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In silico analysis and expression profiling for Resistance Gene Analogues (RGAs) and defence-related genes in early germinating conditions of rice against bakanae disease caused by *Fusarium fujikuroi*

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Rice is a popular staple food in the world. Rice production is generally affected by many biotic and abiotic stresses. Among the biotic stresses affecting rice, bakanae has emerged as a serious challenge to Indian rice growers. Plant resistance gene analogues (RGAs) are genes with conserved domains and motifs that contribute to host resistance to disease. RGAs are frequently utilized in breeding programs and play a significant role in plant defence. The present study aimed to find out resistance gene analogues (RGAs) and other defence-related genes which might provide resistance to bakanae during the early germination stage of rice. A total of 32 gene IDs were selected from the previously reported transcriptome study of the bakanae-infected resistant and susceptible plants. The protein structures, active domains and biological functions were predicted *via* bioinformatical analysis. The predicted proteins were identified to possess one or more of the following features - hydrolase activity and carbohydrate metabolism, kinase activity, Rx_N domain, Rx_CC domain, NB-ARC domain, LRR domain, ADP/ATP binding site, and cytochrome site. Representatives of all the highlighted features were selected to prepare a set of 10 genes which were further evaluated for their expression in the inoculated seeds during germination *via* real-time PCR-based quantification. Four different rice genotypes *viz*, C101A51, Pusa 1342, PB1, and PB1121 with respective disease reaction types as highly resistant (HR), resistant (R), susceptible (S) and highly susceptible (HS) to bakanae were inoculated with two isolates of *F. fujikuroi*, F1121 and F1728. Based on the qPCR expression analysis of rice genotypes, RGA 2, RGA 5(b), and RGA 5(c) were found as potential candidate RGAs which might play role in providing resistance against bakanae in the early germination stage. Our study also identified one negative regulator *i.e.* GDSL esterase in the early stages of interaction due to its constant downregulation in different combinations of host-pathogen interactions. The outcome of this work will support the selection of essential RGAs and defence-related genes in resistance breeding programmes against bakanae disease.

Keywords: Rice, Bakanae, *Fusarium fujikuroi*, Defence-related genes, RGAs, qPCR

Rice serves as a staple food for billions of people worldwide, especially in Asia, where it fulfils their calorie and nutritional requirements[1–4]. Rice cultivation and consumption are firmly embedded in the cultural and agricultural traditions of many nations, serving as the foundation for their food systems. The fact that rice is the major food source highlights its strategic relevance in terms of global security. Rice production faces numerous challenges such as climate change, water shortages, and evolving agricultural practices, with diseases and pests adding further complications[5-7].

Rice is susceptible to various diseases that can significantly impact yield and quality. Losses inculcated due to all pathogens of rice had been estimated to be 12-15%[4,8,9]. One notable rice disease of present days is bakanae or 'Foolish seedling', caused by the ascomycetes fungi *Fusarium fujikuroi*[10-13]. Earlier, it was a minor disease; but in recent days with more production of basmati and scented cultivars of rice, the disease has become one major concern, as the fungus has biases towards them than non-scented ones[11,14]. The fungus attacks plant parts like roots, stems, leaf sheaths, and panicles, leading to death during growth. *F. fujikuroi* infection in rice plant shows chlorotic symptoms leading to reduced seed germination and abnormally tall and weak plants thereby resulting in plant death. Infected seedlings are stunted and exhibit rotting symptoms with powdery growth on the lower parts[15,16]. The primary source of infection for bakanae disease is

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infected seeds[17,18]. The plant residue from the previous season can also infect the new plants[19]. The spores (ascopores or conidia) dispersed by air can infect normal plants[13].

Though the disease was noticed from ancient times and the first scientific paper on this disease was published long back by Shotaro Hori[20], very little has been discovered on the mechanism of the disease development and resistance mechanism till date. Matic *et al*.[21] described transcriptome analysis between resistant and susceptible genotypes of rice, Selenio and Dorella, against *F. fujikuroi*. The resistant cultivar exhibited increased expression of several defence genes, including MAP kinases, glycoside hydrolases, germin-like proteins, PR1, and WRKY transcriptional factors. Recently, Bashyal *et al*.[22] found several defence-related genes like cysteine proteinase inhibitor 10, disease resistance protein TAO1-like, oleosin 16 kDa-like, PR1, PR4, BTB/POZ and MATH domain-containing protein 5-like, alpha-amylase isozyme, GDSL esterase/lipase, serine glyoxylate aminotransferase, CASP-like protein 2C1, WAT1-related protein, Cytoplasmic linker associated proteins, xyloglucan endotransglucosylase/hydrolase protein, β-D xylosidase 7 *etc*. in transcriptome analysis of Indian resistant (C101A51) and susceptible genotypes (Rasi) in seedlings against *F. fujikuroi*. Additionally, whole genome sequencing research on *F. fujikuroi* and its associations with susceptible and resistant rice cultivars revealed that several enzymes were up- or down-regulated in resistant cultivars[10]. However, knowledge about the resistance sources and mechanism against bakanae pathogen *F. fujikuroi* is still limited.

Plant resistance gene analogues (RGAs) are a large class of potential defence-related genes or R-genes, possessing conserved domains and motifs and varied structure, function, and evolution that contribute to host resistance to disease[23]. Yang and Hwa (2008)[24] characterised the genetic variation of 44 NBS–LRR RGAs in rice as preserved, diversified, intermediate-diversified and present/absent patterns across 21 cultivars and 17 wild rice accessions. Mago *et al*.[25] classified nine rice RGAs according to their similarity to the NBS region of different disease-resistance genes. RGAs are frequently utilised in breeding programmes to increase crop disease resistance and play a significant role in plant defence.

Bakanae disease, once started appearing, there is hardly any remedy to cure and save the crop. For this reason, it is very important to know the early players of host engaged in pathogen recognition and providing resistance to bakanae. Although numerous studies have investigated potential sources of resistance to bakanae disease, there are very few identified sources, and the mechanisms of pathogen recognition and the subsequent downstream pathways involved in disease establishment or resistance are not well understood. Therefore, the present study aims to perform an *in silico* analysis of various defence-related genes and RGAs associated with bakanae disease and to evaluate the expression of selected candidate genes in different rice genotypes with varying levels of resistance to the disease during early germination stages.

Material and Methods

Location of experiments

Laboratory tests were performed at the Division of Plant Pathology, Fungal Molecular Biology Lab, ICAR-IARI, New Delhi. Also, glasshouse experiments were carried out at the Division of Plant Pathology, ICAR-IARI, New Delhi (28.64°N latitude and 77.17°E longitude).

