J. Hortl. Sci.
Vol. 15(1) : 81-92, 2020

Original Research Paper

Identification of NBS-LRR Resistance Gene Analogues (RGA) from Rose (IIHRR13-4) Resistant to Powdery Mildew (*Podosphaera pannosa* (Wallr.:Fr.) de Bary)

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ABSTRACT

Resistance is the best strategy to manage powdery mildew (*Podosphaera pannosa* (Wallr.:Fr.) de Bary) of rose. Identification of resistant genes (R genes) from plant species will help in breeding programs. Nucleotide Binding Site - Leucine Rich Repeats (NBS-LRR) is a major class of R gene family in plants. This study reports the identification and molecular characterization of resistance gene analogues from roses maintained at ICAR-Indian Institute of Horticultural Research (IIHR). The powdery mildew resistant line IIHRR13-4 was compared with the susceptible commercial cultivar, konfetti. PCR based approaches with degenerative primers based on different conserved motifs of NBS-LRR were employed to isolate resistance gene analogues (RGAs) from rose. Eleven RGAs (IIHRR13-4R1, IIHRR13-4R2, IIHRR13-4R3, IIHRR13-4R4, IIHRR13-4R5, IIHRR13-4R6, IIHRR13-4R7, IIHRR13-4R8 IIHRR13-4R9 and IIHRR13-4R10) were identified from powdery mildew resistant germplasm line, IIHRR13-4, based on the sequence and similarity to RGAs from rosaceae family and other crops. The major similarity to rose RGAs reported are from *Fragaria vesca, Rosa* hybrid cultivar, *Prunus* and *Rosa chinensis.* RGAs isolated from IIHRR13-4 belonged to Toll Interleukin Receptor (TIR)-NBS-LRR and Non-TIR-NBS-LRR RGAs (Lecine Zipper (LZ) type). Different motifs of RGAs identified were P-loop, RNBS A, kinase 2, kinase 3a, RNBS-D and GLPL of NBS domain. This study reports the existence of resistance at genetic level in powdery mildew resistant genotype IIHRR13-4. These RGAs will be useful for mapping and characterization of R genes in IIHRR13-4 and breeding for improved powdery mildew resistance in roses.

Key words: Nucleotide Binding Site-Leucine Rich Repeats (NBS-LRR), *Podosphaera pannosa,* Powdery mildew, Resistance Gene Analogues (RGA) and Rose.

INTRODUCTION

Two major events involved in defense mechanism are recognition of pathogen attack and induction of defense responses from plants against the pathogen.The defense response against particular pathogen is triggered by an interaction between R gene from the host and avirulence gene product of pathogen which restricts the pathogen invasion (Flor, 1971; Holt *et al.,*2000). R genes form a diverse group of related sequences that are widely distributed in plant genome. There are five classes of *R* genes based on their structural characteristics of predicted protein structure and majority of these *R*-genes belong to nucleotide-binding site (NBS) and leucine-rich repeats (LRRs) groups (Ellis and Jones, 1998; Hammond-Kosack and Jones, 1998; Hattendorf and Debener, 2007a; Hattendorf and Debener, 2007b). Putative NBS domains are concerned with signalling and they are characterized by several highly conserved motifs, *viz.* P-loop, Kinase-2 and Gly-Leu-Pro-Leu (GLPL) motifs. Structural domains of LRRs are involved in protein-protein interactions and pathogen recognition (Belkhadir *et al.,* 2004; Ellis *et al.,* 2003; Yung, 2000).

The NBS-LRR domain is classified into two groups based on their N-terminus. First is the amino terminal toll interleukin receptor (TIR)-NBS-LRR and the second is without TIR region known as Non-TIR-NBS-LRR class. TIR-NBS-LRR is characterized by their similarity to toll receptor in *Drosophila* and interleukin 1 receptor of mammals and Non-TIR groups has a leucine zipper (LZ) or coiled coil(CC) motif instead of TIR. Non-TIR group is widely distributed in both monocots and dicots while TIR group is rare in cereals and grasses. So far several R genes have been cloned in different crops(Collier and Moffett, 2009; Dangl and Jones, 2001; Ellis *et al.,* 2003; Hammond-Kosack and Jones, 1997; Jones, 2001; Liu *et al.,* 2007).

Resistance gene analogues are putative derivatives of R genes. The highly conserved domains provide unique identity to R genes in plant genome (Hammond- Kossack *et al.,* 1996). The conserved motifs of NBS-LRR can be used to isolate resistance genes from plants by PCR based approach with oligonucleotide degenerate primers. RGAs have been isolated from wide varieties of plant species (Hattendorf and Debener, 2007a). Characterization of RGA is an effective strategyto identify R genes and develop markers for disease resistance (Mayer *et al.,* 1999; Hattendorf and Debener, 2007a; Biber *et al.,* 2010; Backiyarani *et al.,* 2013; Lei *et al.,*2014; Sekhwal *et al.,* 2015).

