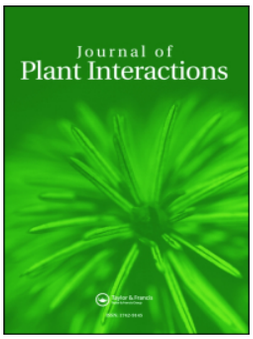

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Comparative transcriptome analysis of resistant, moderately resistant, and susceptible wheat-near-isogenic lines in response to *Puccinia striiformis tritici*

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ABSTRACT

Stripe/yellow rust is caused by a biotrophic fungal pathogen known as *Puccinia striiformis f. sp.tritici (Pst)*. RNA seq technique was used to understand the wheat and stripe rust interaction in three different reaction types. Leave samples of three near-isogenic lines (NILs) of wheat showing Resistant/immune (R), Moderately Resistant (MR), and Susceptible (S) (inoculated with *Puccinia striiformis* race 574232) responses were collected 48 and 72 h after inoculation (hai). Overall, 8,595 DEGs were upregulated and 8,741 were downregulated with high numbers (4,357) of DEGs identified S-R in comparison at 72hai. Subsequent Gene Ontology (GO) enrichment analysis suggests protein phosphorylation, biological process, and cellular process of internal components of membrane enriched in all three comparisons at both time points. DEGs identified as serine/threonine receptor-like kinases, peroxidases, Mitogen-phosphate Kinases (MAPK), Phenylalanine ammonia-lyase (PAL), and Pathogenesis-related protein (PR) were high in resistant NIL in all comparisons at both time points. The results show that although the responses of the near isogenic lines are similar, it is the magnitude of the response that plays a role in the differences in their resistance to *Puccinia striiformis*.

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KEYWORDS
Wheat isogenic lines; resistant; moderately resistant; susceptible; *Puccinia striiformis*; RNA-seq analysis

List of abbreviations

Near Isogenic Lines	NILs
Resistant	R
moderately resistant	MR
susceptible	S
hours after inoculation	hai
differentially expressed genes	DEGs
Gene Ontology	GO
Mitogen phosphate Kinases	MAPK
Phenylalanine ammonia-lyase	PAL
Pathogenesis-related protein	PR
hypersensitive response	HR

Introduction

Puccinia striiformis f. sp.tritici (Pst) is a pathogen that causes wheat stripe (yellow) rust, hinders wheat production in more than 60 countries, and results in high crop losses annually (Wamalwa et al. 2022). Depending on the genetic makeup of the wheat cultivar, initial infection timing, growth rate, and the environment, the PST infection may result in yield losses of 10 to 70%. Potentially, severe epidemics could result in yield losses of up to 100% of their production (Roelfs et al. 1992; Chen 2005).

Pst is an obligate biotroph pathogen. It entirely depends on the host for survival and reproduction. When spores of *Pst* land on a leaf, they enter the host through the stomata. If the plant is susceptible, the pathogen will undergo reprogramming, hijack the plant's immune system, and colonize the host tissue by producing a mesh of invasive hyphae in the mesophyll cell layer of the leaf. These hyphae produce haustoria to obtain food from the cells. However, if the plant is resistant, the resistance proteins recognize the effector proteins produced by the pathogen and activate the defense responses. A number of studies are available where the gene expression in both resistant and susceptible varieties has been characterized during infection (Coram et al. 2008; Chen et al. 2013)

RNA-seq is a promising technology in elucidating the molecular mechanisms of resistance to rust, evaluating gene profiles and the transcriptome of different plant organs including candidate resistance genes, and identifying associated markers for marker-assisted breeding Several studies are available in which interaction between wheat and Puccinia is studied using RNA-seq techniques in compatible and incompatible interactions. Yadav et al. (2016) reported that the near-isogenic-line carrying *Lr57*(WL711+*Lr57*) had numerous differentially expressed genes (DEGs) than the susceptible genotype (WL711). Specifically, more protein kinases and pathogenesis-related (PR) proteins, such as chitinases and glucanases, were expressed in the resistant genotype. (Lee et al. 2020) reported high expression of β-1,3-glucanase and peroxidase in two *Ae. tauschii* accessions in incompatible interaction with leaf rust. (Seifi et al. 2021) reported high

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expression of antioxidant enzymes in a near-isogenic line carrying Yr15 with *Puccinia striiformis*. They also studied non-race specific, wheat-stripe rust interaction using recent RNAseq techniques. Their results have shown the activation of certain processes which are directly involved in the activation of defense response at the adult plant stage. These included the biosynthesis of phenylpropanoid and the production of reactive oxygen species. (Dobon et al. 2016) provide further credence to these results. Using RNA-seq, (Das et al. 2023) studied two near-isogenic-line (NILs), resistance line FLW29, and rust-susceptible line PBW343 and identified 10 differentially expressed lncRNA transcripts in resistant near-isogenic line (NIL). The NAC domain protein, disease resistance proteins RPP13 and RPM1, At1g58400, monodehydroascorbate reductase, NBS-LRR-like protein, rust resistance kinase Lr10-like, LRR receptor, serine/threonine-protein kinase, and cysteine proteinase were among the identified targets that are crucial for wheat stripe rust resistance. RNA-seq analysis of Shumai126, a very strong stripe rust-resistant cultivar, has shown the dominance of oxidative phosphorylation, MAPK signaling pathway, and phenylalanine metabolism in response to infection (Wang et al. 2021).

Despite these achievements, no study has so far studied the moderately resistant varieties in conjunction with the resistant and susceptible reaction types. Therefore, this study aims to bridge this gap by analyzing the transcriptome of not only two contrasting stripe rust-resistant and susceptible NILs but also stripe rust moderately resistant NIL. To the best of our knowledge, this is the first use of the RNA-seq approach to study the wheat stripe rust interactions of three NILs showing resistant, moderately resistant, and susceptible reactions to the same *Puccinia striiformis* race. These NILs had the same genetic background and differed only by a few loci. This also ensured reducing background noises that come from different genetic backgrounds (Pumphrey et al. 2007).

Methods

Plant materials

Near-isogenic-line (NILs) in Avocet background were used for this study. Seeds of NILs were obtained from the National Agriculture Research Center (NARC), Islamabad.

Pathogen

Puccinia striiformis race 574232 was used in the study. The inoculum of the race was collected from the National Agricultural Center Islamabad. Following is the avirulence/virulence formula of the race.

*Yr*5, *Yr*10, *Yr*15, *Yr*24, *YrSp*, *YrTr*1, *YrTye*/*Yr*1, *Yr*6, *Yr*7, *Yr*8, *Yr*9, *Yr*17, *Yr*27, *Yr*43, *Yr*44, *YrExp*2.

Transcriptome profiling

Sample preparation

Three wheat NILs, resistant *Yr*5/6*, moderately resistant *Yr*32/6 Avocet S, and susceptible Yr44/6*, were selected for the transcriptome study. Twenty seeds of each NIL were planted in plastic trays within a greenhouse under controlled conditions of 16°C temperature, and a 16-hour light and 8-hour dark cycle. Each NIL was planted in three replicates. At the twelve-day-old seedling stage, fresh urediniospores of *P. striiformis f.* sp. *tritici* (PST race 574232) were used to inoculate the seedlings, which were then incubated in a dew chamber in the dark at 9°C with 100% humidity for 24 h. After the incubation in the dew chamber, the plants were shifted to a glasshouse set at 16°C under a 16 h light /8 h dark photoperiod. For the transcriptome study and expression analysis, random leaves of the three NILs were collected at 48 and 72 h after inoculation (hai). These leaves were preserved in RNA later for further processing and analysis. The remaining plants were used to monitor the development of the reaction type. The sampling was carried out in triplicates.

RNA extraction and RNA-seq analysis

The total RNA of all 18 samples was extracted through the RNeasy® Plant Mini Kit (Qiagen®) protocol. SuperScript™ III First-Strand Synthesis System was used to synthesize cDNA libraries (Invitrogen™). Sequencing of all 18 samples was performed at 50X coverage on the Illumina HiSeq 2500 platform using PE150 run at Macrogen, Korea.

Data processing and quality trimming

The sequenced raw reads/adapter-trimmed reads were processed to obtain high-quality clean reads using the Sickle v1.200 (https://github.com/najoshi/sickle). Reads with an average Phred quality score below 20 were trimmed. Paired-end reads that had a length greater than 50 bp after trimming were retained.

Reference genome and annotation retrieval

The reference genome of *Triticum aestivum* IWGSC refseq V53.0 was downloaded from ENSEMBL (https://plants.ensembl.org/Triticum_aestivum/Info/Index) along with detailed annotation data for all chromosomes in GFF3 format. This annotation contains information about genes, transcripts, exons, and other genomic features.

Transcriptome assembly

The recommendations from the RNA-seq processing workflow, given as a protocol by (Trapnell et al. 2012), were followed. The quality-trimmed reads were aligned against the reference genome using the splice-aware aligner, TopHat v2.1.1 and the annotation file was included during the alignment process. The aligned reads in BAM format from each sample were then used as input for Cufflinks v2.2.1 to perform transcriptome assembly.

Differential expression analysis

The assembled transcripts were merged using Cuffmerge, and along with the information of biological replicates for three wheat NILs (Near-Isogenic Lines) across two-time points, differential expression analysis was performed using Cuffdiff. This step identified genes/transcripts that showed significant differential expression in all three NILs at the two-time points.

