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In silico differential gene expression analysis of biotic stress response: Insights into the underlying defense mechanisms of *Triricum aestivum*

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Several biotic stresses threaten *Triticum aestivum* (wheat) plants, with fungal infections being particularly concerning. In this research, *in silico* data analysis was used to identify the significantly common expressed genes of wheat plants in response to fungal infections regardless of genotypes and source of pathogen as a biotic stress. Transcriptome sequencing datasets were retrieved from the Gene Expression Omnibus on the NCBI GenBank database. The selected five datasets had four different wheat genotypes as follows: two datasets featured *Triticum aestivum* L. genotypes infected with either *Zymoseptoria tritici* or *Blumeria graminis*; another one included the YR08 genotype infected with *Puccinia striiformis*; and the last dataset contained the Nobeokabouzu-komugi and Sumai3 genotypes, both infected with *Fusarium graminearum.* The count matrix of each dataset was employed for the downstream analysis. Furthermore, the findings demonstrated that out of the total 110,428 studied genes in the four experiments, 10,771 (9.8%) were differentially expressed, of which 6,490 (5.9%) were significantly upregulated and the remaining 4,281 (3.9%) were significantly downregulated. Eight of the significantly regulated genes showed different overlaps across the five datasets, depending on the wheat genotype and the type of fungal infection. Six of these eight genes are crucial for regulating the resistance of wheat plants to various plant pathogens. The molecular functional enrichment analysis revealed five key proteins involved in the plant defense response to fungal infections. The candidate genes identified in this study could be bioengineered to enhance the resistance of wheat varieties against fungal pathogens.

Key words: Biotic stresses, *Triticum aestivum,* fungal infections, transcriptome data, *in silico* analysis

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INTRODUCTION

Wheat is the most significant grain crop, accounting for almost 20% of all agricultural imports and 10% of agricultural production value in Egypt (McGill *et al.*, 2015). Also, wheat whole grains contain dietary fiber, vitamins, minerals, and phenolic substances that play a significant role in the diet and human health (Ficco and Borrelli, 2023). Wheat is not only an essential, high-nutrient food source but also a key component of animal feed and an industrial raw material (Katileviciute *et al.*, 2019; Qiu *et al.*, 2019). Bread wheat (*Triticum aestivum*) is a young hexaploid species that belongs to the *Triticeae* tribe within the *Poaceae* family of grasses. It was formed 8,500–9,000 years ago by hybridizing *Aegilops tauschii* and a domesticated free-threshing tetraploid ancestor. With increasing population and prosperity demand, global wheat yields are expected to rise by 1.6% annually until 2050 (Elbeltagi *et al.*, 2020).

Wheat cultivation in Egypt faces multiple biotic stresses, with fungal infections being the most significant. The main diseases threatening global wheat grain production are caused by either biotrophic or necrotrophic fungi. These include powdery mildew, rusts, blotches, head scab, and other diseases that have recently emerged or received little attention, such as wheat blast and spot blotch (Figueroa *et al.*, 2018). Favorable environmental conditions have led to significant fungal infections that can cause yielding losses and quality deterioration in susceptible cultivars. In Egypt, some reports indicate that wheat yield losses have reached as high as 22.52% due to high disease severity levels (Sallam and Awad, 2012; Esmail and Draz, 2018; Shahin *et al*., 2020; El-Mougy *et al.*, 2022). Genetic resistance to fungal infections in wheat can be broadly categorized into two genetically distinct categories. The first is seedling resistance, also known as race-specific resistance or all-stage resistance, which is detected at the seedling stage but is also expressed at all developmental stages. It is short-lived due to frequent changes in virulence in the pathogen population (Bai *et al*., 2022). The second is adult plant resistance (APR), which is expressed at the adult plant stages and is known as race nonspecific resistance. It is generally quantitatively inherited, is often durable, and provides partial and slow resistance (Sthapit Kandel et al., 2017).

The initial plant response to infection is triggered by the interaction between pattern-recognition receptors (PRRs) on the host membrane and pathogen-associated molecular patterns (Doehlemann *et al.*, 2017). Plant pathogenic fungi can form structures such as appressoria and haustoria to facilitate infection of epidermal cells and for nutrient

uptake (Chethana *et al*., 2021). Gene mapping and characterization studies demonstrated that these structures are intense sites where all secreted proteins, including fungal effectors, are expressed (Wang *et al.*, 2023). Fungal pathogens must secrete these effector proteins to suppress the host's defense responses and promote colonization. Furthermore, these effectors initiate plant immune responses after being recognized by cognate host resistance genes (Mapuranga *et al*., 2022a; Mapuranga *et al*., 2022b). Most identified resistance-conveying genes encode classic nucleotide-binding site leucine-rich repeat (NBS-LRR) proteins that recognize effectors and trigger a defense response to resist disease (Bai *et al*., 2022). The presence of susceptible wheat varieties without effective resistance-conveying genes increases their susceptibility to fungal infections (Brown *et al*., 2015). Transcriptome sequencing data serves as a bridge between the genetic information in the genome and the biological processes occurring at the proteome level. Transcriptomics is a valuable bioinformatics tool for understanding the functioning of specific regulatory genes and exploring the mechanisms of genes that are not yet well understood (Rhoads and Au, 2015).

Continuous research efforts are focused on understanding the pathogen's biology (Steinberg, 2015), developing resistant wheat varieties (Brown *et al*., 2015), and implementing integrated disease management strategies (Lynch *et al*., 2016; Kristoffersen *et al*., 2022). Therefore, this research aims to investigate the molecular mechanisms underlying the wheat plant's response to fungal infections by conducting a differential gene expression analysis on the transcriptome sequencing data of different experiments retrieved from the Gene Expression Omnibus (GEO) dataset Home-GEO-NCBI (nih.gov) to uncover the key genes involved in the defense response pathways against fungal pathogens.

