Molecular cloning of a Coiled-Coil-Nucleotide-Binding Site-Leucine-Rich Repeat gene from pearl millet and its expression pattern in response to the downy mildew pathogen

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Abstract

I. **Background:** Downy mildew caused by *Sclerospora graminicola* is a devastating disease of pearl millet. Based on candidate gene approach a set of 22 resistance gene analogues were identified. The clone RGPM 301 (AY117410) containing a partial sequence shared 83% similarity to rice R-proteins.

II. **Methods and Results:** A full-length *R*-gene *RGA RGPM 301* of 3552 bp with 2979 bp open reading frame encoding 992 amino acids was isolated by the degenerate primers and rapid amplification of cDNA ends polymerase chain reaction (RACE-PCR) approach. It had a molecular mass of 113.96 kDa and isoelectric point (pI) of 8.71. The sequence alignment and phylogenetic analysis grouped it to a non-TIR NBS LRR group. The quantitative real-time PCR (qRT-PCR) analysis revealed higher accumulation of the transcripts following inoculation with *S. graminicola* in the resistant cultivar (IP18296) compared to susceptible cultivar (7042S). Further, significant induction in the transcript levels were observed when treated with abiotic elicitor β-aminobutyric acid (BABA) and biotic elicitor *Pseudomonas fluorescens*. Exogenous application of phytohormones jasmonic acid (JA) or salicylic acid (SA) also up-regulated the expression levels of *RGA RGPM 301*. The treatment of cultivar

IP18296 with mitogen-activated protein kinase (MPK) inhibitors (PD98059 and U0126) suppressed the levels of *RGA RGPM 301*.

III. **Conclusions:** A 3.5 kb *RGA RGPM 301* which is a non-TIR NBS-LRR protein was isolated from pearl millet and its upregulation during downy mildew interaction was demonstrated by qRT-PCR. These studies indicate a role for this RGA in pearl millet downy mildew interaction.

Keywords: BABA · CC-NBS-LRR · Elicitors · Inhibitors · qRT-PCR · *R* genes

Introduction

Plants combat pathogen invasion by their innate immune system. The battle between them activates the basal defense by elicitors such as bacterial flagellins, lipopolysaccharides, peptidoglycan, fungal chitin and β-glucans of oomycetes termed microbe- or pathogen-associated molecular patterns (MAMPs or PAMPs). These in turn are recognized by pattern-recognition receptors (PRRs) localized on the membrane leading to pathogen associated molecular pattern triggered immunity (PTI) [1, 2, 3]. One of the reported MAMP-PRR recognition includes perception of Ax21 a *Xanthomonas oryzae pv. oryzae (Xoo)* type I secreted protein by Xa21 [4]. The establishment of resistance (*R*) genes encoding the R-proteins is an outcome of the co-evolution between the host plants and their pathogens. This acts as a second layer of immunity against the pathogen effectors setting up the effector triggered immunity (ETI). The R-proteins mediate a defense reaction culminating in hypersensitive response (HR) and eventually a localized cell death at the site of infection [1, 2]. Further, the pathogenesis-related (PR) genes are induced in the host plant accelerating a systemic disease resistance against the pathogens throughout the plant [5].

Various *R*-genes cloned and characterized have been grouped into eight classes based on the organization of the amino acid motifs and membrane spanning domains which provide information for the structural and functional elucidation of *R*-genes [6]. They are mainly comprised of the nucleotide binding site (NBS) and leucine rich repeat (LRR) domains with specific functional importance [7]. The N-terminal NBS domain lodge the conserved kinase-1a or P-loop, hydrophobic GLPL, kinase-2 and kinase-3a motifs. This domain functions in activation of the signal transduction pathways by binding and hydrolyzing ATP or GTP, leading to defense response. The variable LRR at the C-terminus plays a role in pathogen

recognition and is involved in protein-protein interactions [8, 7]. However, interaction of these domains intramolecularly helps in the pathogen detection.

The *R*-genes in plant genomes are abundantly of NBS-LRR type with around 600 and 125 NBS-LRR genes found in the rice and Arabidopsis genomes respectively [9, 1]. Generally, the NBS-LRRs upon activation leads to the development of defense signal both locally and systemically. This involves generation of reactive oxygen species, networking of hormones and expression of defense genes [1]. The *R*-genes are broadly categorized as TIR NBS-LRR (TNL) and non-TIR NBS-LRR subclasses on the basis of the presence or absence of N-terminal Drosophila Toll and mammalian interleukin-1 receptor (TIR) region [10]. The TIR subclass is found in dicot species whereas the non-TIR subclass with a coiled-coil (CC) structure occurs among both the monocot and dicot plants [11, 10]. The CC domain with a coiled-coil structure is implicated in specific interactions with other proteins and also facilitates dimerization [12]. The CC-NB-LRR protein Rx of potato is found to interact with the *Potato virus X* (PVX) coat protein conferring resistance to PVX [13] and the Arabidopsis *RPS4* gene is involved in resistance to Avr Rps4 of bacterial pathogens [14]. Based on the conserved domains of known *R*-genes across plant species, novel resistance gene analogues (RGAs) have been isolated from diverse plants by degenerate oligonucleotide primer based PCR approach [15]. This also serves as a tool for rapid isolation of full-length resistance genes.

Pearl millet [*Pennisetum glaucum* (L.) R. Br.] is cultivated as a drought- and high-temperature tolerant major warm-season cereal crop for the production of grain and fodder in the arid and semi-arid tropical regions of Asia and Africa [16, 17]. The plant is highly susceptible to the devastating obligate biotrophic oomycete *Sclerospora graminicola* (Sacc.) Schroet., causing downy mildew disease leading to considerable yield loss [18]. A quick breakdown of resistance is often noticed in some of the resistant cultivars of pearl millet due to evolving pathogen. Hence, knowledge of genetic resistance is a prerequisite in understanding the possible defense mechanisms. Earlier studies from our department have identified 22 RGAs in pearl millet using the candidate gene approach based on the conserved sequences of known *R*-genes [19]. Of these, clone RGPM 301 (GenBank accession number: AY117410) shared 83% similarity to rice R-proteins. This clone contained only a partial sequence of the gene.

The present study was carried out to isolate and clone the full-length gene containing *RGA RGPM 301*. The expression pattern of this gene in pearl millet during downy mildew

infection and during elicitation of defense reaction by abiotic and biotic elicitors was also investigated.

Materials and methods

Plant material

Pearl millet seeds of cultivars IP18296 and 7042S were obtained from International Crop Research Institute for the Semi-Arid Tropics, Hyderabad, India. The cultivar IP18296 is highly resistant with 0% downy mildew disease incidence (DMDI) and was used for the isolation of resistance gene candidate. The cultivars 7042S (highly susceptible) with >25% DMDI upon inoculation with *S. graminicola* under field conditions was used along with the IP18296 cultivar for the transcript analysis.

Total RNA extraction and cDNA synthesis

Total RNA of the harvested seedlings was isolated with Spectrum Plant Total RNA Kit (Sigma-Aldrich, Bangalore) according to the manufacturer's instructions and quantified by NanoDrop 2000 spectrophotometer (Thermo Scientific, Bangalore). Isolated RNA was treated with RNase free - DNase I (Thermo Scientific, Bangalore) to remove all the genomic DNA contamination. Total RNA (2 µg) was used in reverse transcription reaction using RevertAid H Minus M-MuLV Reverse Transcriptase (Thermo Scientific, Bangalore) with 18-mer oligo dT primer in 25µL reaction volume for 60min at 42 °C.