Statistical exploration and visualization

The output generated by Cuffdiff was further analyzed using CummeRbund (Trapnell et al. 2012) an R package designed for the visualization and exploration of Cufflinks high-

throughput sequencing data. CummeRbund provides a comprehensive statistical and visualization framework, allowing us to explore differential gene expression results in depth. Additionally, custom-made R scripts were developed to enhance data representation in downstream processes.

Unraveling differentially expressed genes

Differentially expressed genes (DEGs) were identified by calculating the Fragments Per Kilobase of transcript per Million(FPKM) value and the read count of each gene using Cufflinks (Trapnell et al. 2012; Anders et al. 2015). DEGs were determined based on a $\log_2$ fold change of at least 1.5 and a False Discovery Rate (FDR) of $p < 0.05$. To analyze changes in gene expression patterns, hierarchical cluster analysis was performed on these DEGs. *p*-values were adjusted using the Benjamini-Hochberg correction for multiple testing (Benjamini and Hochberg 1995).

Comparative analysis of DEGs was carried out at three levels between samples: resistant (*Yr*5), moderately resistant (*Yr*32), and susceptible (*Yr*44) at two-time points. The three NILs were compared in three pairwise comparisons at two time-points (S48-R48, S48-MR48, R48-MR48, S72-R72, S72-MR72, and R72-MR72).

To summarize the DEGs and visualize the results of all three comparisons at two-time points, particularly identifying the number of transcripts differentially expressed and shared between all comparisons, as well as those consistently upregulated or downregulated across all comparisons, we utilized the ggVennDiagram package (Gao et al. 2021).

Gene Ontology (GO) annotation and enrichment analysis of differentially expressed genes

R's BioMart package (Durinck et al. 2005) was used to obtain the comprehensive Gene Ontology (GO) records associated with differentially expressed transcripts from all three comparisons at two-time points. Subsequently, these GO annotations were categorized into Molecular Functions, Cellular Components, and Biological Functions using the GO.db package. For GO term enrichment analysis, the classic Fisher's statistics algorithm was applied, using the topGO package in R, and the top 50 enriched GO terms with *p*-values less than 0.01 were obtained (Rahnenfuhrer 2022).

Defense-related genes discovery

A custom-made R script was developed to retrieve transcription factors (TF), kinases, and defense-related genes from each comparison.

Validation of differentially expressed genes through quantitative real-time PCR (qRT-PCR)

To evaluate the reliability of the RNA-seq and DEG analysis, 21 candidate genes were selected. The genes were selected on the basis of their transcript levels and role reported in plant defense in previous research. The mRNA levels of these candidate genes were analyzed by qRT-PCR.

RNA was extracted from all three wheat reaction types using RNeasy® Plant Mini Kit from Qiagen® from all three wheat reaction types (R, MR, and S). The genomic DNA contamination was eliminated using DNase I treatment. The RNA concentration was assessed using a Nanodrop 2000 spectrometer (Thermo Scientific). For cDNA synthesis, 2.5µg of RNA was used as a template, and the synthesis was conducted according to the manufacturer's protocol (Thermo Fisher Scientific, Waltham, MA, USA). Gene-specific primers were designed using Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome). Macrogen Seoul, South Korea, synthesized all the primers (Supplementary Table 1).

1:10 diluted first strand cDNA and SYBR Green PCR Master MIX (Solis Biodyne HOT FIREPol® EvaGreen® qPCR Mix Plus with ROX) were used for performing quantitative real-time polymerase chain reaction (qRT-PCR). Each 10 µL reaction contained 60 ng/µL cDNA, 1× EvaGreen qPCR mix plus, 10 pmol/µL of gene primers, and nuclease-free water. Amplification was carried out in an Applied Biosystems Real-Time PCR Instruments with a cycling program consisting of initial activation at 95°C for 12 min, followed by qRT-PCR at 95°C for 1 min, and 40 cycles of 95°C for 15 s, 65°C (30 s), and 75°C (30 s). Melting curve analysis was conducted between 55°C and 95°C to verify PCR reaction specificity. The qRT-PCR included three biological replicate samples, non-template control (without template), and non-reverse transcriptase control (non-RT). Gene expression levels of five genes were calculated relative to their mean expression levels in the mock plants. The expression of the β-actin gene was used for calibration. Relative fold changes were determined using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001).

Availability of data and materials

Sequence data are available from the Sequence Read Archive (SRA) database under BioProject Submission PRJEB70648 with details of the samples provided in Data Table Supplementary 2.xlsx.

Statistical analysis

One-way ANOVA was used to determine significant differences in gene expression between inoculated and mocked plants. The difference with $p < 0.05$ was considered significant.

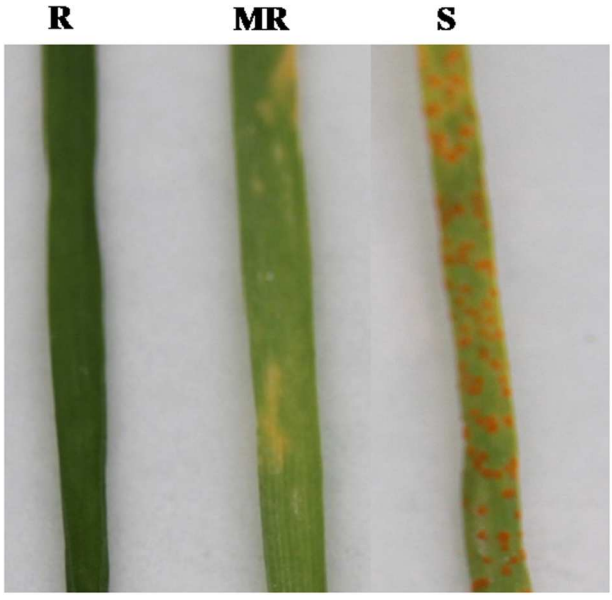

Figure 1. Seedling leaves showing reaction types 20 days after the inoculation i.e. susceptible (S), moderately resistant (MR), and resistant (R).

Table 1. Sequencing and Alignment Statistics of *Triticum aestivum* NILs (S, R, MR) Inoculated with P. striiformis at 48 and 72 hai.

Sample	Raw Reads(M)	Clean Reads(M)	GC%	Q30%	Mapped reads (M)
R48	33.22 ± 2.28	32.64 ± 2.33	53.75 ± 0.67	94.20 ± 0.16	63.57 ± 6.64
R72	42.06 ± 4.03	41.35 ± 3.94	52.59 ± 0.30	94.24 ± 0.29	62.85 ± 8.79
MR48	35.08 ± 3.48	34.52 ± 3.47	52.57 ± 0.50	94.42 ± 0.35	60.42 ± 3.22
MR72	34.49 ± 4.52	33.88 ± 4.36	52.96 ± 0.45	94.11 ± 0.05	75.41 ± 7.23
S48	39.92 ± 1.99	39.30 ± 1.91	52.43 ± 0.37	94.39 ± 0.24	72.92 ± 3.87
S72	39.18 ± 7.83	38.62 ± 7.73	52.80 ± 0.49	94.94 ± 0.91	71.67 ± 14.33

Note: Alignment was performed against the reference genome (*Triticum aestivum* IWGSC refseq V53.0 from ENSEMBL).

Results

The phenotypic response of Triticum aestivum *NILs to* P. striiformis

The selected resistant NIL of *Triticum aestivum* produced an infection type (IT) of '0,' the moderately resistant showed an IT of '4' while the susceptible one displayed an IT of '8.' Leaf tissues from resistant, moderately resistant, and susceptible NILs were sampled 48 h after inoculation (hai) and 72 hai to monitor transcriptome profiling. The plants were maintained for two-week post-inoculation to observe the reaction types (Figure 1).

RNA sequencing and mapping statistics

The RNA-seq generated a total of 67.2 Gbps of data with mean raw reads of 37,326,134 per library. The average length read was 100 base pairs (bp). After filtering out ambiguous and low-quality reads, an average of 36,716,775 high-quality (HQ) clean reads remained per library and were used in the downstream data analysis. The HQ clean reads were mapped to the *T. aestivum* reference genome (Genome length = 17 Gbps). An average of 67,806,215 clean reads were uniquely mapped (Supplementary Table 3).

The highest number of raw paired-end reads were recorded in R72 with a mean value of 42.06 ± 4.03 M followed by S48 (39.92 ± 1.99 M) and S72 (39.18 ± 7.83 M) while the lowest number of reads were obtained for resistance at 48hai with a mean value 33.22 ± 2.28 M. The percentage of GC content was higher in R48 with a mean value of 53.75 ± 0.67%. The number of mapped reads was 75.41 ± 7.23 M in MR at 72hai followed by S48 (72.92 ± 3.87 M) and S at 72hai (71.67 ± 14.33 M) (Table 1).

Overview of differentially expressed genes

To identify potential genes and related metabolic processes/pathways that could be targeted by pathogen effector proteins, differential expression (DE) analysis was performed. For this analysis, a pairwise comparison was conducted between Susceptible vs Resistant, Susceptible vs Moderately Resistant, and Resistant vs Moderately Resistant, respectively. All these comparisons were performed at two-time points (48hai & 72hai). Changes in the transcript expression were analyzed using Cuffdiff2 and genes with at least 1.5 log2-fold changes were retained (Trapnell et al. 2012). These were identified across all three comparisons at 2-time points 48 and 72 hai (S-R, S-MR, R-MR) that are involved in wheat and *Puccina striiformis* interaction (Figure 2). A total of 17,336 genes were differentially expressed in all three comparisons at two-time points. Among these, 8,595 DEGs were downregulated, and 8,741 DEGs were upregulated, respectively. The highest number of 4,357 DEGs was detected in the S72-R72 comparison, followed by S72-MR72 (4,315) (Figure 3(a)).

