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Cloning, expression and purification of resistance gene analogue RGPM 301 from pearl millet in *Escherichia coli*

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ABSTRACT

Plants combat their pathogens with an array of defense responses. One of the key mechanisms involves products of resistance (*R*) genes which are responsible for recognition of effector molecules from pathogens and subsequent triggering of defense responses. Resistance gene analogues (RGAs) containing the specific conserved domains of *R*-genes are isolated from various plants using degenerate oligonucleotide primer based PCR approach. In an earlier study, *RGPM 301* an RGA from pearl millet shown to be involved in resistance mechanism against downy mildew disease was isolated and characterized. In the present study, *RGPM 301* containing an open reading frame (ORF) of 992 amino acids was cloned into pRSET A expression vector and expressed in *Escherichia coli* as a Hig-tag fusion protein. The recombinant RGA RGPM 301 was purified to near homogeneity using the Nickel-CL agarose column. Its molecular mass was found to be 120 kDa when separated on the SDS-PAGE which was confirmed by western blotting analysis using the anti-His antibody. The purified protein was subjected to in-gel trypsin digestion followed by mass spectrometric analysis for the confirmation of its identity. These findings facilitate further studies on the exact role of this RGA in the pearl millet downy mildew host pathogen system.

1. INTRODUCTION

Plants have developed effective and sophisticated mechanisms in pathogen perception and defense against their attack. The resistance (*R*) genes of plants with specific conserved domains and motifs are known to play an important role in disease resistance. The *R*-gene products of the adaptive immune system recognizes the effector molecules from the pathogen and triggers the defense response with a localized host cell death or the hypersensitive response limiting the spread of the pathogen from the site of infection [1]. Based on the structural characteristics of the encoded R-proteins they are distinguished as nucleotide binding site-leucine rich repeats (NBS-LRR), receptor like serine-threonine kinases and extracellular/intracellular receptor like proteins [2]. Majority of the plant *R*-genes are members of NBS-LRR gene family conferring resistance to a wide range of pathogenic bacteria, fungi, viruses and pests such as insects and nematodes [3]. The NBS-LRR can be further categorized as toll/interleukin receptor

TIR-NBS-LRR (TNL) or coiled coil-NBS-LRR (CNL/CC-NBS-LRR) [4]. Most of the NBS-LRR and CNL encoding genes are found in monocots than in the dicots. Plant *R*-genes with such domains have been isolated by following methods such as positional cloning, transposon tagging and homologous cloning [5]. Since specific domains in *R*-genes are conserved across plant species, novel resistance gene analogues (RGAs) have been isolated from various plants using degenerate oligonucleotide primer based polymerase chain reaction (PCR) approach [6]. These, RGAs are useful tools for the isolation of full-length *R*-genes which provides vital information about the organization of these genes, their expression, roles in resistance mechanism and evolution [7]. Cloning of the *R*-genes and their heterologous expression in other host expression systems helps in establishing their functional characteristics. Protein isolation from plants is found to be a tedious process and may result in poor yields during purification. Hence, *in vitro* expression systems of bacteria, yeast, insect cells and *Xenopus laevis* oocytes which are available are used for overexpression of the encoded R-proteins with fusion tags. These methods produce large-scale plant proteins in suitable hosts and are helpful in validation of their biochemical and biophysical features [8].

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Pearl millet [*Pennisetum glaucum* (L.) R. Br.] grown in the arid and semi-arid tropics of Indian subcontinent and Africa serves as a staple food and fodder crop of millions of poor families of these regions [9]. The crop is found to possess high potentiality with tolerance to high temperatures and also drought stress conditions [10]. The biotrophic oomycete *Sclerospora graminicola* (Sacc.) Schroet., causes the downy mildew disease in pearl millet leading to about 60% crop loss [11].

The resistance induced in the plants is often suppressed by pathogens. Such competitive evolution of the pathogen leads to break down of resistance. The pathogen causing downy mildew in pearl millet also evolves rapidly and results in the quick breakdown of resistance [12].