MATERIALS AND METHODS

Data mining

The Gene Expression Omnibus (GEO) dataset Home-GEO-NCBI (nih.gov) was used as the main database to retrieve the transcriptome data from different experiments that fit the following selection criteria. Keywords such as wheat plant (*Triticum aestivum*) exposed to biotic stressors, especially fungal infection, were used to get the datasets. Various filters were applied to aid in the selection of experiments. The first filter focused on study type, specifically selecting studies that used DNA microarrays and high-throughput sequencing for expression profiling. The second filter concerned the number of samples; only experiments with more than nine samples were included. The final filter pertained to the wheat genome used for alignment. Four datasets (accession numbers GSE59721, GSE169298, GSE13346, and GSE12936) fulfilled the previously mentioned criteria. Accordingly, their transcriptome sequence data were retrieved from the GEO on the NCBI GenBank database to be included in this study (accessed on 4 November 2023). All the raw data from the selected datasets were aligned against the wheat genome reference sequence (IWGSC CS RefSeq v2.1) to examine the altered gene expression profiles of wheat genotypes against different species of infectious fungi as follows. In the first experiment with accession GSE169298, *Triticum aestivum* genotype L. was inoculated with *Zymoseptoria tritici* strain T02596, a *Septoria tritici* blotch (STB) disease causal agent. In the second experiment with accession GSE13346, *Triticum aestivum* genotype YR08 was inoculated with *Puccinia striiformis* strain Pst-21, a causal agent of stripe rust disease. Moreover, in the GSE12936 accession number experiment, *Triticum aestivum* genotype L. was inoculated with *Blumeria graminis* f. sp. *tritici*, a powdery mildew disease causal agent. Finally, in the accession GSE59721 experiment, two wheat genotypes (Nobeokabouzu-komugi and Sumai-3) were inoculated with *Fusarium graminearum*, the causal agent of *Fusarium* head blight (FHB). All the pertinent details about each of the chosen datasets used in this investigation are described in Table 1.

Data distribution analysis

Gene expression analysis tool (GEO2R) is an interactive web tool built on the LIMMA (based on R) analysis package GEO2R-GEO-NCBI (nih.gov). This tool was utilized to analyze the selected data sets. The first step included grouping the sample into treated and control groups for each genotype. Each experiment was subjected to individual analysis with GEO2R. The analysis was conducted with the default parameters that apply adjustments to P-values using the Benjamini and Hochberg (false discovery rate) model (Benjamini and Hochberg 1995); the adjusted p-value significance level cutoff was 0.05; and the $\log_2$ fold change threshold was set to zero.

Differential gene expression analysis

The series matrix file was downloaded for each experiment and was used to select the ominously expressed genes at an adjusted p-value significance

level cutoff of 0.05. At this stage, the gene names in the selected experiments were unknown, as the raw data was collected from microarray analysis, which only indicated the names of the probes used. Hence, a different approach using the BLAST: Basic Local Alignment Search Tool (nih.gov) was adopted to search against the GenBank database with the gene sequence as a query, and the name of each gene was successfully found. The top 200 significant genes from each experiment were used for the Venn diagram analysis (Jia *et al*., 2021) using the interactive Venn web tool (InteractiVenn: Interactive Venn Diagrams). Heatmap analysis was used to summarize the variation in the expression level of the significantly expressed shared genes. Gene expression was converted into the $\log_{10}$ values of each experiment. The outcomes were visualized using a color scheme, with red indicating the maximum value and green indicating the minimum value of each expression level using Next-Generation Clustered Heat Map Viewer (ng-chm heatmap generator software, University of Texas MD Anderson Cancer Center).

Protein-protein interaction and gene ontology analysis

The same genes were also used for enrichment analysis, protein-protein interaction, and network analysis using the Search Tool for the Retrieval of Interacting Genes and Proteins (STRING) database (Szklarczyk *et al*., 2021), STRING: functional protein association networks (string-db.org). The STRING database parameters were set to remove repeated genes from the dataset and clustering using K-means legacy 1. Network clustering was performed using K-means clustering calculations with three random starting points. The bioinformatics workflow, which includes detailed steps and software for data analysis, is illustrated in a flowchart (Figure 1).

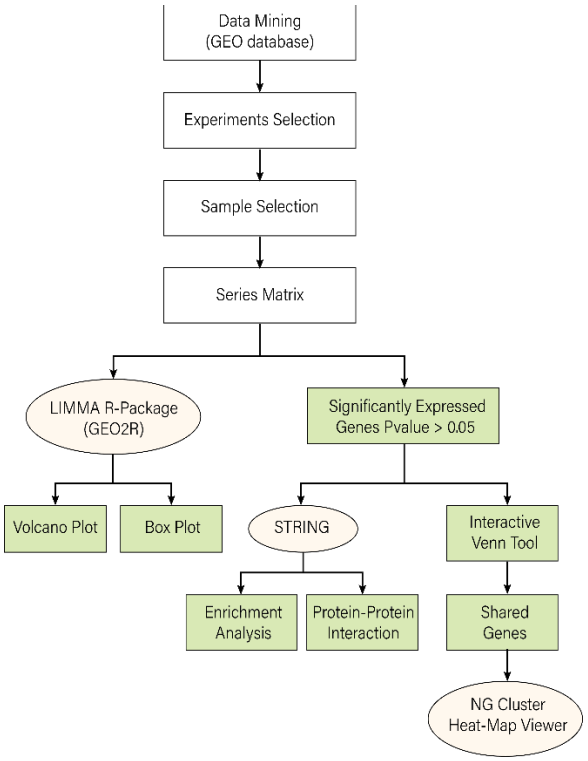

Figure 1. Illustrative flowchart of the bioinformatics workflow involving the detailed steps and software used for data analysis.

