) with some modifications. The quality and quantity of the DNA were determined with a UV-Vis spectrophotometer (model GENESYS 10S, Thermo Scientific, USA). Three R-gene-specific degenerate oligonucleotide primers that had previously been used for other taxa (Liester et al., 1999; Palomino et al., 2006) were selected.

The genes and their conserved motifs, which formed the basis for primer design, are given in Table 1. The selected set of primers were designed on the conserved P-loop (GGVGKTT) and hydrophobic domain (GLPLAL) from the N, L6 and RPS2 genes of the NBS-LRR class specific against pathogens. The primers were designed in such a way that PCR products of 500bp were obtained upon amplification. For isolation of RGAs, PCR amplification was carried out using two combinations of forward and reverse degenerate primers: P-loop-F1/GLPL-R1 and Ploop-F1/GLPL-R2, designed from the conserved motifs of genes encoding known NBS-LRR proteins (Table 1).

PCR reactions were performed in a 50 ml reaction mix containing 200 ng of genomic DNA, 1x PCR buffer with (NH₄)SO₄ (Fermentas, USA), 2.0 mM MgCl₂, 0.2 mM each dNTP mix, 1.2 mM of each forward and reverse degenerate primers and 1.0 unit of Taq polymerase (Fermentas, USA). The PCR reactions were carried out in a thermal cycler (Veriti Thermal Cycler, Applied Biosystems, USA) with following cycling conditions: initial denaturation at 95°C for 1 min, followed by 36 cycles of denaturing at 94°C for 50 sec, primer annealing at 51°C for 1 min and strand extension at 72°C for 1 min, and a final extension step at 72°C for 10 min. All the amplified products along with 100 bp DNA ladder (Fermentas, USA) were separated by electrophoresis on ethidium bromide (0.5 mg/ml) stained 1.0% (w/v) agarose gels.

The resolved amplification products of predicted size were excised from the gel and purified using Wizard SV gel and PCR cleanup system (Promega, USA), cloned with pGEM-T easy vector system (Promega, USA) as per manufacturer’s instructions and transformed into competent cells of E. coli strain JM109 using the heat-shock method. Recombinant clones were selected through blue/white colony screening on LB plates supplemented with antibiotic ampicillin (0.1 mg/ml), IPTG (0.1 M) and X-gal (20 mg/ml). The positive clones were checked for the presence of inserts and custom sequenced (Chromous Biotech, India).

The primary and raw DNA sequences obtained were first processed to remove vector and primer sequences using the VecScreen programme of NCBI. The predicted peptide sequences of the sequenced products were obtained through ExPaSy translate tool (Joshi et al., 2010). Database searches were performed using the NCBI BlastP programme to search the similarity of the RGAs with the NBS-LRR-encoding R genes and the available cloned RGAs. Amino acid sequences of resistance genes from other plant species were added to the set of NBS sequences, and cluster analysis was carried out using the default settings of ClustalW2 tool of EMBL-EBI (Larkin et al., 2007).

The phylogenetic tree was constructed by the neighbor-joining method (Saitou and Nei, 1987) using the NJ algorithm implemented in MEGA software package version 5.1 with Poisson correction (Tamura et al., 2011). The bootstrap method (with 1000 replicates) was used to test the robustness of the NJ tree. Furthermore, V. aconitifolia RGAs along with other RGC (NBS-LRR resistance-like protein) sequences available in GenBank, which were isolated from pulse crops such as Vigna mungo, Cajanus cajan, Phaseolus vulgaris, Vigna unguiculata, Lens culinaris, Pisum sativum, Vicia faba and Cicer arietinum were clustered in a phylogenetic tree using the MEGA5 programme.