In an earlier study at our department, a set of 22 putative RGAs were identified in pearl millet using the conserved sequences of known *R*-genes by candidate gene approach method. Of the clones obtained, *RGPM 301* (GenBank accession number: AY117410) which contained a partial sequence was 83 % similar to rice *R*-proteins [13]. To identify and characterize the protein encoded by this partial sequence the full-length gene was isolated using rapid amplification of cDNA ends PCR approach [14]. This RGA, was 3552 bp long comprising an ORF of 2979 bp (GenBank accession number: KP226586) coding for 992 amino acids. Based on the conserved domains and motifs this CC-NBS-LRR gene was grouped as a non-TIR NBS LRR containing RGA [14]. Quantitative real time-polymerase chain reaction (qRT-PCR) studies revealed higher expression of transcripts of *RGPM 301* in pearl millet cultivar resistant to downy mildew upon infection with the pathogen *S. graminicola*, as well as upon treatment with biotic and abiotic elicitors [14].

These studies indicate a role for this RGA in resistance mechanisms of pearl millet against downy mildew. In order to have a better understanding of the function of *RGPM 301* in pearl millet downy mildew system, in the present study this full-length gene was cloned, expressed as a fusion protein in *E. coli* and purified.

2. MATERIALS AND METHODS

2.1 Bacterial strain, vector and chemicals

The bacterial strain *E. coli* BL21 (DE3) was used for expression of RGA *RGPM 301*. The vector pRSET A plasmid was obtained from Invitrogen, Bangalore and used in expression studies. The restriction enzymes BamHI and NcoI were procured from Merck Biosciences, Bangalore. The molecular biology reagents of the highest grade were purchased from commercial sources.

2.2 Recombinant plasmid construction

The ORF of *R*-gene *RGPM 301* which contained the CC-NBS-LRR region was previously amplified, cloned in pTZ57R/T vector and sequenced (GenBank Accession No. KP226586) [14]. Plasmid DNA was isolated using GeneJET™ plasmid miniprep kit (Fermentas). The primers PMRGPM-RE-F

forward (5'-CGGGATCCATGGGCACCGTTTTGGAGTCTCTTGC TTGG-3') and PMRGPM-RE-R reverse (5'-CATGCCATGG CTAATGATAGAGAGATTCATCTGGAAATATATCTCG-3') bearing BamHI and NcoI restriction sites (underlined) respectively were designed based upon the complete sequence and used for PCR amplification of *RGPM 301*. The PCR conditions included: initial denaturation at 94 °C for 5 min followed by 30 cycles of denaturation at 94 °C for 45 s, annealing at 60 °C for 1 min and polymerization at 72 °C for 1 min with a final extension of 10 min at 72 °C. Following amplification, the purified PCR product was digested with BamHI and NcoI and cloned into pRSET A vector to generate an expression construct. This recombinant vector containing the *RGPM 301* fused to a His-tag was transformed into *E. coli* BL21 (DE3). Presence of the gene in the vector was analyzed by colony PCR and its reading frame was confirmed by DNA sequencing.

2.3 Overexpression of recombinant RGA *RGPM 301*

The BL21 (DE3) strain harboring the recombinant plasmid was grown overnight at 37 °C in the Leuria Bertani medium supplemented with ampicillin (100 µg/mL) with shaking at 250 rpm until A_{600} was 0.8. The recombinant protein was induced by IPTG (isopropylthio-β -D-galactoside) with a final concentration of 1 mM and grown for 6 h at 37 °C [15]. The cells were harvested at 8000 rpm for 10 min at 4 °C and were lysed in 20 mM phosphate buffer pH 7.4 by sonication. The cell lysate was centrifuged at 20,000 g for 20 min at 4 °C. The supernatant and the pellet were further evaluated.

