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In Silico Characterization of Resistance Gene Analog, Ds-DbRCaG-05-Rga5p, expressed under Dieback Disease Stress

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Dalbergia sissoo commonly known as Shisham, is an important timber-producing tree however dieback disease poses considerable challenges to its existence. In this study, the in silico characterization of RGA, Ds-DbRCaG-05-Rga5p, expressed under the dieback challenge was demonstrated. Homology modeling predicted that the identified RGA displayed a similarity with Guanine-N (7)-methyltransferase RID2, an 18S ribosomal RNA protein that plays a role in defense mechanisms in plants. The computational analyses predicted the presence of structural motifs including protein kinase 3, N-glycosylation site, and phosphorylation site that are attributed to proteins containing disease resistance genes. The ligand docking analysis predicted the presence of lysine and arginine residues in active binding sites that suggested its potential as an antimicrobial peptide. Hence, in silico characterization has demonstrated its role in conferring resistance against pathogens.
Keywords: RGAs, Resistance genes, In silico characterization, Dalbergia sissoo, Homology modeling, antimicrobial peptides.

INTRODUCTION

Dalbergia sissoo (Shisham), a member of the *Papilionaceae* family, is a crucial plant with enormous economic significance (Gill *et al*.,2002). The nomenclatural designation of *Dalbergia sissoo* was ascribed by William Roxburgh, a botanist, during the early 19th century. The common names for *D. sissoo* exist all over the globe, including sisu, shisham, tahli, iruguduyam, and Jag. It is indigenous to India, Pakistan, Burma, Sri Lanka, and Mauritius (Sissoo, 2014). In the middle of the nineteenth century, shisham was brought to Pakistan. Widespread plantings of this crop have been seen as linear plantations beside highways, railroads, and in the irrigated region of Punjab (Gill *et al*., 2002). *D. sissoo* is used in traditional medicine to cure various diseases. Extracts of oil from sissoo seeds are often used to alleviate symptoms of including itching, scabies, and burning. The oil may also be useful in treating certain skin conditions (Saini and Sharma, 2012).

Shisham is susceptible to devastating diseases like dieback. Dieback is the progressive demise of branches, which begins at the tips (Javaid *et al*., 2004). Dieback, characterized by the gradual loss of twigs, branches, and shoots, often starting at the plant's extremities, is a symptom or name of a disease affecting mainly woody plants (Ghazali *et al*., 2015). Dieback can be attributed to various factors, including nematodes, stem- or root-boring insects, mechanical injury, root pavement, winter damage from cold temperatures or deicing salts, imbalances in moisture or essential nutrients, and winter damage resulting from the paving over of roots. The ailment is caused by several factors, including environmental pressures and biotic disease organisms that interact to weaken and eventually kill, which work together to damage and end up diminishing the plants (Javaid *et al*., 2004).

The most efficient method of achieving sustainable management of dieback, particularly for new plantations, will be the adoption of resistant germplasm to suppress the disease (Ijaz *et al*., 2022). Plants have evolved certain molecular and chemical characteristics to cope with biotic stresses, owing to their inherent immobility. The immune system of plants relies on receptors that identify widely conserved molecules that are linked to a diverse array of pathogens. The products of resistance genes, commonly referred to as R proteins, are believed to have the ability to detect signal molecules that are produced by pathogens. Upon detection, R proteins trigger a series of rapid changes in the physiology and metabolism of the host cell, which eventually concluded the inhibition of pathogen growth. As of present, over a hundred R genes have

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been successfully cloned (Sanseverino and Ercolano, 2012). Plants cop with ailments by innate immunity. Plants' innate immunity systems are highly evolved, allowing them to detect and react to infectious pathogens (Sekhwal, 2015).

