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RESEARCH ARTICLE

Characterization of the wild rice *Oryza rufipogon* transcriptomes associated with susceptibility to *Burkholderia glumae*

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ABSTRACT

Bacterial panicle blight (BPB), caused by *Burkholderia glumae* (*Bg*) is an emerging rice disease. Characterizing the host's transcriptome at various time points following pathogen infection can provide valuable insights into the defence mechanisms during a compatible interaction. Thus, this study aimed to investigate the interaction between common wild rice, *Oryza rufipogon* Griff., and *Bg* at the transcriptome level upon infection of a susceptible accession, IRGC 86685. Following *Bg* infection on the stems of young plants, the disease responses were observed at 24, 48, 72, and 96 h post inoculation (hpi). After 72 h, visible browning started to emerge around the injection sites. The transcriptome analysis revealed 1014 (468 up-regulated and 546 down-regulated), 830 (665 up-regulated and 165 down-regulated), 2207 (1174 up-regulated and 1033 down-regulated), and 1993 (863 up-regulated and 1130 down-regulated) differentially expressed transcripts (DETs) at different time points. The highest level of differential expression observed at 72 hpi was consistent with the increased level of BPB symptoms at this time point. The gene ontology (GO) term enrichment analysis shows that most of the up-regulated DETs correspond to membrane, transporter activity, and response to stress across all time points in the cellular component, molecular function, and biological process, respectively, suggesting their involvement in IRGC 86685's response to *Bg*. These findings shed light on the molecular mechanisms underlying BPB susceptibility in wild rice during *Bg* infection, aiming to improve rice resistance to this pathogen.

Key words: Bacterial panicle blight, *Burkholderia glumae*, *Oryza rufipogon*, RNA-seq analysis, wild rice.

INTRODUCTION

Rice (*Oryza sativa* L.) is a primary source of calories for nearly half of the world's population. In 2022, approximately 776 million tons of rice was produced globally (FAOSTAT, 2022). Since the start of the Green Revolution, steady increases have been recorded in rice production (Muthayya et al., 2014). However, disease remains a significant cause of yield loss, posing a threat to global food security (Ristaino et al., 2021). One of the emerging diseases of rice is bacterial panicle blight (BPB), caused by a Gram-negative bacterium, *Burkholderia glumae* (*Bg*) (Nandakumar et al., 2009). Warm temperatures and high relative humidity are the ideal growth conditions for this pathogen (Nandakumar et al., 2009). Hence, it is anticipated that BPB will become more prevalent in warm regions worldwide due to climate change (Ham et al., 2011). During severe outbreaks, the infected plants develop seedling rot, sheath brown rot, grain rot or discoloration, and floret sterility. These combined effects lead to a significant reduction in grain weight, with a potential decrease of up to 75% (Nandakumar et al., 2009).

The application of bactericides has proven to be successful in managing BPB populations (Maeda et al., 2004). Nevertheless, this only effective control measure is costly and has adverse effects on the environment. Alternatively, the development of disease-resistant rice varieties, which typically involves the deployment of

dominant resistance (*R*) genes would be a more sustainable approach. However, most of the cultivated rice varieties lack resistance to BPB, with only a few exhibiting partial resistance (Zhou, 2019). Even in cases where there are complete resistant rice varieties available, the rapid variation of virulent races allows the pathogen to eventually overcome the resistance of the corresponding *R* genes (Rimbaud et al., 2018).

Research that examines the gene expression changes in the host plant following the pathogen's infection through transcriptome analysis may provide insights on the pathways involved in the plant-pathogen interaction. The RNA sequencing (RNA-seq) has emerged as an important tool for analysing the transcriptome (Shendure, 2008). However, to date, only one study has been reported on the rice-*Bg* pathosystem using this approach. Magbanua et al. (2014) obtained differentially expressed genes (DEGs) through transcript profiling via RNA-Seq and identified unique transcripts involved with resistance to BPB. Therefore, the elucidation of the transcriptome during compatible interaction between wild rice and *Bg* remains unexplored.

In this study, the interaction between the common wild rice, *Oryza rufipogon* Griff. and *Bg* upon infection of a susceptible accession, IRGC 86685 at the transcriptome level was investigated, with a particular interest in elucidating the key genes involved in this process. The changes in transcript expression patterns at four different time points were analysed and differentially expressed transcripts (DETs) involved in regulating the wild rice-*Bg* compatible interaction have been identified. Collectively, these findings provide insights on the molecular regulatory networks associated with susceptibility in wild rice upon responses to *Bg* infection.

MATERIALS AND METHODS

Plant materials and pathogen inoculation treatment

Five *Oryza rufipogon* Griff. accessions namely IRGC 86685, IRGC 86687, IRGS 86698, IRGC 106083, and IRGC 106092 were obtained from the International Rice Research Institute (IRRI), the Philippines. The *Burkholderia glumae* (*Bg*) strain BGS.AS1 (Genbank number: ON062124) was obtained from the Department of Plant Protection, Faculty of Agriculture, Universiti Putra Malaysia (UPM) and maintained on King's B agar medium. The bacterial suspension was prepared from a fresh culture with an OD600 concentration of 107 to 108 CFU mL^{-1}. The 45-d-old plants of the five accessions were inoculated with BGS.AS1 using the injection method on the main stem (Magbanua et al., 2014). Four replicates were grown for each accession and arranged in a randomized complete block design (RCBD) with a single factor (lesion length) under greenhouse conditions at 35 °C and a relative humidity of over 75%. The data were subjected to one-way ANOVA and the means were compared using Tukey's test in R software. Based on the disease assay, IRGC 86685 was selected for RNA-Seq. The plants were treated with two treatments, mock using distilled water (T0) and *Bg* (T1). Following *Bg* inoculation, the stem samples (consisting of three replicates) were harvested at 24, 48, 72, and 96 h post inoculation (hpi). The stems collected immediately after the inoculation were used as a control.

RNA isolation and sequencing

The RNA was extracted using the RNA Tissue Miniprep System (ReliaPrep, Promega Corporation, Madison, Wisconsin, USA) according to the manufacturer's instructions. The RNA concentration and quality were measured using spectrophotometry (NanoDrop, Thermo Scientific, Waltham, Massachusetts, USA) and electrophoresis (2100 Bioanalyzer, Agilent, Santa Clara, California, USA), respectively. Only RNA samples with an OD260/280 ratio ranging from 2.00 to 2.20 and an RNA integrity number (RIN) > 6.0, were used for the next processing step. For each sub-sample, 1 µg total RNA was used for the following library preparation. Next-generation sequencing library preparations were constructed according to the manufacturer's protocol (NEBNext UltraTM RNA Library Prep Kit for Illumina; New England Biolabs, Ipswich, Massachusetts, USA). The mRNA was purified from the total RNA using poly-T oligo-attached magnetic beads. The purified mRNA was fragmented randomly by adding a fragmentation buffer and followed by first-strand cDNA synthesis using First Strand Synthesis Reaction Buffer and random hexamers primer. Next, Second Strand Synthesis Reaction Buffer, dNTPs, RNase H and DNA polymerase I were added to initiate the second-strand synthesis. After second-strand synthesis, terminal repair and sequencing adaptor ligation were performed. Adaptor-ligated fragments were size-selected and enriched before being sequenced. The library was checked with Qubit and real-time PCR for quantification and a bioanalyzer for size distribution detection. Finally, libraries were multiplexed and loaded on a sequencing system instrument (NovaSeq 6000 System) according to the manufacturer's instructions

(Illumina, San Diego, California, USA). Sequencing was carried out using a 2×150 bp paired-end (PE) configuration. The database was submitted to the National Center for Biotechnology Information (NCBI, Bethesda, Maryland, USA) under accession number PRJNA1166944.