Cloning of full-length *RGA RGPM 301* cDNA

The available partial cDNA of RGPM 301 (GenBank accession number: AY117410) was used for designing the specific primers (Table 1) to carry out 3′ and 5′ rapid amplification of cDNA ends (RACE). The SMART RACE cDNA amplification kit (Clontech, Takara) was used to synthesize the first strand cDNA from two-day-old pearl millet total RNA.

For 3′ RACE, total RNA was transcribed with 1 µl of 12 µM 3′ cDNA synthesizing primer (CDSP) 1 for 90 min at 42 °C and then for 5 min at 72 °C. This was followed by PCR with 1µl of 10µM 3RACE1 and 3RACE2 nested primers along with 2.5 µl of 5′ cDNA and 5 µl 10X universal primer mix (UPM). PCR conditions were: denaturation at 94 ^{o}C for 30 sec, annealing at 58 ^{o}C for 30 sec and polymerization at 72 ^{o}C for 3 min for 30 cycles with

PowerScript Reverse transcriptase enzyme. The RACE products were cloned using pTZ57R/T vector and sequenced.

Table 1 Primers used in the study

Primer name	Sequence (5′- 3′)	Description
Isolation of RGA RGPM 301		
CDSP 1	AAGCAGTGGTATCAACGCAGAGTAC (T) 30 V N	3′ RACE cDNA synthesizing primer
CDSP 2	$(T)_{25}$ V N	5′ RACE cDNA synthesizing primer
3RACE1	ATGACCAAGACCGAACTTCTTCCCCATC	Forward primer for 3′ RACE, outer
3RACE2	CGAGAGATGCTTGAATGCCCATATCCTC	Forward primer for 3′ RACE, nested
5RACE1	GACGAGGATATGGGCATTCAAGCATCTC	Reverse primer for 5′ RACE, outer
5RACE2	GTTGATCGGAGAAGATCGATCCAGACAT	Reverse primer for 5′ RACE, nested
PMRGPM-F1	GTTGGATAGCCTGCCTGTTA	Forward primer for the amplification of gap region
PMRGPM-R1	GCTTGTGAGCACCTTCAATG	Reverse primer for the amplification of gap region
PMRGPM-F2	AAGTCATCGGCACTCTGCAG	Gene specific primer for ORF, Forward
PMRGPM-R2	TGTCAGGGATAGGGACCAAG	Gene specific primer for ORF, Reverse
Relative quantification by qRT-PCR		
RGPM-RT-F	CAATTCGAGCTTTGGGAAATAAG	Forward primer for *RGA RGPM 301*
RGPM-RT-R	GTAGTGAGTATCGGCACGGAACT	Reverse primer for *RGA RGPM 301*
GAPDH-RT-F	GCCCTCCAGAGTGAGGATGTC	Forward primer for *GAPDH*
GAPDH-RT-R	GGTCATGTATTCGGTGGTGATG	Reverse primer for *GAPDH*

Where (N = A, C, G, or T; V = A, G, or C)

5′ RACE was performed by transcribing the total RNA with 1 µl of 12 µM 5′ CDSP 2 for 90 min at 42 °C and then for 5 min at 72 °C. PCR was carried out with 1µl of 10µM 5RACE1 and 5RACE2 nested primers along with 2.5 µl of 5′ cDNA and 5 µl 10X UPM. PCR cycling conditions were: denaturation at 94 ^{o}C for 30 sec, annealing at 58 ^{o}C for 30 sec and polymerization at 72 ^{o}C for 3 min for 30 cycles with PowerScript Reverse transcriptase enzyme. The RACE-PCR products were cloned using pTZ57R/T vector and sequenced.

Sequence processing, alignment and phylogenetic analysis

The 3' and 5' RACE sequences were assembled using Clone Manager Professional 9 (Sci-Ed software) which was non-overlapping indicating the presence of a gap region between the two. Primers were designed for the gap region using the sequences of 3′ and 5′ ends of RACE product (Table 1). The resulting PCR product was used to obtain the full length sequence. Analysis of open reading frame (ORF) was conducted using ORF finder (http: //www.ncbi.nlm.nih.gov/gorf/). The primers PMRGPM-F2 and PMRGPM-R2 (Table 1) were used to amplify ORF and confirmed by sequencing.

The conserved motifs of the *RGA RGPM 301* protein sequence were analyzed at NCBI and the domains were predicted. The multiple alignment was performed using DNAMAN version 8 with protein sequences of *Oryza sativa* Japonica Group *Pit* (AIR72965.1), *Brachypodium distachyon RGA3* (XP_003568667.1) and *Oryza brachyantha RGA4*-like (XP_006658520.1).

The evolutionary relationships of *RGA RGPM 301* protein sequence and known R-protein sequences from other plant species obtained from NCBI were examined by phylogenetic analysis using maximum-likelihood approach at http://www.phylogeny.fr/ [20].

Pathogen and inoculum preparation

The downy mildew pathogen *S. graminicola* isolated from susceptible pearl millet cultivar HB3 and maintained on the same cultivar under greenhouse conditions of temperature 22 ± 2 °C and relative humidity (RH) of 80% was used for all inoculation experiments. The leaves showing profuse sporulation of *S. graminicola* on the abaxial side were collected in the evening and were washed under running tap water. They were blotted dry with blotter paper and were cut to pieces of about 4 inches in length and placed in a moist chamber at 20 °C and >95% RH for sporulation. The fresh zoospores released the next morning were harvested in sterile distilled water and used as a source of inoculum.

Inoculation of seedlings with *S. graminicola* zoospores

The seeds of cultivars IP18296 and 7042S were surface sterilized in 0.1% (w/v) sodium hypochlorite solution for 15 min, followed by washing with sterile distilled water. They were germinated under aseptic conditions on moist filter papers at 25 ± 2 °C in the dark for two days. The two-day-old seedlings were root-dip inoculated with $4x10^4$ *S. graminicola* zoospores mL^{-1} [21]. Seedlings were harvested at different time intervals of 0, 3, 6, 9, 12, 24 and 48 hours post inoculation (h.p.i.) and the uninoculated, water-treated seedlings served as control. The harvested samples were quickly frozen in liquid nitrogen and stored immediately at -80 °C for subsequent analysis.

Treatment of seedlings with elicitors and inhibitors

Different sets of treatments were carried out with the two-day-old seedlings of pearl millet cultivars IP18296 and 7042S. In the first set, the seedlings were treated with 50 mM β-aminobutyric acid [BABA] (Sigma-Aldrich, Bangalore) by vacuum infiltration. The treated

seedlings were harvested after 0, 3, 6, 9, 12, 24 and 48 hours post treatment (h.p.t.) [22] along with water-treated seedlings which were maintained as control in parallel.

The second set included the treatment with the biotic inducer *P. fluorescens* isolate UOM SAR14 obtained from the culture collection of the Department of Biotechnology, University of Mysore, India. A single bacterial colony was inoculated into fresh 250 mL King's B broth followed by incubation for 24 h at 28 °C. The bacterial cell suspension obtained was centrifuged at 6000 rpm for 5 min and the cells were resuspended in sterile water to get a final density of 10^8 cfu mL^{-1} at A_{260}. The bacterial cell suspension was inoculated to the seedlings and harvested at 0, 3, 6, 9, 12, 24 and 48 h.p.t.