The plant defense system involves two additional processes known as indirect and direct interactions. In the case of direct interactions, pathogen Avr effectors establish direct contact with plant R-genes, leading to the induction of signaling pathways (Sekhwal, 2015). In the indirect associations, *R*-gene products interact with pathogenic effectors and then examine how those effectors alter the host proteins which leads to the development of resistance (McDowell and Woffenden, 2003). The availability of high-density genome-wide resistance gene analog (RGA) genetic maps greatly facilitates the development of diagnostic markers and the identification of quantitative trait loci (QTL) or markers associated with plant disease resistance. These resources significantly enhance the effectiveness of research in understanding and combating plant diseases (Sekhwal, 2015). The objective of this study was *in silico* characterization of identified resistance gene analog (Ds-DbRCaG-05-Rga5p) in *Dalbergia sissoo* under dieback disease stress.

MATERIALS AND METHODS

Through the application of computational biology, the identified short-read DNA sequences were meticulously characterized *in silico*. These particular sequences, known as RGAs, exhibited a short length of nucleotides and were found exclusively in *D. sissoo* plants that displayed resistance to dieback. These differentially expressed short-read nucleotide sequences were identified during transcriptomic analysis under dieback stress conditions (Ijaz *et al*., 2022).

***Motif scanning*:** The protein motif of identified RGA Ds-DbRCaG-05-Rga5p was scanned using the ScanProsite program (Hulo *et al*., 2006) and NCBI-CDD database (Marchler-Bauer *et al*., 2015).

***Subcellular localization*:** The subcellular location of identified RGA Ds-DbRCaG-05-Rga5p was predicted by an online web server DeepLoc.2.0 (Thumuluri *et al*., 2022). The deep TMHMM tool was used to predict the topology and location of protein segments across the transmembrane (Hallgren *et al*., 2022).

***Physiochemical characterization*:** The physiochemical characteristics of Ds-DbRCaG-05-Rga5p were determined using the ProtParam tool (Gasteiger *et al*., 2005).

***Secondary structure annotation*:** The protein secondary structure of identified RGA Ds-DbRCaG-05-Rga5p was determined using the SOPMA web server (Geourjon and Deleage, 1995).

***Homology modeling*:** The homology of identified RGA Ds-DbRCaG-05-Rga5p was predicted using the AlphaFold Protein Structure Database (Jumper *et al*., 2021), SWISS MODEL (Waterhouse *et al*., 2018), and Phyre2 (Kelley *et al*., 2015).

***3D modeling*: A** 3D model of Ds-DbRCaG-05-Rga5p was generated using PyMOL (DeLano, 2002).

***Electrostatic potential and Docking study*:** Electrostatic potential of identified RGA Ds-DbRCaG-05-Rga5p is analyzed using a PyMOL web server (DeLano, 2002). Protein docking of identified RGA Ds-DbRCaG-05-Rga5p is carried out using a Hex 5.1 web server (Macindoe *et al*., 2010).

RESULTS

***Structural motif analysis*:** Protein motifs are short conserved sequences or patterns found in proteins that are important for their structure, function, or interaction with other molecules. ScanProsite is a commonly used tool for locating and analyzing patterns and protein motifs in protein sequences. The comprehension of protein motifs holds significant importance in acquiring knowledge about their biological functionalities and identifying their involvement in diverse cellular mechanisms (Ijaz et al., 2023). The sequence Ds-DbRCaG-05-Rga5p was subjected to analysis of structural motifs and functional residues using the ScanProsite web server. The RGA that was identified exhibited a phosphorylation site for protein kinase 3 as well as an N-glycosylation site (Table 1). The protein kinase 3 phosphorylation site plays a significant role in the defense mechanism of plants (Altman and Kong, 2016). The N-linked glycosylation site in plants is of significant importance in facilitating growth under conditions of stress and promoting adaptive immune activation (*Wolfert and Boons, 2013*; *Nagashima et al., 2018*). However, the online search tool NCBI-CDD showed no motif for the translated sequence Ds-DbRCaG-05-Rga5p.