2.4 Affinity purification of RGA *RGPM 301* expressed in BL21 (DE3)

The Histidine-tagged recombinant RGA *RGPM 301* protein was purified using His-Tag Fusion Protein Purification Kit (Merck Biosciences, Bangalore) according to the manufacturer's instructions. The Nickel-CL agarose column (2.5 x 10.0 cm) was equilibrated with the equilibration buffer. The clarified lysate was loaded onto the column and the bound (His)₆-tagged fusion protein was eluted. The eluted fractions were further analyzed for the recombinant protein by polyacrylamide gel electrophoresis.

2.5 Protein analysis

The protein was estimated and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli [16]. Fifty micrograms of bacterial soluble total protein and 10 µg of purified protein were mixed with SDS-PAGE loading dye followed by incubation at 90 °C for 10 min and loaded on a 10 % SDS-PAGE gel. The gel was stained with Coomassie blue and assessed for protein expression.

2.6 Western blot analysis

Following SDS-PAGE, the separated protein was transferred electrophoretically on to a polyvinylidene difluoride (PVDF) membrane using Multiphor II apparatus (LKB, Pharmacia). The blot was incubated with 5 % (w/v) milk powder

of blotting-grade in Tris-buffered saline (TBS: 10 mM Tris HCl pH 8.0, 150 mM NaCl) and washed 3 times for 5 min each with TBST [TBS with 0.05 % (w/v) Tween-20]. The blot was then incubated with Histidine-Tag Mouse Monoclonal Antibody at 37 °C for 90 min and washed 5 times with TBST. The secondary antibody anti-mouse conjugated to horseradish peroxidase (1:1000 dilution in blocking buffer) was added and incubated for 60 min at 37 °C. Finally the blot was washed thrice with TBST and developed with horseradish peroxidase substrates 3, 3'-diaminobenzidine and hydrogen peroxide.

2.7 In-gel trypsin digestion

The in-gel digestion of the recombinant expressed protein separated by SDS-PAGE was processed as described by Ray *et al.* [17] with slight modifications. The gel piece was diced and destained in 100 µl of destaining solution (50 mM ammonium bicarbonate/acetonitrile of 1:1, v/v) with intermittent gentle vortexing until most of the Coomassie dye was removed. The gel was dehydrated with 100 % acetonitrile (ACN) and the shrunken pieces were incubated at 56 °C for 1 h in reducing solution (10 mM Dithiothreitol in 100 mM ammonium bicarbonate) and washed in 100 µl of 25 mM ammonium bicarbonate. After reduction the gel pieces were incubated in alkylation solution (55 mM iodoacetamide in 100 mM ammonium bicarbonate) in dark for 20 min at room temperature, washed with 25 mM ammonium bicarbonate and dehydrated in 100 % acetonitrile. The gel pieces were air-dried and incubated in 25 µl of 12.5 ng/µl trypsin (Proteomics Grade, Sigma) at 37 °C for 17 h.

The supernatant containing the peptide mixtures were collected into a fresh microfuge tube. The remaining peptides in the gel pieces were extracted sequentially using 50 µl each of extraction buffers 1 (60 % ACN with 0.1 % formic acid) and 2 (100 % ACN) respectively. Supernatant was collected from both the peptide extraction steps and were pooled with earlier peptide fraction and dried in SpeedVac.

This was followed by reconstitution of peptides in 20 µl of 0.1 % formic acid and was desalted by custom made desalting tips using C-18 solid phase extraction disks (3M Empore) as described by Rappsilber *et al.* [18].

2.8 Mass Spectrometric Analysis

The peptide mixture which was desalted was subjected to fractionation by Agilent 6550 iFunnel QTOF mass spectrometer (Agilent Technologies) coupled to an Agilent 1260 Infinity Capillary Pump and 1260 Infinity Nanoflow Pump LC system (Agilent). The desalted peptide samples were loaded onto a ProteinID-Chip Zorbax 300SB-C18 5 µm via an Infinity Autosampler (Agilent Technologies). The data was acquired by the Agilent Mass Hunter Workstation Software, LC/MS Data Acquisition B.05.00 (Build 5.01.5125.1) operating in auto MS/MS mode. The data files were processed in Agilent Mass Hunter Spectrum Mill MS Proteomics Workbench software (Version B.04.00.127) and analyzed in MASCOT search engine.