RESULTS

Data distribution analysis

Box plot analysis was used to inspect the distribution of the gene expression values of the selected samples. The samples were colored according to their groups (control and treated). The plots showed data after log transformation and normalization. The data distribution established that the selected samples were suitable for differential expression analysis, as the median-centered values indicated that the data were normalized and cross-comparable (Figure 2), except for sample number GSM1444308 in experiment GSE59721-Nobeokabouzu-komugi, which had a median-centered value that deviated and had to be removed from the data set (Supplementary Figure).

Differential gene expression analysis

In the experiment with accession GSE169298, the wheat plant was infected with the fungus *Z. tritici*. Analyzing the variation in the expression levels between the control and treated samples revealed that the total number of detected genes was 10,471, while the number of significant genes (at 0.05 adjusted p-value) was 214 (Table 2). A total of 54 significantly downregulated genes were counted, whereas 160 genes were detected as upregulated significantly (Figure 3A). The top two most significantly downregulated genes were ribulose bisphosphate carboxylase/oxygenase activase B, chloroplastic-like, and pathogenesis-related protein PRB1-2-like. In contrast, the two most significantly upregulated genes were identified as probable cinnamyl alcohol dehydrogenase 5 and sucrose: sucrose 1-fructosyltransferase-like, chloroplastic.

In the second experiment involving accession GSE13346, the wheat plants were infected by the

fungus *P. striiformis*. The differential gene expression

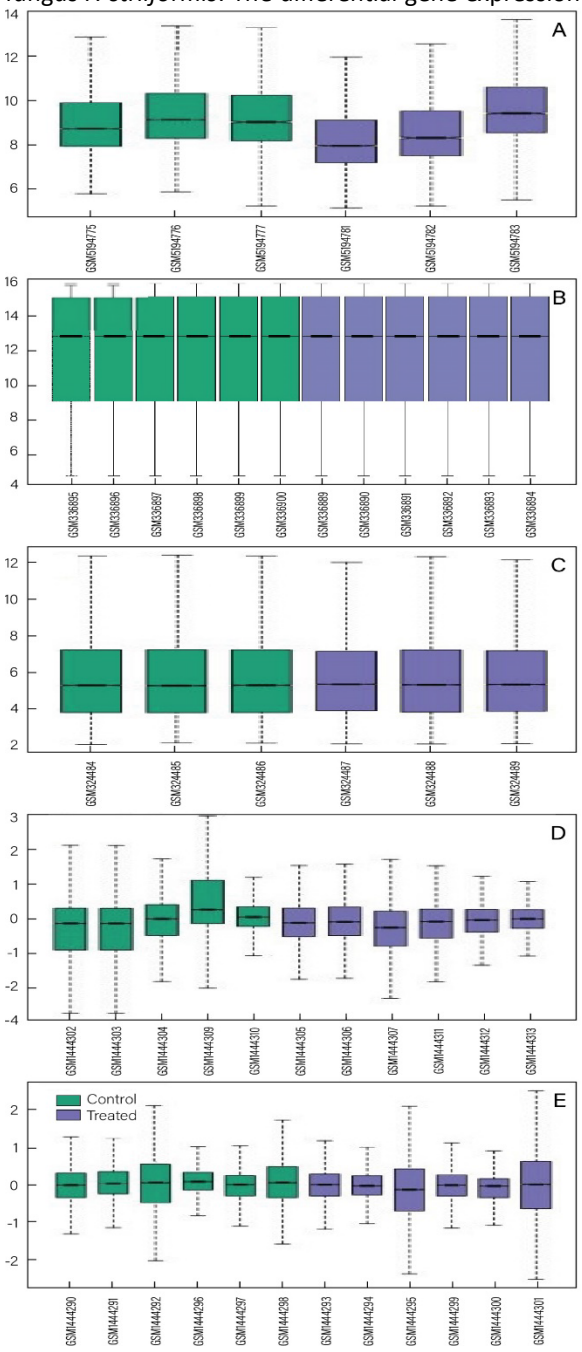

Figure 2. Box plot shows the distribution value between the control and treated samples: (A) the analysis for experiment GSE169298; (B) the analysis for the experiment with accession number GSE13346; (C) the analysis for the experiment with accession number GSE12936; (D) the analysis for the experiment with accession number GSE59721-Nobeokabouzu-komugi genotype; and (E) the analysis for the experiment with accession number GSR59721-Sumai-3 genotype. The green color displays the control samples, and the purple color displays the treated samples. The x-axis demonstrates each sample name, and the y-axis indicates the degree of the distribution value (whether at the minimum, median, or maximum value), which signifies each sample quality.