The number of upregulated DEGs was higher in all three comparisons at 48hai compared to downregulated DEGs. However, the number of downregulated DEGs increased compared to upregulated DEGs in all three comparisons at 72hai. At 48hai, the highest number of upregulated DEGs (1,979) and downregulated DEGs (680) was recorded for the R48-MR48 comparison. However, the number of downregulated DEGs was almost the same for S48-MR48 and S48-R48. At 72hai, the number of downregulated DEGs increased. The number of upregulated DEGs increased only for S72-MR72 (1,792) and R72-MR72 (916) (Figure 3(b)).

Among the upregulated differentially expressed genes (DEGs), 33% (1,124) were shared in the R-MR comparison at 48hai. For downregulated DEGs, a significant number were shared in the R-MR comparison at 48hai, whereas at 72hai, a higher number were shared between the S-R and S-MR comparisons, accounting for 23% (Figure 4(a–f)).

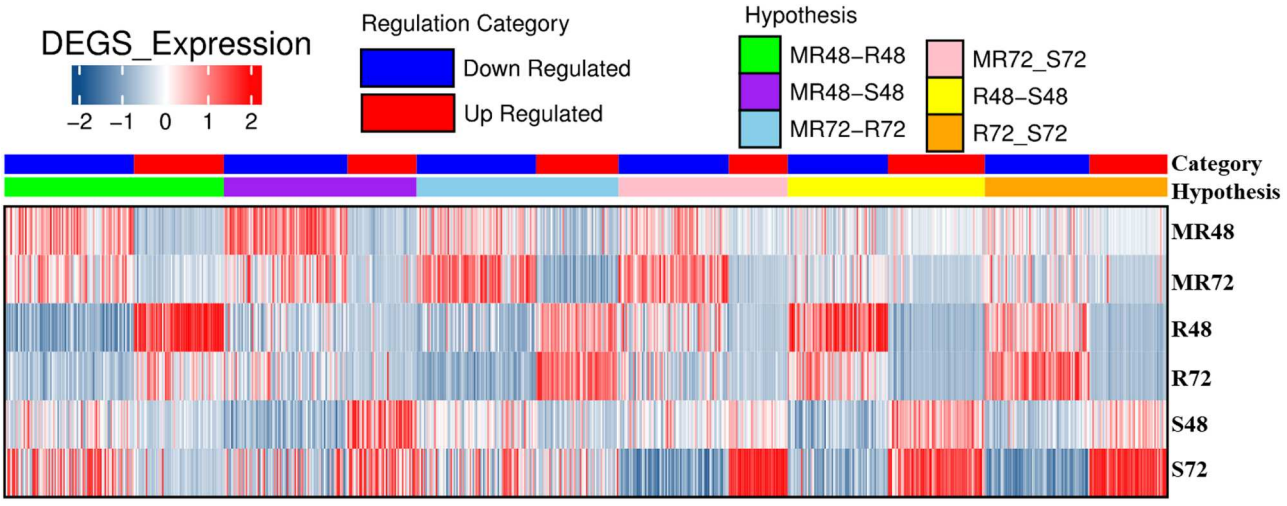

Figure 2. Heatmap of differentially expressed genes in R, MR, and S NILs at two-timepoints i.e. 48 and 72 hai during wheat and *Puccinia striiformis* interaction. The horizontal row represents the gene and vertical columns denote samples.

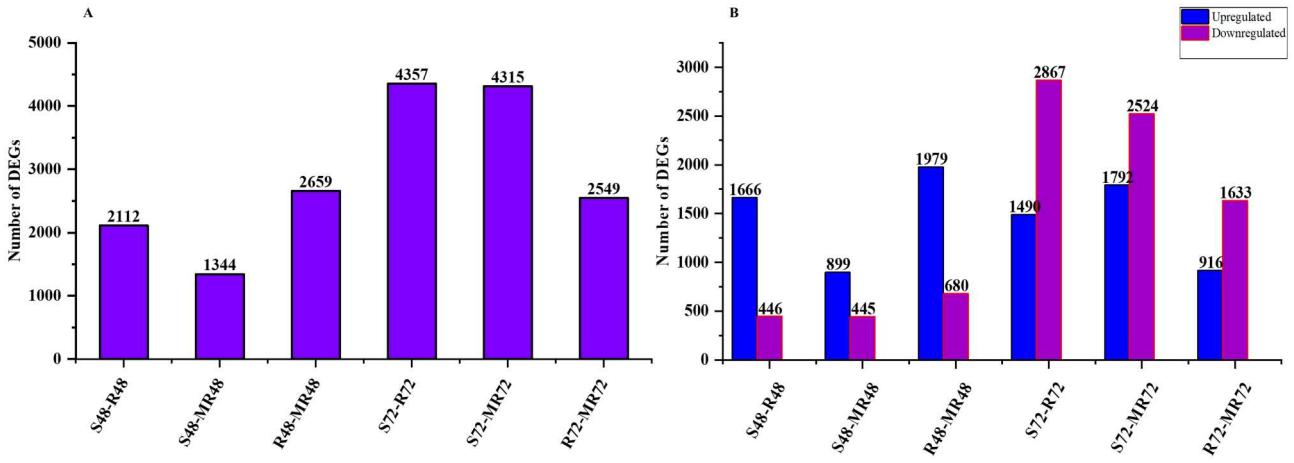

Figure 3. (a) Bar plot showing the total number of DEGs in six comparisons of three Wheat NILs reaction types i.e. Susceptible (S), Moderately Resistant (MR), and Resistant at two-time points (48hai and 72hai) (log^2-fold change>|1.5|, adjusted *p*-value [FDR] 0.05). (b) Bar plot showing comparison in wheat and *Puccinia striiformis* interaction showing the number of Upregulated, and downregulated DEGs in three reaction types at two-time points.

Principle component analysis

Principal component analysis (PCA) was performed to illustrate distinctions among samples based on their expression profiles. Samples with similar expression patterns, such as those from susceptible plants, clustered together, whereas resistant plant samples formed a distinct cluster. PCA revealed clear clustering of samples with a clear division between Resistant (R), Moderately Resistant (MR), and Susceptible (S) samples at both time points 48hai and 72hai (Figure 5).

Gene Ontology

The highest number of GO IDs was obtained in the S72-R72 comparison (10,044). Among these 5,094 GO IDs were associated with the Molecular Function (MF) category and 2,950 GO IDs were related to the Biological Processes (BP). Similarly, 10,072 GO IDs were obtained in S72-MR72.

The enriched DEGs were primarily involved in functions of ATP binding, protein binding, and Protein kinase phosphorylation (Supplementary Figures 1–6).

Screening of genes related to plant defense against Puccinia striiformis

Plant pathogens trigger the upregulation of defense genes, which may produce compounds that are directly antimicrobial or stimulate the biochemical pathways capable of producing antimicrobial metabolites. We focused our analysis on the number of differentially

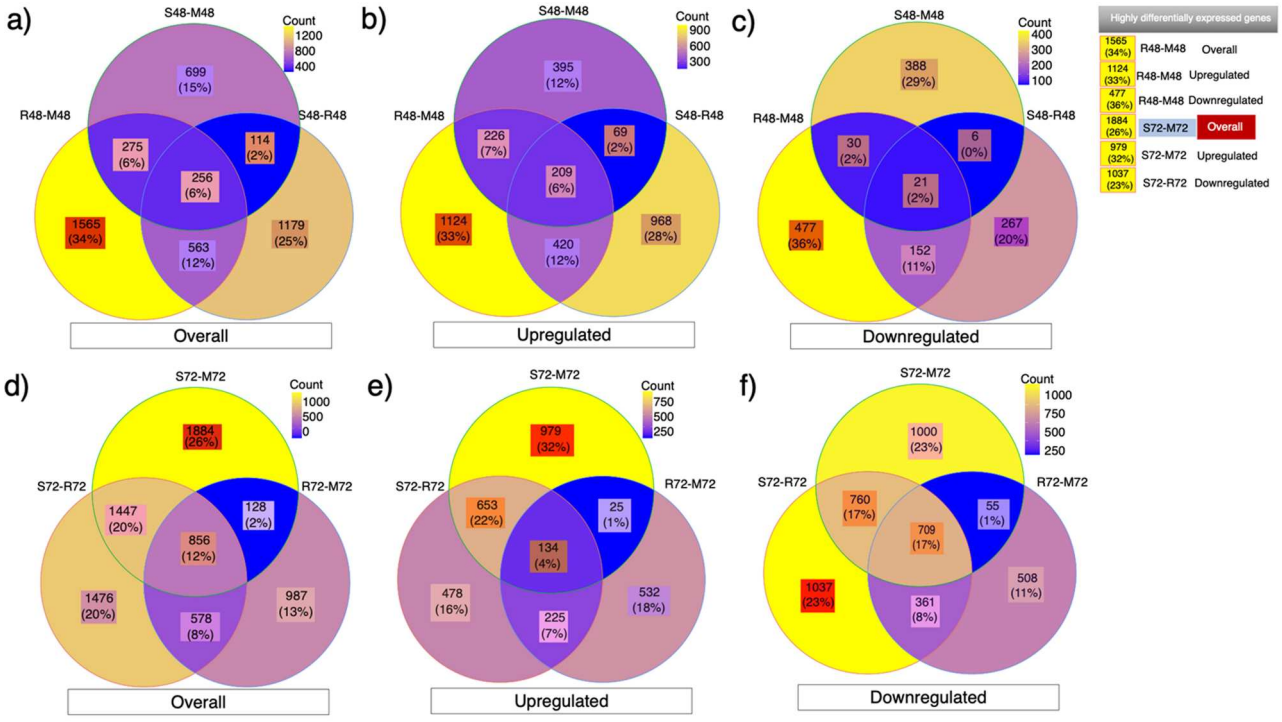

Figure 4. Venn diagram depicting the overlap of DEGs of differentially expressed genes in three comparisons at two-time points. (a,b,c) Comparison in wheat and *Puccinia striiformis* interaction showing a number of overall, upregulated, and downregulated DEGs in three reaction types at 48hai. (d,e,f) Comparison in wheat and *Puccinia striiformis* interaction showing the number of overall, upregulated, and downregulated DEGs in three reaction types at 72hai. ggVennDiagram: A 'ggplot2' Implement of Venn diagram. R packages.

