ORIGINAL ARTICLE

Identification and expression analysis of pathogenicity-related genes of *Rhizoctonia solani* anastomosis groups infecting rice

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Abstract

Sheath blight disease caused by *Rhizoctonia solani* Kuhn (teleomorph; *Thanatephorus cucumeris*) is a major constraint in rice production. Among the different anastomosis groups (AGs) of *Rhizoctonia solani*, AG1-IA causes sheath blight of rice, which induce necrotic lesions on leaf sheaths of the infected plants. Several reports contradict the host specificity of anastomosis groups in *Rhizoctonia solani*. There is lack of information on the pathogenicity genes of these *Rhizoctonia solani* anastomosis groups during sheath blight infection in rice. In the present study, *Rhizoctonia solani* isolates collected from diverse rice growing regions of India were screened for anastomosis groups and two groups namely, AG1-IA, AG2-2 were identified. Accordingly, comparative studies were made with AG1-IA (GenBank ID: 16,395) and AG2-2 (GenBank ID: 2,318,768) group sequences, which enabled the identification of specific gene clusters (119 in AG1-IA and 604 in AG2-2) belonging to these groups. Pathogen Host Interaction (PHI) blast with these specific gene clusters could further identify genes involved in host pathogen interaction (38 in AG1_IA and 150 in AG2-2), which were shortlisted for qRT-PCR validation based on qcov cutoff values representing different phenotypic categories of PHI blast. Expression analysis-based validation in sheath blight susceptible (Pusa Basmati 1) and resistant (Pusa 1908-13-12-5) rice genotypes showed that most of the genes expressed significantly higher in the susceptible variety Pusa Basmati 1. The genes like inorganic phosphate transporter (*AG1_IPT*), Bromodomain containing protein (*AG1_BrD*), Aldehyde dehydrogenase (*AG1_AldD*), AMP binding domain (*AG1_AMP*) and Heme peroxidase (*AG1_HmPr*) were upregulated in the susceptible genotype, PB 1 at 72hpi compared to Pusa 1908–13-12–5. Among these, inorganic phosphate transporter (*AG1_IPT*), Bromodomain containing protein (*AG1_BrD*) and Heme peroxidase (*AG1_HmPr*) were specific to *Rhizoctonia solani* AG1-IA. Through the present study, we could demonstrate the AG1-IA-specific interactions of *Rhizoctonia solani* causing sheath blight disease of rice, which is a step forward in understanding the specificity of *Rhizoctonia solani* with reference to sheath blight disease of rice.

Keywords AG1-IA · AG2-2 · Inorganic phosphate transporter · PB 1 · Sheath blight

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Introduction

Rice is a staple food for millions of people throughout the world. Biotic stress causes major yield losses in rice. Sheath blight of rice is one of the important diseases of rice, caused by *Rhizoctonia solani* Kuhn (teleomorph; *Thanatephorus cucumeris*). It causes around 50% losses in rice yield (Lee and Rush 1983; Groth 2008; Chen et al. 2016). Reduction in rice grain yield around 6 million tons and serious damage to irrigated paddy was reported in Eastern Asia.

Rhizoctonia solani species differ from other fungi with presence of multiple nuclei and right angle branching of the hyphae (Anderson 1982; Ogoshi 1987; Cubeta and Vilgalys l997). The identification and classification methods based on differences in pathogenicity, morphology, cultural appearance and physiology were proposed but the widely used method is the classification of isolates based on anastomosis grouping (Ogoshi 1987). In anastomosis group (AG), isolates are closely related and they are grouped based on the ability of isolates to fuse with other isolates (Carling 1996; Cubeta and Vilgalys 1997). *Rhizoctonia solani* consist of 14 anastomosis groups (Carling 1996; Carling et al. 2002), which are distributed worldwide. The anastomosis group 1 causes diseases such as sheath blight, banded leaf, aerial blight and brown patch in many plants (Ogoshi 1987; Akhtar et al. 2009). Based on colony morphology, biochemical, genetic and pathogenicity, *Rhizoctonia solani* AG-1 has been further divided into six sub-groups, namely IA, IB, IC, ID, IE and IF (Ogoshi 1987; Yang and Li 2012; Wibberg et al. 2013). *Rhizoctonia solani* AG1-IA causes sheath blight of rice, induces necrotic lesion on leaf blades and leaf sheaths of infected plant. AG 2 isolates of *Rhizoctonia solani* causes a root canker and many of them cause root diseases on crucifers. Several studies observed that anastomosis groups in *Rhizoctonia solani* are not host specific (Parmeter et al. 1969; Grisham and Anderson 1983). For example, AG-1 isolates were highly virulent on kidney bean, weakly to moderately virulent on red pepper, soybean and sugar beet but avirulent on soybean leaves, cabbage and radish. AG-4 isolates were more virulent and had a wider host range than isolates belonging to other anastomosis groups. Isolates of AG-2 and AG-3 were shown to possess greater host specificity, whereas, AG-1 and AG-4 lack such host specificity (Bolkan and Ribeiro 1985).

Comparative genomics studies are helpful in understanding the molecular mechanisms of pathogenicity genes like AAA peroxisomal biogenesis protein involved in appressorium and toxin production, GLU glutathiones-s-transferase kappa 1 involved in inactivation and degradation of toxic compounds and these were upregulated in both *Rhizoctonia solani* AG1 and AG3 pathosystems at an early stage of pathogenesis (Rioux et al. 2011). In another study, 3942 genes were identified unique to *Rhizoctonia solani* AG1-IA infecting rice. Among these parvulin like protein *(RS_P1),* NifU like protein (*RS_P3)* and V-SNARE like protein (*RS_P4)* were identified as AG1-IA-specific highly upregulated pathogenicity genes through qRT-PCR (Ghosh et al. 2014). In the infection process, these genes express differentially and performed important molecular and biological function. However, information on interaction and the pathogenicity genes of these *Rhizoctonia solani* anastomosis groups during infection of rice is lacking. Therefore, we attempted to identify the pathogenicity related genes of *Rhizoctonia solani* anastomosis groups (AG1-IA and AG2-2) infecting rice in a set of rice genotypes contrasting for their reaction to sheath blight disease.

Materials and methods

Fungal isolates, rice genotypes

Rhizoctonia solani isolates were collected from diverse rice growing regions of India. Total 27 isolates were screened for disease severity (Supplementary Table 1). Potato Dextrose Agar (PDA) slants were used for culturing and maintaining of isolates and stored at 4 °C. Pusa Basmati 1 (Susceptible variety) and Pusa 1908-13-12-5 (ILS 12-5; tolerant genotype) were used for this study.

Anastomosis grouping

The *Rhizoctonia solani* isolates were classified into anastomosis groups according to the method described by Dubey et al. (2014 Supplementary Fig. 1).