3. RESULTS AND DISCUSSION

3.1 Recombinant protein expression

Previously, the full-length *RGA RGPM 301* was isolated and cloned in pTZ57R/T vector. The PCR amplified gene with primer pairs appropriate for expression studies generated an amplicon of ~2979 bp (Fig 1).

The obtained amplicon and the expression vector pRSET A were subjected to restriction digestion with BamHI and NcoI and the recombinant plasmid construct was generated. The pictorial representation of the construct obtained by SnapGene Viewer tool version 3.0.3 is depicted in Fig 2.

The most widely used *E. coli* strain for protein overexpression is BL21 (DE3) which provides the highest yield of target protein [19]. Hence, the construct was transformed into *E. coli* BL21 (DE3) and was confirmed by colony PCR and found to be in-frame by sequencing (Fig 3).

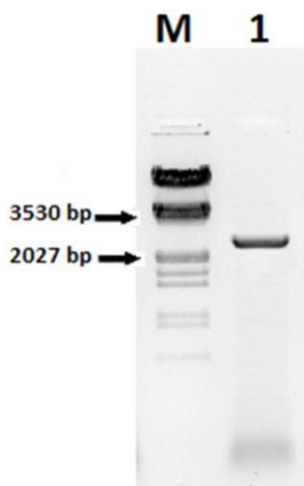


Fig. 1: Agarose gel electrophoresis of *RGA RGPM 301*. M-Lambda DNA/EcoRI+HindIII Marker (Thermo Scientific, Bangalore). Lane 1-PMRGPM-RE-F/ PMRGPM-RE-R amplified PCR product of ~2979 bp..