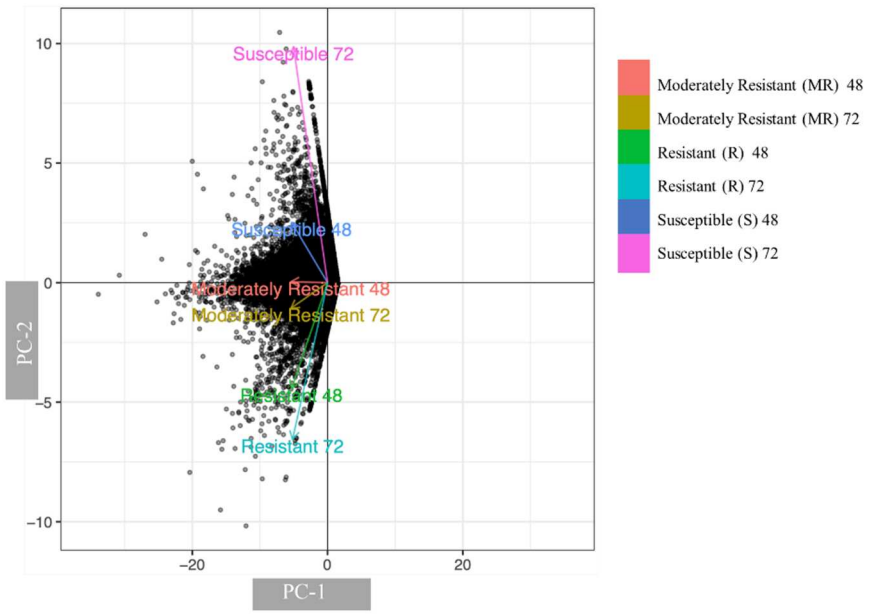

Figure 5. PCA plots of RNA Seq data show the expression of DEGs of all three reaction types at two-time points.

expressed common sets of genes relating to plant immunity such as transcription factors, and protein kinases which play crucial roles in the activation of pathogenesis-related proteins, pathogenesis-related (PR) proteins, ROS-producing genes, and transcript-producing stress hormones.

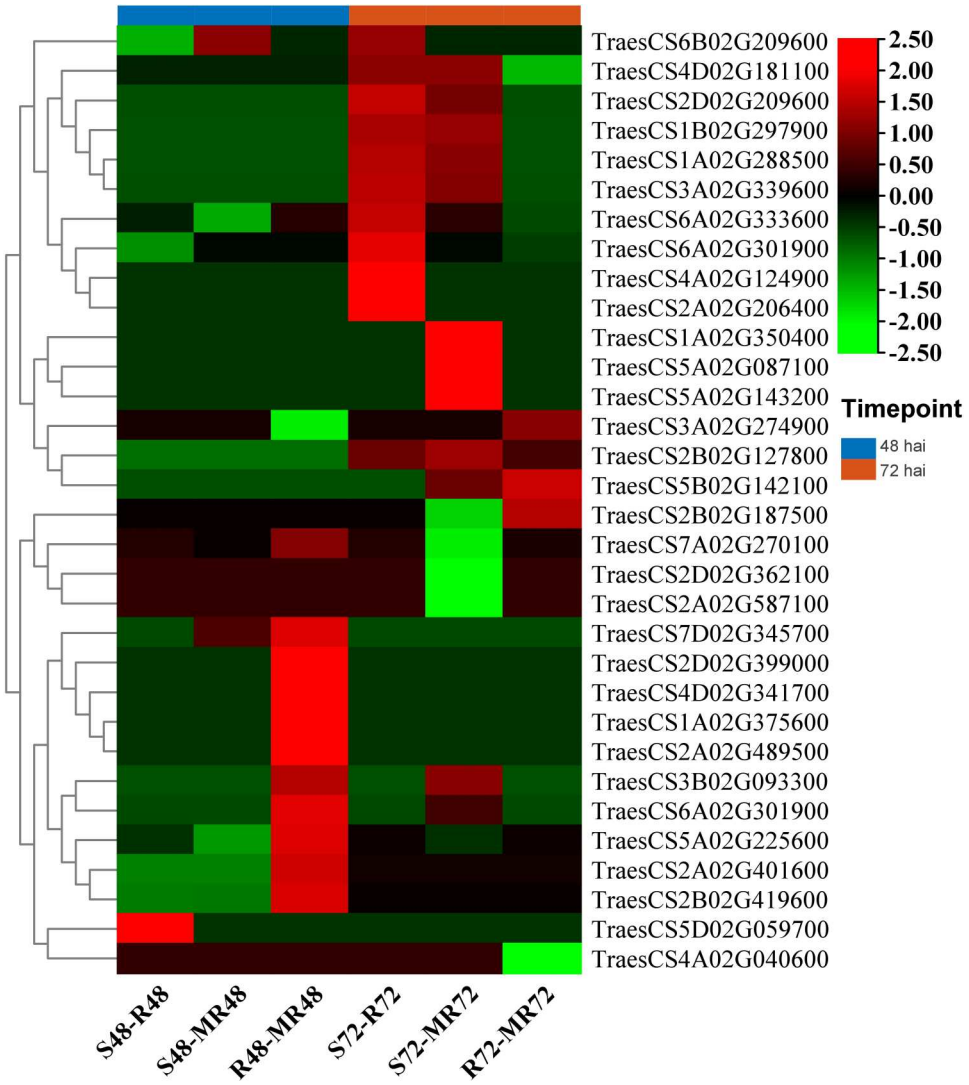

Figure 6. Differentially expressed transcription factors in resistant (R) and moderately resistant (MR) NILs in all comparisons at 48hai and 72hai.

Expression of transcription factor (TF)-encoding genes

Analyzing the transcription factors encoding differentially expressed gene transcripts, distinct expression patterns in different comparisons were observed. A total of 65 transcripts were identified in all three comparisons at two-time points (Supplementary Table 4).

The highest number (17) of transcription factors was identified in the S72-MR72 comparison. Among these, 13 were found to be upregulated in moderately resistant NIL, while 4 were downregulated. Among these, seventeen TF transcripts, four upregulated transcripts of NAC domain transcription factor, and 3 gene transcripts of MYB transcription factor transcripts were identified. All these transcripts were upregulated in moderately resistant NILs.

Similarly, in the R48-MR48 comparison, 13 DEGs were identified, out of which, 12 showed upregulation in resistant NIL, while only 1 was downregulated. Three gene transcripts were associated with WRKY transcription factor 146, WRKY45, and WRKY Transcriptional repressor. The transcripts of the bZIP transcription factor were all upregulated in resistant and moderately resistant NILs at all time-points (Figure 6).

Differentially expressed kinases transcripts

Protein kinases play a critical role in recognizing signals and activating plant defense mechanisms during pathogen infections (Supplementary Table 5).