Pathogenicity assay

Typha (*Typha angustifolia*) stem pieces were cut into 4–5 cm long, loosely filled ¾th of 0.5L conical flask and autoclaved at 121 °C, 15psi for 15 min. The fresh culture of *Rhizoctonia solani* was used for inoculation of the Typha bits in laminar air flow chamber and incubated at 26 ± 1 °C in BOD for 1 week. The Typha bits carrying the culture of *Rhizoctonia solani* were used as inoculum (Bhaktavatsalam et al. 1978). The inoculum (3 to 4 Typha bits) was placed at the base of the tillers in the rice genotypes, PB 1 and Pusa 1908-13-12-5 and typha bits were fastened to the tillers using the thread, thus establishing contact between the mycelium and rice plants. Three replicates were maintained for each isolate along with control (three inoculated hills per pot were considered as one replication and three replicates per isolates

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were evaluated). The relative lesion height (RLH) was calculated using the following formula

$$RLH = \frac{\text{Lesion height}}{\text{Plant height}} x100$$

DNA isolation: Autoclaved Potato Dextrose Broth (50 ml) grown mycelium of *Rhizoctonia solani* UP114 (AG1-IA), UP105 (AG2-2), RPBC 1 (AG3) and RMHM6 (AG5) were harvested and immediately stored at − 20 °C. All materials like pestle and mortar, spatula, eppendorf tubes were autoclaved at 121 °C, 15psi for 15 min. The mycelium of about 200 mg was homogenized with liquid nitrogen in a precooled mortar and pestle. The mycelium powder (100 mg) was taken into 50 ml eppendorf tube. About 10 ml of prewarmed isolation buffer (CTAB) was added to the tubes and incubated for 60 min with occasional stirring followed by Chloroform: Isoamyl alcohol (24:1) extraction for about 10 min and centrifuged at 10000 rpm in room temperature. The upper aqueous liquid was transferred into new tubes, added with 600 µL of ice-cold isopropanol, 100 µL of sodium acetate (3 M) incubated at − 20 °C for 30 min and centrifuged for 10 min in 4 °C at 10,000 rpm. The DNA pellet was retained and supernatant was discarded. The pellets were washed with 5 ml of 75% ethanol and centrifuged for 10 min in 4 °C at 10,000 rpm. The pellets were dried and dissolved in nuclease free water and stored at − 20 °C. The amount of DNA present in samples were recorded through Nano Drop − 2000 Spectrophotometer (Thermo Fisher Scientific, USA).

ITS sequencing and phylogenetic analysis

Internal Transcribed Spacer region of *Rhizoctonia solani* isolates were amplified using universal ITS primers, ITS1 and ITS4 described by White et al. (1990). The ITS sequence of the *Rhizoctonia solani* isolates was submitted to GenBank. Accession no MW479453 (UP114) and MH465659 (UP105) were used to construct the phylogenetic tree. Top ten nucleotide hits of UP114 and UP105 isolates were downloaded from NCBI database. MEGA 6 was used with Clustal W alignment and Neighbor joining method with bootstrap value of 1000 replicates.

Comparative studies for the identification of pathogenicity related genes

The *Rhizoctonia solani* isolates UP114 (AG-1_IA) and UP105 (AG-2_2) were selected (Fig. 1 and Supplementary Table 2) for comparative gene expression studies. The protein database UniProt (http://www.uniprot.org) was used to retrieve the proteome of *Rhizoctonia solani* AG-1_IA (GenBank ID: 16,395) and AG-2_2 (GenBank ID: 2,318,768). The proteomes of these anastomosis groups were classified into unique and common gene clusters through OrthoVenn (www.bioinfogenome.net/OrthoVenn) to compare the proteomes of both AG's and classified into specific and orthologous gene clusters. Specific proteins were functionally annotated as biological, molecular, cellular components and the top ten hits were identified in each category. To know the genes involved in host–pathogen (*Rhizoctonia solani*-rice) interaction Pathogen Host Interaction (PHI)-blast was performed. PHI-base accessions were retrieved from http://www.phi-base.org. Proteins database of *Rhizoctonia solani* AG-1_IA and AG-2_2 was used for local blast search with PHI database. Parameters used in PHI blast were: blastp_db_query with e value 0.001, Max HSPS 1, and output was tabular form. Query coverage (≥ 30) and e-value (1e-3) cut off was used to select the genes. Putative pathogenicity-related genes were identified using PHI database with e-value of $\leq 1e − 0$ in *Rhizoctonia solani* isolates. The phenotypic classes were grouped into increased virulence (Hypervirulence), unaffected pathogenicity, reduced virulence, lethal, loss of pathogenicity and represented in pie chart.

Fig. 1 *Rhizoctonia solani* anastomosis group isolates. **a** AG1-IA: UP114, **b** AG2-2: UP105
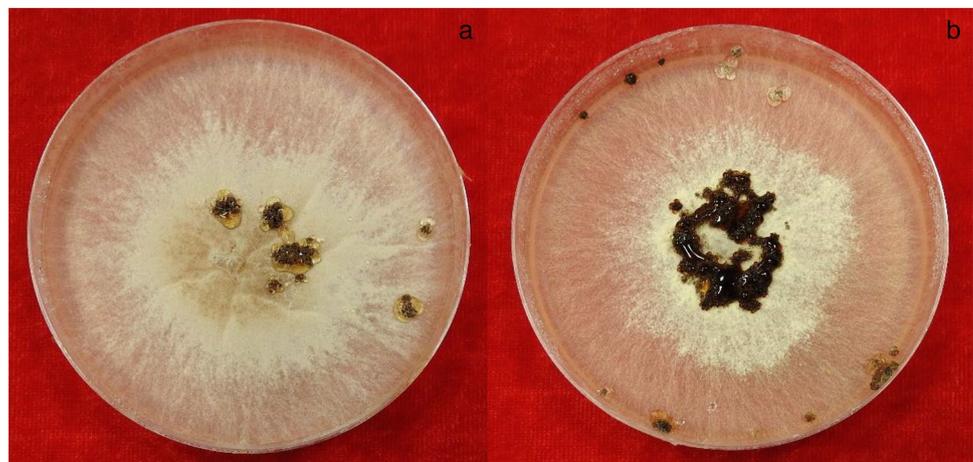
Primer designing

Primer3 Plus software (www.bioinformatics.nl/primer3plus) was used for primer synthesis. Nucleotide sequences were uploaded into the software, the programme was set to qRT–PCR and product size of 150–250 bp was selected. The primers (forward and reverse) showing least variations in different parameters like GC content, melting temperature, self-complementarity were considered and the specificity was confirmed through NCBI nucleotide blast. The primers were synthesized from GCC Biotech Pvt. Ltd., India. Stock primers were stored at − 20 °C and they were diluted in the ratio of 1:10 for the experiment. Total seven primers of AG-1_IA and eight primers of AG-2_2 were used (Supplementary Table 3). The housekeeping gene GADPH was also included and to amplify the *Rhizoctonia solani* cDNA, pathogen-specific 18S rDNA housekeeping gene (Supplementary Table 3, Gosh et al. 2014) was used. Along with these, primers from previous studies were also included (Supplementary Table 3, Rioux et al. 2011).