Rose is one of the economically important ornamental crops from Rosaceae family and it has the highest economic impact in the world. Rose flower industry comprises of local and international marketing of cut flowers, loose flowers, scent, oil and medicines. The worldwide estimated production of rose is 18 billion cut stems, 60-80 million potted-rose and 220 million for landscape purposes (Debener and Byrne, 2014). Apart from roses, other economically important members of Rosaceae family are apples (*Malus*), strawberries (*Fragaria*), stone fruits like peach, plum, apricots (*Prunus*) and pears (*Pyrus*). Most of the species of Rosaceae family are woody perennials. There is a wide range of pathogens *viz.,* fungi, bacteria, virus, phytoplasma and nematodes that attacks rose plants causing its death and thereby reducing the marketability of roses. Powdery mildew is one of the most damaging diseases of Rosaceae family (Xu *et al.,* 2007). *Podosphaera pannosa* (Wallr.:Fr.) de Bary is an obligate (biotrophic) pathogen (order Erysiphales, phylum Ascomycotina) that inhabits numerous economically important plants.Severe powdery mildew infection reduces greenhouse cut flower production (Leus*et al.,* 2003; Xu *et al.,* 2005, Debener and Byrne, 2014).

Characterization of R genes from wild varieties will help in obtaining disease resistant cultivars of roses (Hattendorf *et al.,* 2004; Hattendorf and Debener, 2007b). So far,only two R genes have been characterized in rose *viz*. Rdr 1 for black spot resistance (Von Malek *et al.,* 2000; Ayana *et al.,* 2011) and RPP1 for powdery mildew resistance (Linde and Debener, 2003; Linde *et al.,* 2004) from Institute for Ornamental Plant Breeding, Germany. Diseaseresistance loci have been identified and mapped in apple (Calenge*et al.,* 2005; Perazzolli *et al.,* 2014; Pessina *et al.,* 2014), strawberries (Zamora *et al.,* 2004), peach (Dirlewanger *et al.,* 1996, 2004; Quarta *et al.,* 2000; Dettori *et al.,* 2001; Lalli *et al.,* 2005), *Arabidopsis thaliana* (Aarts *et al.,* 1998; Mayer *et al.,*2003) and soybeans (Yu *et al.,*1996).

Study of R gene and its locus can help to reveal their exact function in pathogen recognition followed by defense and their evolution among particular plant species. This can be used to develop novel disease management strategies (McHale *et al.,* 2006). In this context, the best desirable strategy for disease management is development of resistant varieties as it can be a cost effective alternative for chemical method of disease management. Powdery mildew resistance was observed in rose genotype (IIHRR13-4) during field evaluation but mechanism of disease resistance was unknown. The objective of this study was to identify and characterize resistance gene analogues from IIHRR13-4.

MATERIALS AND METHODS

Rose genotypes used in this study were obtained from Division of Ornamental crops, ICAR- Indian Institute of Horticultural Research (IIHR). Eight rose genotypes (Table 1) were used to identify resistant gene analogues based on earlier reports. Among those genotypes selected, IIHRR13-4 was found to be resistant, *Rosa indica* was immune and remaining were highly susceptible to powdery mildew.

Genomic DNA was isolated from coppery red rose leaves by CTAB method (Doyle and Doyle, 1987). Six sets of degenerative primers (Table 2) were used

Table 1. Rose genotypes maintained at IIHR used in the study

S.No.	Rose genotypes used in the study	Field assessment of Powdery mildew disease
1	R1 - IIHRR13-4 (PMR)	Resistant
2	R2 - Rosa indica	Resistant
3	R3 - 11-3	Susceptible
4	R4 - First Red	Susceptible
5	R5 - Dean De Pointers	Susceptible
6	R6 - Fantasy	Susceptible
7	R7 - Konfetti	Susceptible
8	R8 -13-24	Susceptible

Table 2. List of primers used in this study for the amplification of RGAs

S.No.	Primer name	Sequence (5'-3')	Motif
1	RS1 F	GGIGGIATIGGIAAAACIAC	GGMGKTT
	RS1 F	RAARCAIGCDATRTGIARRAA	FLHIACF
2	RS3 F	GGIGTIGGIAAIACI	GGVGKTT
	RS3 R	RAARCAIGCDATRTGIARRAA	FLHIACF
3	RS4F	GGIGGIATIGGIAAAACIAC	GGMGKTT
	RS4R	RAARCAIGCSATRTCIARRAA	FLDIACF
4	RS10F	GGIGGIATIGGIAAAACIAC	GGMGKTT
	RS10R	YTCIGGRAAIARIGCRCARTA	YCALFPE
5	RS11F	GGIGGIYTIGGIAARACIAC	GGLGKTT
	RS11R	YTIIGGRAAIARIGCRCARTA	YCALFPE
6	RS12F	GGIGGIGTIGGIAAIACI	GGVGKTT
	RS12F	YTCIGGRAAIARIGCRCARTA	YCALFPE