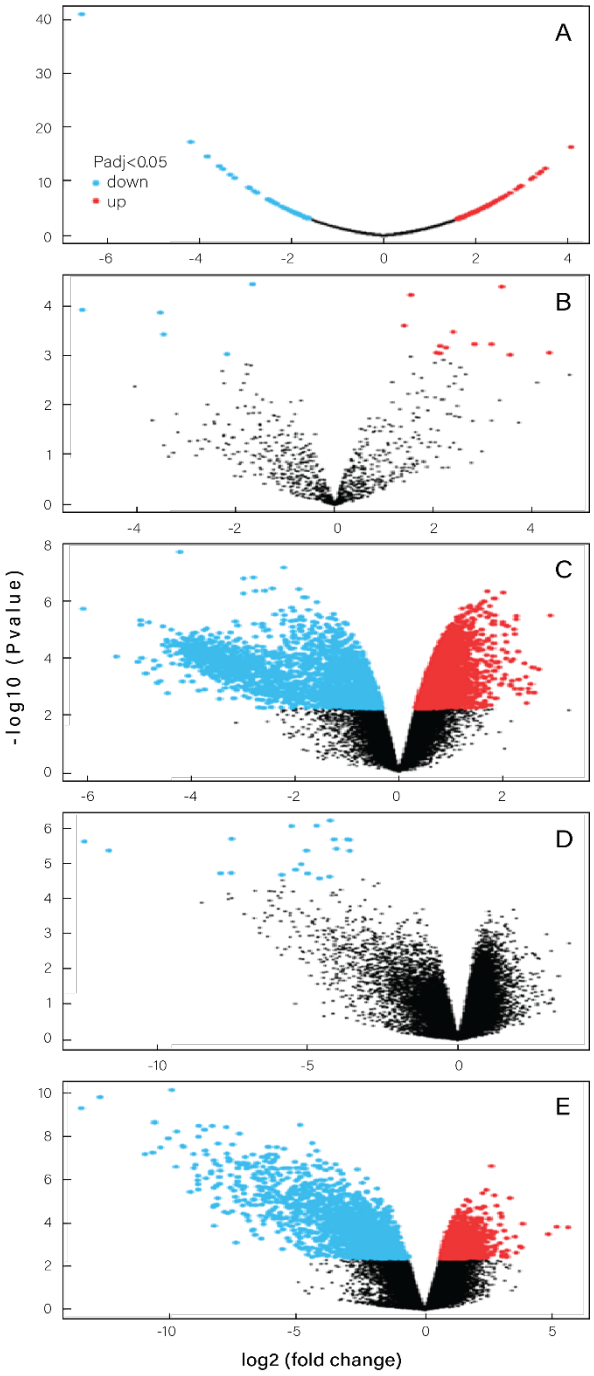

Figure 3. Volcano plot illustrating the significantly expressed genes in response to fungal infection: (A) the analysis for the experiment with accession number GSE169298; (B) the analysis for the experiment with accession number GSE13346; (C) the analysis for the experiment with accession number GSE12936; (D) the analysis for the experiment with accession number GSR59721-Sumai3 genotype; and (E) the analysis for the experiment with accession number GSE 59721-Nobeokabouzu-komugi genotype. Each point represents a gene, plotted based on its fold change (logFC) on the x-axis and its statistical significance (-$\log_{10}$ p-value) on the y-axis. In this plot, upregulated genes are shown in red, while downregulated genes are shown in blue.

analysis displayed a total of 841 genes, of which only 18 were significantly regulated at a 0.05 adjusted p-value (Table 2). Thirteen significant genes were upregulated, whereas five were downregulated, as depicted in the volcano plot (Figure 3B). The two most downregulated genes were peroxidase1 and 9-beta-pimara-7,15-diene synthase, chloroplastic, whereas the two most upregulated genes were mavicyanin-like and dirigent protein 22-like.

In the accession GSE12936 experiment, the effect of biotic stress by *B. graminis* f. sp. *tritici* was studied on wheat plants. The differential gene expression analysis indicated that 61,290 genes were detected, with 7,256 being statistically significant at a 5% adjusted p-value (Table 2). There were 2,630 significant downregulated genes and 4,626 significant upregulated genes, as determined by the log fold change calculation (Figure 3C). Furthermore, the result revealed that the acidic ribosomal protein P2 was the most upregulated gene, whereas 1-acy1-glycerol-3-phosphate acyltransferase was the most downregulated gene in this experiment.

In the accession GSE59721 experiment, the wheat genotype Sumai-3 plants were treated with the fungus *F. graminearum*. The differential gene expression analysis was conducted to compare the gene expression level variation in the treated group to that of the control group. The volcano plot (Figure 3D) illustrated the significantly expressed genes as a function of the fold change to the adjusted P value. The total number of expressed genes was 37,826, among which only 23 were significantly downregulated, with no significantly upregulated genes detected (Table 2). The two most significant downregulated genes in the Sumai-3 genotype were found to be the ones encoding the following proteins: chloroplastic import inner membrane translocase subunit HP30-2-like protein and endo-1,4-beta-xylanase 5-like protein. In the same experiment, the wheat genotype Nobeokabouzu-komugi was infected with the same fungal species as earlier. Consequently, the variations in expression levels were evaluated as previously done. A total of 37,826 genes were identified, of which 3,260 were deemed significant at a p-value cutoff of 0.05 (Table 2). Among these significant genes, 1,691 were upregulated, and 1,569 were downregulated (Figure 3E). However, no significantly upregulated genes were detected, and the two most significantly downregulated genes in the Nobeokabouzu-komugi genotype were those coding the corresponding proteins: putative ribosomal protein L35 and beta-glucosidase 22-like.

This study identified eight intersecting genes significantly differentially regulated during the fungal infection in the four studied experiments. The eight overlapping genes were identified in the datasets using a Venn diagram analysis (Figure 4). The highest number of overlapping genes (five genes) was observed between the two datasets: [GSE169298] and [GSE59721-Nobeokabouzu-komugi genotype]. Each one of the remaining three genes overlapped between the following: [GSE169298] and [GSE12936] datasets; [GSE13346] and [GSE59721-Nobeokabouzu-komugi] datasets; and [GSE59721-Sumai3] and [GSE59721-Nobeokabouzu-komugi] datasets (Figure 4).