Table 1. Residue and Predicted Features of Identified RGA *(Ds-DbRCaG-05-Rga5p), predicted by ScanProsite.*

RGA	Residue	Predicted Feature
Ds-DbRCaG-05-Rga5p	protein kinase 3 phosphorylation site	Phosphothreonine
	N-glycosylation site	N-Linked (GlcNAc) asparagine

***Prediction of subcellular localization*:** The DeepLoc-2.0 server (https://services.healthtech.dtu.dk/services/DeepLoc-1.0/) was used to examine the subcellular localization of the translated protein sequence Ds-DbRCaG-05-Rga5p (Table 2). The Mitochondria exhibited the highest subcellular signals, with a statistically significant value of 0.7384. In a previous study, it was suggested that the AtUSP protein in Arabidopsis thaliana has antifungal action by producing reactive oxygen species (ROS) and altering mitochondrial potential (Park *et al*., 2017). The protein known as Glutathione S-transferase

DHAR1 in Arabidopsis thaliana exhibits mitochondrial localization and plays a crucial role in the removal of reactive oxygen species (ROS). This protein significantly contributes to the plant's capacity to withstand oxidative stresses induced by both biotic and abiotic factors *(*Sasaki-Sekimoto *et al.,* 2005*).*

Determining protein topology is important because it helps in characterizing the protein's functional regions and their interactions with other molecules. DeepTMHMM utilizes machine learning to predict protein topology and the location of transmembrane regions (Ijaz *et al.,* 2023). Accordingly, an in-silico analysis using DeepTMHMM was performed on the sequence Ds-DbRCaG-05-Rga5p after it had been translated (Figure 1). Ds-DbRCaG-05-Rga5p's predicted protein sequence was found to have segments that are membrane-localized and have a globular protein structure. Hemoglobin, a globular protein, is predominantly located within the erythrocytes, commonly known as red blood cells (RBCs). Hemoglobin, particularly in its oxygenated form, can produce reactive oxygen species (ROS) through autoxidation. The generation of ROS by hemoglobin could contribute to its antibacterial activity (Hobson and Hirsch, 1958).

Table 2. ***Subcellular Localization of*** **Ds-DbRCaG-05-Rga5p, predicted by DeepLoc.**

RGA	***Localization***	***Probability***
Ds-DbRCaG-05-Rga5p	Mitochondria	0.7384
	Extracellular	0.5989
	Cytoplasm	0.3475
	Nucleus	0.2947
	Endoplasmic reticulum	0.1969
	Cell membrane	0.1637
	Golgi apparatus	0.1402
	Lysosomes/Vacuole	0.0929
	Peroxisome	0.0263
	Plastid	0.0214

***Prediction of physicochemical characteristics*:** Molecular weight is an essential element in the functional characterization of proteins. It provides valuable information about the size and mass of a protein molecule, which can help to understand its structure, stability, and interactions with other molecules. ProtParam (*https://web.expasy.org/cgi-bin/protparam/protparam*) is an extensively utilized web-based application that allows users to examine various characteristics of a given protein based on its amino acid sequence (Ijaz *et al.,* 2023). The protein with the identifier Ds-DbRCaG-05-Rga5p was anticipated to possess a sequence consisting of 25 amino acid residues. Furthermore, its molecular weight was determined to be approximately 2.937 kilodaltons. The computational analysis conducted determined that the identified RGA is a protein with acidic properties, as indicated by its isoelectric point of 4.41 (Table 3). The predominant characteristic observed in PR1-type proteins present in plants is their acidic nature. PR1 (Pathogenesis-Related Protein 1) is a class of proteins commonly found in plants. They are known for their involvement in plant defense mechanisms against various pathogens, including bacteria, fungi, and viruses. PR1 proteins play a crucial role in the innate immune response of plants (*Stolzenberg et al., 2017*).