for amplification of RGAs. RS1 RS2 and RS3 primer pairs were specific for TIR-NBS-LRR type and RS10, RS11 and RS12 were for LZ type (Hattendorf and Debener, 2007a). PCR assays were performed with genomic DNA in a total volume of 25 µl containing10µM of forward and reverse primers (Sigma Aldrich, India), 3 units of *Taq* polymerase and 2.5 mM*Taq* buffer (Genei, Bengaluru, India).PCR reaction was performed in Eppendorf thermal cycler with initial denaturation at 94^{0}C for 3 min, 35 cycles of 94^{0}C for 1 min, 42^{0}C for 1 min, 72^{0}C for I min followed by final extension step at 72^{0}C for 10 minutes (Hattendorf and Debener, 2007a).

Agarose gel (1.5%) electrophoresis was carried out to view and purify the PCR products.The amplified products were further purified by Nucleospin gel and purification kit by Macherey-Nagel GmbH & Co. KG, Germany. The purified products were ligated into P^{TZ} $^{57R/T}$vector. Cloning was done with Thermo-Fisher Scientific InsTA clone PCR cloning kit (Thermo-Fisher Scientific Baltics UAB, Lithuania). Transformed colonies were selected for plasmid isolation and presence of insert was confirmed by plasmid PCR. Cloned plasmids were sequenced for further identification (Hattendorf and Debener, 2007a).

RGA sequences were analysed using NCBI Vecscreen (https://www.ncbi.nlm.nih.gov/tools/vecscreen/) and BioEdit (Hall, 1999). Sequence similarity search was done using NCBI Blast (https:/

/blast.ncbi.nlm.nih.gov) for RGA sequences. Amino acid sequences were generated using Expert Protein Analysis System (ExPASy) (https://web.expasy.org/translate/) translating tool and conserved motifs were identified by amino acid sequence alignment. Phylogenetic tree was constructed with MEGA-6 (Tamura*et al.,* 2013) with bootstrap analysis with 1000 replications. Sequences of selected RGAs were deposited at NCBI-Gen Bank database.

RESULTS AND DISCUSSION

Genomic RGAs were identified from rose genotypes with various degenerate primer sets with an amplified product of 550-700 bp length (Fig.1).From the different primer sets used only RS1 and RS10 primer combinations amplified in rose plants irrespective of susceptibility or resistance. The PCR amplified products were cloned and 41 colonies were picked for confirmation and sequence analysis. Finally ten RGA clones were selected from IIHRR13-4 by RS1 primer combination and three RGAs identified respectively from IIHRR13-4, Konfetti and First red by RS10 combination. These RGAs were finalised based on the sequence length and similarity to RGAs from Rosaceae family and other plant RGAs. The RGAs sequences with internal stop codons were eliminated. Total eleven RGAs were confirmed from IIHRR13-4 after sequence analysis and similarity to other plant RGAs. Other two RGAs were confirmed

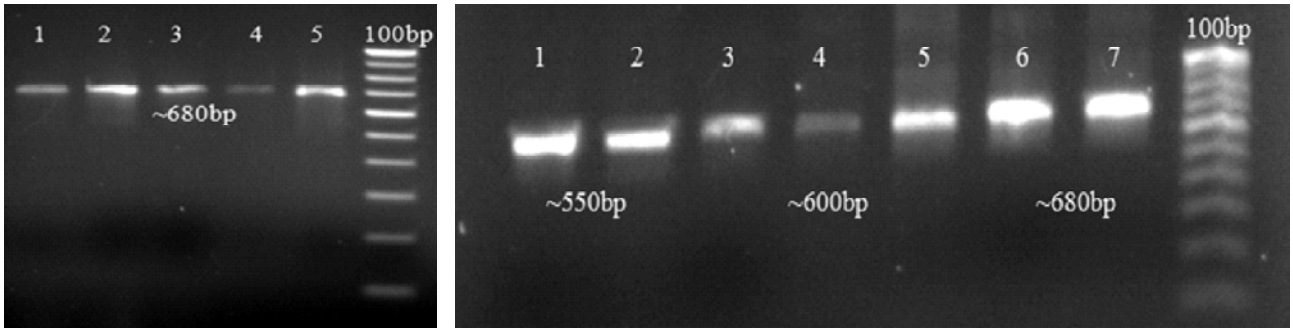

Fig. 1. Agarose gel electrophoresis confirming the amplification of Rose RGAs fragments, 1-5, 1 -7 - Rose RGAs. RGA fragments amplified at 550-700bp.

from powdery mildew susceptible First red and konfetti. All the amplified PCR products were purifiedand cloned in P^{TZ} vector. The sequence homology of rose RGAs to other plant proteins and other known R genes was confirmed by NCBI BLAST search. The list of proteins present in other plants belonging to rosaceae family to which close similarity was observed for the RGAs identified in the present study is given in Table 3.