When examining the gene expression to elicit the effect observed on wheat plants in responses to fungal pathogens, four (sucrose 1-fructosyltransferase-like, aldo-keto reductase 3, aspartic proteinase nepenthesin-1-like, glutathione S-transferase) of the five shared genes between the two datasets [GSE169298] and [GSE59721-Nobeokabouzu-komugi genotype] were significantly upregulated in the first experiment (accession GSE169298) after inoculation of the plants with *Z. tritici* strain T02596. In contrast, gene expression was altered considerably when the plants were inoculated with *F. graminearum* in the second experiment (GSE59721-Nobeokabouzu-komugi genotype), with upregulated genes becoming downregulated. However, the fifth gene (chitinase CLP) was significantly downregulated in both experiments (Figure 5). The most highly upregulated of the five overlapping genes between the two datasets, GSE169298 and GSE59721-Nobeokabouzu-komugi genotype, was sucrose 1-fructosyltransferase-like, with a log fold change (logFC) gene expression level value of 3.2454071, followed by aldo-keto reductase 3, logFC value of 2.0495435 in the GSE169298 experiment (Table 3). Conversely, glutathione S-transferase was the most highly downregulated among the five shared genes, with a logFC value of -7.565892467 in the GSE59721-Nobeokabouzu-komugi genotype experiment (Table 3).

Notably, one (thaumatin-like protein) of the three remaining shared genes was found overexpressed between the two datasets [GSE169298 and GSE12936] with almost twofold higher upregulation level (logFC values of 1.7020893) in the GSE169298 experiment (Table 3). Additionally, between the two datasets [GSE59721-Sumai3] and [GSE59721-Nobeokabouzu-komugi genotype], a downregulation

Table 1. The experimental setup that was applied to the datasets used in the *in silico* differential gene expression analysis of this study.

Dataset	Plant materials used for RNA extraction	Days postinoculation (before RNA extraction)	Gene Expression analysis platform	Wheat growth conditions
Accession GSE169298	Leaf samples	Five days	Agilent-022297 Wheat Gene Expression Microarray	Greenhouse at 21 ± 2°C for three weeks with 16 h of light and 8 h of darkness before inoculation
Accession GSE13346	Shoot tissues	48 hours	Wheat custom oligonucleotide array for stripe rust study	Greenhouse at 25°C for ~10 days with 16 h of light and 8 h of darkness before inoculation
Accession GSE12936	Leaf samples	1 week	Affymetrix Wheat Genome Array	Greenhouse at 18°C–22°C for four weeks with 16 h of light and 8 h of darkness before inoculation
Accession GSE59721 Nobeokabouzu-komugi (Japanese landrace cultivar) Sumai-3 (Chinese variety)	Spikelet samples	72 h (3 days)	Agilent-015520 Custom wheat 38k array	Greenhouse at 25°C for 10 to 14 days with 16 h of light and 8 h of darkness before inoculation

Table 2. Summary of datasets' results used in the *in silico* differential gene expression analysis of this study.

Accession number	Fungus name	Genotype of infected wheat	Size of data MB	Number of detected genes	Number of significant genes (cutoff = 0.05)	Number of significantly downregulated genes	Number of significantly upregulated genes	Matrix link
GSE169298	*Z. tritici*	*T. aestivum* L.	46.0	10471	214	54	160	https://www.ncbi.nlm.nih.gov/geo/geo2r/?acc=GSE169298#
GSE 13346	*P.* striiformis	YR 08	0.977	841	18	5	13	https://www.ncbi.nlm.nih.gov/geo/geo2r/backend/geo2r.cgi
GSE12936	*B. graminis*	*T. aestivum* L.	57.7	61290	7256	2630	4626	https://www.ncbi.nlm.nih.gov/geo/geo2r/?acc=GSE12936
GSE59721	*F. graminearum*	Sumai3	66.1	37826	23	23	0	https://www.ncbi.nlm.nih.gov/geo/geo2r/?acc=GSE59721
GSE59721	*F. graminearum*	Nobeokabouzu-komugi	62.5	37826	3260	1569	1691	GEO2R - GEO - NCBI (nih.gov)

Table 3. Summary of the upregulation and downregulation of the overlapping genes between the five datasets, referred to as a log fold change (logFC) gene expression level value.

Shared genes	Log fold change gene expression level values				
	GSE169298	GSE13346	GSE12936	GSE59721_sumai3	GSE59721_Nobeokabouzu-komugi
Sucrose 1-fructosyltransferase-like	3.2454071				-4.5868512
Aldo-keto reductase 3	2.0495435				-6.0470495
Aspartic proteinase nepenthesin-1-like	1.9023744				-6.6843241
Chitinase CLP	-1.8965857				-4.6820469
Glutathione S-transferase	1.860256				-7.565892467
Thaumatin-like protein	1.7020893		0.9755		
Mavicyanin-like		3.38882			-8.395740467
Flavin-containing monooxygenase 1				-4.5890029	-6.0425572

of flavin-containing monooxygenase 1 occurred, which was associated with a log fold change value equal to -4.2474882 and -6.0425572, respectively, in response to the pathogens (Table 3). Finally, the last of the shared genes (mavicyanin-like protein) expression was significantly upregulated (logFC of 3.38882) after inoculating the plants with *P. striiformis* in the GSE13346 dataset, but it was significantly downregulated (logFC of -8.53252) when inoculated with *F. graminearum* in the second dataset (GSE59721-Nobeokabouzu-komugi genotype) (Figure 5 and Table 3).