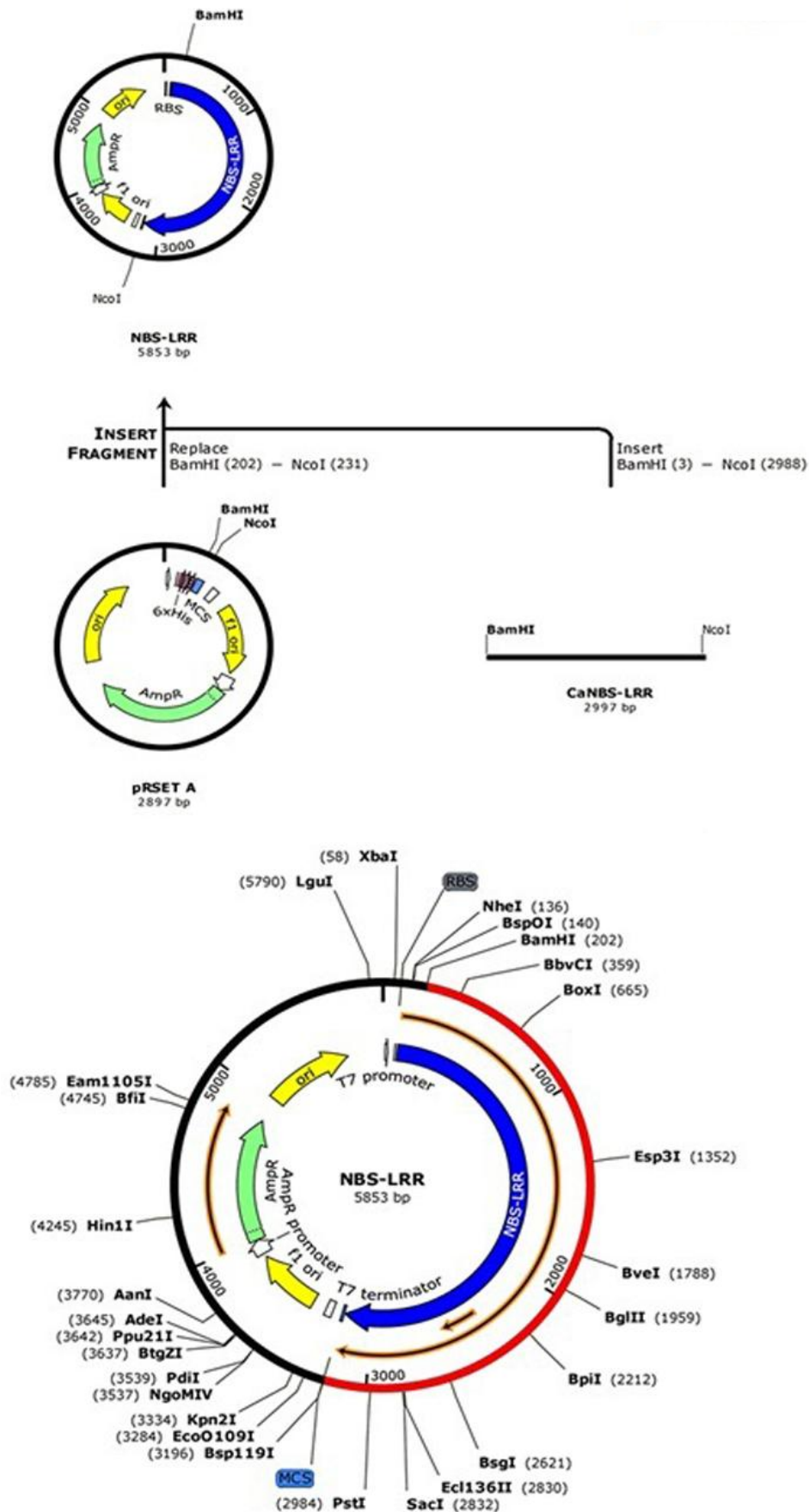


Fig. 2: Recombinant RGA RGPM 301 expression constructs. RGA RGPM 301 and expression vector pRSET A were restriction digested with BamHI and NcoI. These were ligated to form a recombinant RGA RGPM 301 expression construct.

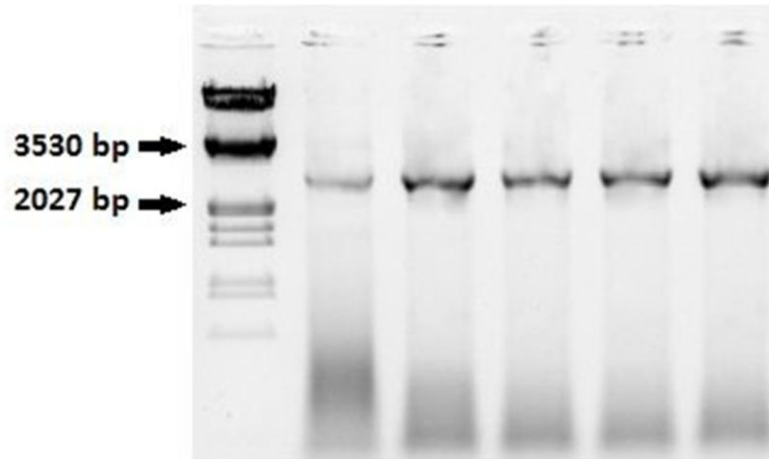


Fig. 3: Colony PCR confirmation of the *RGA RGPM 301* amplified with expression primers.

3.2 Induction of *RGA RGPM 301*

IPTG (1 mM) induction for 6 h induced the expression of recombinant protein. The recombinant *RGA RGPM 301* was expressed with (His)₆-tag derived from the pRSET A expression system. The fusion protein was mainly obtained in the soluble fraction of *E. coli* cell lysate with a molecular mass of approximately 120 kDa resolved on a SDS-PAGE (Fig 4).