In silico analysis of transcriptomic data

With the help of bioinformatics, the experimental data generated to date is stored in several databases available online and can be utilized for prediction-based *in silico* analysis and validation[26-32]. A total of 32 genes related to disease resistance were selected by utilizing the BLASTn results from gene IDs in the published transcriptome data[22] of resistant and susceptible rice genotypes in response to bakanae disease (Table 1). The selected genes were also analysed using BLASTx to confirm their identity and putative protein names were annotated. Amino acid sequences for the respective genes were translated *via* the Expasy translate tool. Then, the protein structures were predicted using amino acid sequences through *de novo* modelling and homology-based modelling. *De novo* modelling was performed using Phyre2, and the visualization of the predicted protein structures was performed using Pymol software. Homology-based structures were generated by SwissModel (accessed on 01/03/2024). Representative domains and function prediction were performed by InterPro (accessed on 14/03/2024). Based on the bioinformatics analysis, 10 genes showing role in defence response with major focus to RGAs were selected for further analysis of gene expression

Table 1 — Structural and functional annotation of the selected gene IDs

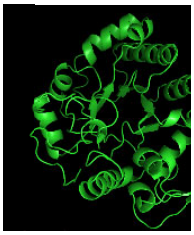
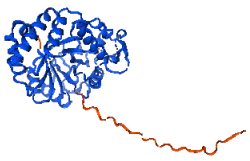
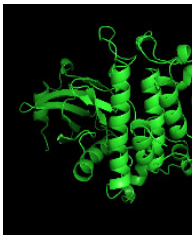
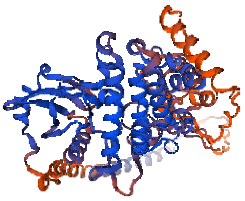
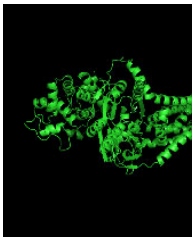
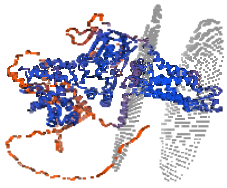
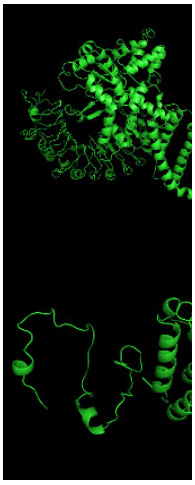
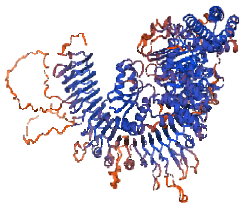
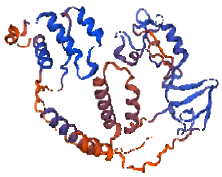
Sl. No.	Gene ID	Putative protein name	Active domains and GO annotations	Structure predicted by de-novo modelling	Structure predicted by homology-based modelling
1.	BGIOSGA000133	Endo-1,3-beta-glucanase	Representative domain- Glyco_hydro_17 (30-334 aa) InterPro GO terms- Biological Process- carbohydrate metabolic process Molecular Function- hydrolase activity, hydrolyzing O-glycosyl compounds PANTHER GO terms- Cellular Component- obsolete anchored component of plasma membrane		
2.	BGIOSGA001477	Protein kinase	Representative domain- GNK2 (19-114 aa) Serine-threonine/tyrosine-protein kinase, catalytic domain (230-499 aa) Protein kinase domain (227-515 aa) serkin_6 (227-500 aa) Active site Serine/threonine-protein kinase (348-360 aa) Binding site Protein kinase, ATP binding site (233-254 aa) InterPro GO terms- Biological Process- protein phosphorylation Molecular Function- protein kinase activity, ATP binding PANTHER GO terms- Cellular Component- plasma membrane		
3.	BGIOSGA001663	Respiratory burst oxidase homolog protein B	Representative domain- EF_HAND_2 (229-264 aa) NOX_Duox_like_FAD_NADP (599-813 aa) NADPH oxidase Respiratory burst (134-231 aa) FAD-binding domain, ferredoxin reductase-type (587-715 aa) Ferric reductase, NAD binding domain (719-887 aa) Ferric reductase transmembrane component-like domain (403-551 aa) Binding site- EF-hand calcium-binding domain (242-254 aa) InterPro GO terms- Molecular Function- calcium ion binding, oxidoreductase activity, peroxidase activity, oxidoreductase activity; acting on NAD(P)H; oxygen as acceptor Cellular Component- plasmamembrane		
4.	BGIOSGA001667	RGA 4	Representative domain- Rx_N(16-95 aa) NB-ARC (171-273 aa, 274-368 aa) InterPro GO terms Biological Process-defence response Molecular Function- protein binding, ADP binding		
5.	BGIOSGA002512	Rust resistance Kinase	Representative domain- GUB_WAK_bind (29-75 aa) InterPro GO terms- Molecular Function- polysaccharide binding, protein kinase activity		

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Table 1 — Structural and functional annotation of the selected gene IDs (*Contd.*)

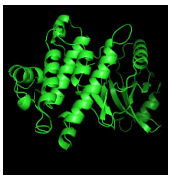
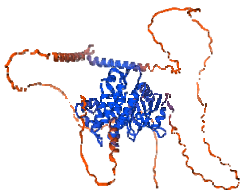
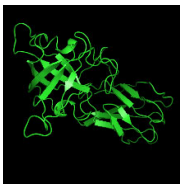
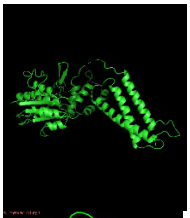
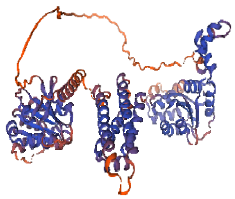
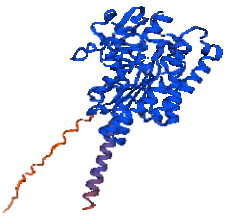
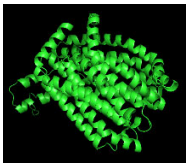
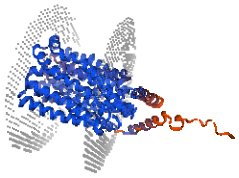
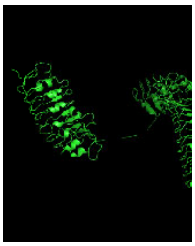
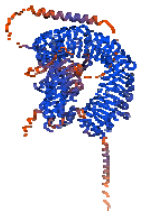
Sl. No.	Gene ID	Putative protein name	Active domains and GO annotations	Structure predicted by de-novo modelling	Structure predicted by homology-based modelling
6.	BGIOSGA003923	Receptor-like protein kinase	Representative domain- serkin_6 (269-547 aa) Active site- Serine/threonine-protein kinase, active site (391-403 aa) Binding site- Protein kinase, ATP binding site (275-297 aa) InterPro GO terms Biological Process- protein phosphorylation Molecular Function- ATP binding, protein kinase activity		
7.	BGIOSGA004707	Expansin	Representative domain- DPBB_EXPA_N (29-154 aa) EXPANSIN_CBD (169-248 aa) Expansin/pollen allergen, DPBB domain (47-159 aa) RlpA-like protein, double-psi beta-barrel domain (64-159 aa) InterPro GO terms- Biological Process- plant-type cell wall organization Cellular Component- extracellular region		
8.	BGIOSGA005356	Pib	Representative domain- RX-CC_like (9-123 aa) NB-ARC (178-339 aa, 407-596 aa) Rx N-terminal domain (13-94 aa) InterPro GO terms- Biological Process- defence response Molecular Function- ADP binding		
9.	BGIOSGA005366	GDSL esterase/lipase	Representative domain- SGNH_plant_lipase_like (29-346 aa) InterPro GO terms- Molecular Function- hydrolase activity, acting on ester bonds		
10.	BGIOSGA006116	Protein detoxification	Representative domain-MATE_eukaryotic (46-480 aa) InterPro GO terms Biological Process- transmembrane transport, xenobiotic detoxification by transmembrane export across the plasma membrane Molecular Function- antiporter activity, xenobiotic transmembrane transporter activity Cellular Component- membrane		
11.	BGIOSGA007118	Receptor kinase	Representative domain- LRR_typ_2 (110-134 aa) LRR_1 (187-208 aa, 212-234 aa) LRR_sd22_2 (258-285 aa, 380-406 aa, 609-635 aa) LRR_8 (458-517 aa) PROTEIN_KINASE_DOM (779-1056 aa) Leucine-rich repeat-containing N-terminal, plant-type (47-84 aa) serkin_6 (770-1052 aa) Active site- Serine/threonine-protein kinase, active site (902-914 aa) Binding site- Protein kinase, ATP binding site (785-808 aa) InterPro GO terms- Biological Process- protein phosphorylation Molecular Function- protein binding, protein kinase activity, ATP binding		