Protein-protein interaction and functional enrichment analysis

The STRING database was utilized to analyze the protein-protein interactions (PPI) network of significant genes to identify key genes from all the datasets during infection. Out of 641 selected (cutoff 200 genes) significantly regulated genes, 47 encoded unidentified proteins and were thus excluded from the PPI analysis. After removing repeated genes and filtering for gene ID, only 72 of the 594 remaining significant genes were included in the PPI and network analysis (Figure 6).

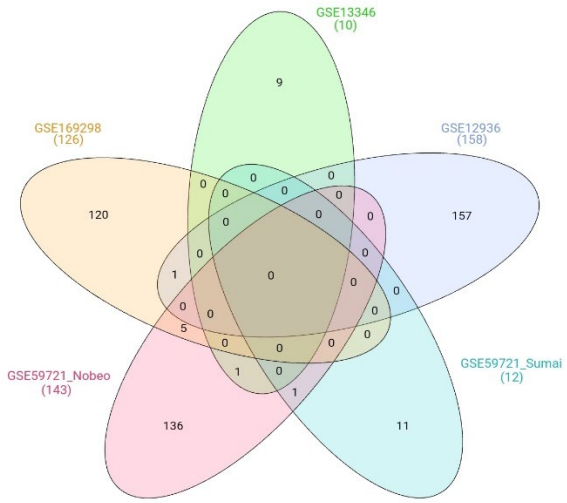

Figure 4: Venn diagram that shows the eight overlapping genes. Each color represents the significantly expressed gene collection from an experiment (GSE59721_Nobeo: Nobeokabouzu-komugi genotype).

Subsequently, the STRING database employed the Kyoto Encyclopedia of Genes and Genomes (KEGG) knowledge base to analyze gene functions systematically, linking genomic information with higher-order functional details of the 72 proteins. This provided insights into the pathways these proteins are involved in and their correlations, leading to the creation of the main protein network map (Figure 6).

Gene ontology and network analysis of the significantly regulated proteins in this study, illustrated in Figure 6, showed three interlinked clusters. The red cluster signifies a group of proteins involved in plant defense mechanisms in response to fungal stress with a false discovery rate of 0.3. This mechanism against fungal infection includes 696 genes (GO: 0050832), of which five—chitinase-1, chitinase-3, xylanase inhibitor TAXI-I, xylanase inhibitor TAXI-III, and defensin-like protein-1—were detected as significantly regulated in this study and related to solid evidence of co-expression as indicated with the black line (Figure 6). The molecular function of those interconnected genes detected by the functional enrichment analysis includes chitinase, hydrolase, and xylanase degrading activities that enable them to identify and degrade chitin in the invading fungal cell wall. In contrast, the xylanase inhibitors (TAXI-I, TAXI-III) inhibit xylanases produced by pathogens to prevent the breakdown of xylan-rich structures, which could compromise the integrity of host tissues or plant defenses and limit pathogen-driven tissue destruction. Moreover, chitinase-3 modulates inflammation and tissue repair to prevent excessive damage to host cells. In contrast, defensin-like protein-1 acts as a broad-spectrum antimicrobial agent, causing pore formation and disrupting the pathogen's cell membranes, particularly in early immune responses.

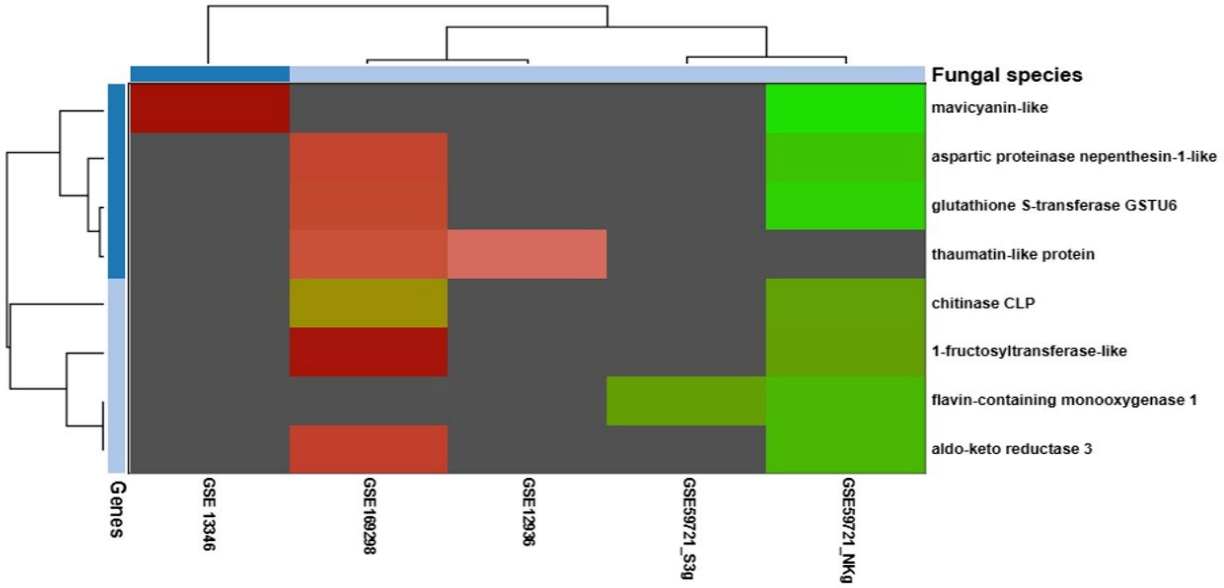

Figure 5. Heatmap shows significantly upregulated or downregulated shared genes in the five studied experiments inoculated with different fungal species. The columns represent contrasts between the studied data sets and the inoculated fungal species. In total, eight genes were commonly identified between the five experiments as differentially expressed ($P < 0.05$ fold change) in at least one contrast. Each gene corresponds to a colored line indicating the transcription level: green corresponds to a $\log_{10}$ ratio of –8.4 (downregulation) and red corresponds to a $\log_{10}$ ratio of 3.4 (upregulation).