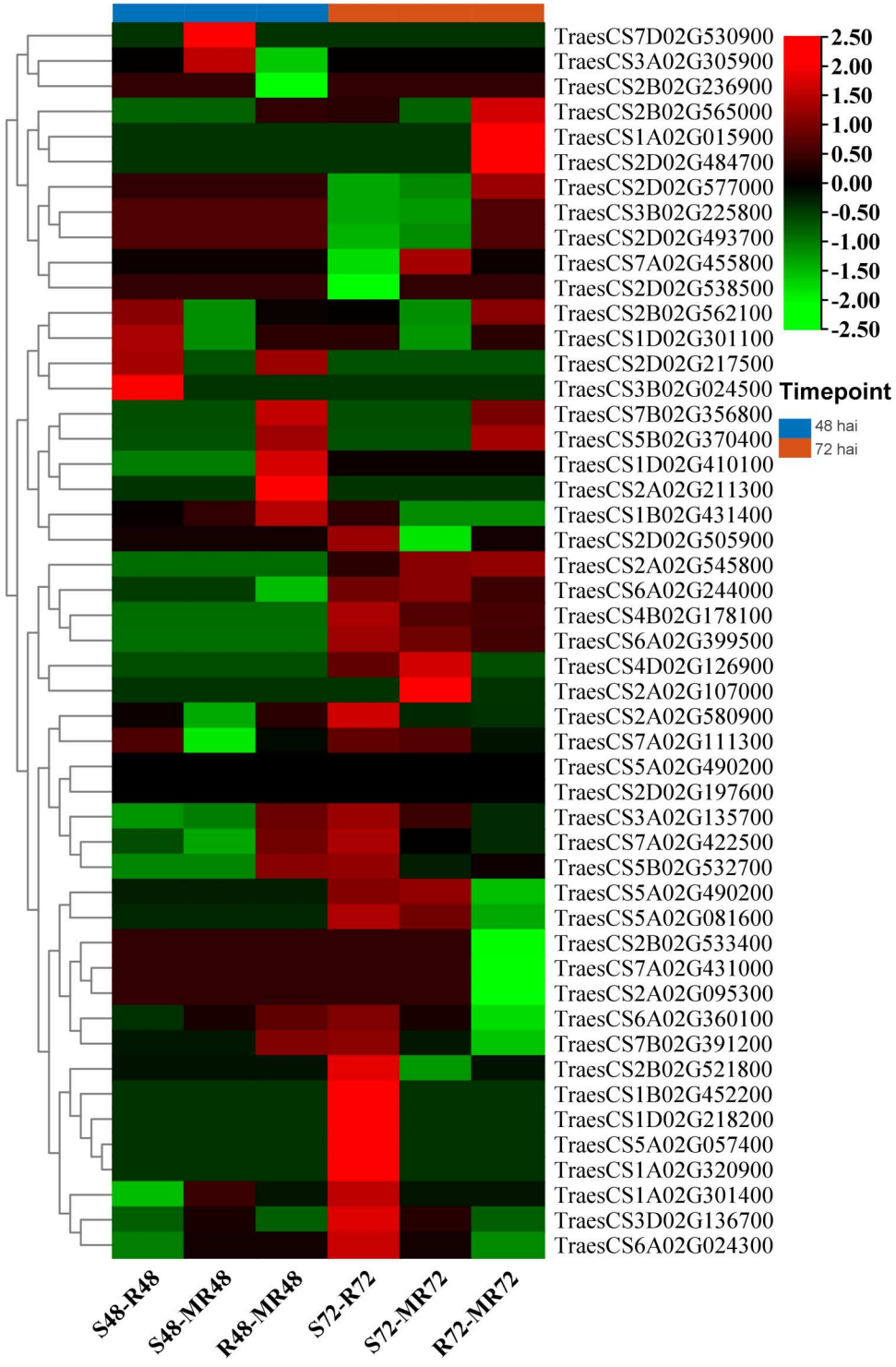

Figure 7. Differentially expressed Protein kinase in susceptible (S) and resistant (R) NILs in all comparisons at 48hai and 72hai.

The highest number of differentially expressed genes (31) was identified in the S72-R72 comparison, Among these, twenty-six genes showed upregulation in resistant NIL. Among these, 31 gene transcripts encoded potential defense-related kinases, twenty-three gene transcripts were related to Serine/threonine-protein kinase, and eighteen of these transcripts were found to be upregulated in resistant NIL at 72hai. Four differentially expressed gene transcripts of Mitogen-activated protein kinase were identified, showing a significant upregulation in resistant NIL (Figure 7).

DEGs Involved in Phenylalanine Ammonia-Lyase (PAL) Synthesis

A total of 66 transcripts encoding PAL were found in all six comparisons, out of which 10 transcripts were identified in the S48-R48 comparison with 6 upregulated in resistant NIL. Eleven transcripts of genes encoding PAL were identified in the S48-MR48 comparison. Out of 11 transcripts, 6 transcripts were upregulated in resistant NIL. In the R48-MR48 comparison, 18 transcript genes were identified. All of these transcripts were upregulated in resistant NIL. Eleven differentially expressed transcripts were identified in the S72-

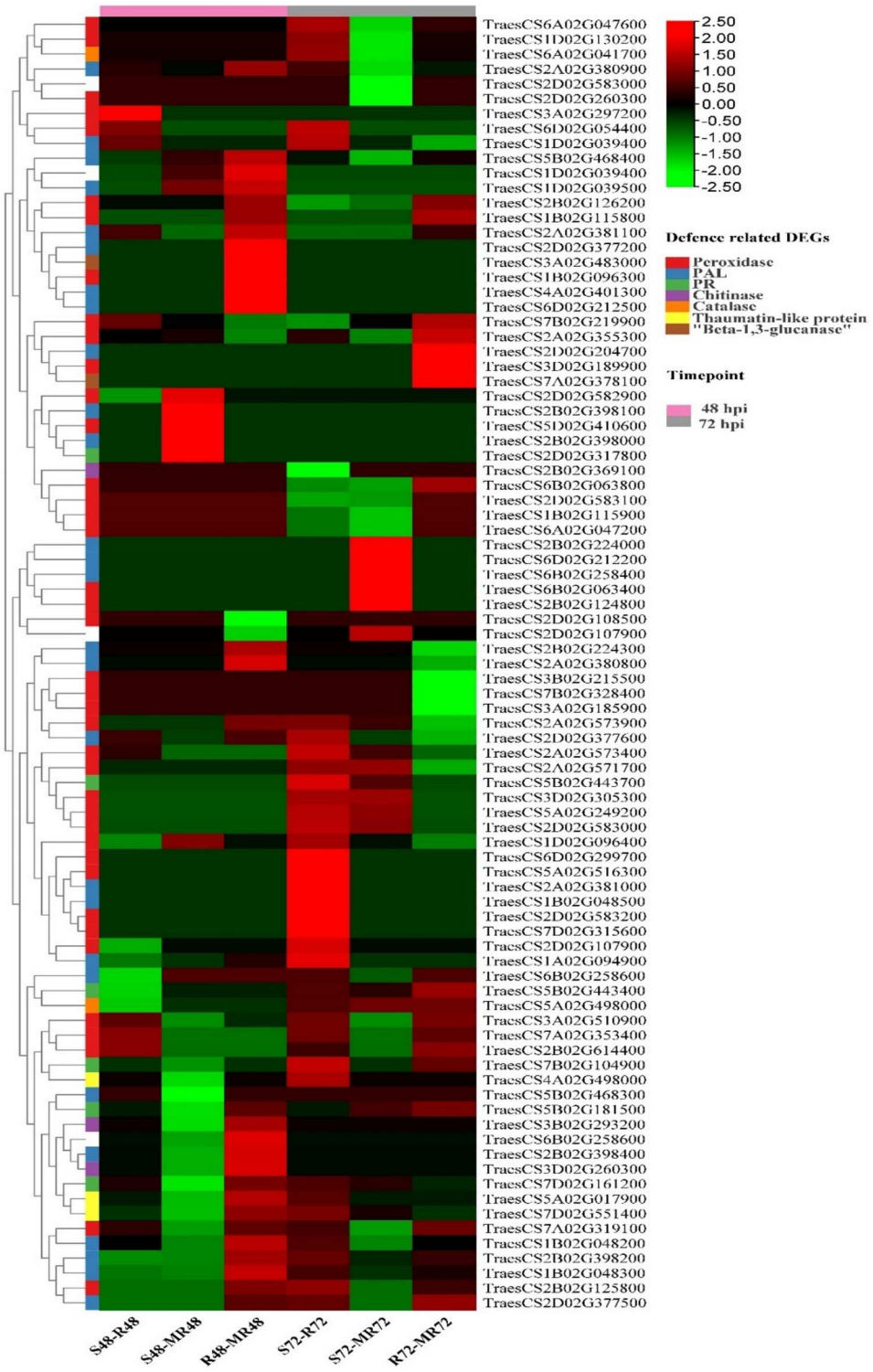

Figure 8. Heatmap showing the expression of defense-related gene transcripts in all three comparisons at two-time points.

Table 2. Pathogenesis-related proteins (PR) gene transcripts.

Transcript ID	Protein	Comparison	Function	Expression
TraesCS2D02G317800	PR1	S48-MR48 (1)	Antifungal and antivirus activity	Upregulated
TraesCS5B02G181500		R48-MR48 (1)		Upregulated
		S48-MR48 (1)		Downregulated
TraesCS3A02G483000	PR2	R48-MR48 (1)	Antibacterial, antifungal, and antivirus activity	Upregulated
TraesCS7A02G378100		R72-MR72(1)		Upregulated
(TraesCS7D02G161200)	PR4	S48-R48(1)	Chitin hydrolysis	Upregulated
		S48-MR48 (1)		Downregulated
		R48-MR48 (1)		Upregulated
		R72-MR72 (1)		Upregulated
gene:TraesCS5A02G017900	PR5	R48-MR48 (1)	Similarities with thaumatin	Upregulated
		S72-R72 (1)	Antifungal activity Causes osmotic rupture of fungus	Upregulated
gene:TraesCS7D02G551400		R48-MR48 (1)		Upregulated
		S72-R72 (1)		Upregulated
gene:TraesCS4A02G498000		S72-R72 (1)		Upregulated
TraesCS5B02G443400	PR7	S48-MR48 (1)	Pathogen cell wall degradation	Upregulated
		R48-MR48 (1)		Upregulated
		R72-MR72 (1)		Upregulated

R72 comparison. All of these transcripts showed upregulation in resistant NIL. Four transcripts were found in the S72-MR72 comparison. Three gene transcripts were upregulated in MR. In the R72-MR72 comparison, 12 differentially expressed PAL encoding transcripts were identified. 8 of these gene transcripts were upregulated in resistant NIL. Overall, the highest number of differentially expressed PAL encoding transcripts was observed in R48-MR48 with no downregulation (Figure 8).