### Table 1. Degenerate primers used to amplify resistance gene analogs from Vigna aconitifolia.

<table>
<thead>
<tr>
<th>Degenerate primer</th>
<th>Primer sequence (5'→3')</th>
<th>Conserved domains</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ploop-F1</td>
<td>GGIIGGIGTGGAAACIAC</td>
<td>GGII/M/V/GKTT</td>
<td>Palomino et al., 2006</td>
</tr>
<tr>
<td>GLPL-R1</td>
<td>CAACGCTAGTGGCAATCC</td>
<td>GLPLAL</td>
<td>Liester et al., 1999</td>
</tr>
<tr>
<td>GLPL-R2</td>
<td>IAGIGCGAGIGGIGAACC</td>
<td>GLPLAL</td>
<td>Palomino et al., 2006</td>
</tr>
</tbody>
</table>
Degenerate primers based on conserved motifs among the NBS-LRR class resistance genes were used in a PCR-based strategy for isolation and characterization of mothbean NBS-LRR and non-TIR NBS-LRR genes in V. aconitifolia (Fig. 1).

Prominent bands of approximately 500-550 bp in size were amplified by the above primer combinations, were purified, cloned and confirmed for the presence of the insert. In earlier studies, co-amplification of non-specific fragments apart from the expected amplicons had been observed in soybean (Yu et al., 1996), rice (Mago et al., 1999), grapes (Di Gaspero and Cipriani, 2002) and sorghum (Totad et al., 2005). DNA fragments of 500-600 bp were considered to be specific for RGA amplicons as earlier attempts with 300 and 900 bp did not produce any significant homology to known R-genes (Aarts et al., 1998; Lopez et al., 2003; Totad et al., 2005).

Nucleotide sequences were translated into peptides using the ExPaSy translate tool and identity search was made with the BLASTp algorithm (Joshi et al., 2010). The pairwise comparison at the amino acid (aa) level is more reliable than nucleotide-nucleotide comparison and it has been exploited to establish the identity of RGAs from different species (Totad et al., 2005; Thirumalalandi et al., 2008). When aa sequences derived from the selected clones were subjected to BLASTp algorithm they revealed the presence of the NBS domain and a significant homology to R genes and RGAs from angiosperms.

Analysis of the sequences using the ORF finder at the NCBI server (nlm.nih.gov/projects/gorf) disclosed that only 23 sequences could be translated into a single open reading frame (ORF) with more than 100 aa. All 23 RGAs had a high identity level with RGAs deposited in GenBank (Table 2). These sequences are deposited in the GenBank under accession no. KF438052 to KF438074.

![Fig. 1. PCR products of a set of degenerate primer targeted to amplify RGs from Vigna aconitifolia genomic DNA. Lanes 1-3 represents the amplification of RGC fragment from three different samples of Vigna aconitifolia DNA. L is 100 bp DNA ladder.](image)

**Table 2.** Results of homology search (Blastp) between VaRGAs and known R-proteins or RGAs from other plant species as in GenBank accessions. *E value refers to the number of matches expected by chance. The lower the E value, the greater the similarity is and stronger the match is.