The third set of seedlings were treated with 0.1 mM jasmonic acid (JA) or 0.5 mM salicylic acid (SA) and harvested at 0, 6, 12, 24 and 48 h.p.t. Another set of seedlings were treated with MPK cascade inhibitors of 100 µM PD98059 [2-(2-amino-3-methyoxyphenyl)-4*H*-1-benzopyran-4-one] (Invitrogen, Bangalore) or 10 µM U0126 [1, 4-diamino-2, 3-dicyano-1, 4-bis (*o*-aminophenylmercapto) butadiene] (Invitrogen, Bangalore) for 6 h. The inhibitor pre-treated seedlings were washed with distilled water and subsequently treated with 0.5 mM SA. Seedlings were harvested at time intervals of 0, 6, 12, 24 and 48 h.p.t. The untreated seedlings were maintained in parallel as control. All the experimental samples were harvested thrice such that three independent biological replicates were maintained.

RGA RGPM 301 specific expression analysis through quantitative real-time PCR (qRT-PCR)

The expression pattern of *RGA RGPM 301* in pearl millet during downy mildew infection was analysed by qRT-PCR. The accumulation of *RGA RGPM 301* transcripts during downy mildew infection, treatment with abiotic (BABA) and biotic (*P. fluorescens*) elicitors, phytohormones (JA and SA) and MPK inhibitors (PD98059 and U0126) was studied. *RGA RGPM 301* specific primers were designed using Primer Express Software v3.0 (Applied Biosystems, Bangalore) (Table 1) and these primers were used for the amplification and quantification of the *RGA RGPM 301* by qRT-PCR. GAPDH (GQ398107) was used as the internal reference for measuring the gene expression. The products were electrophoresed in agarose gel for the confirmation of the primer specificity. qRT-PCR was performed with StepOnePlus™ Real-Time PCR Systems (Applied Biosystems, Bangalore). Each reaction was set in a total volume of 20 µl containing 10 µl of 1x Power SYBR Green PCR master mix reagent (Applied Biosystems, Bangalore), 1 µl of 20 ng each of cDNA and 3 µl of 3

pmol forward and reverse primers. qRT-PCR cycling conditions were: 95 ^{o}C for 10 min (initial denaturation) followed by 40 cycles of denaturation at 95 ^{o}C for 15 s, annealing and extension for 60 s at 60 ^{o}C. A melting curve was created at the end of each reaction, using a cycle consisting of 15 s denaturation at 95 ^{o}C and a 60 s annealing at 60 ^{o}C followed by a slow temperature increase to 95 ^{o}C at the rate of 0.3 ^{o}C s^{-1}. The comparative CT ($2^{-\Delta\Delta C}{}_{T}$) method [23] was used for calculating the relative gene expression.

Statistical analysis

All experiments were carried out with three replicates. Each of the experimental datasets considered for qRT-PCR were analysed and the transformed values were subjected to Arcsine transformation and analysis of variance (ANOVA). These experimental results were further subjected to Duncan's multiple range test (DMRT) ($P < 0.05$) [SPSS tool (version 8)].

Results

Cloning of *RGA RGPM 301* encoding a NBS-LRR in pearl millet

The partial cDNA RGPM 301 of 434 bp (GenBank accession number: AY117410) was obtained from the pearl millet cultivar IP18296. RACE-PCR amplification of 3′- and 5′- ends generated fragments of 781 bp and 2103 bp respectively. The alignment of the two sequences was non-overlapping indicating the presence of a gap region between the two which was amplified with the specific primers (Table 1) to obtain an amplicon of 668 bp. The full-length *RGA RGPM 301* sequence of 3552 bp deduced after sequence assembly comprised an ORF of 2979 nucleotides (GenBank accession number: KP226586) encoding a protein of 992 amino acids (Fig. 1) with an estimated molecular mass of 113.96 kDa and a predicted pI of 8.71 (ExPaSy pI/Mw tool). The initiation codon ATG is located at the position 106 bp and the stop codon TAG at position 3282 bp (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). A 5′-untranslated region (UTR) of 105 bp was present upstream to the start codon and the 3′- UTR of 468 bp was identified downstream of the stop codon. The ORF region was amplified with the gene specific primers (Table 1), cloned in pTZ57R/T vector and sequenced for reconfirmation.

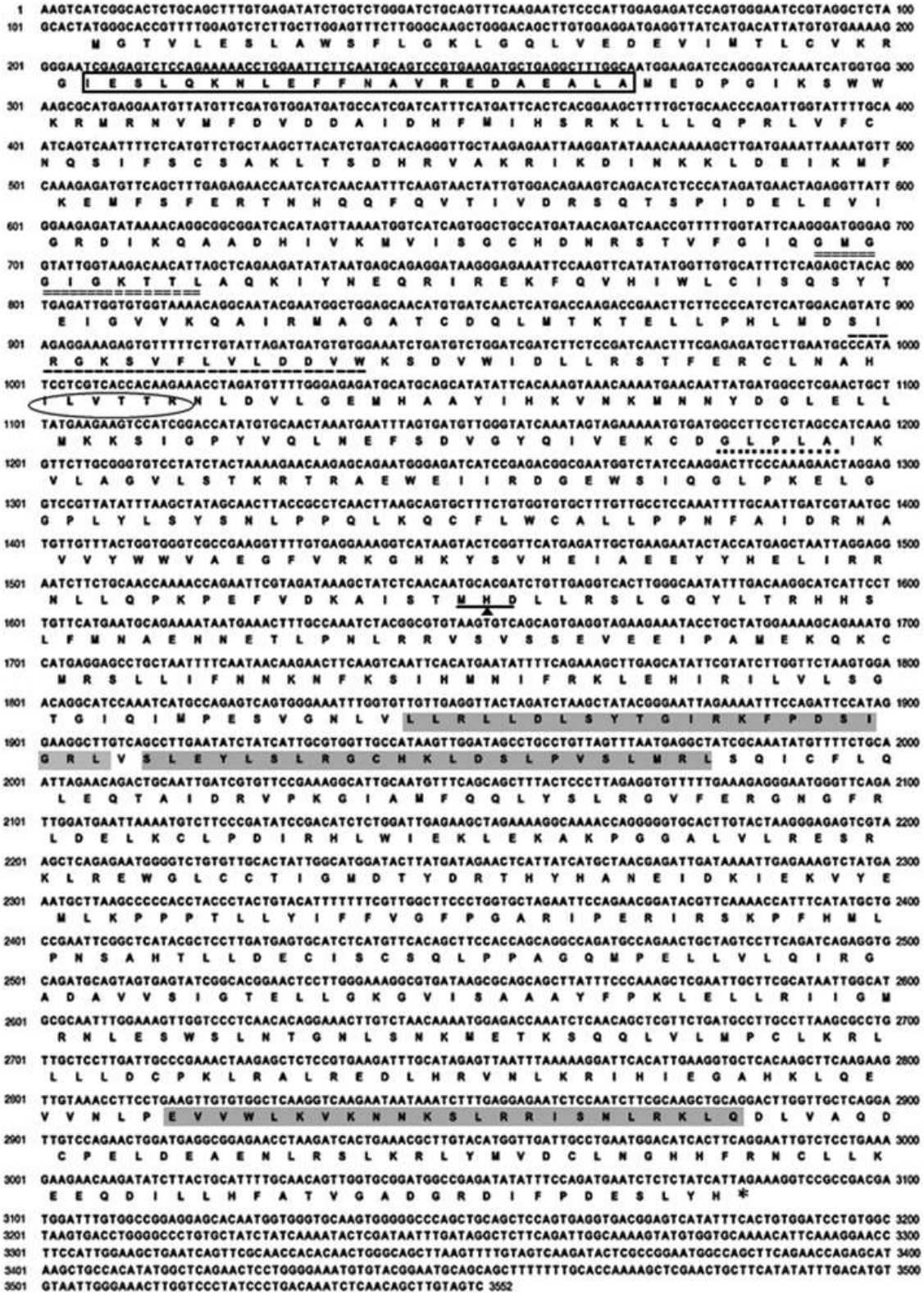

Fig. 1. Nucleotide sequence of the *RGA RGPM 301* and the deduced amino acid sequence. The cDNA sequence is shown in the top lines with its predicted ORF by the single-letter amino acid codes below. The CC motif is shown in the *box* followed by the P-loop with *double underline*. *Single underline* indicates the kinase-2 motif, kinase-3A within the *ellipse*, and GLPL with a *dotted line*. The MHD motif is *underlined* and is marked with a *arrow head*. The leucine rich repeats are represented by the *gray boxes*

Multiple alignment and phylogenetic relationship of *RGA RGPM 301*

The analysis of deduced amino acid sequence of *RGA RGPM 301* revealed the presence of highly conserved N-terminal CC domain (34-55 amino acid residues), the NBS (195-484 amino acid residues) and three LRR motifs at the C-terminal which constitutes the signalling motifs in majority of the plant disease resistance proteins. Within the NBS domain kinase 1a (P-loop), kinase 2, kinase 3a, GLPL and MHD motifs were identified and depicted in Fig. 1.