DEGs involved in ROS-mediated defense in the response to Pst *infection*

Differentially expressed peroxidase transcripts were found in all three comparisons. A total of 88 gene transcripts encoding peroxidases were found. In the S72-R72 comparison, the highest number of 72 transcript genes were found, out of which 21 showed upregulation in resistant NIL, while 6 showed downregulation. A gene encoding Catalases enzyme was found downregulated to −2.32-fold at 48hai in the S48-R48 comparison, while at 72hai comparison, five gene transcripts were obtained, two differentially expressed gene transcripts were upregulated in resistant NIL in the S72-R72 comparison (Figure 8).

DEGs related to pathogenesis-related proteins

A total of 9 transcripts encoding pathogenesis-related protein genes were identified (Table 2). Out of these 9 transcripts, one transcript of PR 1 and PR4 were downregulated, all other transcripts were upregulated in resistant and moderately resistant NILs at 48 and 72hai (Table 2).

DEGs related to stress phytohormones

The expression of ethylene encoding transcripts was found to be prominent around 72hai. A total of 8 transcripts encoding ethylene receptors were identified in all three comparisons. Among 8 transcripts one gene transcript was found at 48hai in R-MR comparison, whereas 7 transcripts were found at 72hai. Four of these transcripts were upregulated in resistant and moderately resistant NILs. Differentially expressed gene transcripts encoding ABA were identified in all comparisons. A total of 14 differentially expressed transcripts encoding ABA 8'-hydroxylase and ABA-induced and plasma membrane protein PM 19 were identified. One gene transcript was identified in S48-R48, three transcripts were in S48-MR48, and two gene transcripts were identified in the R48-MR48 comparison. At 72hai, three gene transcripts were observed in S72-R72, four gene transcripts and transcripts encoding the ABA gene were observed in R48 and MR48, and one gene transcript in R72-MR72. All of these transcripts were upregulated in resistant and moderately resistant NIL. The expression of Jasmonate ZIM-domain protein-encoding transcripts was only found in R-MR comparison at both 48 and 72hai. Six JAZ domains encoding genes were identified with three in each. All of these were upregulated in resistant NIL with the expression ranging from 1.61 to 2.21 and 2.29 to 2.62 fold change at 48 and 72hai, respectively (Figure 9).

Validation of RNA-seq data with qRT-PCR analysis

To study the validity of transcriptome data obtained from the RNA sequencing, transcripts were selected on the following criteria:

1. Five transcripts that consistently exhibited upregulation across almost all six comparisons were selected. These differentially expressed transcripts were identified as follows: Bzip Transcription factor (TraesCS6A02G333600), peroxidase (TraesCS3A02G510900), Mitogen-activated protein kinase (TraesCS7A02G422500), PAL (TraesCS5B02G468400), and Serine/threonine-protein kinase (TraesCS2A02G580900).
2. The transcripts upregulated in 5 comparisons. This included Mitogen-activated protein kinase (TraesCS7A02G422500), TraesCS7A02G111300 (Mitogen-activated protein kinase), Bzip Transcription factor (TraesCS6A02G333600), PAL (TraesCS5B02G468400), peroxidase.
3. The transcripts upregulated in 4 comparisons: peroxidase (TraesCS3A02G510900), TraesCS2B02G562100 (Serine/threonine-protein kinase), TraesCS1B02G431400 (Mitogen-activated protein kinase), and TraesCS5D02G558800 (ABA-induced plasma membrane protein PM 19),
4. The transcripts upregulated only at 72hai. TraesCS5D02G059700 (NAC), TraesCS3B02G093300 (NAC 47), TraesCS3A02G339600 (NAC 4), TraesCS5A02G143200 (NAC), TraesCS5B02G142100 (NAC), and TraesCS4D02G181100 (MYB transcription factor), TraesCS2D02G209600 (MYB transcription factor 74), TraesCS2A02G206400 (MYB transcription factor 80), and TraesCS5A02G087100 (MYB transcription factor 79).

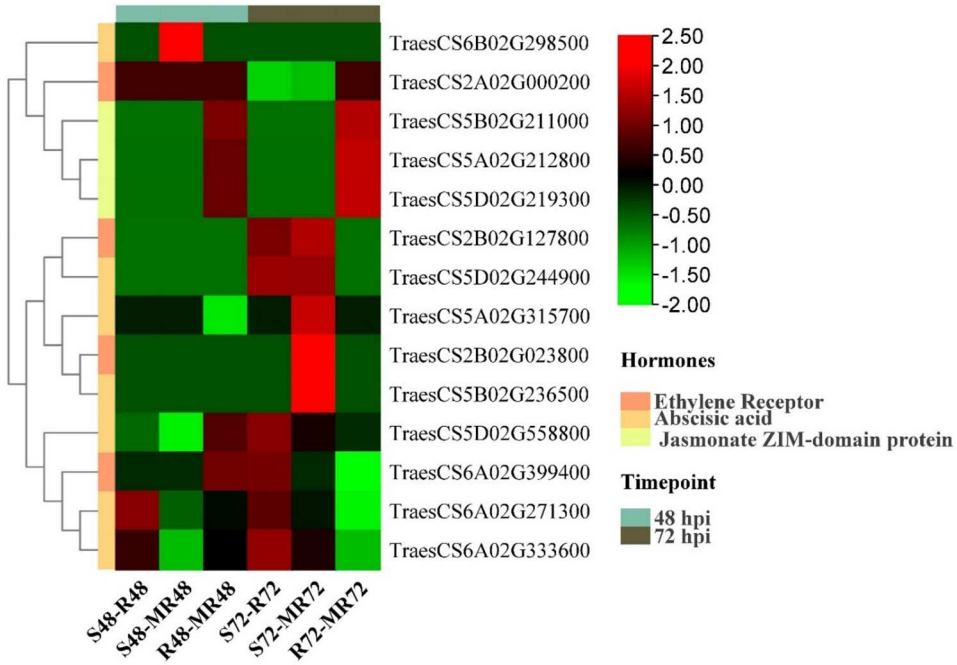

Figure 9. Heatmap showing the differential expression of hormones encoding gene transcripts in all three comparisons at two-time points.

Notably, the expression patterns of these transcripts as observed via RT-qPCR were consistent with the findings from RNA-seq analysis (Figure 10(a)).

To further validate the RNA-seq results, a correlation between differential gene expression levels determined by RNA-seq and qRT-PCR was analyzed after $\log_2$ transformation. The Pearson correlation coefficients for all 21 genes were greater than 0.8. Overall, the expression pattern of the 21 transcripts analyzed by qRT-PCR was consistent with the RNA-seq results, confirming the reliability of the transcriptomic sequencing analysis (Figure 10(b)).

Discussion

When a Pst urediniospore penetrates the plant cell, the plant exhibits a diverse range of a diversity of physiological and biochemical responses. The responses can be seen in the form of symptoms/responses that appear on the host plant. On the basis of these responses, the infected plant will either be resistant, susceptible or moderately resistant to the pathogen (Farrakh et al. 2018). A key difference between these responses is the prompt recognition of the colonizing pathogen and the activation of suitable defense mechanisms. Recent advances in RNA sequencing have revolutionized the transcriptome analysis. With the help of these advancements, differential gene expression in resistant and susceptible crops has been studied in various plant-pathogen interactions to dissect the molecular basis of plant defense systems (Kamber et al. 2016; Poretti et al. 2021). In the current study, apart from Resistant and Susceptible genotypes, a Moderately Resistant genotype was also included in RNA-seq. analysis. The sampling was carried out at two-time points i.e. 48hai and 72hai. The data were analyzed in three comparisons (S-R, S-MR, and R-MR) at both time points. Near-Isogenic-lines (NILs) were selected for the study. The number of DEGs was different at both time points. At 48hai, a high number of DEGs was found in R-MR comparisons followed by S-R and S-MR. However, the number increased at 72hai in S-R and S-MR with no significant difference, while DEGs decreased in R-MR. It is a well-known fact that gene expression is linked with the recognition of the pathogen. At 48hai, the number of differentially expressed genes was higher in R-MR, indicating the early recognition of pathogen and mobilization of effective defense machinery. At 72hai, in S-R and S-MR comparisons the number of highly expressed DEGs was found in susceptible NIL, indicating the late recognition of the pathogen and activation of defense mechanism. As a consequence of delayed pathogen recognition in susceptible NIL a greater number of genes were differentially expressed compared to the resistant and moderate NILs. A number of studies show an increased number of DEGs in plants showing the susceptible reaction type compared to resistant and moderately resistant. A greater number of DEGs was induced in susceptible wheat lines than resistant wheat lines in response to *Puccinia striiformis* infection (Geng et al. 2022). The results of the study showed the number of differentially expressed genes, upregulated genes, and gene activities was higher in susceptible NIL than the resistant and moderately resistant NIL (Figure 2). The number of DEGs is also reported to be linked with the degree of infection as the degree of infection increases the number of DEGs also increases. More DEGs were found in susceptible rice infected with *F. fujikuroi* pathogen, and the number of DEGs was consistent with the degree of infection of rice varieties (Matic et al. 2016). This finding suggests that the susceptible plant undergoes extensive transcriptional reprogramming in response to pathogen attack potentially as a compensatory mechanism to counteract infection. In contrast, resistant and moderately resistant lines may rely on a more efficient and targeted activation of defense pathways, minimizing excessive gene expression changes while still effectively restricting pathogen proliferation.