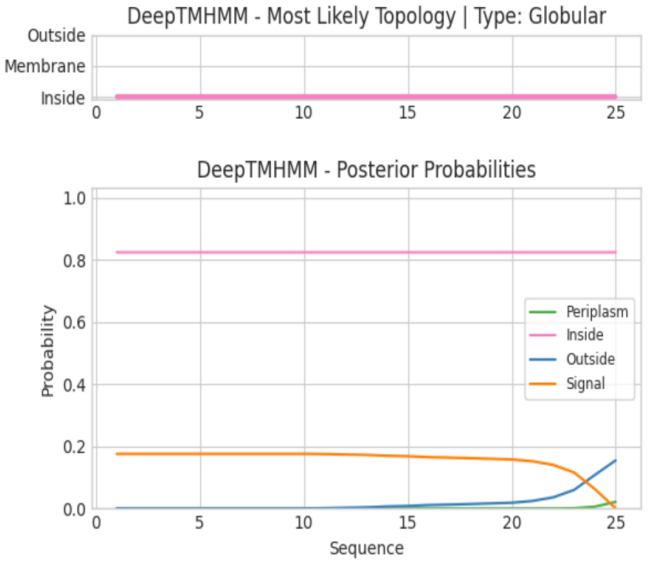

Figure 1. DeepTMHMM analyzed the topology of identified RGA *Ds-DbRCaG-05-Rga5p. Ds-DbRCaG-05-Rga5p* showed the presence of a segment of protein inside the transmembrane.

The protein's extinction coefficient was estimated to be 6,990 M^{-1} cm^{-1}, a crucial parameter for quantifying its concentration in aqueous solution at a wavelength of 280 nm. The protein was predicted to be stable based on an instability index value of 17.36. A high aliphatic index of 116.80 in the translated protein Ds-DbRCaG-05-Rga5p suggested that it is enriched in aliphatic amino acids, particularly alanine (Ala), valine (Val), leucine (Leu), and isoleucine (Ile). These amino acids have hydrophobic side chains and are known to contribute to the stability and structural integrity of proteins. Additionally, it has been suggested that this entity may exhibit certain characteristics that confer upon it the ability to endure extreme temperatures and thrive in hostile environmental circumstances (Mizuguchi *et al.,* 2007). The discovered protein was found to have a GRAVY score of 0.088, indicating that the translated protein sequence is hydrophobic (Insoluble in water).

Table 3. Identification of physicochemical properties by the web server ProtParam and PepCalc.

Web server	**Physicochemical properties**	
	Length of Amino acid	25
	Molecular weight	2937.38

	Isoelectric point	4.41
	Molecular Formula	$C_{137}H_{214}N_{30}O_{41}$
ProtParam	Instability Index	17.36
	Aliphatic Index	116.80
	Grand Average of Hydropathicity	0.088
	Extinction Coefficient	6990
PepCalc	Estimated solubility	Poor water solubility
	Net Charge at pH 7	-2

Protein secondary structure annotation: The SOPMA (Self-Optimized Prediction Method with Alignment) is a bioinformatics tool commonly used to predict the secondary structure of proteins (Table 4). In the translated protein sequence (Ds-DbRCaG-05-Rga5p) of the discovered RGAs, the findings revealed proportions of an alpha helix (Hh), a random coil (Cc), and an extended strand (Ee). It was estimated that 36% of the Ee and 32% of the Hh in Ds-DbRCaG-05-Rga5p came from Cc.

Table 4. Prediction of protein structure by the SOPMA

Secondary structure prediction	
Alpha helix (Hh)	32%
Random coil (Cc)	32%
Extended strand (Ee)	36%