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Table 1 — Structural and functional annotation of the selected gene IDs (*Contd.*)

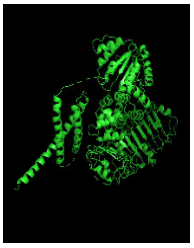
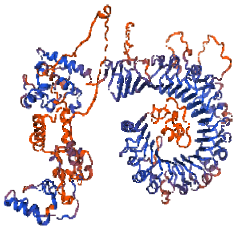
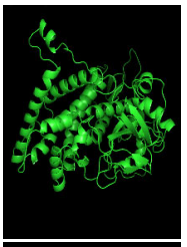
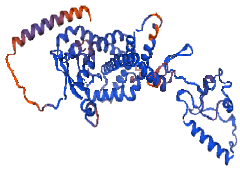
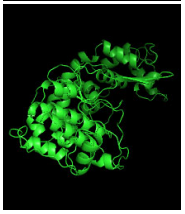
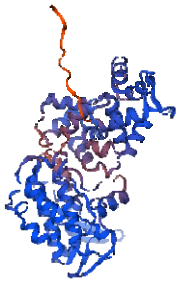
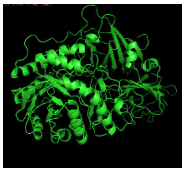
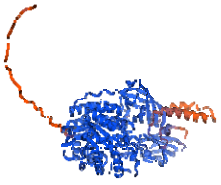
Sl. No.	Gene ID	Putative protein name	Active domains and GO annotations	Structure predicted by de-novo modelling	Structure predicted by homology-based modelling
12.	BGIOSGA007396	RGA2	Representative domain- NB-ARC (94-339 aa, 1966-2209 aa) Rx_N (1759-1841 aa) AAA+ ATPase domain (111-253 aa, 1981-2124 aa) InterPro GO terms- Biological Process- defence response Molecular Function- ADP binding, ATP hydrolysis activity		
13.	BGIOSGA007962	ent-cassadiene hydroxylase-like	Representative domain- p450 (46-359 aa, 371-479 aa) Conserved site- Cytochrome P450, conserved site (429 – 438 aa) InterPro GO terms- Molecular Function- oxidoreductase activity; acting on paired donors; with incorporation or reduction of molecular oxygen, iron ion binding, heme binding, monooxygenase activity		
14.	BGIOSGA017475	Calcium-dependent protein kinase	Representative domain- PROTEIN_KINASE_DOM (1-207 aa) EF-hand_7 (255-315 aa, 324-387 aa) serkin_6 (1-207 aa) EF-hand calcium-binding domain profile (250-285 aa, 286-321 aa, 322-356 aa, 357-392 aa) efh_1 (254-282 aa, 290-318 aa, 326-254 aa, 361-389 aa) Active site- Serine/threonine-protein kinase, active site (69-81 aa) Binding site- EF-Hand 1, calcium-binding site (263-275 aa, 299-311 aa, 370-382 aa) InterPro GO terms- Biological Process- protein phosphorylation Molecular Function- protein kinase activity, ATP binding, calcium ion binding PANTHER GO terms- Biological Process- peptidyl-serine phosphorylation, intracellular signal transduction, protein autophosphorylation Molecular Function- calcium-dependent protein serine/threonine kinase activity, calmodulin-dependent protein kinase activity, calmodulin binding Cellular Component- cytoplasm, nucleus		
15.	BGIOSGA017654	Beta-galactosidase	Representative domain- Glyco_hydro_35 (41-366 aa) BetaGal_ABD_1 (414-533 aa) BetaGal_gal-bd (581-645 aa) Conserved site- Glycoside hydrolase, family 35, conserved site (178-190 aa) InterPro GO terms- Biological Process- carbohydrate metabolic process Molecular Function- hydrolase activity, hydrolyzing O-glycosyl compounds, beta-galactosidase activity PANTHER GO terms- Molecular Function- beta-galactosidase activity Cellular Component- vacuole		

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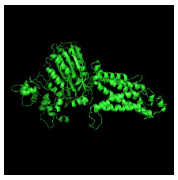
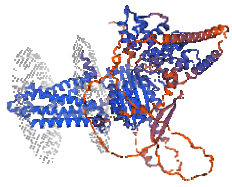
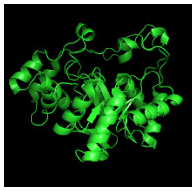
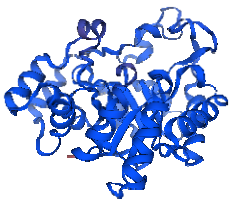
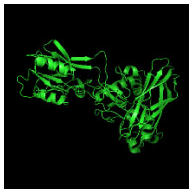
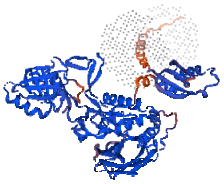
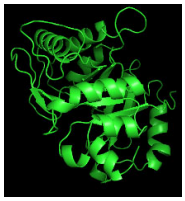
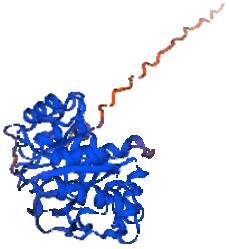
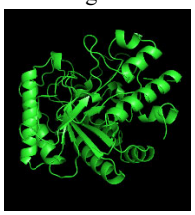
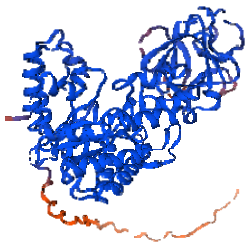
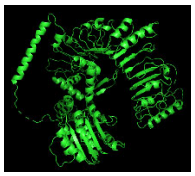
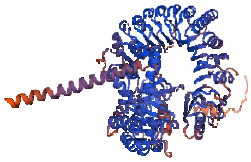
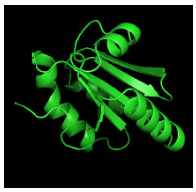
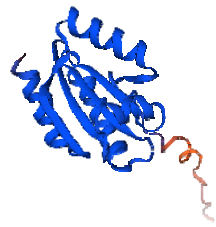
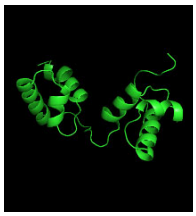
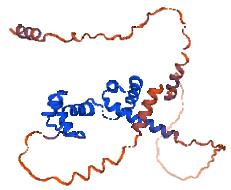
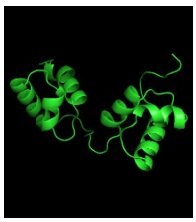
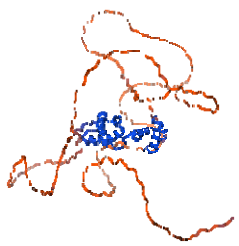
Table 1 — Structural and functional annotation of the selected gene IDs (*Contd.*)