Multiple alignment of the *RGA RGPM 301* protein sequence with RGA proteins of *Oryza sativa* Japonica Group *Pit* (AIR72965.1), *Brachypodium distachyon RGA3* (XP_003568667.1) and *Oryza brachyantha RGA4*-like (XP_006658520.1) performed using DNAMAN version 8.0 indicated the consensus sequences positioned appropriately (Fig. 2).

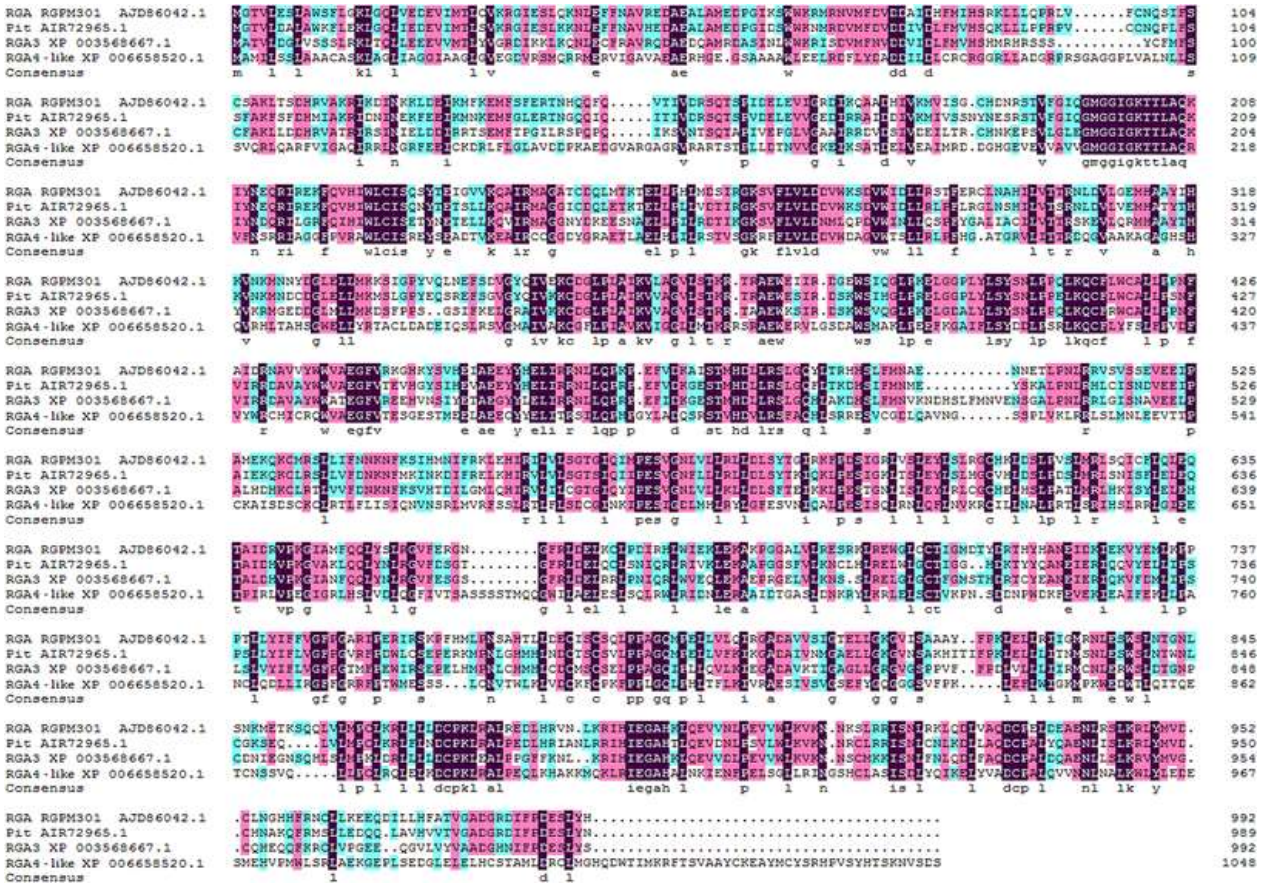

Fig. 2. Alignment of the deduced amino acid sequence of RGA RGPM 301 (AJD86042.1) with other plant resistance genes generated using DNAMAN version 8.0. The protein sequences used in the analysis are as follows: *Oryza sativa* Japonica Group Pit (AIR72965.1), *Brachypodium distachyon* RGA3 (XP_003568667.1) and *Oryza brachyantha* RGA4-like (XP_006658520.1). Completely conserved residues are indicated below the alignment as consensus sequence and the *dots* have been introduced to optimize the alignment

The NCBI BLASTp search of the *RGA RGPM 301* protein showed significant similarities of 73% with *Oryza sativa* Japonica *NBSt1* (BAH20866.1), *Pit (*AIR72965.1) and

NBS-LRR disease resistance protein (BAD61137.1). *Oryza brachyantha RPP13-like protein 1-like isoform X1* (XP_006643756.1) showed 71% similarity. 67% similarity was observed with *Aegilops tauschii RGA3* (EMT03703.1) and *RGA4* (EMT10171.1). *Brachypodium distachyon RGA3* (XP_003568667.1) shared 60% similarity and *Hordeum vulgare rga s-reg19* (CAD45035.1) exhibited 71% similarity.

The phylogenetic analysis to elucidate the relationship of *RGA RGPM 301* based on the maximum likelihood approach was conducted using the R-proteins retrieved by BLASTp analysis and further compared with the two known R-proteins of *Nicotiana glutinosa N* (AAA50763) and *Linum usitatissimum M* (U73916). The tree generated, consisted of two major branches-TIR and non-TIR-NBS-LRR disease resistance proteins. The *RGA RGPM*