RNA isolation

Forty-five-day-old plants of the rice genotypes, PB 1 and Pusa 1908-13-12-5 were inoculated as described in the above section. Plants were watered twice a day and inoculated in the evening hours. The inoculated plants were collected at 24, 48 and 72 h post inoculation. The sampled plants were stored at − 80 °C, labelled and wrapped with aluminium foil. Autoclaved potato dextrose broth(50 ml) cultivated with UP114 (AG-1_IA) and UP105 (AG-2_2) mycelium was collected at 24, 48 and 72 h of growth and immediately stored at − 80 °C for comparison of the pathogen *in planta* with control. Total RNA was isolated from infected rice sheaths and mycelium according to Trizol method (Invitrogen). All laboratory materials like eppendorf tubes, spatula, microcentrifuge tubes, pestle and mortar were sterilized with DEPC (Diethyl pyro carbonate) treatment @ 0.1 percent to inactivate the RNase enzymes. These materials autoclaved at 121 °C, 15psi for 15 min. Infected Pusa Basmati 1, Pusa 1908–13-12–5 sheaths and separately mycelium of *Rhizoctonia solani* isolates were homogenized with liquid nitrogen in a pre-cooled mortar and pestle. The fine powder (about 100 mg) was taken into to 1.5 ml eppendorf tube containing 1 ml of Trizol reagent and incubated for 5 min at room temperature to allow nucleoprotein complex to be fully dissociated. Chloroform (0.2 ml per 1 ml of Trizol) was added to the tubes for phase separation. The tubes were vigorously hand shaken for 15 s, kept at room temperature for 2 to 3 min and centrifuged at 12,000*g* for 15 min in 4 °C. The aqueous phase was transferred to the new tubes, precipitated with 0.5 ml of pre-chilled isopropanol and centrifuged at 12000*g* for 10 min in 4 °C. RNA pellet was retained by removing the supernatant solution from the tubes. The pellets were washed with 1 ml of 75 percent ethanol by centrifugation at 7500*g* for 5 min in 4 °C. The wash was removed and the pellets were air dried for 10 min. The RNA pellets were dissolved in 30μL DEPC-treated water and stored at − 80 °C. Nano Drop-2000 Spectrophotometer (Thermo Fisher Scientific, USA) was used for quantification of RNA.

RNA samples were treated with DNase I, RNase free enzyme (Thermo scientific, USA) using one μg of total RNA in DEPC treated microcentrifuge tubes. RNA template was converted into cDNA using Verso cDNA synthesis kit according to manufacturer's protocol (Verso cDNA, Thermo scientific, USA). To synthesize first strand cDNA anchored Oligo dT primers were used. Reverse transcription conditions consist of one cycle at 42 °C for 30 min and one cycle of inactivation at 95 °C for 2 min performed in C1000TM thermal cycler (Bio rad, USA). The cDNA was stored at − 20 °C.

Validation of *Rhizoctonia solani* primers

The primers of *Rhizoctonia solani* AG-1_IA and AG-2_2 were validated through PCR. DNA from the *R. solani* strains, UP114 (AG1-IA) and UP105 (AG2-2) was used as a template to validate the primers. Primers were verified with *Rhizoctonia solani* anastomosis groups such as AG1, AG2, AG3 and AG5 (Supplementary Table 4). The programme conditions of thermal cycling consist of initial denaturation at 95 °C for 10 min followed by 34 cycles of denaturation at 95 °C for 30 s, annealing at 51–54 °C (different for each primer pair) for 1 min and extension at 72 °C for 1 min was setup in a C1000TM thermal cycler (Bio rad, USA). The amplified products were resolved in 1.5% agarose gel electrophoresis at 90 V for 30 min and gel was analyzed in Gel doc imaging system (UVITEC Cambridge, UK). The amplified primers in the above PCR conditions were taken for quantitative real-time PCR.

In addition to the above primers, study also included the pathogenicity genes of *Rhizoctonia solani*, which were identified during infection of rice and potato (Rioux et al. 2011). The selected primers were given in Supplementary Table 3. The PCR conditions to validate these primers were similar as described in above section.

Specific 18SrDNA housekeeping gene was used (Ghosh et al. 2014) to amplify the cDNA of *Rhizoctonia solani*. The PCR programme consist of initial denaturation at 95 °C for 10 min with 34 cycles of denaturation at 95 °C for 30 s, annealing at 56 °C for 1 min, and extension at 68 °C for 1 min carried out in a C1000™ Thermal cycler (Bio rad). The amplified products were resolved in 1.5 percent agarose gel electrophoresis at 90 V for 30 min and gel was analyzed in gel doc imaging system (UVITEC Cambridge, UK).

Real time-PCR assays

The samples for the real time PCR were setup in three replicates along with non-template control. qRT-PCR plates (Thermo Scientific, USA) consist of 96 wells were used for reactions. Light Cycler 480 Instrument (Roche Life Science, Germany) was used for real time PCR. DyNAmo flash SYBR (2X) green mix dye (Thermo Scientific, USA), was used for monitoring the target gene amplification. The SYBR green mix dye was used in dark room conditions to ensure the efficiency of the fluorescent dye. The final reaction volume was 20 μL, which include 10 μL of SYBR, forward and reverse primer of 1 μL, 1 μL template cDNA and 7 μL of nuclease free water. Real-time PCR programme consist of initial denaturation at 95 °C for 10 min with 40 cycles of denaturation at 95 °C for 15 s, annealing at 51–54 °C (different for each primer pair) for 30 s, extension at 72 °C for 1 min and default dissociation curve analysis to check amplicon specificity. The quantification of the target gene expression was calculated using reference gene GAPDH through $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). The average Ct values of GAPDH gene were subtracted from that of pathogenicity genes. The subtracted values obtained are ΔCt values. Thereafter, $\Delta\Delta Ct$ values were calculated for each treatment by subtracting their respective ΔCt values from negative control ΔCt value (calibrator). The following formula was used to calculate $\Delta\Delta Ct$.

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

where, Time x = any time point and Time 0 = 1 × expression of the target gene normalized to reference gene.

Finally, the fold change expression data were obtained using the formula $2^{-\Delta\Delta Ct}$. Error bars represent standard deviation calculated from three technical replicates on each of three independent biological experiments.

Results

### Assessment of rice genotypes for sheath blight disease severity

The *Rhizoctonia solani* isolates were classified into anastomosis groups and evaluated for disease severity (Supplementary Table 1). UP114 (AG1-IA) and UP105 (AG2-2) isolates were evaluated in Pusa Basmati 1 and Pusa 1908-13-12-5 genotypes for Relative Lesion Height (RLH %, Supplementary Fig. 2). Susceptible genotype Pusa Basmati 1 showed highest disease severity (UP114: 29.51; UP105: 31.10) while comparing with tolerant genotype Pusa 1908-13-12-5 (UP114: 26.95; UP105: 21.51) at 15 days after inoculation (Fig. 2). *Rhizoctonia solani* isolates UP114 (AG1-IA) and UP105 (AG2-2) were selected for comparative studies. ITS region of these isolates was sequenced, aligned with respective *Rhizoctonia solani* anastomosis groups in NCBI nucleotide blast. Similarly, the two isolates were also clustered with their respective anastomosis groups in phylogenetic analysis (Fig. 3).