<table>
<thead>
<tr>
<th>RGAs</th>
<th>Description and Genbank accession no.</th>
<th>Max identity</th>
<th>E values*</th>
</tr>
</thead>
<tbody>
<tr>
<td>VaRGa01</td>
<td>NBS-LRR type disease resistance protein [Vicia faba] (ABB85184)</td>
<td>97%</td>
<td>2e-123</td>
</tr>
<tr>
<td>VaRGa03</td>
<td>Resistance protein nbs-lrr [Vigna unguiculata] (AAL14232)</td>
<td>97%</td>
<td>5e-113</td>
</tr>
<tr>
<td>VaRGa07</td>
<td>NBS-like putative resistance protein [Phaseolus vulgaris] (AAK48437)</td>
<td>89%</td>
<td>3e-99</td>
</tr>
<tr>
<td>VaRGa09</td>
<td>Putative disease resistance protein [Vigna mungo] (ABY27013)</td>
<td>79%</td>
<td>6e-79</td>
</tr>
<tr>
<td>VaRGa11</td>
<td>Disease resistance protein homolog [Glycine max] AAC49503</td>
<td>69%</td>
<td>7e-76</td>
</tr>
<tr>
<td>VaRGa13</td>
<td>Putative resistance gene analogue protein [Lens ervoides] (CAD56849)</td>
<td>77%</td>
<td>3e-90</td>
</tr>
<tr>
<td>VaRGa18</td>
<td>NBS-LRR type disease resistance protein [Vicia faba] (ABB88171)</td>
<td>94%</td>
<td>2e-119</td>
</tr>
<tr>
<td>VaRGa26</td>
<td>NBS-LRR-Toll resistance gene analogue protein [Medicago sativa] (AF487949)</td>
<td>76%</td>
<td>1e-89</td>
</tr>
<tr>
<td>VaRGa27</td>
<td>NBS-LRR type disease resistance protein [Vicia faba] (ABB82222)</td>
<td>96%</td>
<td>6e-119</td>
</tr>
<tr>
<td>VaRGa32</td>
<td>NBS resistance protein [Phaseolus vulgaris] (ABE61539)</td>
<td>58%</td>
<td>2e-63</td>
</tr>
<tr>
<td>VaRGa38</td>
<td>NBS-LRR type disease resistance protein [Cicer arietinum] (ABB85205)</td>
<td>97%</td>
<td>1e-117</td>
</tr>
<tr>
<td>VaRGa41</td>
<td>Putative NBS-LRR type disease resistance protein, partial [Pisum sativum] (AFZ13795)</td>
<td>87%</td>
<td>5e-100</td>
</tr>
<tr>
<td>VaRGa42</td>
<td>Putative resistance gene analogue protein [Lens culinaris] (CAD56815)</td>
<td>77%</td>
<td>4e-90</td>
</tr>
<tr>
<td>VaRGa46</td>
<td>NBS-LRR type disease resistance protein [Vicia faba] (ABB85169)</td>
<td>98%</td>
<td>4e-120</td>
</tr>
<tr>
<td>VaRGa49</td>
<td>NBS-LRR type disease resistance protein [Medicago truncatula] (XP_003599426)</td>
<td>82%</td>
<td>1e-93</td>
</tr>
<tr>
<td>VaRGa50</td>
<td>Putative NBS-LRR type disease resistance protein, partial [Pisum sativum] (AGM38896)</td>
<td>98%</td>
<td>2e-127</td>
</tr>
<tr>
<td>VaRGa53</td>
<td>Putative NBS-LRR type disease resistance protein [Pisum sativum] (AAD92711)</td>
<td>96%</td>
<td>2e-114</td>
</tr>
<tr>
<td>VaRGa58</td>
<td>Putative resistance gene analogue protein [Lentil sativum] (CAD56823)</td>
<td>78%</td>
<td>3e-91</td>
</tr>
<tr>
<td>VaRGa65</td>
<td>TIR-NBS-LRR RCT1 resistance protein, partial [Medicago truncatula] (XP_003609592)</td>
<td>80%</td>
<td>2e-87</td>
</tr>
<tr>
<td>VaRGa68</td>
<td>NBS-LRR type disease resistance protein [Cicer arietinum] (ABB85194)</td>
<td>98%</td>
<td>6e-121</td>
</tr>
<tr>
<td>VaRGa69</td>
<td>Putative NBS-LRR type disease resistance protein [Pisum sativum] (AAD27515)</td>
<td>78%</td>
<td>5e-87</td>
</tr>
<tr>
<td>VaRGa70</td>
<td>NBS-LRR type disease resistance protein [Cicer arietinum] (ABB85176)</td>
<td>98%</td>
<td>1e-120</td>
</tr>
<tr>
<td>VaRGa77</td>
<td>TIR-NBS-LRR type disease resistance protein [Medicago truncatula] (XP_003610657)</td>
<td>72%</td>
<td>2e-67</td>
</tr>
</tbody>
</table>
The multiple sequence alignment of deduced aa sequences of the 23 VaRGAs and representative R-protein genes representing TIR (N, L6) and non-TIR (AtRPM1, Mla-1 and Sw-5) class revealed the presence of various conserved regions, which are P-loop, RNBS-A, Kinase-2, Kinase-3A and GLPL motifs (Fig. 2A, 2B). Furthermore, 16 and 7 VaRGAs were found to contain N-Methyl-D-aspartate and tryptophan residues (W), respectively, at the end of the Kinase-2 domain, corroborating the typical feature of the TIR and non-TIR class of NBS-LRR genes.