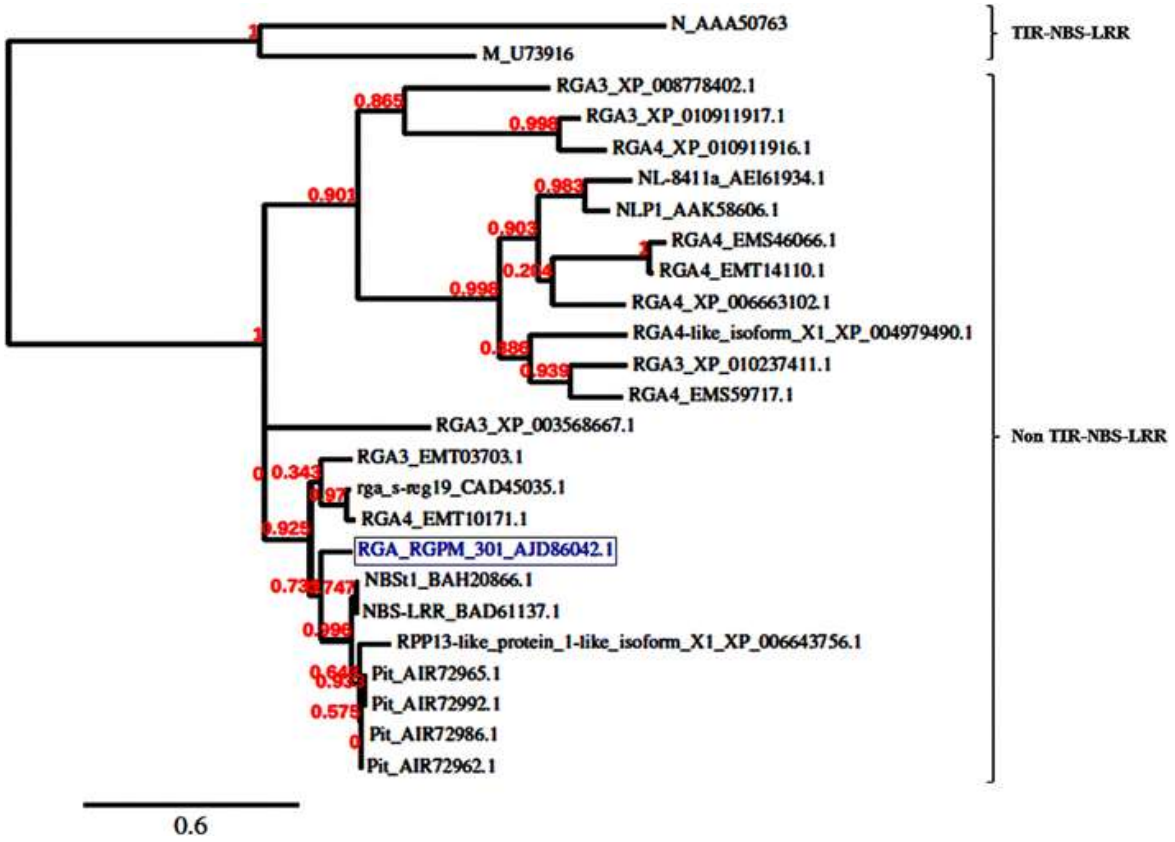

Fig. 3. Phylogenetic relationship of RGA RGPM 301 (AJD86042.1) with protein sequence of other *R*-genes and proteins of two *R*-gene representatives of TIR-NBS-LRRs. The tree was constructed using the maximum-likelihood method provided in the platform at www.phylogeny.fr. Protein sequences used were: *Oryza sativa* Japonica NBSt1 (BAH20866.1), Pit (AIR72965.1) and NBS-LRR disease resistance protein (BAD61137.1); *Oryza sativa* NL-8411a (AEI61934.1) and NLP1 (AAK58606.1); *Oryza brachyantha* RPP13-like protein 1-like isoform X1 (XP_006643756.1) and RGA4 (XP_006663102.1); *Oryza sativa* Indica Pit (AIR72962.1); *Oryza nivara* Pit (AIR72986.1); *Oryza rufipogon* Pit (AIR72992.1); *Triticum urartu* RGA4 (EMS46066.1) and RGA4 (EMS59717.1); *Aegilops tauschii* RGA3 (EMT03703.1), RGA4 (EMT10171.1) and RGA4 (EMT14110.1); *Brachypodium distachyon* RGA3 (XP_003568667.1) and RGA3 (XP_010237411.1); *Hordeum vulgare* rga s-reg19 (CAD45035.1); *Setaria italica* RGA4-like isoform X1 (XP_004979490.1); *Elaeis guineensis* RGA3 (XP_010911917.1) and RGA4 (XP_010911916.1); *Phoenix dactylifera* RGA3 (XP_008778402.1); *Nicotiana glutinosa* N (AAA50763) and *Linum usitatissimum* M (U73916). TIR and non-TIR-NBS-LRR classes are indicated in the figure with the RGA RGPM 301 marked with a *box*

301 clearly grouped with the non-TIR-NBS-LRR disease resistance proteins, while the *N* and *M* genes grouped separately as TIR-NBS-LRR (Fig 3). This was confirmed by inspecting the sequence of kinase 2 motif for the presence of tryptophan (W) at the C-terminus [11].

RGA RGPM 301 expression is induced in response to downy mildew pathogen in resistant cultivar IP18296

The relative expression pattern of *RGA RGPM 301* in cultivars IP18296 and 7042S in response to *S. graminicola* was determined by qRT-PCR. A significant higher transcript at basal level was observed in IP18296 compared to 7042S control. An increase in the accumulation of *RGA RGPM 301* transcripts induced by the pathogen was observed only in IP18296 cultivar. No relative changes were seen in the 7042S samples upon inoculation with *S. graminicola*. The expression profile revealed an increase in mRNA accumulation in IP18296 which was nearly two-fold at 3 h.p.i. and peaked at 6 h.p.i. with more than two and half-fold. The levels at later time points declined gradually and remained higher than the IP18296 control (Fig. 4). A remarkable difference in expression levels of *RGA RGPM 301*

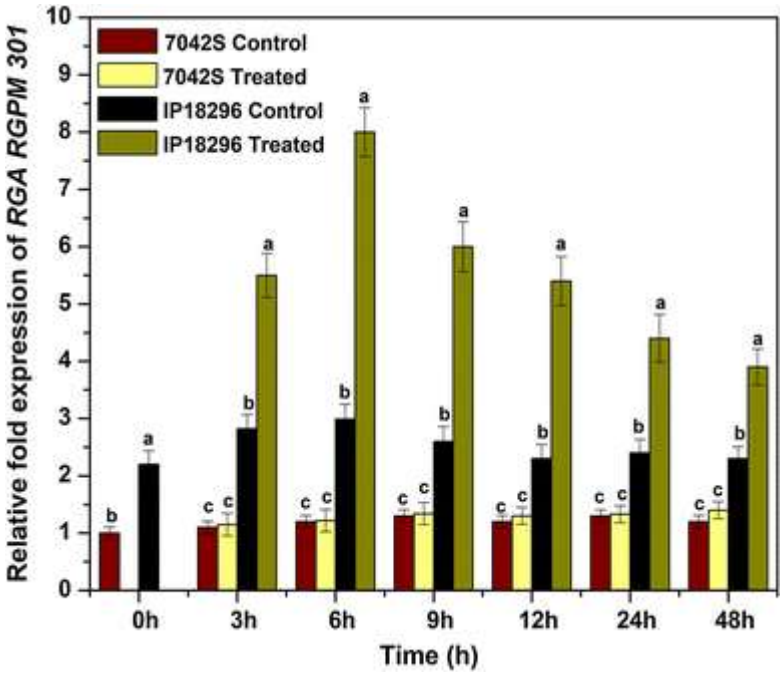

Fig. 4. Relative expression levels of *RGA RGPM 301* transcript in 7042S and IP18296 cultivars of pearl millet seedlings in response to *S. graminicola* inoculation. The *RGA RGPM 301* expression levels were measured by qRT-PCR and were normalized to the expression of constitutive *GAPDH* gene. Where control (0 h) was taken as onefold relative expression. The values were calculated as means of three replicates of the experiment with *error bars* representing the standard error. Means designated with the *same letter* are not significantly different according to DMRT at $P < 0.05$

between the IP18296 (highly resistant) and 7042S (highly susceptible) pearl millet cultivars in response to the downy mildew pathogen indicates a role of this RGA in pearl millet downy mildew interaction.