### *Rhizoctonia solani* candidate genes and functional annotation

A total of 5483 gene clusters were present in AG-IA and 5968 in AG2-2, out of which 119 unique gene clusters specific to AG1-IA and 604 specific to AG2-2 were identified based on the comparative studies (Fig. 4). About 5364 gene clusters are common to both anastomosis groups. The specific proteins were functionally

Fig. 2 Pathogenicity test in susceptible and resistant rice genotype. Relative lesion height was observed highest in susceptible variety Pusa Basmati 1 as compared to tolerant genotype Pusa 1908-13-12-5

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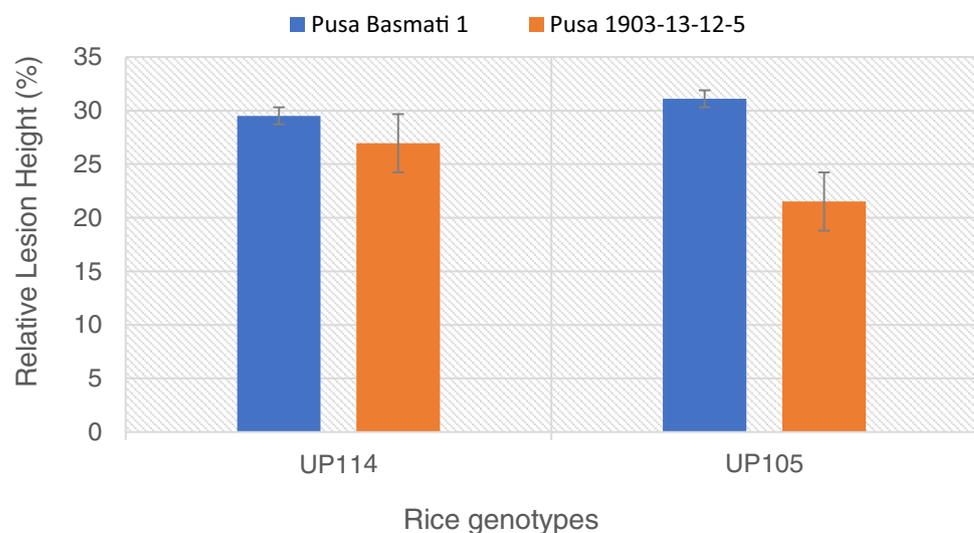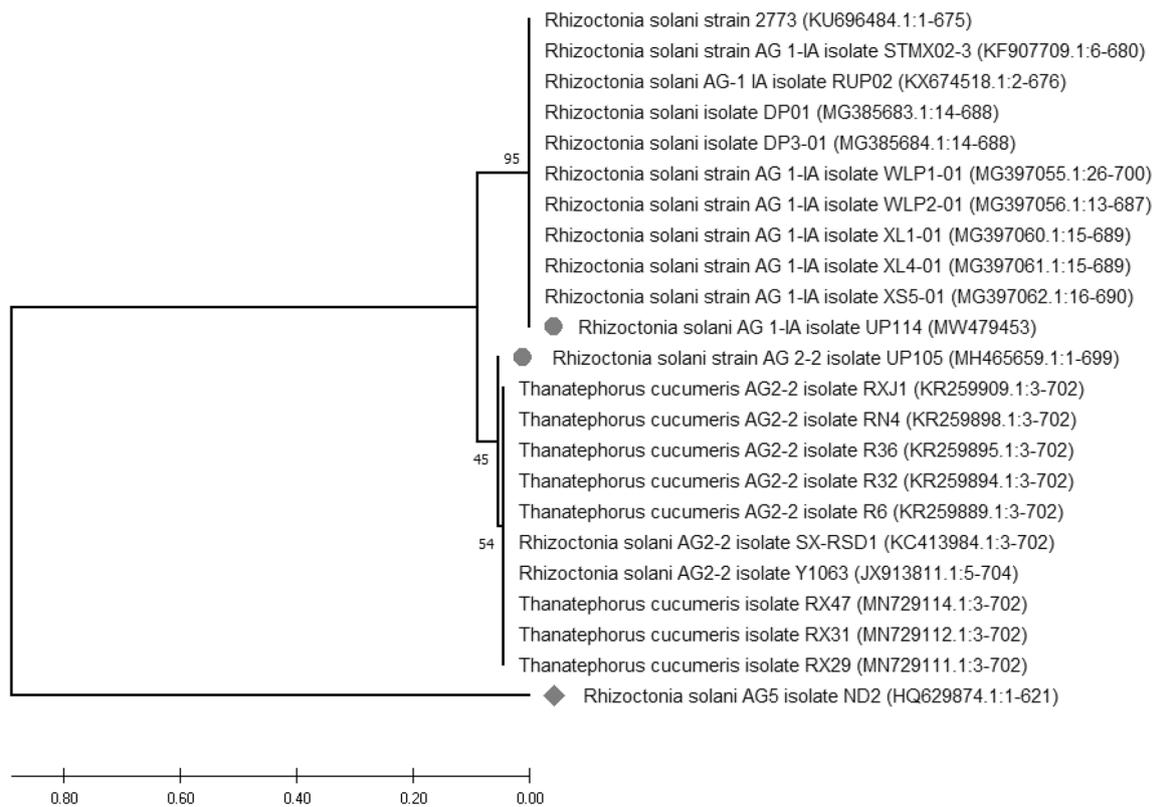
Fig. 3 Phylogenetic tree showing the clusters of *Rhizoctonia solani* isolates. UP114 in AG1-IA group and UP105 in AG2-2. AG5 ND2 taken as outgroup. ClustalW-based alignment was used to construct the tree based on Neighbor Joining method with bootstrap value of 1000 replicates
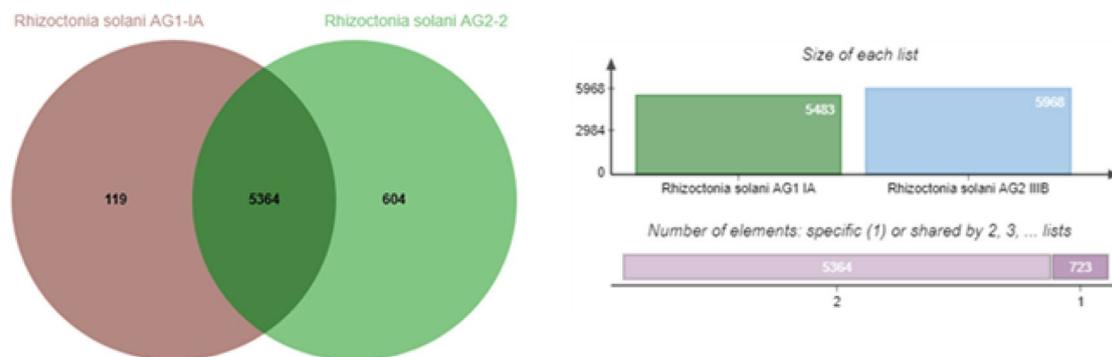
Fig. 4 Unique, orthologous and size of gene clusters in *Rhizoctonia solani* AG1-IA and AG2-2 isolates