Sl. No.	Gene ID	Putative protein name	Active domains and GO annotations	Structure predicted by de-novo modelling	Structure predicted by homology-based modelling
16.	BGIOSGA017692	Respiratory burst oxidase	Representative domain- EFh (249-296 aa) NOX_Duox_like_FAD_NADP (630-893 aa) NADPH oxidase Respiratory burst (170-269 aa) Ferric reductase-like transmembrane component (437-583 aa) FAD-binding 8 (626-741 aa) FAD-binding domain, ferredoxin reductase-type (623-743 aa) Ferric reductase, NAD binding domain (747-933 aa) Binding site- EF-Hand 1, calcium-binding site (279-291 aa) InterPro GO terms- Molecular Function- oxidoreductase activity, acting on NAD(P)H, oxygen as acceptor; peroxidase activity, calcium ion binding Cellular Component- membrane PANTHER GO terms- Molecular Function- NAD(P)H oxidase H2O2-forming activity Cellular Component- plasma membrane		
17.	BGIOSGA017928	Putative oxidase reductase/ aldo-keto reductase	Representative domain- AKR_AKR4C1-15 (3-290 aa) NADP-dependent oxidoreductase domain (17-283 aa) Aldo/keto reductase family (17-283 aa) Conserved site- Aldo/keto reductase, conserved site (39-56 aa, 139-156 aa, 252-267 aa) InterPro GO terms- Molecular Function- oxidoreductase activity PANTHER GO terms- Molecular Function- alditol:NADP+ 1-oxidoreductase activity Cellular Component- cytosol		
18.	BGIOSGA017986	Subtilisin like protease	Representative domain- SUBTILASE (1-405 aa) fn3_6 (451-553 aa) Peptidase S8 propeptide/proteinase inhibitor I9 (38-108 aa) Peptidase S8/S53 domain (108-374 aa) Subtilisin-like protease, fibronectin type-III domain (451-553 aa) Active site- Serine proteases, subtilase family, serine active site (334-344 aa) InterPro GO terms- Biological Process- proteolysis Molecular Function- serine-type endopeptidase activity, serine-type peptidase activity		
19.	BGIOSGA018439	Xylanase inhibitor/chitinase	Representative domain- GH18_2 (25-297 aa) Glycoside hydrolase family 18, catalytic domain (25-297 aa) Glycosyl hydrolases family 18 (27-189 aa) Chitinase Cts1-like (25-281 aa) GH18_hevamine_XipI_class_III (25-281 aa) InterPro GO terms- Biological Process- carbohydrate metabolic process PANTHER GO terms- Molecular Function- chitinase activity Cellular Component- extracellular region		

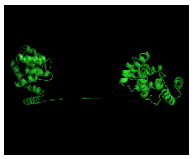
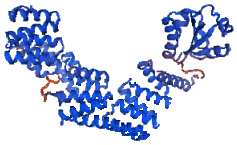
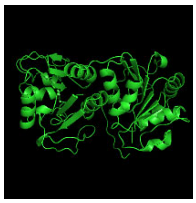
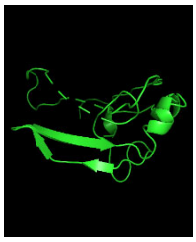
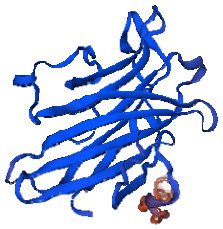
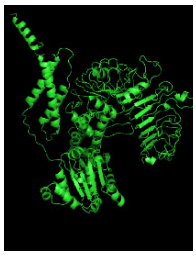
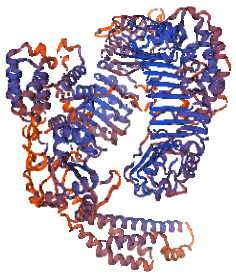
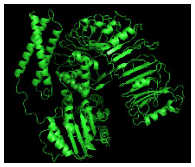
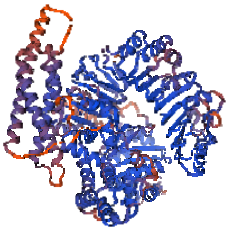
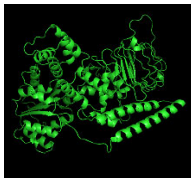
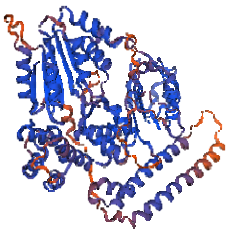
(*Contd.*)

Table 1 — Structural and functional annotation of the selected gene IDs (*Contd.*)

Sl. No.	Gene ID	Putative protein name	Active domains and GO annotations	Structure predicted by de-novo modelling	Structure predicted by homology-based modelling
20.	BGIOSGA018452	1,3 beta-glucosidase	Representative domain- Cellulase (235-505 aa) Glycoside hydrolase, family 5 (235-505 aa) Conserved site- Glycoside hydrolase, family 5, conserved site (354-363 aa) InterPro GO terms- Biological Process- organic substance metabolic process, carbohydrate metabolic process, actin filament organization Molecular Function- hydrolase activity, hydrolyzing O-glycosyl compounds, actin filament binding PANTHER GO terms- Biological Process- actin filament bundle assembly, establishment or maintenance of cell polarity, cell migration Molecular Function- actin filament binding Cellular Component- actin cytoskeleton, cytoplasm		
21.	BGIOSGA018518	RGA 5 (b)	Representative domain- NB-ARC (71-309 aa) InterPro GO terms- Biological Process- defence response Molecular Function- ADP binding		
22.	BGIOSGA018734	Thioredoxin H	Representative domain- THIOREDOXIN_2 (18-130 aa) Thioredoxin (26-126 aa) Conserved site- Thioredoxin, conserved site (48 – 66 aa)		
23.	BGIOSGA019054	MYB transcription factor	Representative domain- HTH_MYB (97-151 aa) SANT/Myb domain (44 – 98 aa, 101-149 aa) SANT (51-96 aa, 106-147 aa) Myb-like domain profile (44-96 aa, 97-147 aa) Myb-like DNA-binding domain (49-96 aa, 102-146 aa) Myb-type HTH DNA-binding domain profile (48 – 96 aa, 97-151 aa) InterPro GO terms- Biological Process- regulation of DNA-templated transcription Molecular Function- DNA-binding transcription factor activity, sequence-specific DNA binding PANTHER GO terms Cellular Component- nucleus		
24.	BGIOSGAO19160	MYB transcription factor	Representative domain- HTH_MYB (62-116 aa) SANT/Myb domain (9-63 aa, 66-114 aa) SANT (17 – 61 aa, 69-109 aa) Myb-like DNA-binding domain (14-61 aa, 67-110 aa) Myb domain (9-61 aa, 62-116 aa)		