The first step of the plant defense system is the timely recognition of pathogens, which is mainly mediated by specialized receptors. Among these, Receptor-Like Protein Kinases

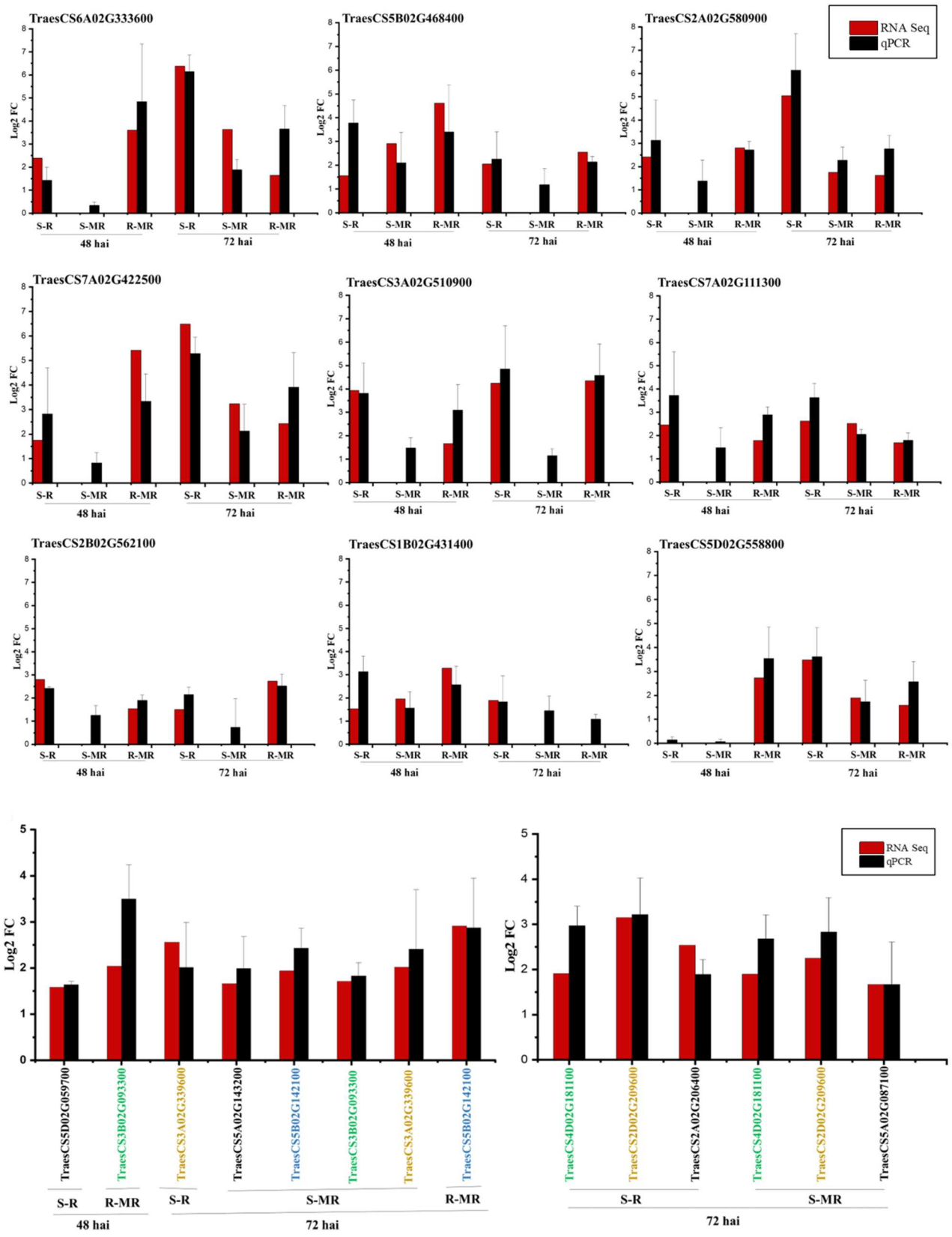

Figure 10. (a) Expression patterns between qPCR and RNA-seq for the five genes. The heights of the columns and points stand for the $\log^2$ (fold change) computed from both qRT-PCR and RNA-seq profiles. (b) Correlation analysis of DEGs between RNA-seq and qRT-PCR data, the colors show the Pearson correlation.

(RLKs) play a crucial role in both plant development and defense against phytopathogens. RLKs function as intracellular signaling molecules that perceive pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) and initiate defense signaling cascades. Since most RLKs are present at the crossroads of many pathways, they also formed a complex network. The accumulation of these kinases results in specific phosphorylation and dephosphorylation of genes, which results in metabolic changes in the host. These metabolomic changes then lead to specific plant responses. Plant defense response is also one of those metabolic changes. The majority of RLKs in

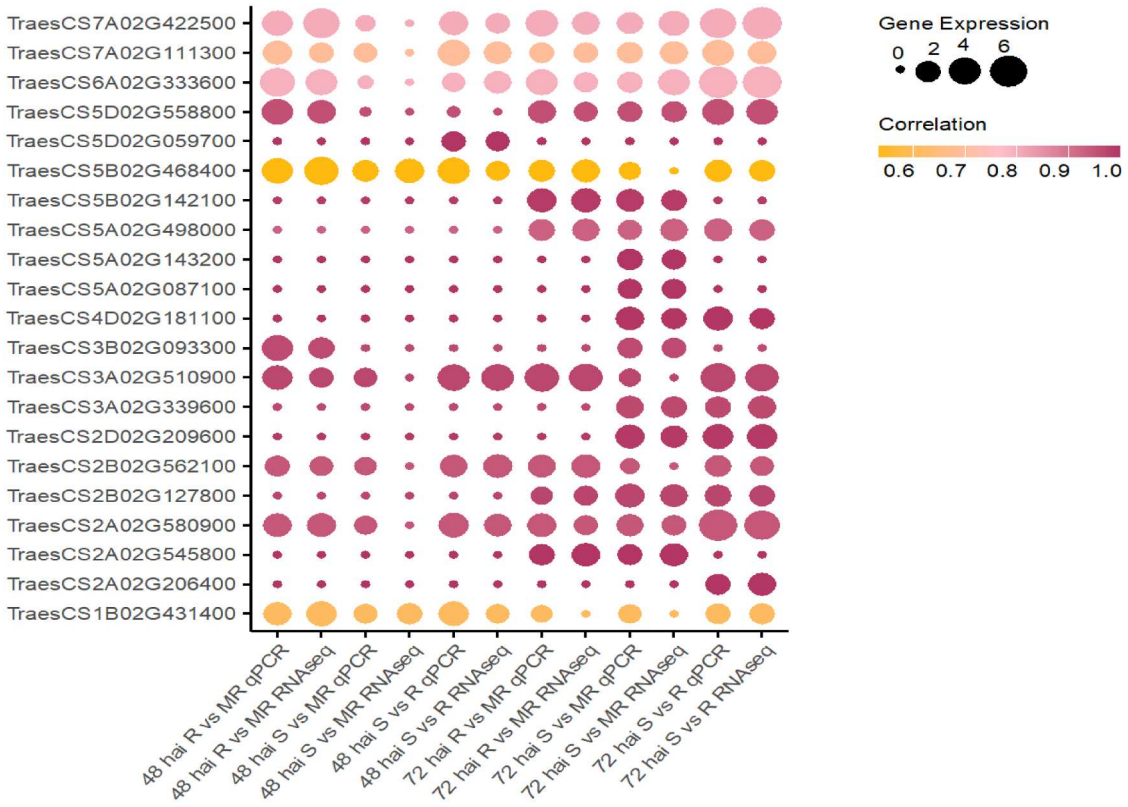

Figure 10. *Continued.*

plants are serine/threonine kinases (Song et al. 1995; Hwang et al. 2011; Zhang, Su, et al. 2018). In our study, 38 serine/threonine protein kinase genes in three comparisons at two-time points were identified. In resistant and moderately resistant comparison high number of serine/threonine protein kinase (13) DEGs were identified. A number of studies indicate the involvement of serine/threonine protein kinase (Ser/Thr_kinase) in the timely recognition of pathogens (Wang, Luan, et al. 2010). In a study of wheat-stem rust interaction, the majority of host proteins were serine/threonine (Ser/Thr) protein kinase identified in resistant cultivar (Kataria and Kaundal 2022).

The number of MAP kinases (MAPK) was relatively higher in resistant NIL than moderately resistant and susceptible NILs in all three comparisons at both time-points. High expression of genes encoding MAPKs was found in resistant wheat near-isogenic lines with or without *Lr28* compared to susceptible lines (Chandra et al. 2016). In the current study, only one transcript of calcium-dependent kinases was upregulated at 72hai in resistant NIL in S-R comparison, and in the same comparison, the high fold change of MAPKs was also observed. MAPKs are involved in activating multiple defense responses which include the biosynthesis of plant stress/defense hormones, ROS production, the closing of stomata, activation of pathogenesis-related protein genes, phytoalexin biosynthesis, cell wall lignification, and hypersensitive response (HR) (Tang et al. 2017). Phosphorylation of ERF6 transcription factor by MPK3 and MPK6 was found to be a major defense response against fungal pathogens (Meng et al. 2013). In cotton, GhNTF6 is the only member of the MPK (Mitogen-Activated Protein Kinase) family that is phosphorylated under the pathogen attack. Overexpression of this gene in Arabidopsis enhances resistance against *Verticillium dahliae*, suggesting that GhNTF6 plays a crucial role in cotton's immune signaling, likely by facilitating downstream defense responses upon phosphorylation (Zhou et al. 2022).