***Homology modeling*:** The Swiss model and Phyre2 web tools were used to assess the accuracy of 3D models. This was done by creating Ramachandran plots, which visually represent the phi and psi angles of amino acids in a protein sequence (Figure 2). Both the Swiss Model and Phyre2 are popular web tools for protein structure prediction and modeling. While they employ different algorithms and approaches, they aim to generate accurate 3D models based on the input protein sequence and available template structures. Homology modeling of identified RGA Ds-DbRCaG-05-Rga5p displayed a similarity with 18S Ribosomal RNA. The Guanine-N(7)-methyltransferase RID2 (also known as DNG7) is an 18S ribosomal RNA protein that plays a role in defense mechanisms in plants (Ohbayashi *et al*., 2011**).** The Ramachandran plot is a visual depiction that illustrates the distribution of amino acids in a protein sequence based on their respective phi and psi angles. The model quality assessment revealed that the 3D model of Ds-DbRCaG-05-Rga5p exhibited 36.4% and 63.6% residues in the most favored region of the Ramachandran plots (Fig. 3). QMEAN Z values of DbRCaG-05-Rga5p was−4.20 which validated this model of high quality.

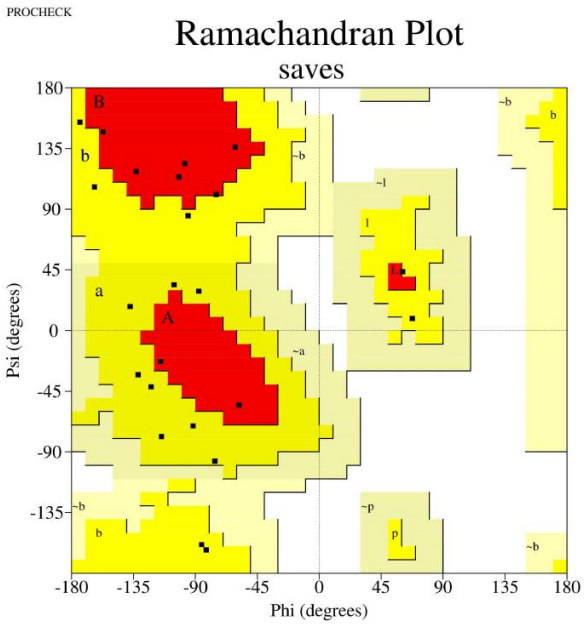

Figure 2. Ramachandran plots of *Ds-DbRCaG-05-Rga5p*. Validation of the protein structure of *Ds-DbRCaG-05-Rga5p* using the PROCHECK server. In Ramachandran plots of *Ds-DbRCaG-05-Rga5p*, the most favored region is shown in red, the additionally allowed region in yellow, the generously allowed region in pale yellow, and the additionally allowed region in white.

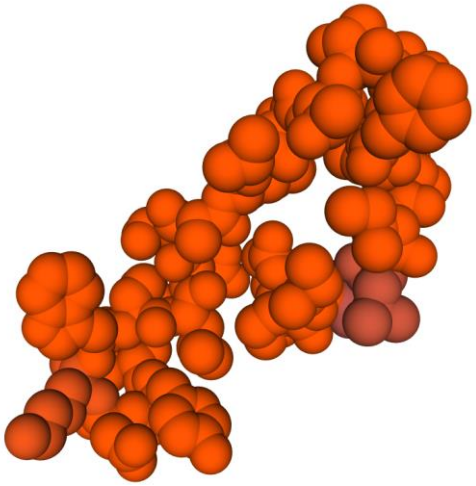

Figure 3. Three-dimensional homology model of Ds-DbRCaG-05-Rga5p 4.6 Electrostatic potential.

PyMOL was utilized to assess the electrostatic potential of Ds-DbRCaG-05-Rga5p**.** PyMOL uses a color scheme where red represents negative potential and blue represents positive potential. This color scheme is chosen to be consistent with the convention that negatively charged regions attract positively charged molecules or ions. The observed RGA Ds-

DbRCaG-05-Rga5p, which exhibits a greater blue hue and reduced white coloration, indicates that the electrostatic potential of Ds-DbRCaG-05-Rga5p demonstrates a higher positive charge and a lower neutral charge within the modeled active region (Figure 4).