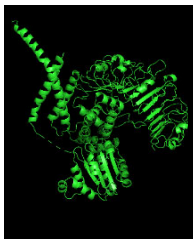
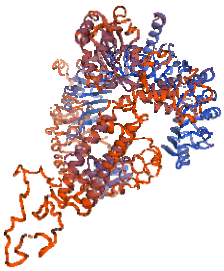
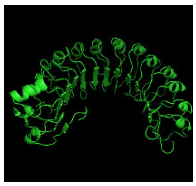
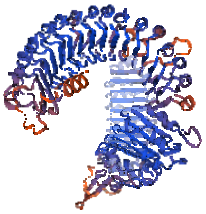
(*Contd.*)

Table 1 — Structural and functional annotation of the selected gene IDs (*Contd.*)

Sl. No.	Gene ID	Putative protein name	Active domains and GO annotations	Structure predicted by de-novo modelling	Structure predicted by homology-based modelling
25.	BGIOSGA019365	TPR repeat-containing thioredoxin TTL	Representative domain- tpr_5 (9 – 42 aa, 153 – 186 aa, 201 – 234 aa, 247 – 280 aa, 315 – 348 aa) TPR_19 (57 – 103 aa) TPR (281 – 314 aa) TRX_family (386 – 473 aa) Thioredoxin domain (397 – 466 aa) InterPro GO terms- Molecular Function- protein binding PANTHER GO terms- Cellular Component- cytoplasm		
26.	BGIOSGA020206	DJ-1 domain-containing protein	Representative domain- DJ-1_PfpI (25 – 189 aa) GATase1_DJ-1 (230 – 392 aa) PANTHER GO terms Biological Process- glyoxal metabolic process Cellular Component- cytoplasm		
27.	BGIOSGA028477	Cytochrome b561 and DOMON domain-containing protein	Representative domain- PROKAR_LIPOPROTEIN (1 – 35 aa) DOMON_CIL1_like (46 – 203 aa) AIR12, DOMON domain (46 – 203 aa) Protein of unknown function (DUF568) (97 – 202 aa) DOMON domain (55 – 176 aa)		
28.	BGIOSGA035067	RGA 2	Representative domain- Rx_N (29 – 99 aa) NB-ARC (188 – 396 aa) InterPro GO terms- Biological Process- defence response Molecular Function- ADP binding		
29.	BGIOSGA028177	RGA 5 (a)	Representative domain- RX-CC_like (14 – 136 aa) NB-ARC (181 – 421 aa) Disease resistance, N-terminal (15 – 105 aa) InterPro GO terms- Biological Process- defence response Molecular Function- ADP binding		
30.	BGIOSGA036088	RGA 5 (c)	Representative domain- RX-CC_like (1 – 86 aa) NB-ARC (124 – 368 aa) Disease resistance, N-terminal (1 – 53 aa) InterPro GO terms Biological Process- defence response Molecular Function- ADP binding		

(*Contd.*)

Table 1 — Structural and functional annotation of the selected gene IDs (*Contd.*)

Sl. No.	Gene ID	Putative protein name	Active domains and GO annotations	Structure predicted by de-novo modelling	Structure predicted by homology-based modelling
31.	LOC4328098	RGA 3	Representative domain- Rx_N (20 – 96 aa) NB-ARC (234 – 477 aa) InterPro GO terms Biological Process- defence response Molecular Function- ADP binding		
32.	LOC4350068	RGA 1	Representative domain- RX-CC_like (87 – 208 aa) NB-ARC (248 – 487 aa) LRR_typ_2 (644 – 666 aa, 923 – 947 aa, 1285 – 1308 aa) LRR_8 (803 – 845 aa) LRR_CC_2 (1356 – 1383 aa) Disease resistance, N-terminal (90 – 165 aa) InterPro GO terms Biological Process- defence response to fungus Molecular Function- protein binding, ADP binding		

(Genes selected for gene expression study are highlighted in light green colour)

Table 2 — Primers designed for performing RT-PCR of the ten selected genes for gene expression studies

S. No.	Gene	Forward Primer	Sequence (5′-3′)	Reverse Primer	Sequence (5′-3′)
1	RGA 1	RGA 1F	CCGCGAAATTGGTTACTACG	RGA 1R	CTGCAGTGCTTGATTTGTGC
2	RGA 2	RGA 2F	ACAATGATGAGCGTGTGGTG	RGA 2R	TCCATGATCTCGGCTGTATG
3	RGA 3	RGA 3 F	CATTAAAGGGCAACCCACTG	RGA 3R	TTTCCTCCAGTGCTCAAAGC
4	RGA 4	RGA 4 F	TTGGCTGGGAGATTATGGAC	RGA 4 R	AATACGGCCCTCCATTCTTC
5	RGA 5(a)	RGA 5(a) F	TCGGCAATTATCCAGAGGAC	RGA 5(a) R	CATATCATGCACCCTGCAAC
6	RGA 5(b)	RGA 5(b) F	GGTGGTCAATTGTGCTTTCC	RGA 5(b) R	TGCATGCTGTGAAGATAGCC
7	RGA 5(c)	RGA 5(c) F	TGTCCTGCTGATTTGTGTGG	RGA 5(c) R	TTGACCTTGTCCCATTCCTC
8	RUST R	RUST R _F	GCATAATCGGGCTACATTGC	RUST R _R	AATGGTCTTGGAGGCATCTG
9	GDSL E	GDSL E _F	AAGCTTATGTGCTGGGCAAG	GDSL E _R	TTTGGGAACCCTTGTCGTAG
10	Cb561	Cb561 _F	TCCTCCTCATCGAGTTCGTC	Cb561 _R	AGAACCGTGTCCAGGTCAAC

profiling. The function of Cytochrome b561 is not understood properly yet, so we have included that gene as well under our study to see whether this gene is related to bakanae defence response or not.

Primer designing for RT-PCR

The selected genes (Table 1) were used to design forward and reverse primers using the software Primer 3 plus (www.bioinformatics.nl/primer3plus). By analysing the melting temperature (60°C or more) and the GC content (50% or more), suitable primers were selected (Table 2). Primer size was limited to 20 base pairs (bp). Self-complementarity was checked using the Oligo Calc tool. The primers were synthesised by Eurofins Genomics India Pvt. Ltd. in India. The primer stock (100µM) was diluted in a 1:10 ratio for the experiment and stored at -20°C until further use.

Rice genotypes and fungal isolates

Seeds of four different rice genotypes, *viz*., C101A51, Pusa 1342, PB1, and PB1121 with disease reaction type as highly resistant (HR), resistant (R), susceptible (S) and highly susceptible (HS) to bakanae were selected[22,33]. Two isolates of *Fusarium fujikuroi,* F1121 and F1728 were used for artificial inoculation. The isolates belong to the same pathotype, *i.e*., pathotype 2, one of the most prevalent pathotypes of the fungus in Indian context[34].