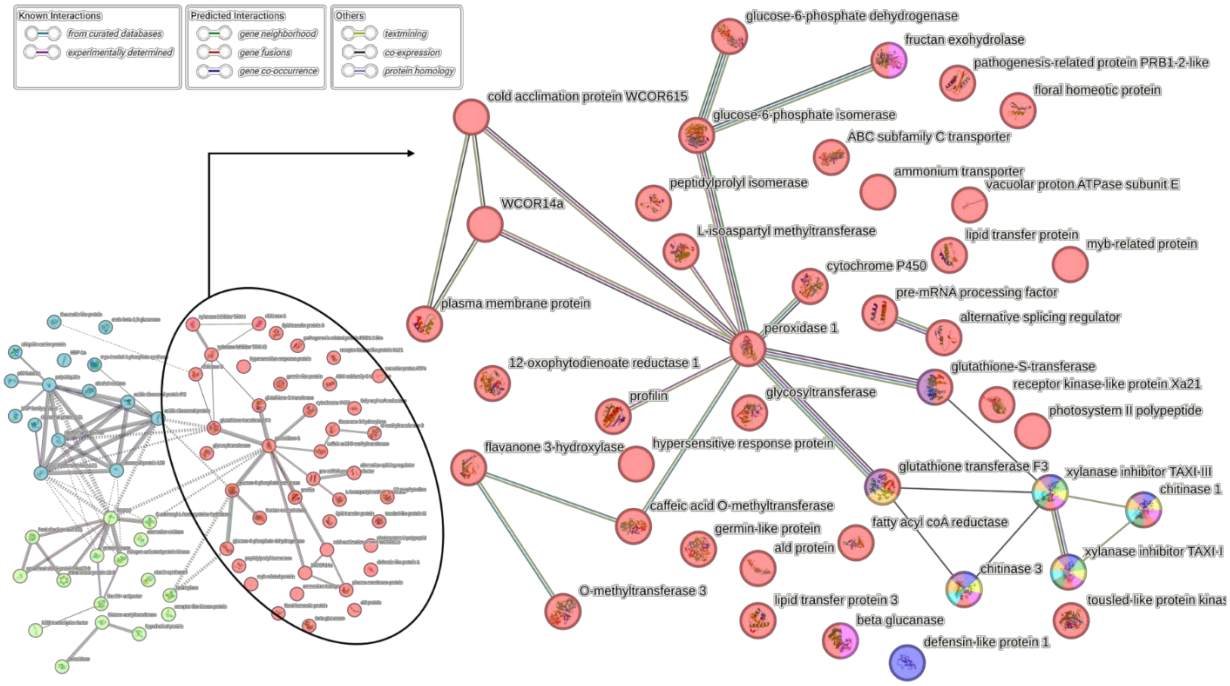

Figure 6. Protein-protein interaction network includes 72 nodes and 77 edges, PPI enrichment p-value: 6.632^{e-05}. Each node represents a different protein; each edge indicates the functional and physical protein association; each line represents an association; the line thickness indicates the strength of the data support; and the line color indicates the type of interaction evidence. The functional enrichment analysis of the network showed two genes with endo-chitinase activity (light green color code), four genes with chitinase activity (yellow color code), and six genes with hydrolase activity (fuchsia color code). The five genes involved in the defense response to fungal infection are represented in purple.

Additionally, the beta-glucanase enzyme that breaks down β-glucan, a major component of the fungal cell wall, is a significantly regulated protein in this study. This enzyme helps the plant cell recognize fungal infection at the molecular level. This triggers signal transduction pathways, leading to the activation of plant immune responses. The other two enzymes differentially expressed significantly in response to fungal infection are glutathione transferases F3 and glutathione-S-transferases. According to the functional enrichment descriptive analysis, both are involved in detoxifying reactive oxygen species (ROS) generated during biotic stress responses. They are directly involved in the plant's defense against fungal pathogens by enhancing their ability to manage oxidative stress.

DISCUSSION

This research aimed to understand the underlying mechanisms of wheat plant defense initiated through their stress-response genes against biotic stresses. It was conducted as a comparative transcriptome analysis on the sequence results of previous experimental studies (accession numbers: GSE169298, GSE13346, GSE66772, GSE12936, and GSE59721) that involved four wheat genotypes: *Triticum aesitvum*, YR 08, Sumai 3, and Nobeokabouzu-komugi. This transcriptome analysis revealed significant changes in gene expression due to biotic fungal infections by *Zymoseptoria tritici, Puccinia striiformis, Blumeria graminis,* and *Fusarium graminearum*.

Deferential analysis of gene expression levels

Ribulose bisphosphate carboxylase/oxygenase activase B (Rubisco activase) is one of the most significantly affected gene expressions from the accession GSE169298 experiment, which was significantly downregulated in response to *Zymoseptoria tritici* infection. Rubisco activase plays a crucial role in the function of the enzyme ribulose bisphosphate carboxylase/oxygenase (Rubisco), which is responsible for the initial step of carbon fixation in photosynthesis (Flecken *et al*., 2020). The significant downregulation of Rubisco activase gene expression decreases wheat plants' potential to fix carbon dioxide and perform optimal photosynthetic performance, which is particularly important for

wheat yield, growth, and biomass production (Wijewardene *et al*., 2021). The gene expression of Rubisco activase was previously reported to be significantly affected in *Triticum aestivum* by several abiotic stresses, especially drought and heat stress (Degen *et al*., 2021). Nevertheless, to the best of the authors' knowledge, this study is the first report on the negative effect of fungal infection on Rubisco activase gene expression.