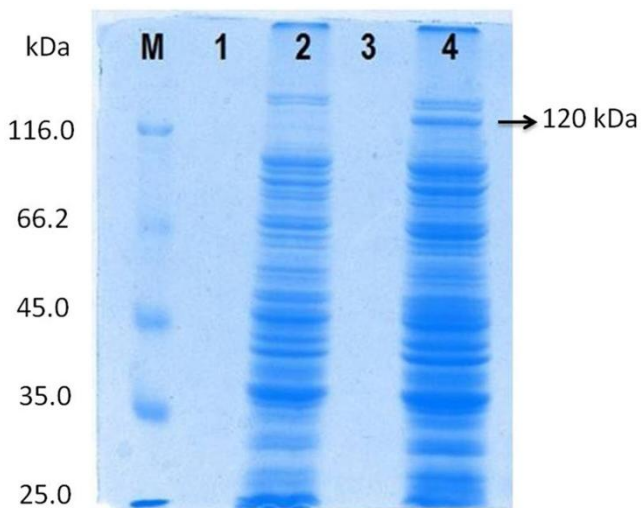


Fig. 4: Expression of recombinant *RGA RGPM 301* in *E. coli*. Lane M: Molecular weight protein marker, Lane 1: uninduced pellet, Lane 2: induced pellet, Lane 3: uninduced supernatant, Lane 4: induced supernatant.

3.3 Purification of His-tagged recombinant protein

The recombinant *RGA RGPM 301* was purified from the *E. coli* lysate using Nickel-CL agarose column chromatography. The non-specifically bound proteins of recombinant *RGA RGPM 301* were washed with wash buffer and removed. The (His)₆-tagged fusion protein bound to the agarose column was eluted and the fraction was analysed on SDS-PAGE for confirmation of the 120 kDa recombinant protein to near homogeneity (Fig 5A). The molecular mass of the purified protein corresponded with the

theoretically predicted molecular mass. Further, the identity of the (His)₆-tagged protein was confirmed by probing the western blot with anti-His antibody (Fig 5B). In a similar study carried out by Ranjini *et al.* [20], the cDNA *RGPM213* of pearl millet with NBS-LRR region was expressed in pRSET-A and transformed in BL21 *E. coli* cells. The expressed His-tag recombinant protein of 35 kDa was purified using nickel purification system.

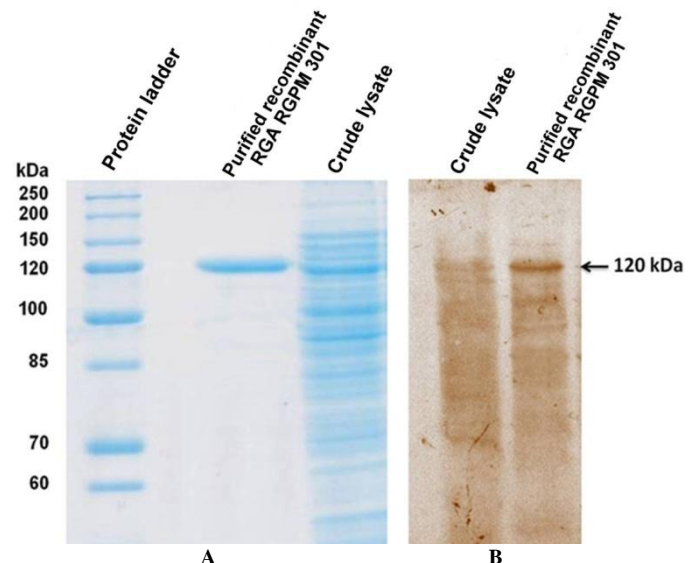


Fig. 5: Recombinant *RGA RGPM 301* production and purification. (A) SDS-PAGE profile of the recombinant protein (1 µg) fraction purified by Nickel-CL agarose column and the soluble total protein (50 µg) from *E. coli* strain BL21 (DE3) harvested 6 h post induction with 1 mM IPTG. (B) Western blot showing the reaction of purified recombinant *RGA RGPM 301* probed for the histidine-tag.