Inoculation and sample collection

The pathogen was subcultured on Potato Dextrose Agar (potato 200 g, dextrose 20 g, agar 20 g, and water 1000 mL) medium. The cultured plates were incubated in BOD incubator (Thermotech) at 25±1°C in the dark for 8 days to allow for growth and

sporulation[10,12,35]. The *F. fujikuroi* isolates (F1121 and F1728) were inoculated for mass multiplication in sterilized sorghum grains and incubated at 25°C. Spore suspension was prepared with sterile distilled water using 15 days old culture of *F. fujikuroi* (F1121 and F1728) and filtered through two layers of sterile muslin cloth and brought to a final concentration of 10^6 spores mL^{-1} for the seed inoculation. The seeds of rice cultivars were first surface sterilized with 1% (*v/v*) sodium hypochlorite solution followed by washing with sterile distilled water and soaked into the *F. fujikuroi* inoculum suspension for 24 h at room temperature (25°C). They were further transferred to moist chambers with germination paper in Petri plates For real-time PCR, samples were collected at specified time points of 5, 7, and 10-days post-inoculation (dpi) for qPCR-based expression studies. Seed samples were carefully wrapped in aluminium foil and dippped in Liquid nitrogen. Subsequently, the samples were stored at -80°C.

RNA extraction and quantification

Total RNA was extracted from infected leaves using the Trizol method (Invitrogen). All lab equipment, including Eppendorf tubes, spatulas, microcentrifuge tubes, pestles, and mortars, was sterilized with 0.1 percent DEPC (Diethyl Pyro Carbonate) treatment to deactivate RNase enzymes. These materials were then autoclaved at 121°C and 15 psi for 15 min. Infected samples were homogenized in a pre-cooled mortar and pestle with liquid nitrogen until the nucleoprotein complex was fully dissolved. Approximately 100 mg of the finely ground powder was added to a 1.5 mL Eppendorf tube with 1 ml of Trizol reagent and incubated at room temperature for 5 min. Phase separation was achieved by adding 0.2 ml of chloroform per 1 mL of Trizol, followed by vigorous shaking for 15 seconds and centrifugation at 12,000 g for 15 min at 4°C, after a 2-3 minute chill. The aqueous phase was transferred to fresh, pre-chilled tubes containing 0.5 ml of isopropanol and centrifuged at 12,000 g for 10 min at 4°C. The RNA pellet was retained after removing the supernatant. The pellet was washed with 1 ml of 75% ethanol and centrifuged at 7,500 g for 5 min at 4°C. After removing the wash, the pellet was air-dried for 10 min and then dissolved in 30 µL of DEPC-treated water and stored at -80°C. The RNA concentration was measured using a NanoDrop-2000 Spectrophotometer (Thermo Fisher Scientific, USA).

c-DNA preparation, quantification and dilution

The RNA template was converted into cDNA using Verso cDNA synthesis kit (Thermo Scientific, USA; Verso cDNA) following the instructions manual. The first strand cDNA was synthesised with the use of oligo dT primers.

RT-PCR and gene expression analysis

RT-PCRs were performed for each selected gene IDs with the respective designed forward and reverse primers using the SYBR green technique in the Bio-Rad thermal cycler. The total reaction volume was 20 µL, which consists of 10 µL SYBR, 1 µL each of forward and reverse primers, 1 µL template cDNA, and 7 µL nuclease-free water. The reactions were performed in the following conditions- a single cycle of denaturation for 10 mins at 95°C, followed by 40 cycles of 3 steps of PCR; denaturation at 95°C for 15 sec, annealing at 58°C for 30 sec, and finally extension at 72°C for 30 sec. After performing RT-PCR to analyze gene expression, the mean values of the biological replicates were calculated for each sample. Housekeeping gene GAPDH was taken as a control and the target gene expression was measured using the $2^{-\Delta\Delta Ct}$ method[36]. Then, ΔΔCt values for each treatment were calculated by deducting each individual's ΔCt value from the ΔCt value of the calibrator, which served as the negative control. ΔΔCt was evaluated using the formula below:

$$\Delta\Delta Ct = (C_{t\ target} - C_{t\ reference})_{\ Time\ x} - (C_{t\ target} - C_{t\ reference})_{\ Time\ 0}$$

where time x can be any time point and Time 0 is the 1x expression of the target gene normalised to the reference gene. Error bars represent the standard deviation of each of the three independent biological experiments, with each experiment including two technical replicates.

A fold change greater than 1 indicates upregulation (*i.e.*, the gene is expressed at a higher level in 7 and 10 dpi compared to the 5 dpi), while a fold change less than 1 indicates downregulation.

Results

Bioinformatics analysis for the selected genes

The selected 32 gene IDs were confirmed for their putative nature and function *via* BLAST and InterPro analysis (Table 1). They were primarily confirmed to be associated with defence responses. Ten out of the thirty-two proteins were selected for the gene expression analysis based on their importance

putatively annotated to them, with a major focus on RGAs. The most important groups coming up from the BLAST analysis are discussed below.

Genes involved in hydrolase activity and carbohydrate metabolism

It has been long established that enzymes showing hydrolase activities like chitinase or glucanases have important role in defence against fungi *via* direct attack on their cell wall[37]. Gene IDs BGIOSGA000133, BGIOSGA005366, BGIOSGA017654, and BGIOSGA018452 were predicted to have hydrolase activity. The genes code for Endo-1,3-beta-glucanase, GDSL esterase/lipase, beta-galactosidase, and 1,3 beta-glucosidase respectively. Except for GDSL esterase/lipase, rest three predicted proteins are supposed to have carbohydrate metabolism activity as well. Plant lipases are important in synthesizing signalling lipids that contribute to plant defence[38]. Earlier studies indicated that GDSL esterase/lipase might act as negative regulators of resistance against fungus and bacteria in rice[39]. Considering all these factors, we selected BGIOSGA005366 for gene expression study to define its plausible role in defence against bakanae disease.

Genes having kinase activity

Protein kinases play a central role in signalling during pathogen recognition and the subsequent activation of plant defence mechanisms[40]. Five gene IDs were predicted to possess kinase domains, *viz*., BGIOSGA001477, BGIOSGA002512, BGIOSGA003923, BGIOSGA007118 and BGIOSGA017475. Among these genes, BGIOSGA002512 is putatively annotated as rust resistance kinase indicating a probability of its strong influence in the defence action. Therefore, this gene has been selected for gene expression analysis against *F. fujikuroi* in rice.

Genes containing Rx_N domain

Rx_N domain has an important role in inducing programmed cell death leading to hypersensitive plant resistance responses[41]. Five gene IDs were found to have Rx_N domains, *i.e*., BGIOSGA001667, BGIOSGA005356, BGIOSGA007396, BGIOSGA035067, and LOC4328098. Among them, BGIOSGA001667, BGIOSGA035067 and LOC4328098 coding for RGA 4, RGA 2 and RGA 3 were selected for further gene expression studies. They possess only Rx_N domain and not the Rx_CC domain, which would help us to interpret the individual role of Rx_N domain and Rx_CC domain separately, if any, towards defence against *F. fujikuroi*.