categorized for biological functions, molecular processes, cellular components and the top ten hits were identified in each category (Fig. 5). The unique proteins of both anastomosis groups were selected for PHI blast analysis. Total proteins in AG1-IA categorized as unaffected pathogenicity (66%), reduced virulence (20%), loss of pathogenicity (6%), lethal (4%) and increased virulence (4%) (Fig. 6). In AG2-2, the proteins classified as reduced virulence (58%), loss of pathogenicity (28%), unaffected pathogenicity (8%), lethal (5%) and increased virulence (1%) (Fig. 6). Query coverage and e-value was used for sorting the PHI-base blast results in descending order (Supplementary Table 5 and 6). For qRT-PCR, *Rhizoctonia solani* genes having highest query coverage with cut-off value 31 were selected from each phenotypic category (Supplementary Table 7).
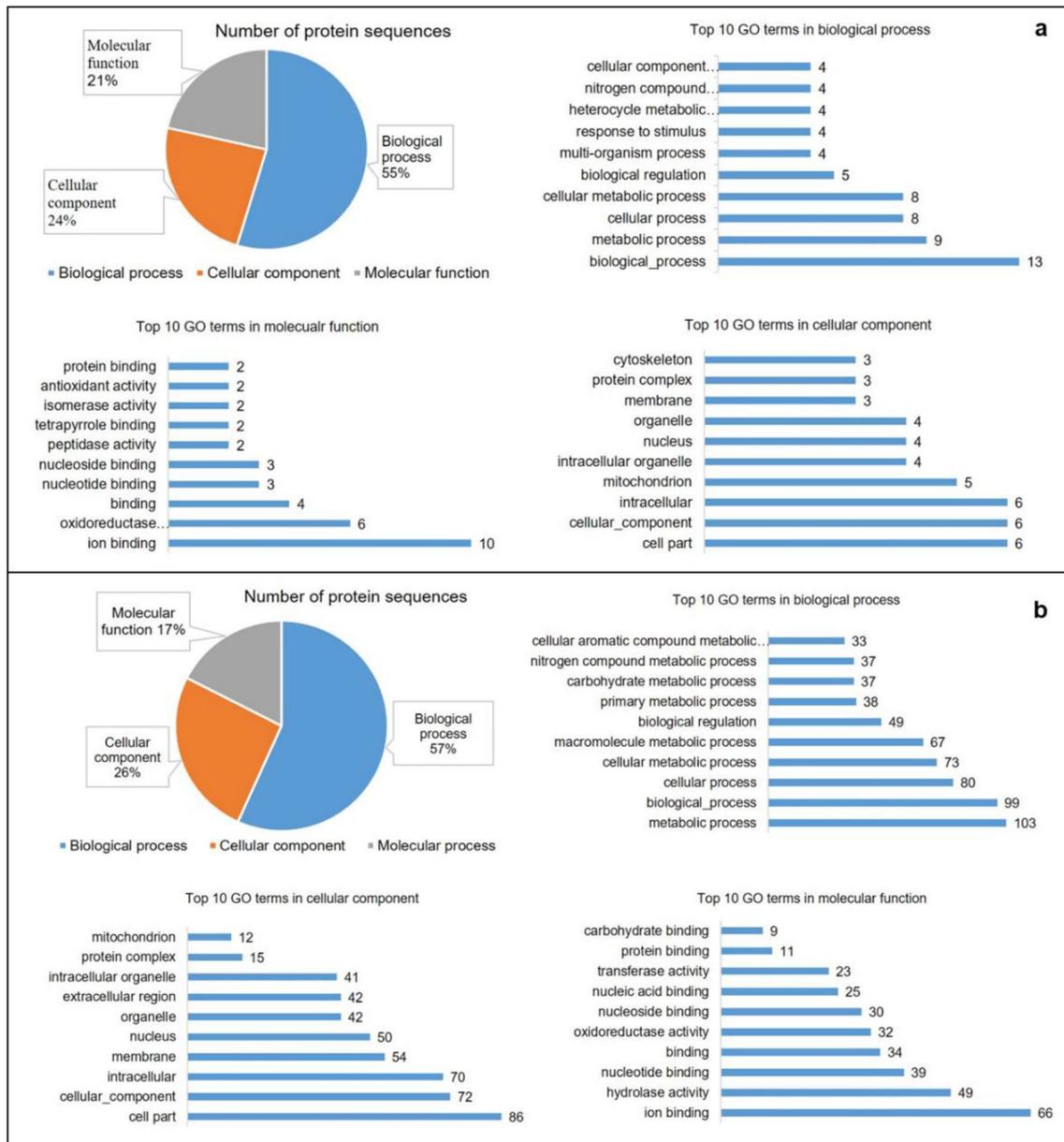
Fig. 5 Functional annotation of genes in *Rhizoctonia solani* AG1-IA and AG2-2. Top ten hits of biological process, molecular function and cellular component were annotated. **a** *Rhizoctonia solani* AG1-IA, **b** *Rhizoctonia solani* AG2-2

Anastomosis group-specific genes and pathogenicity genes of *Rhizoctonia solani*

To amplify the *Rhizoctonia solani* in cDNA, pathogen-specific 18S rDNA housekeeping gene was used. All cDNA samples produced a product size of 184 bp, indicating that rice samples consisted of *Rhizoctonia solani* cDNA (Fig. 7a). Out of seven genes of AG1-IA, inorganic phosphate transporter (*AG1_IPT*), Bromodomain containing protein (*AG1_BrD*), Aldehyde dehydrogenase (*AG1_AldD*), AMP binding domain (*AG1_AMP*) and