Transcriptome analysis

A quality control check was performed on the samples by FastQC. HISAT2 (Kim et al., 2019) was used to align high-quality reads to the *O. rufipogon* IRGC 106162 GSA genome assembly (Xie et al., 2021). The assembly of the mapped reads into their respective transcripts was conducted by Cufflinks (Trapnell et al., 2010) and the fragment reads were normalized in Fragments per Kilobase of exon per Million fragments mapped (FPKM). Cuffdiff, a package in the Cufflinks, was used to determine significant transcripts that were expressed differentially. The T1 samples were compared to the control with the *p*-value cut-off < 0.05 and absolute log2 ratio (Log2 FC) ≥ | 2 | were set as the threshold.

Functional annotation of differentially expressed transcripts (DETs) was performed for homology searches in the NCBI non-redundant (Nr) database through BLASTx searches via the Blast2GO program (Götz et al., 2008) and eggNOG-mapper (Cantalapiedra et al., 2021). The identification of Gene Ontology (GO) term of DETs was also performed via the Blast2GO program and eggNOG-mapper.

Validation of RNA-Seq

Six DETs were selected for expression profiling, including the T0, T1, and control samples to verify the reliability of the RNA-Seq data by quantitative real-time PCR (qRT-PCR). Primers for amplification of the selected transcripts were designed using Primer-BLAST in NCBI (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) (Table 1). The cDNA synthesis was prepared from the extracted RNA using Promega GoTaq 2-Step RT-qPCR System (Promega Corporation). The qRT-PCR was performed in 96-well plates on an Heal Force X960 Real Time PCR system (Heal Force Bio-meditech Holdings, Qingpu District, Shanghai, China) using SYBR Green I assays with GoTaq qPCR Master Mix (Promega Corporation). Actin-1 and OsGADPH2 were used as internal controls. The thermal cycler conditions were 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The relative expression values for each transcript were calculated by the delta-delta C2 (2-Ct) method (Livak and Schmittgen, 2001).

Table 1. Information on primer sequences. Tm: Melting temperature; GC: ratio of the sum of guanine and cytosine; bp: base pair.

Primer name	Sequence (5' to 3')	Tm	GC%	Amplicon size (bp)
LOC127769352_F	GCCTTTGTTGTTGTCGTGATTAG	58.78	43.48	366
LOC127769352_R	AACTGAGCCACTTCCATTCC	57.79	50.00	
LOC127780511_F	GACGCTGGAGGAGATGAG	56.54	61.11	142
LOC127780511_R	AACCCTTAGGATTTGGTGATTT	55.56	36.36	
LOC127786326_F	GGAGTCCAAGTACCCGTCG	59.49	63.16	238
LOC127786326_R	CTTCGCCATGATCCTCGTCA	59.90	55.00	
LOC127768761_F	GCTTCCATTGGTGGGAGAA	57.33	52.63	174
LOC127768761_R	AACAGCCACAGCAGTGAA	57.02	50.00	
LOC127779929_F	AACTACATCCCCTCGCTGTC	59.18	55.00	246
LOC127779929_R	GATTCTTCCTGTGCCGTTGA	58.19	50.00	
LOC4352759_F	CGATGGCGGCATTGTCGG	61.94	66.67	121
LOC4352759_R	GTGGCTGAGGTGCTTGTTG	59.34	57.89	
Actin1_F	ACATCGCCCTGGACTATGACCA	63.21	54.55	437
Actin1_R	GTCGTACTCAGCCTTGGCAAT	60.68	52.38	
OsGADPH2_F	CCACAGACTTCCAGGGTGAC	59.96	60.00	292
OsGADPH2_R	CATCATGCGAAAAGCCAGCA	59.83	50.00	

RESULTS

Infection assay of *O. rufipogon* accessions against *Bg* strain BGS.AS1

While *Bg* mostly infects the panicle, symptoms can also be observed in young plants (Nandakumar et al., 2009; Weny et al., 2019). Therefore, we screened five *O. rufipogon* accessions at tillering stage to determine their susceptibility level to BPB disease. The young stems were injected with 0.5 mL inoculum and observed the response 7 d after inoculation. The lesion length of the infected plants was measured and compared with cultivated rice (*O. sativa* L.) 'MR219', which served as a susceptible control. All five accessions exhibited high susceptibility to the *Bg* strain, BGS.AS1, 7 d after inoculation with no difference between them (Figure 1). The most susceptible accession was IRGC 86685, and the least susceptible accession was IRGC 86698.

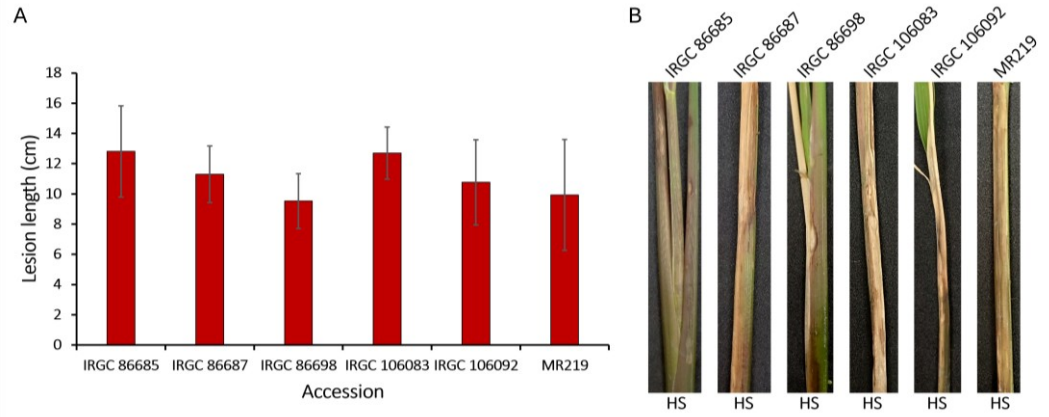

Figure 1. Disease response of five *Oryza rufipogon* accessions at 7 d post-inoculation with *Burkholderia glumae* strain BGS.AS1. HS: Highly susceptible.

Response of a susceptible *O. rufipogon* accession to inoculation with *Bg*

Based on the disease assay, IRGC 86685 was selected to generate a differential gene expression profile that pertains to the wild rice-*Bg* pathosystem during susceptible interaction. Following *Bg* inoculation, the disease responses were observed at four different time points. After 24 and 48 h, the inoculation marks were visible in all plants and there was no difference between the T0 and T1. However, after 72 h, visible browning started to emerge around the injection sites on the T1-treated plants, while the plants under T0 treatment exhibited similar responses at the 48 h observation. At 96 h, the browning around the injection sites had significantly increased and necrosis developed on the T1-treated plants (Figure 2).