The R gene sequences retrieved from NCBI database used in the phylogenetic analysis are listed in the Table 4. RGAs identified in the present study showed similarity to both TIR class of NBS-LRR RGAs and Non-TIR (LZ) class of NBS-LRR. RGAs identified from susceptible varieties showed similarity to RGAs of *Rosaceae* family but some of them excluded after amino acid translation because of the presence of internal stop codons. Finally thirteen RGA sequences *viz.,* IIHRR13-4R1, IIHRR13-4R2, IIHRR13-4R3, IIHRR13-4R4, IIHRR13-4R5, IIHRR13-4R6, IIHRR13-4R7, IIHRR13-4R 8, IIHRR13-4R9, IIHRR13-4R10, IIHRR13-4RS10 (IIHRR13-4), IIHRSFRR10 (First Red) and IIHRRRIRS10 (*Rosa indica*) were identified in this study.

Multiple sequence alignment identified highly conserved amino acid motifs present in the RGAs of IIHRR13-4. Multiple sequence alignment of IIHRR13-4 RGAs was performed with other R genes of Rose, *Arabidopsis, Solanum*, *Nicotiana*, *Malus*, *Prunus, Fragaria* and apoptotic protease activating factor(APAF) gene (Fig. 2). Six highly conserved amino acids motifs of NBS domain identified were P-loop, RNBS (Resistance nucleotide binding Site)-A, Kinase-2, Kinase-3a, RNBS-D, and GLPL. NCBI CD-search (Conserved Domain software) was used to find and confirm the conserved domains of RGAs and presence of nucleotide binding domain (NBARC domain) and LRR3 super family domain. The selected RGAs were further analysed for their phylogenetic relationships among Rosaceae family and other plant R genes.

Table 3. List of RGAs identified from the present study, their GenBank accession numbers and sequence similarity with RGAs of other Rosaceae family

Rose RGAs	NCBI Accession number	Protein to which closer similarity observed	Plant species	Identity (%)
IIHRR13-4R1	MG958641	TMV resistance protein N-like isoform X2	*Fragaria vesca* sub sp. *vesca*	77
		Putative NBS-LRR resistance protein	*Rosa* hybrid cultivar	80
IIHRR13-4R2	MG970527	Putative NBS-LRR resistance protein	*Rosa* hybrid cultivar	98
		Putative winged helix-turn-helix DNA-binding domain, leucine-rich repeat domain	*Rosa chinensis*	
IIHRR13-	MG970528	Putative transcription factor WRKY family	*Rosa chinensis*	94
4R3		Putative TIR-NBS-LRR resistance protein	*Rosa* hybrid cultivar	82
IIHRR13-	MG970529	Putative transcription factor WRKY family	*Rosa chinensis*	100
4R4		Putative TIR-NBS-LRR resistance protein	*Rosa* hybrid cultivar	83
IIHRR 13-4R5	MG970530	TMV resistance protein N-like	*Fragaria vesca* subsp. *vesca*	67
		TMV resistance protein N-like	*Prunus avium*	62
IIHRR13-	MG970531	Putative NBS-LRR resistance protein	*Rosa* hybrid cultivar	87
4R6		Putative toll-like receptor, P-loop containing nucleoside triphosphate hydrolase	*Rosa chinensis*	88
IIHRR13-	MG970532	Putative TIR-NBS-LRR resistance protein	*Rosa* hybrid cultivar	100
4R7		TMV resistance protein N-like	*Fragaria vesca*su bsp. *vesca*	73
IIHRR13-	MG970533	Putative TIR-NBS-LRR resistance protein	*Rosa* hybrid cultivar	83
4R8		Putative transcription factor WRKY family	*Rosa chinensis*	72
IIHRR13-	MG970534	Putative TIR-NBS-LRR resistance protein	*Rosa* hybrid cultivar	88
4R9		Putative transcription factor WRKY family	*Rosa chinensis*	75
IIHRR13-4R10	MG970535	NBS-LRR resistance protein	*Rosa* hybridc ultivar	88
		Putative TIR-NBS-LRR resistance protein	*Rosa* hybrid cultivar	84
IIHRR13-4RS10	MK704433	Putative disease resistance protein RGA3 and RGA4	*Rosa chinensis*	74
IIHRSFR R10 (First	MK704434	Putative disease resistance protein RGA1, RGA2, RGA3 and RGA4	*Rosa chinensis*	86, 85, 86 and 90
Red)		Isolate F11P2-4F NBS-LRR resistance protein gene	*Rosa* hybrid cultivar	87
IIHRRRI RS10	MK689860	Putative disease resistance protein RGA2 RGA3 and RGA4	*Rosa chinensis*	97, 88 and 87
(*Rosa indica*)		NBS-LRR resistance protein gene	*Rosa* hybrid cultivar	88