Heme peroxidase (*AG1_HmPr*) were amplified. Among these, inorganic phosphate transporter (*AG1_IPT*), Bromodomain containing protein (*AG1_BrD*) and Heme peroxidase (*AG1_HmPr*) has been found specific to *Rhizoctonia solani* AG1-IA (Fig. 7b). Similarly, in AG2-2, out of eight genes, Lissencephaly-1 homolog (*AG2_LisH*) and Ephrin type-A receptor (*AG2_HyMt*) were amplified (Fig. 7c). All these amplified genes along with two pathogenicity genes namely Glutathione-s-transferase kappa 1 (*RS_GLU*) and Nuclear pore protein, Nic96 (*RS_NIC*) (Fig. 7d), which were reported to be present in AG1 and AG3 pathosystems
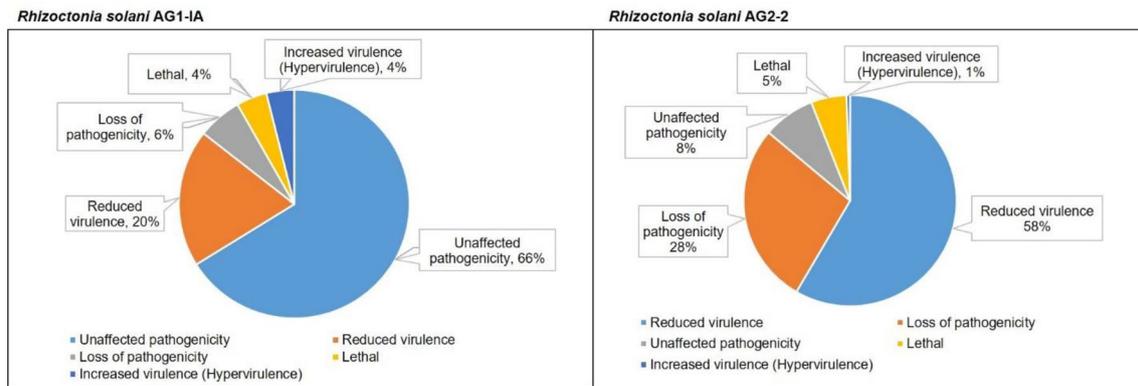
Fig. 6 Pathogen–host interactions (PHI-base) database phenotypic categories of *Rhizoctonia solani* AG1-IA and AG2-2
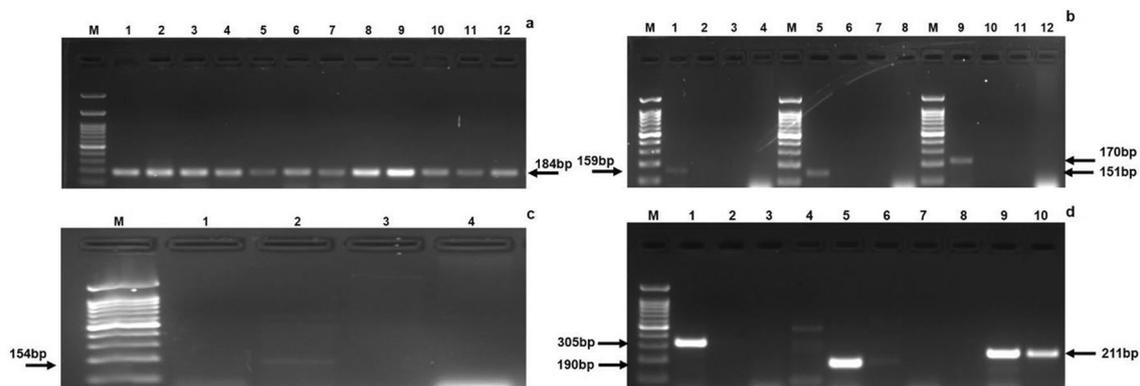
Fig. 7 PCR analysis of *Rhizoctonia solani* 18S rDNA and pathogenicity genes. **a** rice cDNA samples amplified with pathogen specific 18S rDNA: M=Molecular marker:100 bp, Lane 1–3: PB1 inoculated with UP114 (AG1-IA) at 24, 48 and 72 h post inoculation, respectively, Lane 4–6: Pusa 1908–13-12–5 inoculated with UP114 (AG1-IA) at 24, 48 and 72 h post inoculation, respectively, Lane 7–9: PB1 inoculated with UP105 (AG2-2) at 24, 48 and 72 h post inoculation, respectively, Lane 10–12: Pusa 1908–1 inoculated with UP105 (AG2-2) at 24, 48 and 72 h post inoculation, respectively. **b** Pathogenicity genes found specific to *Rhizoctonia solani* AG1-IA: M=Molecular marker:10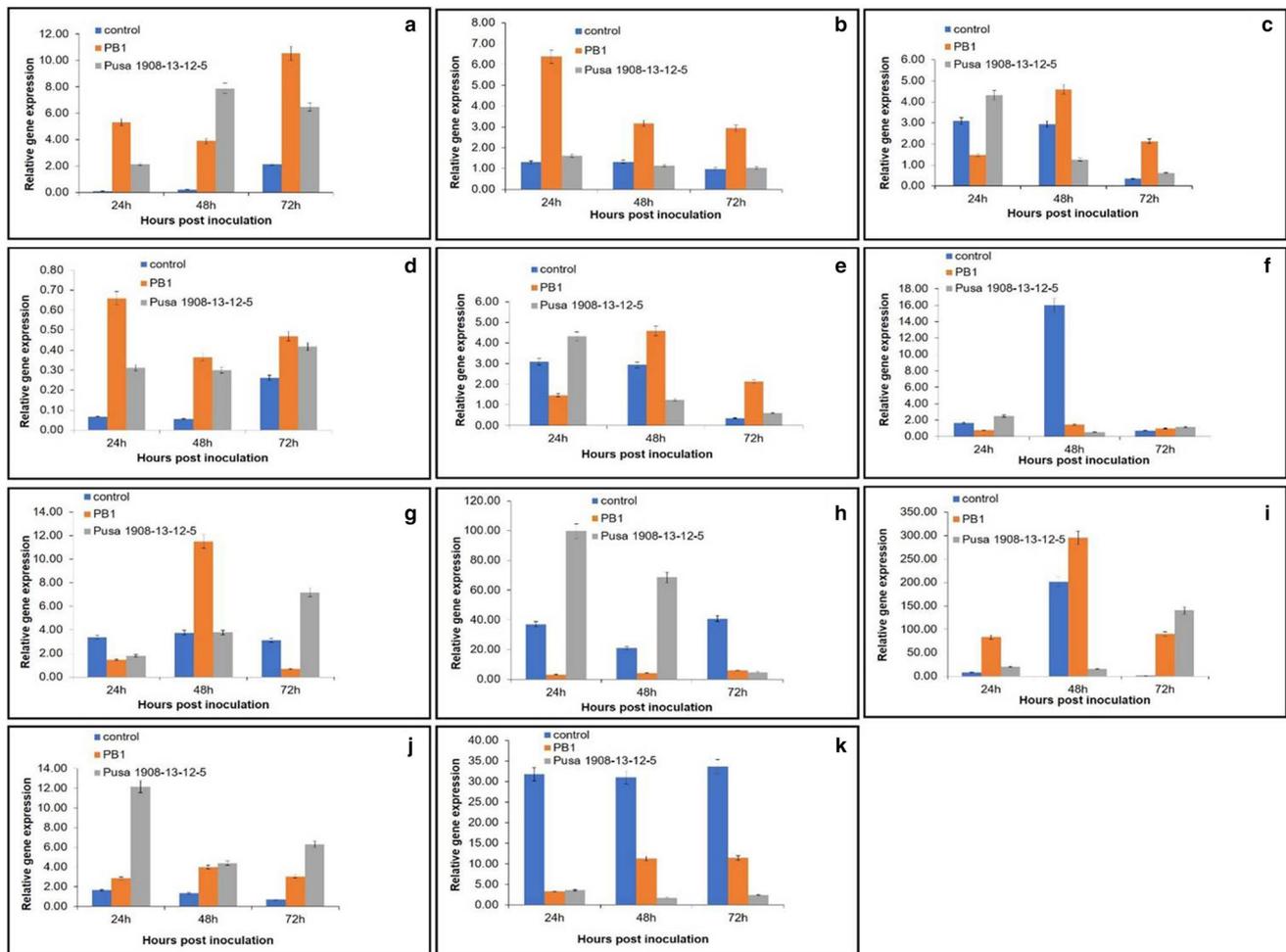0 bp, Lane 1–4: Gene inorganic phosphate transporter with DNA of AG1 (UP114), AG2 (UP105), AG3 (RPBC 1), AG5 (RMHM 6), respectively, Lane 5–8: Bromodomain containing protein with DNA of AG1, AG2, AG3, AG5 respectively, Lane 9–12: Gene Heme peroxidase with DNA of AG1, AG2, AG3 and AG5, respectively. **c** Pathogenicity gene found specific to *Rhizoctonia solani* AG2-2: M=Molecular marker:100 bp, Lane 1–4: Gene Lissencephaly-1 homolog with AG1, AG2, AG3, AG5, respectively. **d** Pathogenicity genes selected from previous studies (Rioux et al. 2011): M: Molecular marker 100 bp; lane 1–2: gene *GLU* with AG1,AG2; lane 5–6: gene *AAA* with AG1, AG2; lane 9–10: gene *NIC* with AG1, AG2