Transcriptome analysis of the susceptible *O. rufipogon* accession upon inoculation of *Bg*

Total RNAs from the young stems of T1-treated plants were isolated and used for high-throughput sequencing. The raw reads varied between 40 694 392 and 63 486 314. Following a quality check, the adapter and low-quality reads were removed from the data. The clean data were ranged from 30 280 204 to 46 936 724 reads. The sequence reads were mapped to the *O. rufipogon* reference genome using HISAT2. The percentage of reads, ranging from 93.52% to 95.41%, were successfully mapped to the reference genome. Furthermore, the percentage of Q30 bases were 89.72% to 90.42% and the GC contents were ranged from 53.85% to 55.71%, indicating that the quality of transcriptome sequencing was relatively high for subsequent bioinformatics analysis (Table 2).

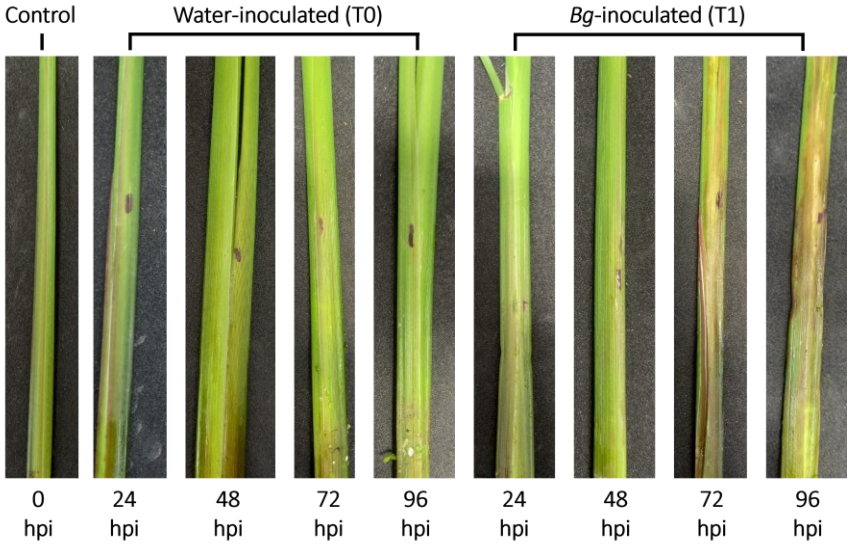

Figure 2. Bacterial panicle blight disease progression. Symptoms were allowed to develop after 24, 48, 72, and 96 h post inoculation (hpi) on plants inoculated with either water (T0) or *Burkholderia glumae* (*Bg*) suspension (T1) on a susceptible *Oryza rufipogon* accession, IRGC 86685.

Table 2. An overview of sequencing and assembly of *Oryza rufipogon* transcriptome in response to *Burkholderia glumae* infection. Q30: Quality percentage > 30; GC: ratio of the sum of guanine and cytosine.

Replicate	Sample	Raw reads	Clean reads	Mapped reads	Q30 (%)	GC (%)
1	Control	49 670 282	36 216 642	34 395 210	89.90	55.46
	24H_T1	40 694 392	30 280 204	28 700 771	90.42	54.60
	48H_T1	55 237 522	40 544 526	38 370 167	90.09	55.03
	72H_T1	51 525 406	37 034 640	35 169 594	89.72	54.34
	96H_T1	44 999 864	33 981 886	32 268 478	90.29	55.15
2	Control	50 329 786	36 813 170	35 122 685	89.96	55.71
	24H_T1	63 486 314	46 936 724	44 253 299	90.16	53.85
	48H_T1	52 103 870	38 336 362	36 116 108	90.17	54.89
	72H_T1	44 586 954	32 782 356	30 866 465	90.01	54.27
	96H_T1	46 841 824	34 646 004	32 488 555	90.10	53.97
3	Control	46 896 014	35 020 502	33 171 580	90.25	55.22
	24H_T1	58 885 520	43 974 608	41 591 953	90.19	53.96
	48H_T1	47 250 466	35 210 240	33 182 069	90.35	54.66
	72H_T1	49 285 984	35 300 722	33 011 496	89.72	54.34
	96H_T1	49 478 908	36 067 604	34 153 450	89.84	54.38

Identification of DETs in response to *Bg* infection

The analysis revealed 1014, 830, 2207, and 1993 differentially expressed transcripts (DETs) at 24, 48, 72, and 96 h post inoculation (hpi), respectively. At 72 hpi, out of 2207 transcripts, 1033 were down-regulated whereas 1174 were up-regulated (Figure 3A). This suggests that the highest level of differential expression occurred at or after 72 hpi. The phenotypic observation of the *Bg*-treated (T1) plants also exhibited growing lesions at 72 hpi (Figure 2). In contrast, the 48 hpi time point demonstrated the least number of DETs (830), in which only 165 DETs were down-regulated and 665 DETs were up-regulated (Figure 3A). This data is also in line with the disease progression whereby no lesion was observed on the plants treated with *Bg* (T1) at 48 hpi (Figure 2).