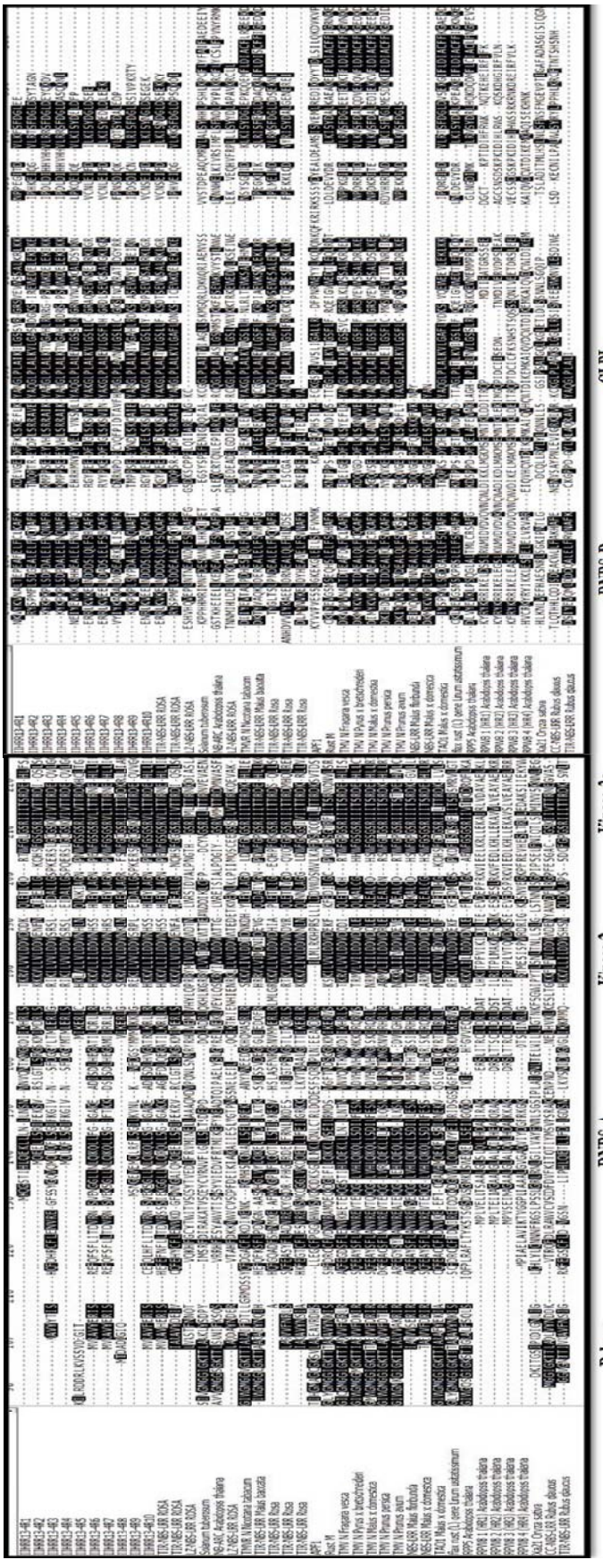

Fig. 2. Multiple sequence alignment showing conserved motifs in the amino acid alignment of IIHRR13-4 RGAs with other R genes of Rose, *Arabidopsis*, *Solanum*, *Nicotiana*, *Malus*, *Prunus*, *Fragaria* and APF gene. Highly conserved amino acids of motifs (P-loop, RNBS-A, Kinase-2, Kinase-3a, RNBS- D, and GLPL) are shaded

Table 4. List of R genes retrieved from NCBI database used in the phylogenetic analysis that were compared with RGAs of rose

Sl.No.	R gene	Host	Accession No.
1	RGC1	*Solanum tuberosum*	AF266747.1
2	NB-ARC domain disease resistance protein	*Arabidopsis thaliana*	NP187360.1
3	Virus resistance (N) gene	*Nicotiana glutinosa*	U15605.1
4	TIR-NBS-LRR type R protein 7	*Malus baccata*	AAQ93075.1
5	TIR-NBS-LRR resistance protein	*Rosa* hybrid	AM075235.1
7	Rust resistance protein M gene	*Linum usitatissimum*	U73916.1
8	RPP5	*Arabidopsis thaliana*	NM114316.3
9	L6	*Linum usitatissimum*	U27081.1
10	RPW8.1, RPW8.2	*Arabidopsis thaliana*	AF273059.1
11	Xa21	*Oryza sativa*	AY885788.1
12	LZ-NBS-LRR resistance protein	*Rosa hybrid*	AM075248.1
13	TMV resistance protein N-like	*Fragaria vesca* subsp. *vesca*	XM011459053.1
14	TMV resistance protein N-like	*Prunus avium*	XM021944693.1
15	Apoptotic protease activating factor 1 (APAF1) mRNA (out group)	*Homo sapiens*	AF149794.1