of *Rhizoctonia solani* (Rioux et al. 2011) were taken up for further qRT-PCR analysis.

Expression analysis of *R. solani* genes by qRT-PCR

The expression pattern of *Rhizoctonia solani* pathogenicity genes varied across different time points of infection in two genotypes. The inorganic phosphate transporter (*AG1_IPT*) was upregulated in PB 1 at 24hpi (5.31) and 72hpi (10.56), but expression was increased in Pusa 1908–13-12–5 (7.88; Fig. 8a) at 48hpi. Expression pattern of Bromodomain containing protein (*AG1_Brd*) was highest in PB 1 at all time intervals (6.38-24hpi, 3.16-48hpi, 2.93-72hpi) compared to control (pathogen) and Pusa 1908-13-12-5 (Fig. 8b).

Aldehyde dehydrogenase (*AG1_AldD*) expression trend was almost similar in control and Pusa 1908–13-12–5. But in Pusa Basmati 1, expression level was reduced at 24hpi (1.02) and upregulated at 48hpi (1.37) and 72hpi (1.14) compared to control and Pusa 1908-13-12-5 (Fig. 8c). The AMP binding domain (*AG1_AMP*) expression was highest in PB 1 (0.65–24hpi, 0.36–48hpi, 0.46–72hpi) at all time intervals compared to control and Pusa 1908-13-12-5. In Pusa 1908-13-12-5, its expression pattern remained constant at 24hpi (0.31) and 48hpi (0.30) but slightly increased at 72hpi (0.41) as compared to control (Fig. 8d). Heme peroxidase (*AG1_HmPr*) expression was less in PB 1 at 24hpi (1.47) but highly upregulated at 48 (4.57) and 72hpi (2.12) as compared to control and Pusa 1908-13-12-5. But in Pusa

Fig. 8 qRT PCR expression analysis of *Rhizoctonia solani* genes in susceptible (PB1) and resistant (Pusa 1908-13-12-5) genotypes at different hours of post inoculation. **a** Inorganic phosphate transporter (*AG1_IPT*), **b** Bromodomain function (*AG1_BrD*), **c** Aldehyde dehydrogenase (*AG1_AldD*), **d** AMP binding domain (*AG1_AMP*), **e** Heme peroxidase (*AG1_HmPr*), **f** Lissencephaly-1 homolog (*AG2_LisH*), **g** Ephrin type-A receptor (*AG2_HyMt*), **h** Glutathione-s-transferase kappa 1 (*RS_GLU*) expression pattern by AG1-IA, **i** Glutathione-s-transferase kappa 1 (*RS_GLU*) expression pattern by AG2-2, **j** Nuclear pore protein, Nic96 (*RS_NIC*) induced by AG1-IA, **k** Nuclear pore protein, Nic96 (*RS_NIC*) in AG2-2. **a–e** Expression pattern of *Rhizoctonia solani*_AG1-IA pathogenicity genes, **a**, **b** and **e**: Expression pattern of AG1-IA-specific pathogenicity genes of *Rhizoctonia solani*. **f–g** Expression pattern of *Rhizoctonia solani*_AG2-2 pathogenicity genes. **h–k** Expression pattern of *Rhizoctonia solani* pathogenicity genes which were previously studied in rice and potato pathosystems by Rioux and coworkers, 2011

1908-13-12-5, it upregulated at 24hpi (4.31) and reduced at 48 (1.25) and 72hpi (0.60) compared to control (Fig. 8e).

The Lissencephaly-1 homolog (*AG2_LisH*) was differed from other pathogenicity genes because it was not induced at any time points in PB 1 and Pusa 1908-13-12-5. However, at 48hpi, the highest expression was observed in control (one-fold, Fig. 8f). Ephrin type-A receptor (*AG2_HyMt*) gene expressed significantly higher in Pusa Basmati 1 at 48hpi (11.51). In control it remains constant at all intervals. On the other hand, its expression in Pusa 1908-13-12-5 was highest at 72hpi (7.18) as compared to control (Fig. 8g).

Among the genes belonging to AG1 and AG3 pathosystems of *Rhizoctonia solani* (Rioux et al. 2011),

Glutathione-s-transferase kappa 1 (*RS_GLU*) in AG1 was induced significantly higher in Pusa 1908-13-12-5 at 24hpi (99.73) and 48hpi (68.59) while comparing with control and PB 1 (Fig. 8h). However, expression level was reduced at 72hpi (4.70). In Pusa Basmati 1, it was not expressed at all at any-time points of infection. *RS_GLU* inactivates the toxic compounds directed by host against the pathogen. Hence, upregulation and activation of this gene in Pusa 1908-13-12-5 can be correlated with defense response of the tolerant genotype, which has the ability to produce antimicrobial compounds in an early interaction. Similarly, with AG2, *RS_GLU* (Glutathione-s-transferase kappa 1) expression was highest in PB 1 at 24hpi (83.28) and 48hpi (295.08) but at

72hpi, it was increased in Pusa 1908-13-12-5 (140, Fig. 8i). We can observe here the contrasting expression activities of AG1 and AG2 for the gene Glutathione-s-transferase kappa 1 (*RS_GLU*) in Pusa 1908-13-12-5 and Pusa Basmati 1.

In AG1, expression of Nuclear pore protein, Nic96 (*RS_NIC*) was highest in Pusa 1908-13-12-5 at all time points of infection (12.16–24hpi, 4.40–48hpi, 6.29–72hpi) compared to control and PB 1 (Fig. 8j) but at 48hpi, expression was almost equal to PB 1. Similarly in AG2, Nuclear pore protein, Nic96 was highly upregulated in control (pathogen) at all time points of infection (31.79–24hpi, 31.04–48hpi, 33.70–72hpi) compared to PB 1 and Pusa 1908-13-12-5 (Fig. 8k). Summary of these genes were given in supplementary file (Supplementary Table 8).