Abiotic and biotic elicitors induce accumulation of *RGA RGPM 301* mRNA in the susceptible 7042S cultivar

The expression pattern obtained previously implies a relatively low-level expression of *RGA RGPM 301* in 7042S compared to IP18296 cultivar. In this regard, 7042S seedlings were treated with BABA and *P. fluorescens* UOM SAR14 to evaluate their effectiveness in up-regulating the *RGA RGPM 301* expression. A significant increase in the level of expression was observed in the susceptible 7042S cultivar at different time points upon treatment with the elicitors. Particularly, the two-day-old 7042S seedlings treated with BABA as well as *P. fluorescens* UOM SAR14 exhibited temporal changes in the level of expression of eight-fold and seven-fold over their respective untreated 7042S control at 3 h.p.t and decreased gradually at the later time points. The IP18296 seedlings treated with BABA or *P. fluorescens* exhibited a higher expression level of *RGA RGPM 301* (Fig. 5). These significant changes infer the effective role of BABA and *P. fluorescens* UOM SAR14 in enhancing the *RGA RGPM 301* in the 7042S cultivar thereby inducing resistance.

***RGA RGPM 301* involvement in JA- and SA- mediated defense response**

The induction of *RGA RGPM 301* transcripts by the defense signalling molecules JA and SA was examined by qRT-PCR. Following the treatment of two-day-old IP18296 and 7042S seedlings with JA or SA, the JA as well as the SA treated seedlings exhibited an increase in the transcript level in both the cultivars over their control samples at all period of time (Fig. 6). Significant elevation of approximately three-fold and four-fold in the transcript levels of *RGA RGPM 301* was depicted in the 7042S seedlings upon treatment with JA or SA respectively at all the considered time points. These expression levels were comparatively higher than the susceptible control and slightly lesser than elicitor treated IP18296 seedlings. Interestingly, more than four-fold increase in the expression of *RGA RGPM 301* was observed in the IP18296 seedlings treated with the same elicitors. In comparison, SA was found to be a better inducer than JA in inducing *RGA RGPM 301* expression. Thus implying *RGA RGPM 301* involvement in JA and SA mediated signaling in defense mechanism.

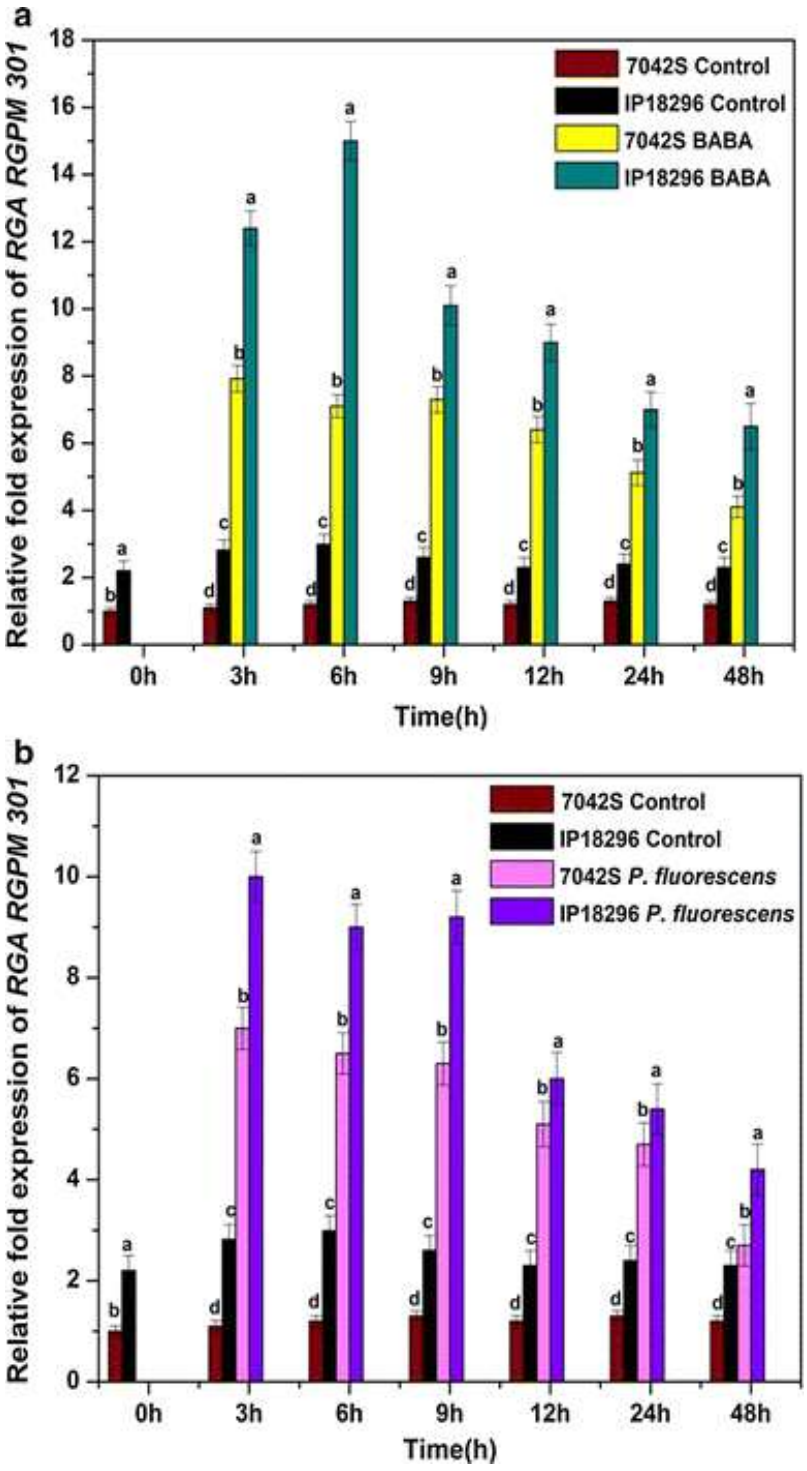

Fig. 5. Relative expression levels of *RGA RGPM 301* transcript in 7042S and IP18296 cultivars of pearl millet seedlings in response to BABA (**a**) and *P. fluorescens* UOM SAR14 (**b**). The *RGA RGPM 301* expression levels were measured by qRT-PCR and were normalized to the expression of constitutive *GAPDH* gene. Where control (0 h) was taken as onefold relative expression. The values were calculated as means of three replicates of the experiment with *error bars* representing the standard error. Means designated with the *same letter* are not significantly different according to DMRT at $P < 0.05$