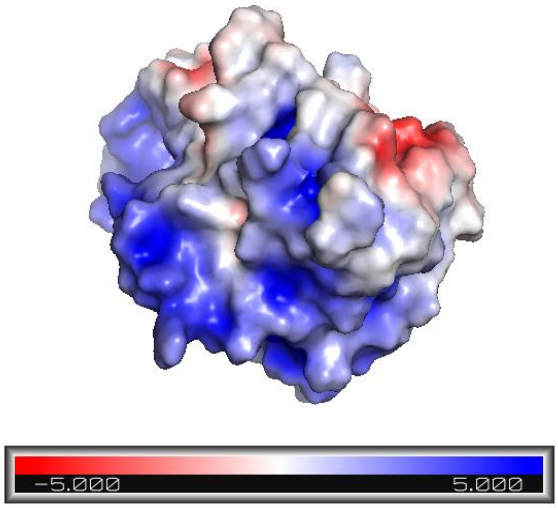

Figure 4. Electrostatic potential: surface electrostatic charge of Ds-DbRCaG-05-Rga5p calculated by PyMOL. Positive charges are shown in the blue region, negative charges in the red region, and neutral charges in the white region.

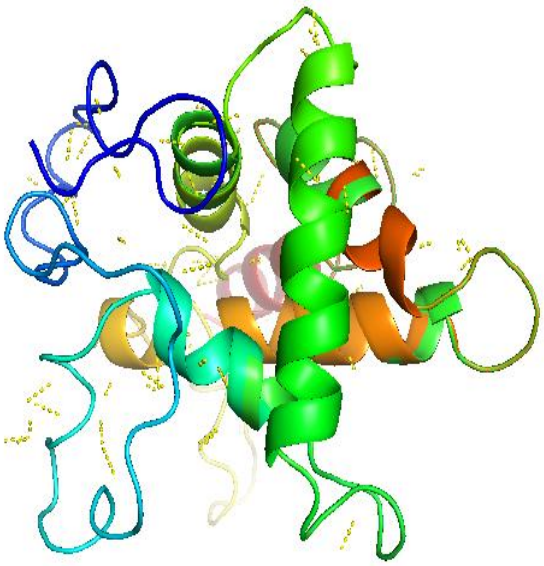

Figure 5. Ligand docking: A representative diagram of Ds-DbRCaG-05-Rga5p displays the active ligand obtained by the PyMOL by using the PDB files generated by the Hex server. Ligand-binding sites analyzed in Ds-DbRCaG-05-Rga5p were shown to have residues such as Arg, proline (Pro), and Lys.

***Potential and docking analysis*:** We conducted ligand docking of the identified RGA (Ds-DbRCaG-07-Rga1p) using Hex 5.1 (Figure 5). The analysis of ligand-binding sites in Ds-DbRCaG-07-Rga1p revealed the presence of specific residues, including Arginine (Arg), Proline (Pro), and Lysine (Lys). Arginine and lysine residues have been consistently associated with the strong antimicrobial activity of peptides (Cutrona *et al*., 2015).

DISCUSSION

Plants have evolved intricate immune systems to protect themselves against both biotic (pathogens) and abiotic stresses. Plant-pathogen interactions involve the recognition of specific molecules such as lipopolysaccharides, proteins, and sugars, which act as elicitors or pathogen-associated molecular patterns (PAMPS). The initial interaction occurs in the apoplast, where plant receptors identify these elicitors or PAMPS. These receptors, known as pattern recognition receptors (PRRS), are localized in the plant's membrane. When PRRS detect PAMPS, they initiate a first line of defense called PAMP-triggered immunity (PTI). PTI involves a series of defense responses, including the activation of defense-related genes, the production of antimicrobial compounds, and the reinforcement of the cell wall. However, some pathogens can secrete effector proteins that suppress or evade PTI. In response, plants have developed a second line of defense called effector-triggered immunity (ETI). ETI is activated when specific plant resistance proteins (r proteins) recognize the presence or activity of these effector proteins. The hypersensitive reaction (HR) is part of the powerful immune response triggered by this identification. The HR manifests as restricted pathogen transmission due to localized cell death in infected plant tissue. In general, the plant immune system has a tiered defensive approach, with PTI serving as the initial line of protection. If the pathogen can overcome PTI, the plant's ETI immune response kicks in to help destroy it and prevent it from spreading (Gupta *et al*., 2015) The pathogen is killed when the R gene from the plant interacts with the avr gene from the pathogen, setting off a chain reaction that activates the plant's immune system (Dangl and jones, 2001).