Discussion

Rhizoctonia solani Kuhn is a soil borne necrotrophic, polyphagous pathogen which causes damage to important food crops. Sheath blight of rice caused by *Rhizoctonia solani* Kuhn is one of the economically important disease. It damages sheaths, leaf and grains resulting in reduction of the yield. *Rhizoctonia solani* has been classified into 14 anastomosis groups and also consist of subgroups. Sheath blight of rice is caused by *Rhizoctonia solani* AG1-IA.

We conducted comparative gene expression studies of *Rhizoctonia solani* AG1-IA and *Rhizoctonia solani* AG2-2 infecting rice. In PHI blast, the percentage of functional categories of *Rhizoctonia solani* AG1-IA and AG2-2 differed significantly. In AG1-IA, unaffected pathogenicity was 56%, whereas 8% in AG2-2, which has been observed earlier as well (Wibberg et al. 2016). As Wibberg et al. (2016) analysis was based on the draft genome sequence of AG2-2IIIB, the percentage data were not exactly similar in our study as we used proteome of the isolate. Similarly, in AG1-IA, the ranking of functional categories from highest to lowest was in agreement with the report of Ghosh et al. (2014). Majority of the genes were expressed significantly high during rice infection in susceptible variety Pusa Basmati 1 and their expression varied across different time intervals. Inorganic phosphate transporter (*AG1_IPT*) expression was found highest in Pusa Basmati 1 and phosphate transporter function is involved in virulence of the pathogen. In *Cryptococcus neoformans*, deletion of the high-affinity uptake system interrupted the growth on low-phosphate medium, affected the formation of virulence factors (capsule and melanin) and reduced virulence in a mouse model of cryptococcosis. Similarly, analysis of nutrient sensing functions of *C. neoformans* revealed regulatory connections between phosphate acquisition and storage (Kretschmer et al. 2014). Bromodomain containing protein (*AG1_Brd*) expressed significantly high fold in PB 1 at all time intervals. Bromodomains

are present in many chromatin-associated proteins and in nuclear histone acetyltransferases. They interact specifically with acetylated lysine (Zheng et al. 2013). Bromodomain containing protein is the evolutionary conserved, having the role in assembly of protein complexes through lysine acetylation, regulates the transcription of genes. This gene also has the important cell proliferation function. Aldehyde dehydrogenases (ALDHs) are oxidizing enzymes that are involved in detoxification of both exogenous and endogenous aldehydes. The expression pattern of gene NAD-dependent Aldehyde dehydrogenase (*AG1_AldD*) increased in PB 1 at 48 and 72hpi. Aldehyde dehydrogenase is important for the growth of the pathogen in *Saccharomyces* sp. (Wang et al. 1998). The gene AMP binding domain (*AG1_AMP*) expression was highest in Pusa Basmati 1 across all time points of infection while comparing with control and Pusa 1908-13-12-5. It is acyl-activating enzyme (AAE) consensus motif (Zheng et al. 2013). Heme peroxidase (*AG1_HmPr*) expression was highly upregulated in PB 1 at 48hpi and 72hpi in comparison with control and Pusa 1908-13-12-5. Heme peroxidase has role in deactivation of hydrogen peroxide. In pathogen, it helps in pathogenicity and in host, it helps in resistance. Upregulation and activation of this gene in Pusa 1908-13-12-5 indicates the defense response of the genotype during early interaction. Lissencephaly-1 homolog (*AG2_LisH*) is required for nuclear migration during cell division and mitotic spindle formation through dynein and microtubule dependent processes. Therefore, the upregulation of the gene at 48hpi in pathogen indicates the gene has the role in active growth of the pathogen. It also has role in signal transduction through regulatory/adaptor systems, present in several eukaryotic proteins, involved in pre-mRNA processing and cytoskeleton assembly (Wibberg et al. 2016). Ephrin type-A receptor (*AG2_HyMt*) is involved in cell communications and interactions through membrane associated proteins. It was induced in susceptible variety Pusa Basmati 1 at 48hpi and expression level was constant during pathogen growth at all time points. These receptors are the major one in Receptor Tyrosine Kinase (RTK) family and involved in signal transduction from the outer cell surface to inner part by kinase domain activation through ligands (Lisabeth et al. 2013).

The AG1 isolate Glutathione-s-transferase kappa 1 (*RS_GLU*) expression was highest in Pusa 1908-13-12-5 at 24hpi and 48hpi. It remained unexpressed in Pusa Basmati 1 across all time intervals. It targets and degrade the toxic compounds generated by the host (Rioux et al. 2011). Therefore, the increased expression level in Pusa 1908-13-12-5 can be correlated with host defense response in an early interaction leading to resistance reaction. However, in AG2 isolate, similar expression pattern was observed in susceptible variety Pusa Basmati 1 at 24hpi and 48hpi.

The expression of Nuclear pore protein, Nic96 (*RS_NIC*) in the AG1 isolate, was highest in Pusa 1908-13-12-5 at all time points of infection, but at 48hpi expression was almost equal to PB 1. In the other case, AG2 isolate Nuclear pore protein, Nic96 expression was highly upregulated in control (pathogen) at all time points of infection. Similar comparative gene expression studies of *Rhizoctonia solani* AG1 and AG3 genome showed that *RS_GLU* and *RS_NIC* genes were upregulated at 72hpi (Rioux et al. 2011). Nuclear pore protein is involved regulation of transcription and cell growth.

To conclude, the comparative gene expression of *Rhizoctonia solani* AG-1_IA and *Rhizoctonia solani* AG-2_2 infecting rice showed that most of the genes expressed significantly high fold in susceptible genotype Pusa Basmati 1. The genes inorganic phosphate transporter (*AG1_IPT*), Bromodomain containing protein (*AG1_BrD*), Aldehyde dehydrogenase (*AG1_AldD*), AMP binding domain (*AG1_AMP*) and Heme peroxidase (*AG1_HmPr*) were upregulated in PB 1 at 72hpi compared to Pusa 1908-13-12-5. Among these, inorganic phosphate transporter (*AG1_IPT*), Bromodomain containing protein (*AG1_BrD*) and Heme peroxidase (*AG1_HmPr*) were found specific to *Rhizoctonia solani* AG-1_IA. Further detailed studies on *Rhizoctonia solani* anastomosis groups and pathogenicity genes will be helpful to understand the molecular mechanisms of its pathogenicity in rice.

Author contributions BM, RA, GK, GP, PST conceptualized the problem and designed the experiment. PST conducted the experiments. PST, HD, AS, BM analyzed the data. PST, BM written the manuscript. BM, RA, GK, AS edited the manuscript. Manuscript was finalized by all the authors.

Declarations

Conflict of interest The authors declare that they have no conflict of interest in the publication.

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