Phylogenetic tree was constructed using MEGA-6 software to identify genetic relationship and diversity among rose RGAs and other known plant R genes from *Rosaceae* and other species (Table 3). Different R genes from different crops available in GenBank were selected to analyse the phylogenic relationship among R genes. The phylogeny was constructed using Neighbour Joining method with1000 boot strap replications (Fig. 3). The two distinct groups of RGAs, TIR and LZ types were clearly separated in phylogram. Apoptotic protease activating factor 1 (APAF1) related to human cell death was used as out-group to construct phylogentic tree because of its NBS domain with greater protein sequence similarity to NBS-LRR proteins of plants. Highest degree of similarity of IIHRR13-4 RGAs was observed with *Malus domestica*, *Rosa chinensis*, *F. vesca* and *Rosa* hybrid cultivar. Comparative sequence analysis specifies the clustering of rose RGAs with certain Rosaceae RGAs. IIHRR13-4R2, IIHRR13-4R3, IIHRR13-4R4, IIHRR13-4R8 andIIHRR13-4R9 clustered together with TIR-NBS-LRR *Rosa* hybrid cultivar. IIHRR13-4R7 clustered with TMV resistance N like protein of *Fragaria vesca*. IIHRR13-4R5 clustered with TMV resistance N like protein of *Prunus avium* and TIR-NBS-LRR *Rosa* hybrid cultivar. IIHRR13-4R1 grouped with TMV of *Nicotiana tabaccum*. Other RGA IIHRRCONRS10 grouped with LZ-NBS-LRR of Rosa hybrid cultivar, NBARC of *Arabidopsis thaliana*, RGC1 of *Solanum* and RGA 4 of *Rosa chinensis*. RGA identified from First Red IIHRSFRRS10 were grouped with Xa21 of *Oryza sativa*. Neighbour Joining phylogenetic tree confirms the similarity of TIR and LZ type of RGAs from IIHR rose genotypes to RGAs of Rosaceae family.

Identification of RGAs can assist in breeding program for superior disease resistance because of the specific gene feature. RGA fragments are generated from different motifs of conserved domains of R genes that code for resistance against particular pathogen. RGA based markers that linked to R genes are more specific and facilitate selection of desirable disease resistant lines (Ellis *et al.,* 2000; Biber *et al.,* 2010, Hattendorf and Debener, 2007a). PCR based approach with degenerate primer is an efficient method for identification and cloning of RGAs from plants (Hattendorf and Debener, 2007a; Vossen *et al.,* 2013; Yu *et al.,* 1996).

PCR based approach was used in the study to identify potential RGAs linked to powdery mildew resistance in IIHR line of rose (IIHRR13-4) for which molecular basis for mechanism of disease resistance had not been earlier identified. Based on previous observations under field evaluation, genotype IIHRR13-4 that wasfound resistant was selected along with wild rose species *R. indica* which was