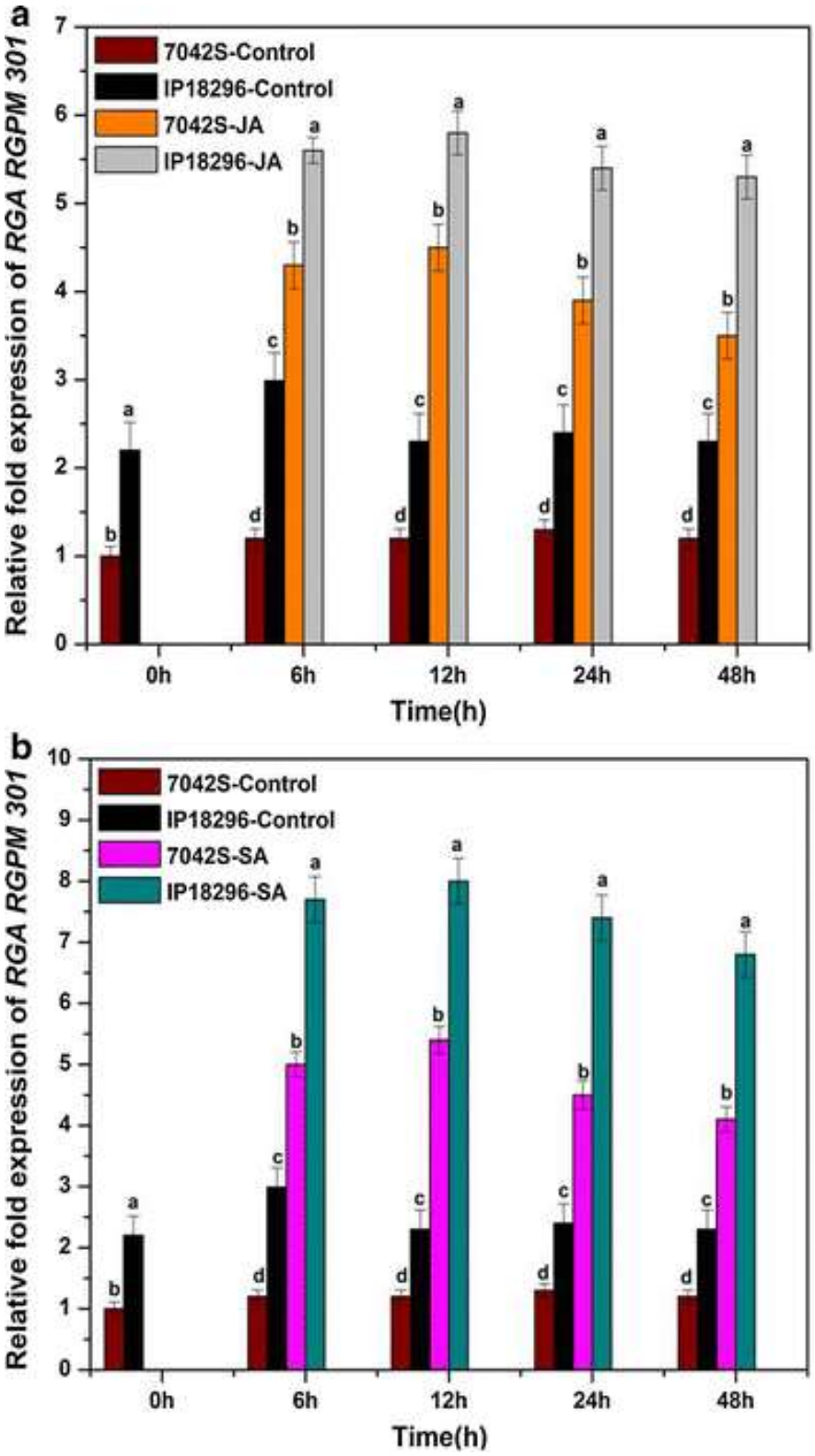

Fig. 6. Relative expression levels of *RGA RGPM 301* transcript in 7042S and IP18296 cultivars of pearl millet seedlings in response to treatment with phytohormones-JA (**a**) and SA (**b**). The *RGA RGPM 301* expression levels were measured by qRT-PCR and were normalized to the expression of constitutive *GAPDH* gene. Where control (0 h) was taken as onefold relative expression. The values were calculated as means of three replicates of the experiment with *error bars* representing the standard error. Means designated with the *same letter* are not significantly different according to DMRT at $P < 0.05$

MPK inhibitors regulate the expression of *RGA RGPM 301*

Earlier studies from our department had identified that MPK cascade plays an important role in defense signalling in pearl millet against downy mildew [22]. To assess the role of MPK signalling in the expression of *RGA RGPM 301*, MPK cascade inhibitors were used in the present study. From the previous expression pattern, SA treatment was found to induce a better transcript level in the IP18296 cultivar than JA. Hence, transcript accumulation levels of *RGA RGPM 301* in IP18296 pearl millet cultivar was assessed with the treatment of MPK cascade inhibitors PD98059 or U0126 prior to with and without SA treatment by qRT-PCR. The level of expression was recorded maximum at 12 h.p.t. with more than three-fold higher accumulation of *RGA RGPM 301* in the samples treated with SA. This level was found to remain higher throughout, in comparison to the cultivar treated with MPK cascade inhibitors and SA (Fig. 7). The temporal accumulation of *RGA RGPM 301* in the inhibitor-treated IP18296 seedlings showed that MPK cascade inhibitors [PD98059 (100 μM) or U0126 (10 μM)] effectively blocked their activation mediated by SA. These results implicate the MPK pathway in the expression of *RGA RGPM 301* in pearl millet.

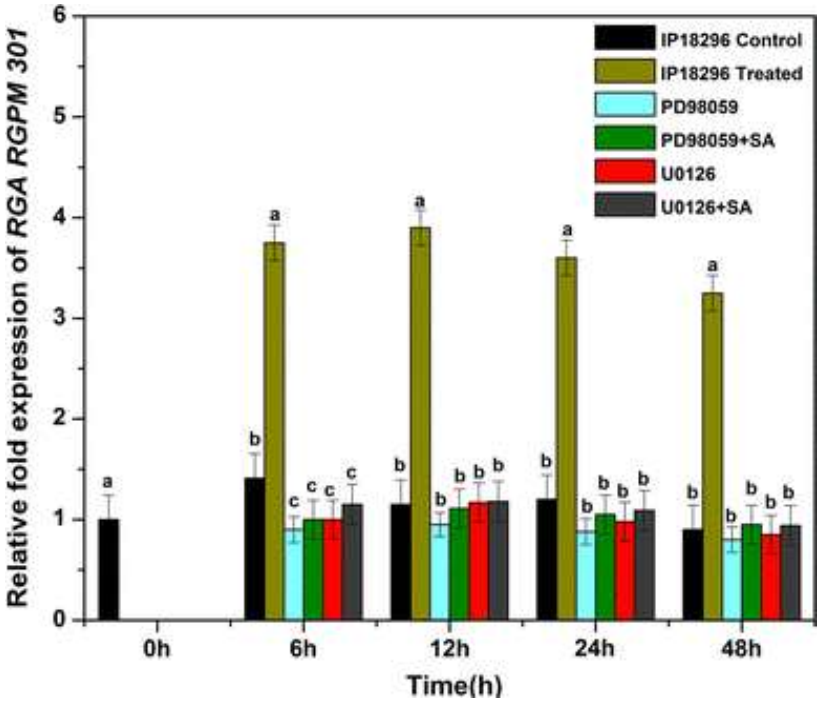

Fig. 7. Relative expression levels of *RGA RGPM 301* transcript in IP18296 cultivar of pearl millet seedlings in response to treatment with SA and MPK cascade inhibitors (PD98059 and U0126). The *RGA RGPM 301* expression levels were measured by qRT-PCR and were normalized to the expression of constitutive *GAPDH* gene. Where control (0 h) was taken as onefold relative expression. The values were calculated as means of three replicates of the experiment with *error bars* representing the standard error. Means designated with the *same letter* are not significantly different according to DMRT at $P < 0.05$

Discussion

Plant defense in curbing the invading pathogens is regulated by the *R*-genes and their products. These are necessary and are crucial in the prevention of diseases occurring in plants. In our study, a full-length *R*-gene designated *RGA RGPM 301* of pearl millet was isolated and cloned based on the PCR approach with degenerate oligonucleotide primers. The obtained sequence was analysed for homology with the known *R*-genes in the NCBI database. Sharma et al. [24] have grouped the *R*-genes encoding the cytoplasmic proteins with CC-NBS-LRR domains under class two of the eight classes of *R*-genes. Similarly, the CC-NB-LRR R-proteins in *A. thaliana* also has characteristics of class two *R*-genes [25]. The cloned *RGA RGPM 301* is found to possess the above tripartite characteristic features and probably belongs to the class two. A similar CC-NB-LRR proteins like TmMla1 of wheat showing resistance against *Blumeria graminis* f.sp. *hordei* and a gene cluster encoding CC-NB-LRR conferring resistance to *Setosphaeria turcica* in sorghum have been identified [26, 27]. These full-length R-proteins act as sensor against the pathogen effectors and elicit the activation of signals in the plant.