Genes containing Rx_CC domain

The CC domain has been proposed to initiate signalling for defence responses[41]. Four gene IDs were predicted to have Rx_CC domain, among them three IDs, *viz*., BGIOSGA028177, BGIOSGA036088, and LOC4350068 coding for RGA 5(a),RGA 5(c) and RGA 1 respectively, had only Rx_CC domain and not the Rx_N domain. So, we have taken them into account to clearly establish whether Rx_CC domain has some role in defence against bakanae disease.

Genes having NB-ARC domain

The NB-ARC domain is a functional ATPase domain, and its nucleotide-binding state is proposed to regulate activity of the R protein[42]. The gene IDs which are having either Rx_CC and/or Rx_N domains also possess NB-ARC domain. The gene ID BGIOSGA018518 predicted to code for RGA 5(b) is a unique gene that only possesses NB-ARC domain but neither Rx_N nor Rx_CC domain. A total of seven out of the selected ten genes have NB-ARC domain.

Genes having LRR domain

The LRR domain has long been hypothesized to be involved in specific recognition of pathogen effect or molecules[43]. Gene IDs BGIOSGA007118 and LOC4350068 were predicted to have LRR domain, among them the later one coding for RGA 1 has been taken for further gene expression studies.

Genes having ADP/ATP binding site

The activation and oligomerization of resistance genes is energy requiring process and thus ADP-ATP exchange plays an important role in defence response[44]. All the seven selected RGAs under study possess ADP binding site.

Genes having cytochrome site

Gene ID BGIOSGA007962 coding for ent-cassadiene hydroxylase-like protein possesses conserved site of Cytochrome P450. This site has an established role in plant metabolism and thus helping in production of large number of secondary metabolites helping in plant defence[45]. One more gene ID BGIOSGA028477 is found to code for Cytochrome

b561 and DOMON domain-containing protein. Physiological functions supported by such proteins include stress defence, cell wall modifications, iron metabolism, *etc*.[46] which might have good role providing defence against bakanae disease in rice. Thus, this is also incorporated in the list of selected genes for further gene expression studies.

Real time PCR based gene expression analysis

Gene expression analysis was performed with designed primers for each selected gene The obtained data for the individual genes are discussed below along with graphical (Fig. 1A, I-J.iv) and heat map representation (Fig. 2).

Interestingly, in the case of isolate F1121, RGA 5(b) was highly upregulated in 10 dpi (229-fold increase) in C101A51, with instant downregulation at 7 dpi followed by upregulation at 10 dpi in P1342 and PB1 and complete downregulation in PB1121 (Fig. 1F and Fig. 2A). An almost similar pattern was followed by RGA 5(c) where C101A51, PB1 and P1342 showed downregulation at 7 dpi followed by upregulation at 10 dpi, whereas in the case of PB1121, it showed constant downregulation (Fig. 1G and Fig. 2A). RGA 4 got upregulated in 7 dpi and 10 dpi in all genotypes irrespective of their resistance level (Fig. 1D and Fig. 2A). An almost similar pattern can be observed for RGA 5(a) as well except for cultivar PB1 (Fig. 1E and Fig. 2A).

RUST R gene showed constant upregulation at 10 dpi in all genotypes; except for PB1, the upregulation followed a drop of expression level at 5 dpi (Fig. 1H and Fig. 2A). RGA 1 is getting upregulated in 10 dpi in PB1121 (4.5-fold increase) and C101A51 (6.3-fold

Fig. 1 — Differential expression plots of 10 RGA loci among four rice genotypes (with reliable R 2 and E-values) on 7 days and 10 days as compared to 5 days post inoculation with the *F. fujikuroi* isolates, F1121 and F1728 represented with the blue bar and the orange bar, respectively. Alphabets denote the ten selected genes studies for gene expression [a= RGA 1, b=RGA 2, c=RGA 3, d=RGA 4, e= RGA 5(a), f=RGA 5(b), g=RGA 5(c), h=RUST R, i=GDSL E, j=Cb561] and Roman numbers denote rice germplasms [i=PB1121 (HS), ii=C101A51 (HR), iii=PB1 (S), iv=Pusa 1342 (R)] (*Contd*.)

Fig. 1 — Differential expression plots of 10 RGA loci among four rice genotypes (with reliable R 2 and E-values) on 7 days and 10 days as compared to 5 days post inoculation with the *F. fujikuroi* isolates, F1121 and F1728 represented with the blue bar and the orange bar, respectively. Alphabets denote the ten selected genes studies for gene expression [a= RGA 1, b=RGA 2, c=RGA 3, d=RGA 4, e= RGA 5(a), f=RGA 5(b), g=RGA 5(c), h=RUST R, i=GDSL E, j=Cb561] and Roman numbers denote rice germplasms [i=PB1121 (HS), ii=C101A51 (HR), iii=PB1 (S), iv=Pusa 1342 (R)] (*Contd*.)

increase), whereas, it showed 3.8-fold upregulation at 7 dpi in the case of P1342 followed by equivalent expression at 10 dpi as compared to 5 dpi. It is showing subsequent downregulation in the case of PB1 (Fig. 1A and Fig. 2A). RGA2 showed downregulation on the 10 dpi for both PB1121 (0.03-fold) and P1342 (0.46-fold) whereas, it showed upregulation in C101A51 and PB1 by 12.6-fold and 17.4-fold, respectively (Fig. 1B and Fig. 2A). RGA3 showed strong upregulation in PB1 on 7 (4.25-fold) and 10 dpi (35-fold), but upregulation only on 10 dpi in C101A51 (25-fold) and P1342 (7.4-fold) (Fig. 1C and Fig. 2A). GDSL E was found to be constantly downregulated in P1342 and PB1121, whereas it showed upregulation on 10 dpi in C101A51 and PB1 by 1.9-fold and 5.5-fold (Fig. 1I and Fig. 2A). The Cb561gene showed constant downregulation in all the genotypes selected except for C101A51 at 5 dpi showing upregulation of the particular gene by 3-fold (Fig. 1J and Fig. 2A).

In case of isolate F1728, RGA 2 showed upregulation on 10 dpi in all genotypes except PB1121 (Fig. 1B and Fig. 2B). RGA 3, RGA 5(c), GDSL E, and Cb561 showed mostly downregulation of expression with contrasting upregulation in PB1 (Fig. 1 and Fig. 2B). RGA 5(b) was upregulated irrespective of the cultivars except for downregulation of 0.21-fold at 10 dpi in C101A51 (Fig. 1F and Fig. 2B).

The progression of expression of RGA 4, RGA 5(b), RUST R, and Cb561 for both the isolates were similar in cultivars PB1 and P1342. The results of RGA 5(c) showed consistency of pattern between both the isolates in cultivar PB1121 and PB1. RGA 3

and RGA 5(a) showed a similar pattern of expression against both the isolates in cultivar P1342 only. RGA 2 showed the correlation of results between two isolates in C101A51, whereas, the same happened for GDSL E in the PB1121 cultivar (Fig. 1). Bakanae disease, caused by *Fusarium fujikuroi*, is prevalent in rice-growing regions around the world, including South and East Asian countries such as India[12], Pakistan[47], Bangladesh[48], and South Korea[49].