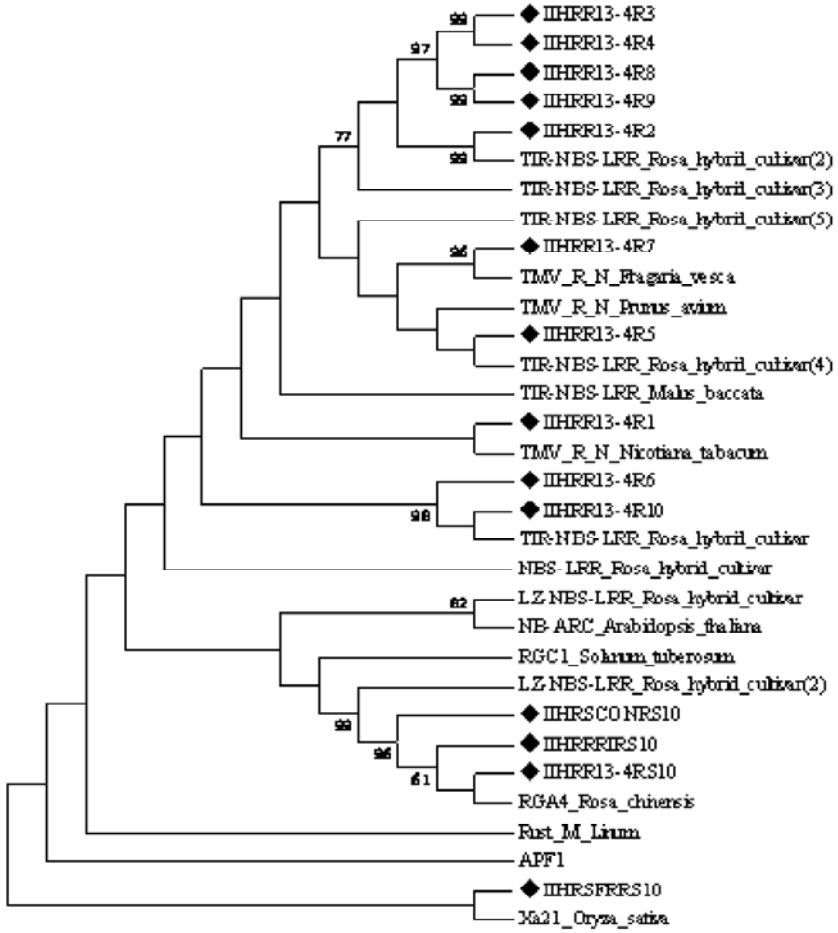

Fig. 3. The phylogenetic tree constructed with Neighbor-Joining method based on the amino acid sequences of rose RGAs, along with RGAs and R genes from *Rosaceae* and other plant species. The bootstrap values obtained from 1000 replications Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed.

immune to powdery mildew. Molecular profiling of RGAs with NBS conserved motifs helps in diversity studies of R gene families and identification of molecular markers for disease resistance (R) genes (Vossen *et al.,* 2013). Six degenerate primers of conserved NBS motifs used in the present study were selected from Hattendorf and Debener (2007) for isolation of genomic RGAs from rose. RS1, RS2 and RS3 primers helped in identifying TIR class of genomic RGAs and RS4, RS5 and RS6 aided amplification of non-TIR class (LZ) of RGAs from rose.The difference in each primer set relies on single amino acid in the motif sequence. Primers of P-loop motif sequence (GG.GKTT) differed in the third amino acid of the motif (GG (M/V/L) GKTT). In the same way, primers designed on NBS-IX motif also differed in single amino acid change. Motif sequence of NBS-IX was FL.IACF and change was on the third amino acid (FL(H/D)IACF). These variation in primer sequences helps to identify complete set of RGAs present in the rose genome.

Genomic RGAs isolated from rose belonged to TIR and non - TIR class (LZ) of NBS-LRR resistance genes. Non-TIR classes of RGAs are present in monocotyledons (wheat, rice, maize) and di-cotyledons but TIR class of RGAs mostly found in dicotyledonous plants. Hattendorf and Debener (2007a) reported that rose genome contained more TIR class of RGAs rather than non-TIR class with respect to number and diversity of RGAs in rose genome. The clear distinction between TIR and LZ class is based on the motif sequences of NBS domain (Xu *et al.,* 2005).

RGAs identified from all genotypes of rose (Table 1) irrespective of powdery mildew resistance and susceptibility. Previous investigations showed constitutive expression of RGAs but their transcription was induced by different factors in different crops

(Hammond-Kosack and Jones, 1997). Expression of RGAs specifically after pathogen attack explains their role particularly in defense response. Hattendorf and Debener (2007b) explained relative expression of RGAs in rose by checking the expression of RGAs in *Diplocarpon rosae* (black spot disease) inoculated and control rose leaves. Enhanced expressions of TIR-RGAs were observed in rose after black spot inoculation than untreated control, indicating direct function of TIR-RGAs in disease resistance against black spot. Genomic RGAs isolated from powdery mildew susceptible lines of rose (Table 1) were excluded because of presence of premature stop codons. RGA isolated from First Red (IIHRRSFRRS10) was without stop codons and that grouped with Xa21 of *O. sativa* in the phylogram. The genomic RGA of First Red probably may not express during powdery mildew infection process to prevent the disease and leads to the susceptibility to powdery mildew. NCBI blast results indicate that IIHRSFRR10 was showing similarity to *Rosa* hybrid cultivar NBS-LRR resistance protein pseudogene by 86.59%. Therefore, the RGA was identified from First Red may be pseudogene. These results gave information regarding the expression levels of RGAs in rose with respect to disease resistance. Some of the RGAs were identified as pseudogenes in many crops (potato, *Arabidopsis,* cotton, lotus and tomato). Non-functional pseudogene paralogs of R-genes (*Xa21*, *Cf9*, *Pto* and *Dm3*) were identified and have strong identity with other NBS protein but their sequences are short and presence of premature stop codons was observed. Pseudogenes are assumed to be involved in the *R* gene evolution process (Sekhwal *et al.,* 2005; Song*et al.,* 1997).

The NCBI blast results showed that IIHRR13-4R4 were 100% similar to putative transcription factor WRKY family of *R. chinensis*. WRKY super family of plant transcription factors plays important role in plant defense. The plant immune receptors detect pathogen effector proteins through WRKY transcription factors and activate defense (Phukan*et al.,* 2016). The NBS-LRR usually connects with other protein domains. *Arabidopsis* RRS1-RNB-LRR protein carries C-terminal WRKY DNA binding domain that enables formation of receptor complex with another NBS-LRR protein, RPS4 that helps in detection of bacterial effectors. This ligand-receptor binding initiates activation of defense mechanisms this indicates that the plants defense depends on intra-cellular immune receptors.