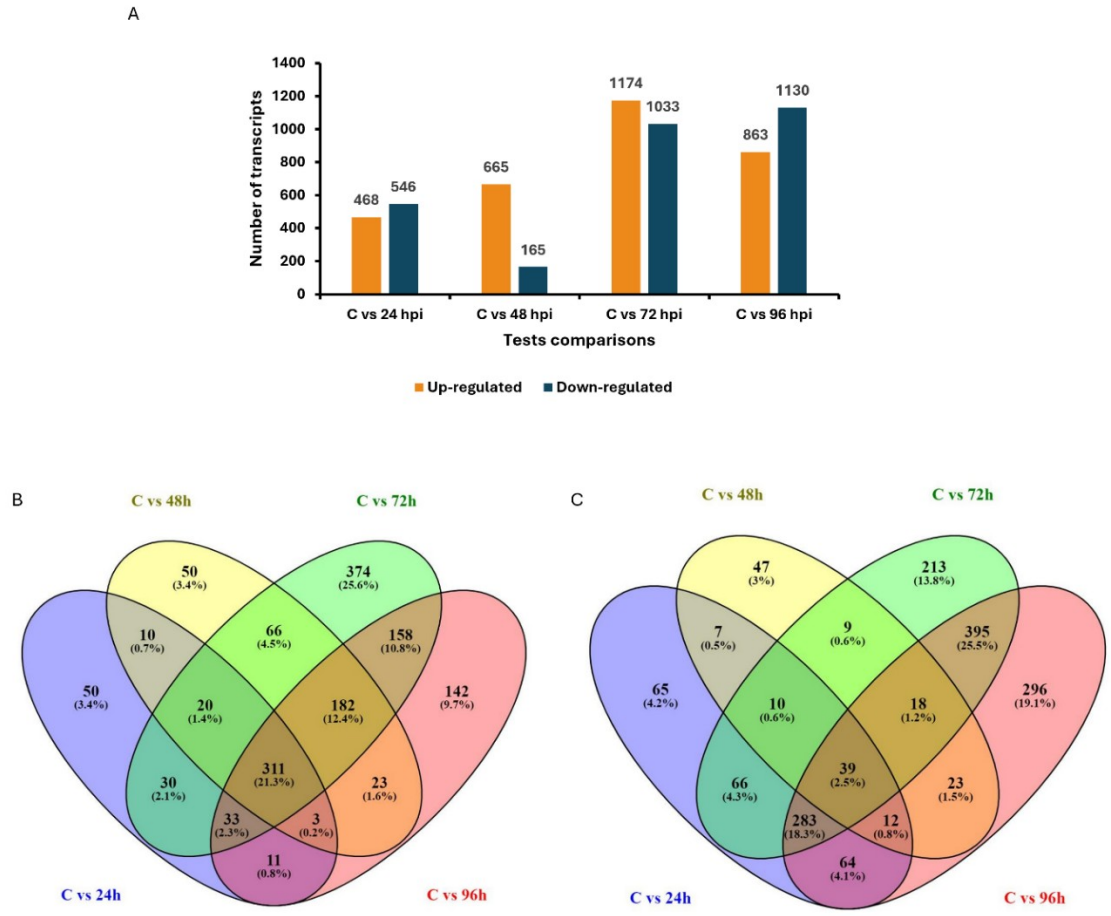

Figure 3. Statistical chart and Venn diagram of the differentially expressed transcripts (DETs) at four time points. (A) Statistical chart of the DETs. The rectangles represent the number of up-regulated (orange) and down-regulated (blue) transcripts. (B) Venn diagram of the up-regulated and (C) down-regulated DETs of *Burkholderia glumae* (*Bg*)-treated (T1) plants. These transcripts were obtained from the C (control) *vs*. 24 h, C *vs*. 48 h, C *vs*. 72 h, and C *vs*. 96 h comparisons.

The identified DETs were then compared using Venny 2.1.0 (https://bioinfogp.cnb.csic.es/tools/venny/) to determine the relationship between them at different time points. There were 21.3% (311 DETs) of the up-regulated and 2.5% (39 DETs) of the down-regulated DETs expressed consistently throughout all the time points (Figures 3B and 3C). The up-regulated DETs exhibited the highest number of overlapping DETs across all time points, followed by DETs that overlap at 48, 72, and 96 hpi only (12.4%) and DETs that overlap at 72 and 96 hpi only (10.8%) (Figure 3B). For down-regulated DETs, the highest number of overlapping DETs was observed at 72

and 96 hpi only (25.5%), followed by DETs at the overlapping time points of 24, 72, and 96 hpi only (18.3%), and the overlapping time points of 24 and 96 hpi only (4.1%) (Figure 3C). There were 616 up-regulated DETs that were uniquely expressed at 24 (50 DETs), 48 (50 DETs), 72 (374 DETs), and 96 hpi (142 DETs) (Figure 3B). Out of these, Os04t0526600-01 which encodes for alpha-amylase/subtilisin inhibitor and defence of the seed against microorganisms exhibited the highest up-regulation at 72 hpi (Table 3). Among the down-regulated DETs, 621 of them were uniquely expressed at each time point (Figure 3C). The most down-regulated DET at 72 hpi was Os03t0157300-01, which encodes a Ca^{2+}-permeable mechanosensitive channel, regulation of plasma membrane Ca^{2+} influx, and reactive oxygen species (ROS) generation induced by hypo-osmotic stress (Table 4).

Table 3. List of selected uniquely expressed genes that were up-regulated at four time points. hpi: Hours post inoculation.

Sequence name	Gene ID	Description	Time point (hpi)	Log 2 (fold-change)
OsG0000006662.01.T01	Os06t0557700-01	Protein kinase, catalytic domain containing protein	24	2.97
OsG0000010985.01.T01	Os07t0618400-02	Leucine-rich repeat transmembrane protein kinase 1 (Fragment)	24	2.68
OsG0000034383.01.T01	Os03t0401100-01	Protein kinase domain containing protein, expressed	24	2.14
OsG0000016174.01.T01	Os11t0173800-01	Serine/threonine protein kinase-related domain containing protein	48	2.59
OsG0000003890.01.T01	Os08t0433400-02	MYB transcription factor, response to salt stress	48	2.53
OsG0000019223.01.T01	Os04t0522500-01	Gibberellin (GA) 2-oxidase, regulation of plant stature, abiotic and biotic stress	48	2.27
OsG0000024006.01.T01	Os02t0726700-02	Basic helix-loop-helix (bHLH)-type transcription factor, JA-mediated resistance response against rice bacterial blight	48	2.16
OsG0000019136.01.T01	Os04t0546800-01	Ethylene response factor (ERF) protein, transcription factor, ethylene response	48	2.09
OsG0000019169.01.T01	Os04t0526600-01	Alpha-amylase/subtilisin inhibitor, defense of the seed against microorganisms	72	6.99
OsG0000024214.01.T01	Os02t0702100-01	Transcription factor GT-3b	72	4.71
OsG0000009856.01.T01	Os10t0464000-01	Hypersensitive-induced response protein	72	3.47
OsG0000001302.01.T01	Os05t0405000-02	Cytosolic pyruvate orthophosphate dikinase (PPDK, EC 2.7.9.1), starch metabolism and structure, modulation of carbon flow for starch and lipid biosynthesis during grain filling	72	3.18
OsG0000034901.01.T01	Os03t0322900-01	Late embryogenesis abundant (LEA) protein, abiotic stress tolerance	72	2.79
OsG0000012399.01.T01	Os07t0283125-00	Lectin-like receptor kinase 7	96	3.24
OsG0000032375.01.T01	Os10t0442000-01	Lectin-like receptor kinase 7;2	96	3.18
OsG0000008066.01.T01	Os02t0787200-01	Serine/threonine protein kinase-related domain containing protein	96	2.80
OsG0000013906.01.T01	Os12t0567300-00	Myb transcription factor domain containing protein	96	2.70
OsG0000036273.01.T01	Os03t0113900-02	A member of S40 gene family, leaf senescence, response to pathogen infection	96	2.51

Table 4. List of selected uniquely expressed genes that were down-regulated at four time points. hpi: Hours post inoculation.