The NBS-LRR classes of genes are classified into two subfamilies: (i) The TIR-NBS-LRR subfamily characterized by the presence of a highly conserved N-Methyl-D-aspartate as the last residue of the kinase-2 domain; (ii) the non-TIR-NBS-LRR subfamily with a highly conserved tryptophan (W) as the last residue of the kinase-2 domain (Pan et al., 2000). Moreover, both TIR and non-TIR-NBS-LRR subclasses are found in dicots, whereas only the
non-TIR subclass is present in monocots (Meyers et al., 1999; Cannon et al., 2002). This supports the hypothesis that *V. aconitifolia*, being dicotyledonous has resistance genes of both the TIR and non-TIR-NBS-LRR subclasses.

A phylogenetic analysis of the 23 VaRGAs was carried out using the MEGA5 programme to understand the relationships among themselves in comparison with nine other known R-genes representing TIR (N, L6, KR1) and non-TIR class (RPM1, Mla1, Sw-5, Hero, RPS2, RPS5) from other plant species. The NJ tree was generated using the aa sequence of the NBS region between the P-loop and Kinase-2 motifs, since this region is present in both the TIR and non-TIR class genes. The resulting phylogenetic tree clearly grouped the 23 VaRGAs and nine R-genes into two major classes, TIR NBS and non-TIR NBS, which were identified based on the homology with the representative R-genes of both classes (Fig. 3).

Out of the 23 VaRGAs, seven (VaRGA1, VaRGA18, VaRGA32, VaRGA38, VaRGA49, VaRGA50, VaRGA53) were comprised in non-TIR group, while the rest clustered into the TIR group. The bootstrap analysis using 1000 replicates supported the separations of the major branches of VaRGAs. The resulting phylogenetic tree clearly grouped the 23 VaRGAs and nine R-genes into two major classes, TIR NBS and non-TIR NBS, which were identified based on the homology with the representative R-genes of both classes (Fig. 3).

Three of the non-TIR class of VaRGAs (VaRGA1, VaRGA49 and VaRGA18) grouped in a clade comprising non-TIR NBS-LRR (RGCs) from *V. faba*, two VaRGAs (VaRGA50, VaRGA53) grouped in a clade with NBS-LRR genes from *P. sativum* while the other two VaRGAs (VaRGA32, VaRGA38) grouped in a mixed clade consisting of non-TIR NBS-LRR genes from *C. arietinum*, *Hordeum vulgare* and *P. sativum*. The TIR VaRGAs constituted a mixed clade consisting of other NBS-LRRs from *V. unguiculata*, *C. arietinum*, *P. sativum*, *L. culinaris* and *V. faba*.

Research on the nature of R-genes and RGAs in different plant species has greatly accelerated in recent times. Information on more resistance gene sequences is necessary to detect additional structural motifs, which are the basis for the search of resistance gene analogs in crop plants. In this paper, we report the amplification, cloning, and characterization of NBS-LRR class resistance-gene candidate sequences from *V. aconitifolia*, using degenerate primers based on conserved motifs of known R genes. This is the first report on the isolation of RGAs from *V. aconitifolia* using a PCR-based approach. In general, the NBS-LRR class of R-genes consists of hundreds of paralogs in plant species. With the help of the identified *V. aconitifolia* RGAs, different primer sets can be designed for analysis of *V. aconitifolia* wild relatives to target novel genomic resources for the genetic improvement of this crop. Studies on R-genes and RGAs are still explorative in

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**Fig. 3.** Phylogenetic tree of plant NBS-LRR-type resistance genes and their homologues constructed using the amino acid sequences in the NBS region by the neighbor-joining method (Saitou and Nei, 1987) using the NJ algorithm implemented in MEGA software package version 5.1 with the Poisson correction (Tamura et al., 2011). The bootstrap method (with 1000 replicates) was used to test the robustness of the N-J tree.
nature. Information on more R-gene sequences is necessary to delineate more structural domains, which is the basis for the search of RGAs in any crop plant.

REFERENCES


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