The phylogenetic tree showed clear separation of two different classes of NBS-LRR RGAs (TIR and LZ). Resistance gene analogues identified in the present study were closely related to *Rosa* hybrid cultivar, *R. chinensis* and other species of *Rosaceae* family (*Fragaria, Prunus* and *Malus)*. The conserved motifs identified from RGAs of IIHR13-4were similar to *Rosa multiflora* hybrid (Hattendorf and Debener, 2007a), chestnut rose (Xu *et al.,* 2005), strawberry (Zamora *et al.,* 2004) and other plant NBS-LRR R genes. These conserved motifs of NBS domain codesfor ATP or GTP binding proteins and hydrolysis activity (Phukan *et al.,* 2016; Saraste *et al.,* 1997; Xu *et al.,* 2005). IIHRR13-4 RGAs were showing more homology with TIR-NBS-LRR disease resistance gene of *Rosa hybrid* and *TMV*- N like disease resistance gene of *F. vesca*. The two major clades observed were TIR group and non-TIR group (LZ-NBS-LRR) of RGAs. TIR group of NBS-LRR genes of *Rosa* hybrid was clustered together with IIHRR13-4 RGAs. Phylogenetic studies revealed the relationship between RGAs identified from rose and other R genes/RGAs of Rosaceae and other family also.

Multiple sequence alignment by ClustalW showed that different motifs of rose RGAs were P-loop, RNBS-A, kinase 2, kinase 3a, RNBS-C and GLPL of NBS domain. Rose TIR - RGAs carried an aspartic acid residue (D) at the end of kinase 2 region (NBS III). Usually LZ-RGAs (Leucine Zipper) possess tryptophan residue (W) instead of aspartic acid residue. Other R genes with LRR3 super family domain reported earlier were NBARC domain of putative rp3 protein from *Zea mays*, RPP5 disease resistance protein of *A. thaliana* and NBARC domain of R genes of Solanaceae, *O. sativa*, Rosids*, Vitis vinifera* (RX-CC-NBARC), *Malus domestica*, *Capsicum annuum* and *Citrus* sp. LRR domain of known R genes (rp3, RPP5, NBARC) was present in IIHRR13-4 RGAs. The presence of conserved domain LRR 3 super family will give unique identity to RGAs identified from the genome of powdery mildew resistance germplasm line IIHRR13-4.

Several RGAs present in each plant genome may or may not link to resistance. The IIHRR13-4 was found resistant to powdery mildew in field evaluations. The

powdery mildew resistant line IIHRR13-4 was studied along with other resistant and susceptible rose lines. The RGAs were identified from all rose genotypes and some were excluded because of stop codons. Finally eleven RGAs selected from the IIHRR13-4 that might be linked to resistance mechanisms. This is the initial study on resistance mechanisms in the rose line IIHRR13-4 against powdery mildew disease. The expression analysis studies (N. K Chandran, Personal communication) revealed more expression of RGAs and resistance related transcription factors in IIHRR13-4 compared to susceptible cultivar konfetti upon powdery mildew infection. The comparison between IIHRR13-4 and konfetti revealed that expression level of RGA transcripts might not be sufficient to elicit resistance in konfetti. This indicates the importance of proper and required expression of disease resistance gene against particular pathogen. This study indicates that several R gene candidates (RGAs) are present in rose plants but only few are linked to disease resistance. These RGAs identified from IIHRR13-4 might be putative derivatives for R gene(s) against powdery mildew and may help in future research on mapping and characterization of R genes from IIHRR13-4.

Map based cloning approach is used to isolate R genes and that requires high-density genetic maps. Genome-wide RGA identification would assist to develop markers and mapping resistance genes and further possible cloning.

CONCLUSION

The present study explains the putative molecular mechanism behind resistance to powdery mildew resistance in IIHRR13-4 through different motifs present in the NBS domain of NBS-LRR group of R genes. This can be used as a basis for further studies related to molecular mechanism of resistance since RGAs are potential candidates for functional resistance gene and marker development in various breeding programs. The results of present study will help to develop RGA based markers linked to powdery mildew resistance in rose and this will help in rose resistant breeding and disease resistance screening programs using R gene profiling. Further study related to expression level of RGAs will provide more insight into molecular basis of disease resistance.

ACKNOWLED'GEMENT

The authors thank the Director, ICAR-IIHR and Head, Division of Crop Protection, ICAR-IIHR, Bangalore for providing the facilities to conduct the research work**.**

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(Received on 17.01.2020 and accepted on 18.03.2020)