Sequence name	Gene ID	Description	Time point (hpi)	Log 2 (fold-change)
OsG0000017205.01.T01	Os11t0523700-01	bHLH transcription factor, positive regulation of chilling tolerance, control of stomatal initiation, regulation of mature stoma differentiation	24	-6.06
OsG0000019174.01.T01	Os04t0526000-01	Auxin-induced basic helix-loop-helix transcription factor	24	-2.90
OsG0000010848.01.T01	Os07t0637700-00	Disease resistance-responsive family protein	24	-2.34
OsG0000006642.01.T01	Os06t0562200-01	Pyrabactin resistance-like (PYL) abscisic acid receptor family protein, ABA-mediated inhibition of seed germination, drought and cold stress tolerance	24	-2.26
OsG0000011065.01.T01	Os07t0614700-02	SPX domain (SYG1, Pho81 and XPR1)-containing protein, negative regulation of phosphate starvation signalling	24	-2.10
OsG0000024704.01.T01	Os02t0631700-01	A-type response regulator, cytokinin signalling	48	-3.24
OsG0000031054.01.T01	Os01t0278000-01	Valine-glutamine (VQ) protein family, disease resistance, control of flowering time	48	-2.28
OsG0000000815.01.T01	Os05t0476700-01	U-box E3ubiquitin ligase, positive regulation of cold stress response	48	-2.15
OsG0000031301.01.T01	Os01t0225600-01	Atypical 5C LEA (late embryogenesis abundant) protein, multiple stress tolerance, Hg accumulation	48	-2.04
OsG0000030049.01.T01	Os01t0547600-01	High-affinity nitrate transporter, nitrate transport, auxin signalling	48	-2.03
OsG0000030226.01.T01	Os03t0157300-01	Ca^{2+}-permeable mechanosensitive channel, regulation of plasma membrane Ca^{2+} influx, ROS generation induced by hypo-osmotic stress	72	-14.16
OsG0000007162.01.T01	Os02t0706400-01	MYB-related transcription factor, regulation of leaf senescence and salt-stress response	72	-2.84
OsG0000003720.01.T01	Os12t0610200-01	Basic helix-loop-helix transcription factor, regulation of grain size	72	-2.65
OsG0000000368.01.T01	Os05t0543600-01	R2R3-type MYB transcription factor, regulation of the development of secondary cell wall	72	-2.62
OsG0000007514.01.T01	Os06t0291600-01	Protein kinase G11A (EC 2.7.1.-) (Fragment)	72	-2.26
OsG0000025450.01.T01	Os02t0514500-01	Glycerophosphodiester phosphodiesterase, response to Pi deficiency, low Pi acclimation	96	-2.79
OsG0000023995.01.T01	Os02t0731200-01	MADS-box transcription factor, cold tolerance, control of tillering	96	-2.76
OsG0000014103.01.T01	Os12t0515300-01	MYB transcription factor, regulation of secondary wall biosynthesis	96	-2.74
OsG0000022783.01.T01	Os09t0491532-01	Squamosa promoter-binding-like protein 17, transcription factor, target gene of microRNA156, SBP-box family protein, modulation of root elongation in response to nitrate supply	96	-2.35
OsG0000031493.01.T01	Os01t0196300-01	Basic helix-loop-helix (bHLH) transcription factor, diterpenoid phytoalexin factor, biosynthesis of diterpenoid phytoalexins, stress response	96	-2.35

Gene Ontology annotation of DETs

The functional annotation of identified DETs revealed the involved Gene Ontology (GO) terms in the cellular component, molecular function, and biological process categories. Bubble plots were generated using R to compare all the identified DETs in the three categories of the GO enriched at each time point (Figures 4, 5, and 6).

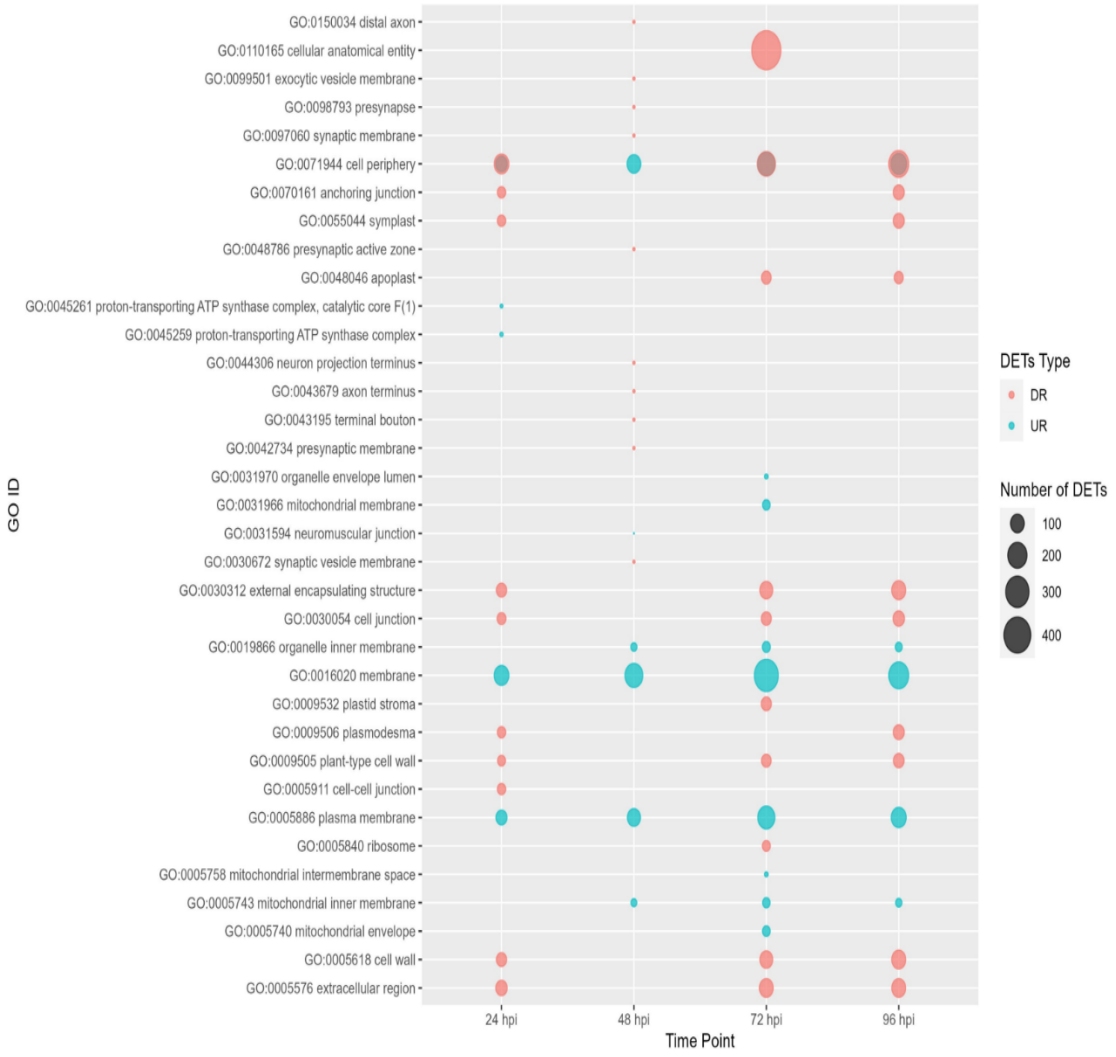

Figure 4. Statistical gene ontology (GO) annotation of the up-regulated and down-regulated differentially expressed transcripts (DETs) at four time points in cellular component category. DR: Down-regulated; UR: up-regulated; hpi: hours post inoculation.