3.4 MS/MS Analysis

The recombinant expressed purified protein band of 120 kDa obtained after SDS-PAGE was excised, trypsin digested and subjected to LC/MS/MS analysis. The 120 kDa protein after LC/MS/MS and MASCOT search showed several peptides with similarities to disease resistance proteins of different species. The

peptide sequence RYFIHEDLWASSMWDIVSR showed high similarity to *Oryza sativa* partial NBS-LRR resistance protein (Accession No. 383844187) and WLSLLQHMK peptide sequence had a similarity with the *Oryza sativa* putative NB-ARC domain-containing protein (Accession No. 146394070). LVTNQLTSIAR peptide was similar to *Zea mays* putative leucine-rich repeat transmembrane protein kinase family protein (Accession No. 413945125). The peptide sequence VLADVSLISVSSNGEIVMHSLQRQMGK was found to be similar to *Arabidopsis thaliana* disease resistance protein SLH1 (Accession No. 51555866). Two of the peptide sequences VQVLPKSLGK and LASPDFVDLILGK were similar to *Glycine max* disease resistance protein RPM1-like (Accession No. 356515314) and NB-LRR type disease resistance protein Rps1-k-1 (Accession No. 62632823) respectively. These results confirm the identity of the recombinant RGPM 301 as a RGA.

Isolation, expression and characterization of full-length *R*-genes is essential for gaining better understanding of the functions of proteins encoded by these genes in plants. Several studies have reported this approach. Two tomato *R* proteins, I-2 which conferred resistance to *Fusarium oxysporum* and Mi-1 conferring resistance to root-knot nematodes and potato aphids were purified as glutathione *S*-transferase fusions in *E. coli* expression system [21]. Similarly, a rice R1-NB a 809 amino acid orphan *R*-protein with N-terminal extension containing a CC domain was expressed as a soluble recombinant protein in *E. coli* and co-purified with a single contaminating protein by affinity purification [22]. Also, DNA fragments which encoded the ORF of the Pi-ta isolated from pCB1906, LRD and LRDA918S isolated and purified from the two-hybrid bait vector were cloned into pET29a and expressed in BL21 (DE3) for expression of the fusion proteins [23]. In the soybean *R*-protein RHG1-LRR, the LRR domain believed to recognize the elicitor was expressed, purified and refolded from *E. coli* [24]. A rust *R*-protein (M) of flax expressed in *Pichia pastoris* as a cytosolic protein was obtained in the soluble fraction which was purified to near homogeneity using ion exchange, metal affinity and size exclusion chromatography [25].

4. CONCLUSIONS

In this study, we report the successful heterologous expression of the recombinant fusion protein RGA RGPM 301 in *E. coli*. The full-length RGA RGPM 301 with 2979 bp encoding 992 amino acids was amplified by PCR and cloned into the expression vector pRSET A. The obtained recombinant plasmid construct was transformed into BL21 (DE3) cells and overexpressed with IPTG induction at 6 h. The (His)₆ tagged recombinant protein was obtained in the soluble fraction and purified using the Nickel-CL agarose column. The purified fusion protein of approximately 120 kDa was confirmed by western blotting with mouse anti-His antibody. Finally, the purified protein was subjected to in-gel trypsin digestion and mass spectrometric analysis. The purified RGPM 301 can be used further for structural

and functional characterization of this protein. Antibodies can be raised against this protein which will be helpful for isolation of the corresponding *R*-protein from pearl millet extracts and its localization in plant tissue. Such a study would pave a way for understanding the biochemical and molecular events that occur during the host pathogen interactions. These events will also contribute to understanding the role of *R*-genes in the plant disease resistance mechanism.

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