The sequence alignment of the deduced amino acids of *RGA RGPM 301* contained the consensus sequence of kinase 1a, kinase 2, kinase 3a, GLPL and MHD motifs characteristic of most of the plant *R*-genes [28]. These domains and motifs which are highly conserved, assists in the prediction of *R*-genes to either TIR or non-TIR subclasses [11]. The BLASTp results revealed 73% identity with *Oryza sativa* Japonica *NBSt1* (BAH20866.1) and *Pit* (AIR72965.1) which are CC-NB-LRR protein of non-TIR subclass. The *Pit* gene has been demonstrated to confer resistance in rice to blast caused by *Magnaporthe oryzae* in a gene for gene manner [29]. In Northern China the rice germplasm accession K59 with *Pit* was found to be an important line in developing resistance against blast disease [30, 31]. The phylogenetic analysis grouped the *RGA RGPM 301* to non-TIR-NBS-LRR subclass based on the distinctive feature of presence of tryptophan (W) residue at the end of the kinase-2 motif which is in line with the findings in sugarcane *SNLR* disease resistance [32]. Whereas, presence of aspartic acid (D) renders the R-proteins to TIR-NBS-LRR subclass [10].

In the absence of pathogen infection or in the unchallenged state the NBS-LRR *R*-genes are expressed at a relatively low level. Upon pathogen perception the *R*-genes are induced with accumulation of higher transcript levels [33]. Our studies on the quantification

of *RGA RGPM 301* by qRT-PCR exhibited low level expression of transcript in the pearl millet highly resistant (IP18296) and highly susceptible (7042S) cultivars without pathogen challenge. The *CzR1* of *Curcuma zedoaria* also revealed low transcript level prior to *Pythium aphanidermatum* infection which is in concurrence with our results [34]. Further, inoculation with downy mildew pathogen *S. graminicola* significantly up-regulated the transcript levels of *RGA RGPM 301* in the resistant pearl millet cultivar. Conversely, the susceptible cultivar showed no changes in the expression levels upon pathogen inoculation. This suggests the involvement of *RGA RGPM 301* in recognition of the downy mildew pathogen invasion and induction of downstream host defense response. The sunflower *RGC260* showed a similar pattern of expression upon infection with *Puccinia helianthi* [35] and *Phytophthora capsici* infection enhanced the expression level of *CaRGA2* in pepper [36].

Plants are challenged by a multitude of abiotic and biotic stress factors in the environment. These challenges are perceived by the plant and they in turn respond to these stress related factors in an efficient manner involving a highly complex and adaptive mechanism [37]. Priming plants with compounds inducing resistance activates a robust and stronger induction leading to enhanced defense against further stress conditions [38]. The particular abiotic and biotic elicitors BABA and *P. fluorescens* are found to initiate cellular defense responses in plants. Application of BABA, a non-protein amino acid has revealed induction of resistance and accumulation of a few defense-related proteins in pearl millet as reported by Shailasree et al. [39]. Priming of pearl millet seedlings with *P. fluorescens* has shown to induce and enhance systemic resistance against *S. graminicola* [40]. Hence, in this study, both the elicitors BABA and *P. fluorescens* UOM SAR14 were used to analyse the expression level of *RGA RGPM 301* in the pearl millet cultivars. These treatments were found to be potent inducers of resistance in the susceptible pearl millet cultivar 7042S. They significantly enhanced the *RGA RGPM 301* levels at an early point of time against the downy mildew pathogen *S. graminicola*.

The signalling molecules as well as phytohormones play a critical role in up-regulation of *R*-genes in plants. Pathogen induction triggers the signalling cascades in the host plant effectively. The propagation of the signal and regulation of defense response is dependent on the phytohormones produced by the plant. Upon perception of the invading pathogen the phytohormones such as SA and JA regulates the network of defense response [41]. In contrast to the studies carried out by Thomma et al. [42] the SA treatment has revealed the establishment of resistance towards the biotrophic pathogens. Whereas, the

exogenous treatment of JA developed resistance against the necrotrophic pathogens such as *Alternaria brassicicola* other than the biotroph *Peronospora parasitica*. Studies have demonstrated that exogenous application of these phytohormones result in the induction of PR genes and in the enhancement of disease resistance against a wide range of plant pathogens. For example, exogenous application of SA induced the expression of a NBS-LRR gene *KR4* in soybean [43]. Similarly, *AsRGA29* of garlic revealed enhanced transcript accumulation in response to treatment with SA and methyl jasmonate [44]. The treatment of two-day old pearl millet seedlings with JA or SA for a specific period of time indicated a marked response to SA treatment than JA in both the cultivars. Increased expression levels of *RGA RGPM 301* were found to be induced with SA treatment in comparison to JA in the susceptible cultivar 7042S and a still higher levels of response in the IP18296 resistant cultivar. Hence, our studies show that the exogenous application of inducers JA or SA are responsible for the activation of signalling pathway which leads to *RGA RGPM 301* mediated defense response in pearl millet against downy mildew.

The plant MPK cascade plays a role in cell signalling and is also involved in defense mechanism. The use of MPK cascade inhibitors PD98059 and U0126 is helpful in understanding the signalling mechanisms involved in host-pathogen interaction. These inhibitors were found to suppress the activity of PgMPK1 and PgMPK2 with reduced POX, PAL, β-1,3-glucanase and SOD enzyme activities in pearl millet IP18296 cultivar [21]. Also, MPK cascade inhibitors PD98059 and U0126 were exploited in realizing their role in the expression of PgMPK4 involved in JA- and SA-induced activation of *LOX*, *CAT3* and *PGIP* genes in pearl millet cultivars [45]. In maize, the PD98059 and U0126 MPK inhibitors were used to study the abscisic acid-induced expression of antioxidant genes *CAT3, cAPX and GR1* mediated by MPK [46]. In the present study, the IP18296 resistant cultivar of pearl millet upon PD98059 or U0126 treatment showed inhibition of MPK activity and suppression of *RGA RGPM 301* levels even after SA treatment. These levels were lower at all time points when compared to the IP18296 cultivar treated with only SA and also in comparison with the control sample. Hence, MPK signalling activity may be necessary for the up-regulation of the *RGA RGPM 301* in pearl millet.

In conclusion, we have isolated a 3.5 kb CC-NBS-LRR class *R*-gene *RGA RGPM 301* from pearl millet which is a non-TIR NBS-LRR protein that may belong to class two of the eight classes. The expression profiles in response to various signalling molecules indicate a role for *RGA RGPM 301* in defense response against the downy mildew pathogen

S. graminicola. These studies indicate that, further cloning and expression of *R*-genes would provide an opportunity for better understanding of its function in pearl millet.

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Author contributions

MV performed the experiments and wrote the manuscript. KRK supervised the research design and reframed the manuscript. PM and SAP along with MV performed the real time experiments, discussed the results and commented on the manuscript. SS conceived and designed the study. HSS provided the inoculum maintained in the pearl millet sick plot.

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