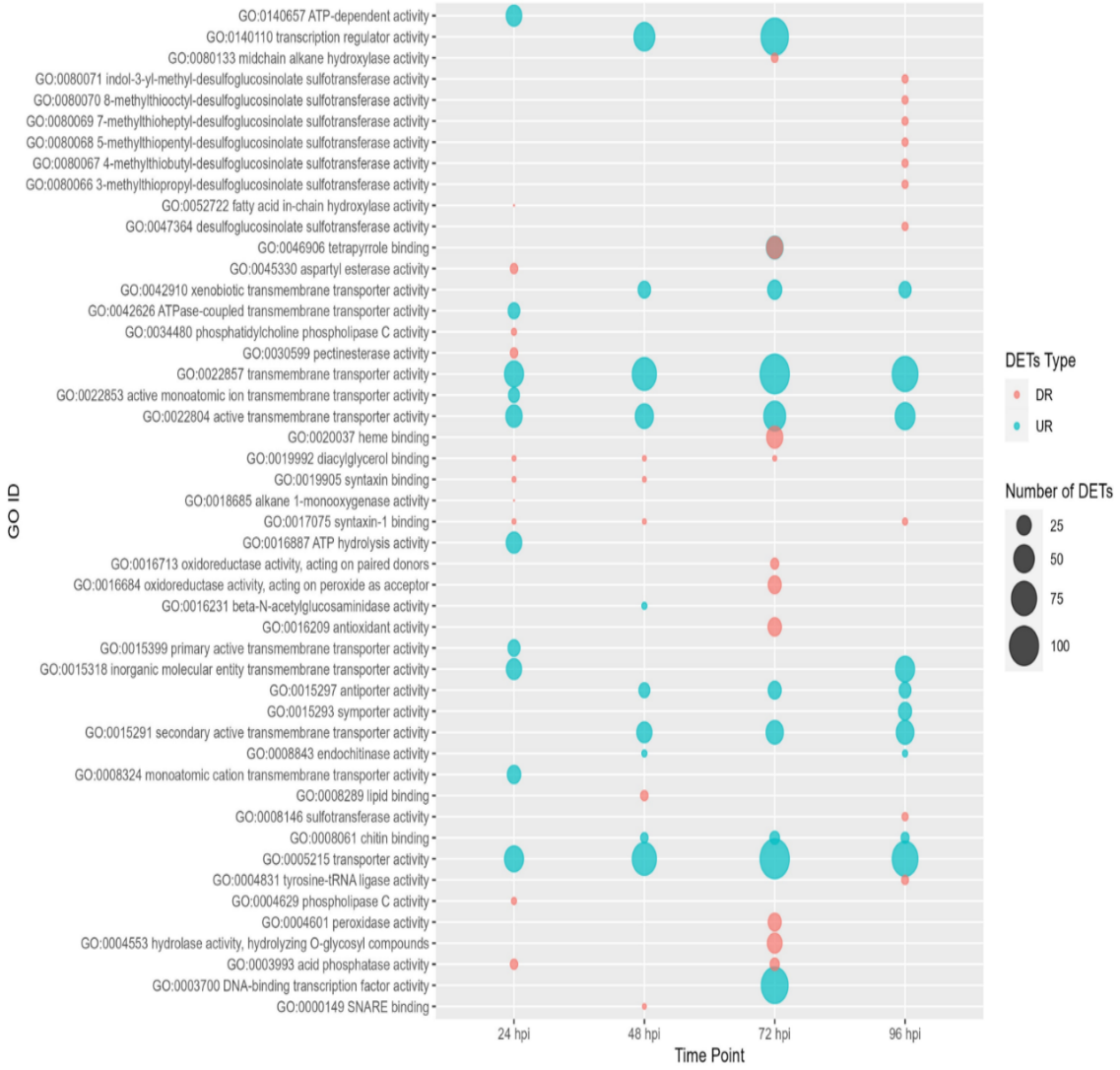

Figure 5. Statistical gene ontology (GO) annotation of the up-regulated and down-regulated differentially expressed transcripts (DETs) at four time points in molecular function category. DR: Down-regulated; UR: up-regulated; hpi: hours post inoculation.

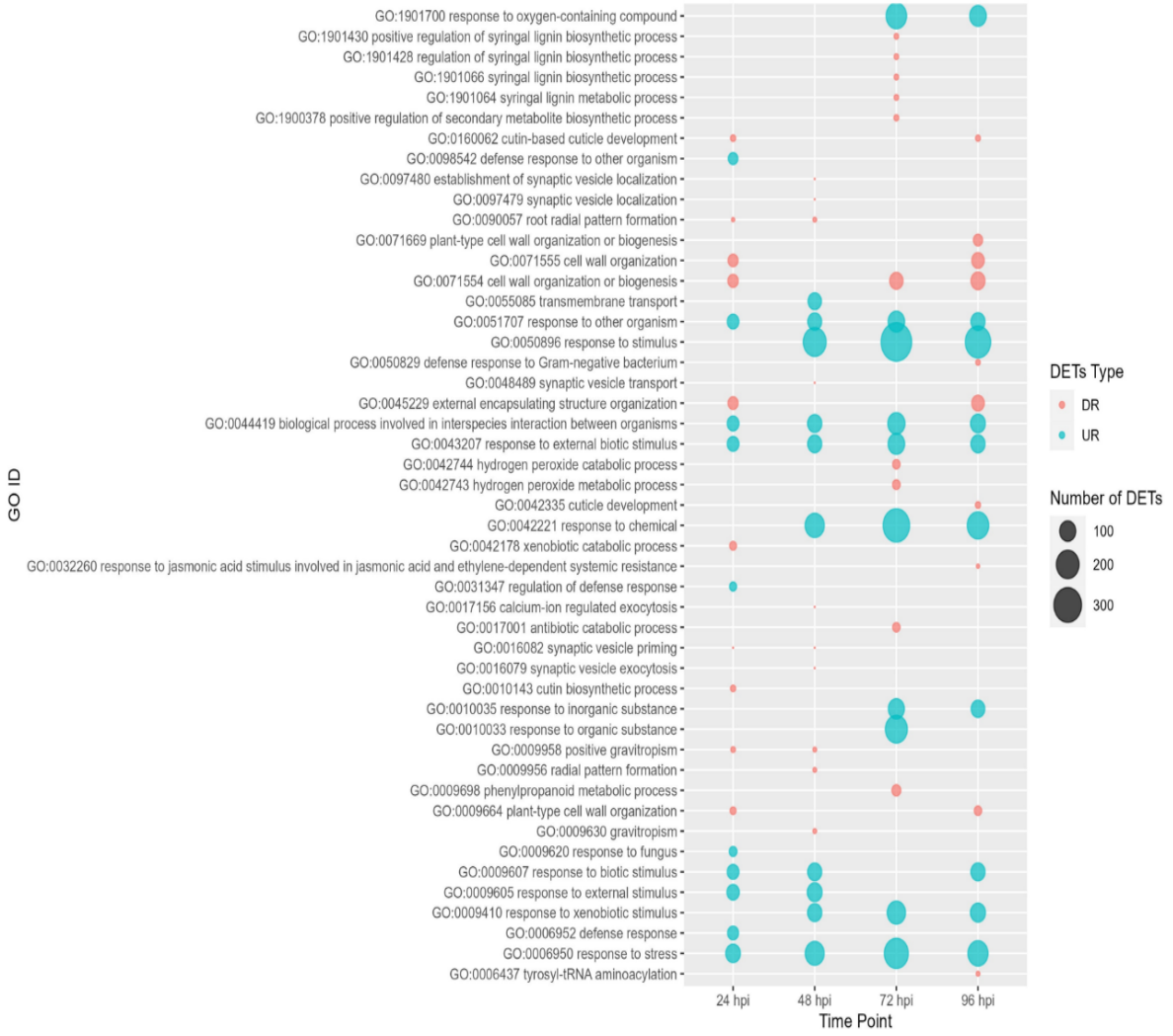

Figure 6. Statistical gene ontology (GO) annotation of the up-regulated and down-regulated differentially expressed transcripts (DETs) at four time points in biological processes category. DR: Down-regulated; UR: up-regulated; hpi: hours post inoculation.