Upon the successful penetration of a pathogen, some transcription factors (TFs) in the host plant activate the defense-related genes by binding to the specific *cis*-regulatory elements present in promoters of the target gene (Singh et al. 2002). WRKY, MYB, AP2/ERF, and bZIP are the main TF families involved in activating and regulating plant defense mechanisms against invading pathogens (Alves et al. 2014; Tsuda and Somssich 2015).

The basic leucine zipper (bZIP) transcription factors activate the target genes by recognizing *cis*-elements in the promoters. (Lebel et al. 1998). Only one transcript of bZIP was found to be upregulated in resistant NIL in both S-R and R-MR comparisons at 72hai. DNA microarray assays of maize infected with *Ustilago maydis showed* the expression of 100 bZIP genes. The expression of bZIP TFs was observed during *Colletotrichum graminicola* and maize interaction and high expression of ZmbZIP65, ZmbZIP21, and ZmbZIP 53 genes was observed at 96hai (Liu et al. 2014). High expression of RT42C09 and RT57A09 bZIP was also high in *Moniliophthora perniciosa* and cocoa interaction (Lopes et al. 2010). High expression of TabZIP1 transcripts was observed during the wheat-stripe rust incompatible interaction showing the role of TabZIP1 in defense response against colonizing fungal pathogen (Zhang et al. 2008). TabZIP74 was also found to be positively regulating wheat resistance to stripe rust pathogen and contribute to root development by mRNA splicing (Wang et al. 2019).

A number of NAC genes have been shown to play significant roles in plant defense by regulating hypersensitive response (HR), stomatal closer, and targeting pathogen elicitors (Yuan et al. 2019). In the current study, NAC 4 and NAC

47 transcription factors were upregulated in resistant and moderately resistant NILs compared to susceptible. The role of rice OsNAC4 in HR cell death is well-established (Kaneda et al. 2009). The expression of the OsNAC4 gene was high during the non-host defense response of rice and was involved in the regulation of the HR response of the cell. A decrease in HR cell death was observed in the OsNAC4- knockdown plant infected with an avirulent strain of *Acidovorax avenae* (Coll et al. 2011), while the enhanced HR cell death was observed in OsNAC4-overexpressing plants infected with an avirulent strain of Pst DC3000. These findings confirmed that NAC4 plays a significant role in regulating HR cell death (Lee et al. 2017).

MYB is another family of TFs involved in plant defense against various pathogens (Dubos et al. 2010). Systemic acquired resistance (SAR) was triggered by the overexpression of R2R3-MYB, which involved the activation of pathogenesis-related (Bostock 2005). Similarly, the overexpression of AtMYB96 resulted in increased disease resistance in transgenic plants of *Arabidopsis by* upregulating the expression of Pathogenesis-related genes (Seo et al. 2009). High expression of SpMYB expression was observed in tomatoes inoculated with *F. oxysporum* and *B. cinerea*. The overexpression of SpMYB in tobacco-transgenic plants showed significantly high resistance to *F. oxysporum* and *B. cinerea* compared to non-itransgenic plants (Liu et al. 2016).

A total of 5 DEGs of MYB TF were identified in the R-S comparison and 3 DEGs were identified in the S-MR comparison at 72hai. In both comparisons, MYB74 was upregulated in resistant and moderately resistant NILs. MYB74 is known to be involved in ABA-dependent and ABA-independent signaling regulates in plant responses to water stress and induces stomatal closing. MYB74oe overexpressing mutants increase water loss due to increased stomatal aperture (Ortiz-García et al. 2022). We assume that MYB74 might be involved in making wheat line resistant against the *Puccinia striiformis* by closing the stomata.

The accumulation of reactive oxygen species (ROS) around the infection site is one of the earliest defense responses of the infected cell (Wojtaszek 1997). Peroxidases are a class of enzymes that are known to play a role in the resistance of the infected cells by synthesizing structural barriers like lignin or by generating ROS and reactive nitrogen species that halt pathogen growth inside the host. In the current study, at 48hai, 12 DEGs of peroxidase were identified in R and S comparison. Out of these 12, nine DEGs were upregulated in resistant NIL. In S-MR comparison at the same time point, only 2 DEGs were identified, both of which were upregulated in moderately resistant reaction. R- MR comparison at the same time point showed expression of 10 DEGs, with 7 being upregulated in Resistant NIL. Interestingly, the number of DEGs increased in all three comparisons at 72hai. In the S-R comparison, 27 DEGs were identified, 20 of which were upregulated in resistant NIL. Similarly, 18 DEGs were identified in the S-MR comparison, with 9 upregulated in moderately resistant NIL. In the R-MR comparison, again 18 DEGs were identified, 12 of which were upregulated in resistant NIL. High expression of peroxidase transcripts was also reported by (Wang, Luan, et al. 2010) in the incompatible interaction of wheat and *Puccinia striiformis* (Pst). High expression of peroxidases (PR9) was associated with leaf rust resistance (Prasad et al. 2019). Peroxidases are known to develop ROS-mediates resistance and contribute to the development of barriers. Since Pst is a biotrophic fungus it needs a living host cell to survive. Production of ROS is linked with cell death which may limit the pathogen before the development of haustoria (Lata et al. 2022). Closing of stomata is one of the early responses of plants to both biotic and abiotic stress. The stomal closure ensures the retention of water under water stress and also provides defense against pathogens. Peroxidases are known to be involved in stomatal closure via the ABA pathway (Agurla et al. 2018).

Pathogenesis-related (PR) proteins are a diverse group of proteins that are structurally or functionally related to each other. These proteins play a vital role in plant defense responses against pathogens. Some PR proteins are known as chitinases and β-1,3-glucanases, peroxidases with catalytic roles against fungi (Lebel et al. 1998). PR proteins inhibit the growth of bacterial and fungal pathogens by degrading their cell walls and by producing ROS. Overexpression of these proteins has been shown to increase tolerance to various pathogenic fungi, suggesting their role in plant defense. In the current study, DEGs of several PR proteins were identified at different time points in three comparisons. Among the PR protein transcripts, 3 DEGs of PR5 were identified. All 3 transcripts were strongly upregulated in resistant NIL in the S-R comparison at 72hai. A number of studies support the role of PR-5 protein in resistance mechanisms in cereals. The TaPR5 transcript was significantly upregulated in the incompatible wheat-stripe rust interaction (Wang, Tang, et al. 2010). TaLr35PR5 was induced by leaf rust pathogen in the incompatible interaction of wheat-leaf rust (Li et al. 2015; Zhang, Wang, et al. 2018).

In the current study, a total of 66 DEGs of PAL were identified in all three comparisons at both time points. However, the most of DEGs were upregulated in resistant NIL. PALs are the central enzymes in the phenylpropanoid pathway, by catalyzing the deamination of L- phenylalanine to form trans-cinnamic acid (Dixon et al. 2002). Trans-cinnamic acid is a precursor for the biosynthesis of various phenylpropanoid compounds, such as lignin a very important constituent of the plant cell wall, salicylic acid (SA) a stress phytohormone SA, and flavonoid phytoalexins. Lignification of cell walls provides the first structural barrier for the pathogens to crosse. Lignin also prevents the movement of nutrients and water from the plant cell to the pathogen thus halting the pathogen growth in the resistant plants; however, due to decreased lignin synthesis in susceptible plants, the pathogen will successfully colonize (Hu et al. 2018). The production of SA hormones is chemical barriers to activate defense genes (Yadav et al. 2020). A total of nine OsPAL genes were identified in the rice (*Oryza sativa*) genome. However, eight of these genes were significantly activated by *Magnaporthe oryzae* infection and seven were associated with quantitative trait loci (QTL) for resistance to *Rhizoctonia solani*, *M. oryzae*, and *Xanthomonas oryzae pv. oryzae* (Xoo) (Duan et al. 2014). The ospal4 mutant rice plants showed enhanced susceptibility to these three pathogens (*R. solani, M. oryzae*, and *Xanthomonas oryzae pv. Oryzae*) (Tonnessen et al. 2015) indicating the significant role of PAL in plant defense.

Conclusions

In plant-pathogen interaction, if the plant is well prepared and being able to mobile its defense machinery have increased the chances of overcoming the infection. In the case of the three lines used in our study, Resistant NIL (Yr5) was well prepared compared to moderately resistant and susceptible NIL in terms of its ability to recognize the pathogen early and activate the defense response. The moderately resistant line was better than the susceptible line in terms of its ability to recognize the pathogen early and activate the defense response. Through a comprehensive RNA sequencing analysis, we uncovered the complex coordination of genes and different processes that underlie the differing responses of wheat genotypes to the pathogen in resistant, moderately resistant, and susceptible reaction types. The serine/threonine protein kinases, MAP kinases, NAC, MYB, bZIP, and peroxidases, emerged as central players in regulating defense responses. These factors triggered cascades of events, including hypersensitive reactions, modulation of stomatal closure, and the induction of pathogenesis-related proteins. Similarly, SA-responsive PR proteins PR1, and PR5 and high expression PAL, indicate that SA and its downstream regulation network may be a major defense process.

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Author contributions

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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Umer Zeeshan Ijaz Since 1999, Dr *Umer Zeeshan Ijaz* has been developing scientific software and engineered systems on mathematical modeling and multivariate statistical analysis of 'Big' datasets. In

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