A review of previously documented literature reveals that *Ds-DbRCaG-07-Rga1p, Ds-DbRCaG-08-Rga04, Ds-DbRCaG-09-Rga9p, Ds-DbRCaG-11-Rga15p and Ds-DbRCaG-10-Rga13p* displayed homology with NADH-quinone oxidoreductase subunit H, NADH-quinone oxidoreductase subunit K, defensin-like protein A, ribonuclease R protein and defensins respectively (Ijaz *et al*., 2023). In the present study, the translated sequence Ds-DbRCaG-05-Rga5p showed a protein kinase 3 phosphorylation site which has a role in signal transduction pathways (Altman and Kong, 2016). The presence of an N-glycosylation site has been observed in the identified protein, which is known to have a crucial function

in promoting growth during periods of stress and facilitating adaptive immune activation (Wolfert and Boons, 2013**;** Nagashima *et al*., 2018). Ds-DbRCaG-05-Rga5p showed the homology with the VAACI which has a crucial role in *defense* against avirulence pathogens (Tateda *et al.,* 2011**).** Ds-DbRCaG-05-Rga5p displayed the homology with DHAR1 that is localized in the mitochondria and contributes to the plant's ability to cope with oxidative stresses caused by biotic and abiotic inducers (Luti et al., 2016).

A previous study showed the results of physiochemical properties of identified RGAs Ds-DbRCaG-07-Rga1p, Ds-DbRCaG-08-Rga04, Ds-DbRCaG-09-Rga9p, Ds-DbRCaG-11-Rga15p and Ds-DbRCaG-10-Rga13p which revealed all are basic. While in the present study identified physiochemical properties using ProtParam and unveiled that Ds-DbRCaG-05-Rga5p is an acidic protein. PR1 proteins play a crucial role in the innate immune response of plants. Moreover, they are known for their involvement in plant defense mechanisms against various pathogens, including bacteria, fungi, and viruses (Stolzenberg *et al*., 2017). The results indicate that the identified RGA (Resistance Gene Analog) Ds-dbrcag-05-Rga5p has a role in providing defense to plants against pathogenic microorganisms.

***Conclusion*:** This research study focused on the characterization and analysis of a specific Resistance Gene Analog (RGA) in plants, referred to as Ds-DbRCaG-05-Rga5p. The protein's physicochemical characteristics were determined and notably, the protein was found to be hydrophobic and acidic. Protein Secondary Structure Prediction revealed the proportions of the alpha helix, random coil, and extended strand in the protein's structure. Homology Modeling and Structure Assessment indicated a high-quality model. Homology with 18S Ribosomal RNA and a similarity to the Guanine-N(7)-methyltransferase RID2 was observed. Further, the electrostatic Potential Analysis demonstrated a predominance of positive charge in the modeled active region while ligand Docking Analysis identified specific residues (Arginine, Proline, Lysine) that might contribute to antimicrobial activity. This research contributes valuable insights into plant molecular biology and immunology, particularly in understanding how specific proteins like Ds-DbRCaG-05-Rga5p contribute to the complex defense mechanisms in plants against various pathogens.

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***Conflict of Interest*:** We clarify that the submitted manuscript is our original research work and has not been published previously. Additionally, there is no competing interest.

Ethical statement: This article does not contain any research with human participants or animals performed by any of the authors.

***Availability of data and material*:**We declare that the submitted research article is our own work which has not been published before and is not currently considered for elsewhere publication.

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***Code availability*:** N/A

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