In the cellular component category, the GO terms with the highest number of up-regulated and down-regulated DETs were membrane (GO:0016020) and cellular anatomical entity (GO:0110165), respectively (Figure 4). When we compared the GO terms enriched to four time points, membrane (GO:0016020), plasma membrane (GO:0005886), and cell periphery (GO:0071944) were identified to be common for up-regulated DETs. However, no GO terms were enriched across all time points for down-regulated DETs (Figure 4).

The most significantly enriched molecular function associated with up-regulated DETs were transmembrane transporter activity (GO:0022857) and transporter activity (GO:0005215). While heme binding (GO:0020037) and tetrapyrrole binding (GO:0046906) were the most significantly down-regulated molecular function (Figure 5). Across all time points, transmembrane transporter activity (GO:0022857), transporter activity (GO:0005215), and active transmembrane transporter activity (GO:0022804) were the enriched GO terms for up-regulated DETs (Figure 5). However, none of the GO terms were found to be enriched at all time points for down-regulated DETs (Figure 5).

The most significant biological process associated with up-regulated DETs was the response to stimulus (GO:0050896), followed by the response to chemical (GO:0042221), both observed at 72 hpi (Figure 6). On the other hand, the most down-regulated DETs in this category were enriched in cell wall organization or biogenesis (GO:0071554) at 96 and 72 hpi, followed by external encapsulating structure organization (GO:0045229) at 96 hpi. The response to stress (GO:0006950), biological process involved in interspecies interaction between organisms (GO:0044419), response to external biotic stimulus (GO:0043207), and response to other organisms (GO:0051707) were found to be enriched at all time points for up-regulated DETs (Figure 6). Whereas no GO terms were enriched at all time points for down-regulated DETs.

Validation of RNA-Seq data via RT-qPCR

To validate the accuracy and reproducibility of the *in-silico* RNA-seq expression data for the selected DETs at four time points, the expression levels of these DETs were further verified using RT-qPCR analysis. The analysis validated the expression data of RNA-seq DETs obtained from the comparative transcriptome analysis of *Bg*-treated (T1) plants at 24, 48, 72, and 96 hpi.

The RT-qPCR results showed that the expression of the six randomly selected genes was mostly in line with the data generated from the RNA-seq analysis (Figure 7). The expression levels of the three selected up-regulated genes were significantly higher in the T1 samples as compared to the T0 samples. The gene expression of the T0 samples exhibited variability across all time points, namely LOC127769352 and LOC127786326. Both LOC127769352 and LOC127780511 exhibited minimal difference between the RNA-seq data and RT-qPCR. However, the expression levels of LOC127786326 in T1 samples were lower at 24 and 48 hpi when measured by RT-qPCR compared to RNA-seq, with a factor change of more than 2-fold (Figure 7).

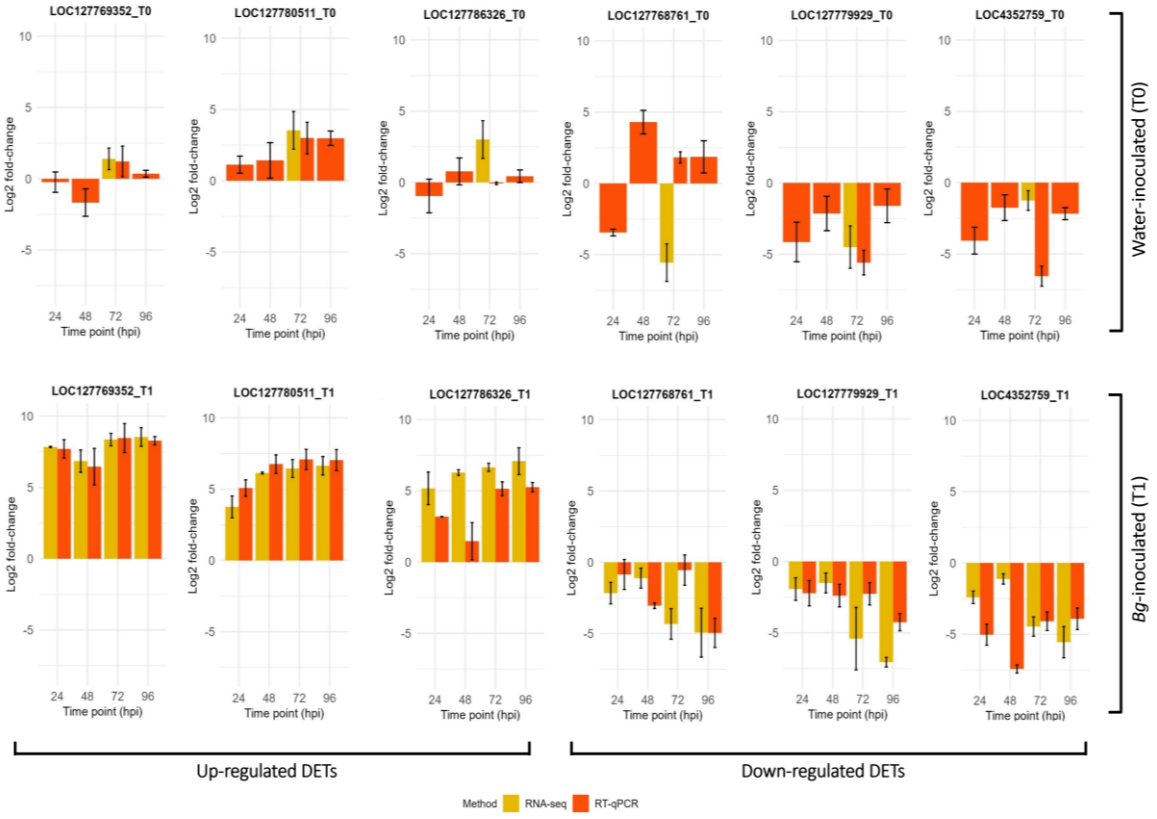

Figure 7. Validation of differential expression of six selected transcripts in mock-treated plants (T0) and *Burkholderia glumae* (*Bg*)-treated plants (T1) using RT-qPCR. Yellow and orange colours indicate RNA-seq and RT-qPCR, respectively. Bars show the standard deviations from three replicates. hpi: Hours post inoculation.

The analysis also revealed that the expression of the three selected down-regulated genes exhibited a slightly significant difference in T1 samples compared to the T0 samples. The gene expression of LOC127779929 and LOC4352759 showed consistent patterns with RNA-seq data when analysed using RT-qPCR at all time points. The expression level of LOC127779929 was significantly higher in the T0 samples compared to the T1 samples at 24 and 72 hpi. Only LOC127768761 showed different expression levels at 48, 72, and 96 hpi in comparison to the RNA-seq data (Figure 7).