Although numerous studies have investigated potential sources of resistance to bakanae disease, there are very few identified sources, and the mechanisms of pathogen recognition and the subsequent downstream pathways involved in disease establishment or resistance remain largely unclear. Therefore, the present study was undertaken to identify potential resistance gene analogues (RGAs) and other critical defence-related genes involved in the early infection of *Fusarium fujikuroi* in rice seeds. The RGAs and defence-related genes were selected from the published literature of transcriptome analysis of resistant and susceptible cultivars against bakanae disease[22]. The study was performed using resistant source C101A51 and susceptible genotype Rasi using F250 isolate of *F. fujikuroi* at different time point of the vegetative stage of growth. Our study was performed at seed germination stage with two different isolates belonging to same pathotype, F1121 and F1728, along with four different types of genotypes of different disease reactions.

Based upon the literature which indicated the disease appearance starts from 7 dpi[10,50], we took three different time intervals, *i.e*., 5 dpi, 7 dpi, and 10 dpi. Different criteria were taken into consideration while selecting the genes, *viz*.- hydrolase and carbohydrate metabolism, kinase activity, possessing different domains like Rx_N, Rx_CC, NB-ARC, LRR, ATP/ADP binding sites and cytochrome sites. From the qRT-PCR data across various combinations of different genotypes of varying disease response and two different isolates, RGA 2, RGA 5(b) and RGA 5(c) can be concluded as potential candidate RGAs involved in providing resistance against bakanae in the early germination stage. GDSL E can play role as negative regulation in early stages of rice-*F. fujikuroi* interaction.

Rx_N domain has an important role in inducing programmed cell death leading to hypersensitive plant resistance responses[41]. The selected RGA 2 for the molecular study was predicted to have Rx_N, NB-ARC, and ADP binding sites. RGA2 showed downregulation on the 10 dpi for both PB1121 (0.03-fold) and P1342 (0.46-fold) whereas, it showed upregulation in C101A51 and PB1 by 12.6-fold and 17.4-fold, respectively. Cb561 got upregulated on 5 dpi in resistant genotype C101A51 against F1121 isolate and on 5 and 10 dpi in susceptible PB1 against F1728 isolate. Bashyal *et al*.[22] reported upregulation of these genes in inoculated susceptible cultivar when compared to inoculated resistant genotypes. This indicates that RGA 2 and Cb561 might change the level of expression based on the growth stages of the host and might behave differentially in regulating the disease.

The CC domain has been proposed to initiate signalling for defence responses[41]. RGA 5(c) was supposed to have Rx_CC domain instead of Rx_N, and RGA 5(b) has neither of them. So, this might be correlated that the presence of NB-ARC and ADP binding sites might have important role in conferring resistance against the disease. Gene expression analysis revealed that for isolate F1121, RGA 5(b) exhibited a notable 229-fold increase at 10 dpi in C101A51, with an initial drop at 7 dpi followed by a rise at 10 dpi in P1342 and PB1, and complete downregulation in PB1121. RGA 5(c) showed a similar pattern, with downregulation at 7 dpi in C101A51, PB1, and P1342, followed by upregulation at 10 dpi, while remaining constantly downregulated in PB1121. It has been previously reported in cavendish banana that transgenic expression of RGA2 provides resistance against Fusarium wilt tropical race 4[51]. Pepper CaRGA2 shows resistance towards Phytophthora blight[52]. RGA2 was also found to be essential for the functioning of *Lr10*, the leaf rust-resistant gene of wheat[53]. This was also identified as the strongest candidate gene for the peach powdery mildew resistance gene *Vr3*[54].

RGA 5 are reported to recognize effectors like AVR-Pia and AVR1-CO39 secreted by *Magnaporthe oryzae* causing rice blast[55]. It is also reported to interact with other effectors like AVR-PikD and AvrPib[56]. RGA4/RGA5 hetero pair is also reported to confer resistance against rice bacterial leaf blight and leaf streak[57].

RGA 3 and RGA 4 possess only Rx_N domain and not the Rx_CC domain[41]. Expression of RGA 3 was found more in resistant genotype C101A51 in earlier studies[22]. In our case, the results were found relatable

in resistant cultivar P1342 in both the isolates and in C101A51 against isolate F1121. RGA 4 showed upregulation of expression against both the isolates in all cultivars except the resistant one (C101A51). This result can be highly supported by the previous transcriptome study where RGA 4 showed upregulation in comparison between inoculated conditions of resistant and susceptible genotypes[22].

Plant lipases are important in synthesizing signalling lipids that contribute to plant defence[38]. Earlier studies indicated that GDSL esterase/lipase might act as negative regulators of resistance against fungus and bacteria in rice[39]. The gene coding for GDSL E, was mostly downregulated in all genotypes. This might be correlated with the study done with two GDSL esterases/lipases, *OsGLIP1* and *OsGLIP2,* where inoculation of rice with bacterial and fungal pathogens led to downregulation of these genes, proving them as potential negative regulators of rice immunity[39].

Further, The RUST R gene consistently showed upregulation at 10 days post-inoculation (dpi) across all genotypes. However, in the PB1 genotype, this upregulation was preceded by a decrease in expression at 5 dpi. This gene is putatively annotated as rust resistance kinase indicating a probability of its strong influence in the defence action.

Bashyal *et al*.[8] examined the expression of plant defence-related enzymes and pathogenesis-related genes using various biocontrol agents. They found higher activity of these enzymes and less expression of pathogenicity-related genes in biocontrol treatments compared to inoculated control against rice sheath blight disease. Bashyal *et al*.[58] also evaluated biocontrol agents against drought stress[59] in resistant and susceptible rice varieties. *Trichoderma harzianum* 2 (IRRI 2) was found to delay drought by reducing Malondialdehyde, reactive oxygen species, electrical conductivity, and proline concentrations.

Utilising qRT-PCR data across various combinations of different genotypes of varying disease response and two different isolates, RGA 2, RGA 5(b), and RGA 5(c) can be concluded as potential candidate RGAs involved in providing resistance against bakanae in the early germination stage. GDSL E can play role as negative regulation in the early stages of rice-*F. fujikuroi* interaction.

These RGAs may be specific to the seed and germination stages, whereas other genes might be linked only to the vegetative stage and disease progression. This discovery highlights the need for further research into evaluating defence-related gene expression over time, according to the host's growth stage. The RGAs and other defence-related genes along with negative regulator found in the study can be further utilized in genome editing approaches[9,60] for achieving effective and affordable management of rice bakanae.

Conclusion

This study focused on 32 genes selected from transcriptome data of rice genotypes inoculated with bakanae disease, including both resistant and susceptible types. These genes were analyzed using *in silico* methods to examine their structure and identify potential domains. The predicted functions of these genes could provide useful information for further investigation into their involvement in the rice plant's defence mechanisms against the pathogen. Further, gene expression studies of ten genes comprising seven RGAs and three other defence-related genes, and could correlate the expression of RGA 2, RGA 5(b) and RGA 5(c) with the varietal resistance at the early germination stage. Our study also identified one negative regulator, GDSL easterase. This information requires further validation at other growth stages to assess disease progression comprehensively. Such validation will offer additional evidence for selecting key RGAs in resistance breeding programs. Further functional studies on the identified genes, along with their application through various methodologies, will be valuable for developing effective strategies for managing bakanae disease.

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Conflict of interest

All authors declare no conflict of interest.

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