DISCUSSION

Bacterial panicle blight significantly affects global rice production and quality. The development and deployment of resistant rice varieties is widely accepted as the most efficient approach to control the disease. However, many of the cultivated varieties in producing countries exhibit susceptible responses, with only a few cultivars being moderately resistant (Pinson et al., 2010; Mizobuchi et al., 2013; 2016; 2018; Wahidah et al., 2019; Zhou, 2019). Hence, wild rice species could provide an invaluable and unexplored source of genetic diversity for BPB resistance (Azmi et al., 2024). *Oryza rufipogon*, a common wild rice, has been one of the most important sources of disease resistance in rice (Wang et al., 2015). In this study, however, all five *O. rufipogon* accessions were highly susceptible to the *Bg* strain, BGS.AS1, like the susceptible control, MR219 (Md Zali et al., 2022). While transcriptomic study of rice under *Bg* infection has been previously reported by Magbanua et al. (2014), the response of wild rice species to *Bg* infection remains unexplored. Thus, we characterized the transcript expression patterns of a susceptible *O. rufipogon* accession throughout different time points.

Similar to Magbanua et al. (2014), stems of young plants were inoculated rather than panicles for a faster evaluation of the symptoms. The earliest time point at which significant browning and lesion formation was observed occurred after 72 hpi. Interestingly, the majority of DETs were found at 72 hpi, displaying either up-regulated or down-regulated expression. This finding was consistent with the increased level of BPB symptoms observed at this time point. The overall functional annotation of the identified DETs is aligned with a previous report (Magbanua et al., 2014) of DETs in susceptible host under the *Bg* infection. Some of them were uniquely expressed at each time point. The most significantly up-regulated DET found at 72 hpi was Os04t0526600-01, which encodes for alpha-amylase/subtilisin inhibitor and defence of the seed against microorganisms. Many plant pathogens secrete subtilisin-like proteases to degrade immune-related host proteins, hence promoting colonization. Plants counteract these proteases using proteases inhibitors, but pathogens may manipulate the expression of these inhibitors to benefit themselves (Jashni et al., 2015). On the other hand, the highly down-regulated DET at 72 hpi was Os03t0157300-01, which encodes Ca^{2+}-permeable mechanosensitive channel, regulation of plasma membrane Ca^{2+} influx, and reactive oxygen species (ROS) generation induced by hypo-osmotic stress. This gene is crucial for regulating calcium homeostasis and ROS production during stress conditions including pathogen attacks. The down-regulation of this gene may help prevent excessive Ca^{2+} influx and uncontrolled ROS accumulation that can lead to potential cytotoxicity (Kurusu et al., 2013; Seybold et al., 2014).

The GO analysis for cellular component category revealed that many transcripts related to membrane were highly differentially expressed across all time points (Figure 4). This could be the central role of membrane in recognizing essential components of whole classes of pathogens such as pathogen-associated molecular patterns (PAMPs), which leads to PAMP-triggered immunity (PTI) (Schellenberger et al., 2019). Even the lowly differentially expressed transcripts were observed to be related to cellular anatomical entity which could also related to membrane. This observation is similar to the previous study by Magbanua et al. (2014), in which they also observed an up-regulation of transcripts enriched in plasma membrane and membrane parts during susceptible interaction of rice and *Bg.* Cell periphery which includes cell wall also have transcripts that were highly and lowly differentially expressed. The cell wall serves as the primary barrier in the plant's defence system. If the cell wall is breached, the plant then senses invading microbes by recognizing PAMPs and activating the PTI (Malinovsky et al., 2014). This indicates that the wild rice cell wall has been compromised, leading to the activation of PTI as *Bg* exhibits its biotrophic characteristics.

In the GO analysis of molecular function category, transmembrane transporter activity and active transmembrane transporter activity were significantly enriched across all time points (Figure 4). The proteins of the membrane transporters (MTs), which are embedded within membrane-bounded organelles, play a

crucial role in maintaining cellular homeostasis, xylem loading, and remobilization of sugar molecules via phloem loading (Gill et al., 2021). Some MTs like sugars will eventually be exported transporters (SWEET), multidrug, and toxic compound extrusion (MATE) transporters, ATP-binding cassette (ABC) transporters, natural resistance-associated macrophage proteins (NRAMP), and sugar transporter proteins (STPs) have a significant role in plant disease resistance. However, transcription activator-like effectors (TALEs) from some pathogen species tend to target the gene encoding SWEET transporters that release sugar into the apoplast to cause plant susceptibility (Devanna et al., 2021). By using transcription activator-like effector nuclease (TALEN), OsSWEET14 promoter was mutagenized to disrupt the binding site of the *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) effector proteins, which ultimately resulted in bacterial blight-resistant rice (Li et al., 2012). It is worth noting that in rice-*Bg* pathosystem, signal transducer, receptor activities, and carbohydrate binding were significantly enriched for up-regulated DETs during compatible interaction (Magbanua et al., 2014), suggesting that susceptibility of wild rice to BPB may be governed by a different set of genes.

Most of the up-regulated transcripts enriched in biological processes were related to response to stimulus (GO:0050896), response to stress (GO:0006950), interspecies interaction between organisms (GO:0044419), response to external biotic stimulus (GO:0043207), and response to other organisms (GO:0051707). This transcriptional reprogramming demonstrates the broad, general responses of *O. rufipogon* to stress and pathogen stimuli upon *Bg* attack (Zhang and Sonnewald, 2017; Saijo and Loo, 2020). While in the rice-*Bg* susceptible interaction, Magbanua et al. (2014) reported that the biological processes enriched for up-regulated transcripts were lipid metabolic process, defence response, and programmed cell death, indicating a more specific immune response, including lipid metabolism and defence-related pathways.

CONCLUSIONS

In conclusion, most of the differentially expressed transcripts were observed after 72 h, and the functional annotation of these transcripts revealed that the highly susceptible *Oryza rufipogon* accession may respond to bacterial panicle blight (BPB) infection by expressing genes associated with membrane and transmembrane transporters. We hypothesize that the expression of differentially expressed transcripts (DETs) related to these gene ontology (GO) terms reflects both the basic resistance exhibited by the highly susceptible accession upon pathogen invasion and the negative regulatory effects of certain genes within these GO terms that suppress resistance as well as increase disease susceptibility. Our findings provided useful insights into the transcriptional response of the highly susceptible *O. rufipogon* accession to *Burkholderia glumae* infection and established a theoretical foundation for enhancing rice resistance to the BPB disease.

Author contribution

Conceptualization: M.A.M.H. Methodology: M.I.H.M.A., D.Z., S.I.R., S.N.A.A. Software: M.I.H.M.A. Formal analysis: M.I.H.M.A. Investigation: M.I.H.M.A. Data curation: M.I.H.M.A. Writing-original draft: M.I.H.M.A. Writing-review & editing: M.A.M.H. Supervision: D.Z., S.I.R., M.A.M.H. Project administration: M.A.M.H. Funding acquisition: M.R.I., S.N.A.A., M.A.M.H. All co-authors reviewed the final version and approved the manuscript before submission.

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