Moreover, the chloroplastic import inner membrane translocase subunit HP30-2-like protein was significantly downregulated in Sumai 3 wheat, according to the GSE59721 experiment. Prior research has largely overlooked this protein, apart from the fact that nuclear-encoded proteins cannot move through the inner membrane of the chloroplast without its function (Bushnell *et al*., 2009; Rossig *et al*., 2013). Therefore, its downregulation is expected to cause malfunctioning of chloroplast-based immune responses, primarily because it works in close association with ubiquitin E3 ligases, which act as regulators for the synthesis of immunologically secreted substances such as jasmonic acid and cytokinin (Kelley, 2018). The downregulation of the translocase protein could be a result of *Fusarium graminearum* trichothecene and fumonisin mycotoxins, proven to photooxidate chlorophyll and cause a significant loss of electrolytes from the chloroplastic medium (Rossig *et al*., 2017; Kretschmer *et al*., 2019). The downregulation of this gene and the downregulation of Rubisco activase led the authors to conclude that numerous fungal infections may target wheat chloroplasts, either directly or as a side effect.

Another significantly downregulated gene in the GSE59721 experiment Sumai 3 wheat genotype was the endo-1,4-beta-xylanase 5-like protein, which is a xylan-hydrolyzing enzyme acting on complex substrates such as hemicellulose in the wheat cell wall. Its function contributes to cell elongation and protoplast synthesis. The endo-1,4-beta-xylanase 5-like protein can also cleave the β-xylosidic bonds and, therefore, is expected to have a role in the fatty acylation and glycosylation of sterols (De Backer *et al*., 2010). Such downregulation of endo-1,4-beta-xylanase 5-like protein could be attributed to the fact that *Fusarium graminearum* also produces endo-1,4-xylanases, triggering an immune response that causes wheat to upregulate three classes (TaXI-I, TaXI-III, and thaumatin-like) of *Triticum aestivum* xylanase inhibitors. Apart from GH10 classes, all three inhibitors can efficiently inhibit various xylanase classes from inflicting cell death. Both TaXI-I and TaXI-III (with >60% similarity to thaumatin-like proteins) compete with the substrates to bind to the active site of xylanase, while thaumatin-like functions as a noncompetitive inhibitor (Sun *et al*., 2020).

Furthermore, in the GSE169298 experiment, the gene expression of cinnamyl alcohol dehydrogenase 5 (CAD) has been identified as one of the most significantly upregulated genes in wheat due to the *Z. tritici* infection. Cinnamyl alcohol dehydrogenase (CAD) is an enzyme involved in the last step of monolignol biosynthesis, a crucial process in lignin polymerization. CAD catalyzes the conversion of hydroxy-cinnamaldehydes into their corresponding cinnamyl alcohols, which are then polymerized to form lignin (Li *et al*., 2022). Lignin is essential for the structural integrity of plant tissues, including stem strength and resistance to environmental stresses (Chao *et al*., 2022). Therefore, the upregulation of that gene during fungal infections will result in lignin accumulation and could be interpreted as a defense action by wheat against biotic stresses (Ma and Li, 2023).

Similarly, the dirigent protein 22-like gene (DIR) is a disease resistance gene, which is the most upregulated gene in wheat against *Puccinia striiformis* infection in the GSE13346 experiment. This gene induces the constitutive or inducible formation of lignan, which has antifungal properties. The gene is also involved in forming lignin, which forms a physical barrier in response to pathogen attacks (Khan *et al*., 2018).

The peroxidase1 gene (also known as class III peroxidase, glutathione peroxidase, ascorbate peroxidase, and thioredoxin-dependent peroxidase) was among the most significantly downregulated in the experiment with accession number GSE13346. The peroxidase1 gene is responsible for generating reactive oxidative species (ROS), which act as signaling molecules involved in triggering hypersensitive responses (HR) and activating signal transduction processes associated with cell death responses during the interaction between wheat and *Puccinia striiformis* races (Yang *et al*., 2016). Therefore, the downregulation of this gene in response to *P. striiformis* infection in the accession GSE13346 experiment serves as a mechanism for wheat to migtate cell death, as fungal mycotoxins already promote cellular necrosis.

The beta-glucosidase 22-like gene, which regulates cell wall lignification and cell wall turnover in plants, was significantly downregulated in Nobeokabouzu-

komugi wheat genotype, according to the GSE59721 experiment. The expression of this gene is responsible for the immobilization of callose, which acts as a physical defense barrier against both spores and hyphae. Besides contributing to a local antimicrobial defense barrier, these enzymes hydrolyze the β-glucans in the walls of the invading microbes (Perrot *et al*., 2022). Several β-glucosidase genes are induced as a response to glycoside secondary metabolism, which generally releases toxic compounds such as cyanide and hydroxamic acids, where the β-glucosidase detonating effect eliminates the production of those toxins (Ketudat Cairns and Esen, 2010). Nevertheless, overcoming this induction of β-glucosidase genes by generating signaling molecules for their downregulation, as observed in the Nobeokabouzu-komugi wheat genotype (accession GSE59721 experiment), is sometimes crucial for improving wheat resistance against pathogen attack. Various plants usually maintain toxic metabolites in nonactive forms to minimize their risk of harming plant cells. These toxic metabolites are activated as a chemical defense upon fungal infection, producing broad-spectrum and potent antifungal molecules (Ma *et al*., 2024).

Additionally, ribosomal proteins are involved in several processes, such as replication, transcription, DNA repair, and the regulation of plant cell development (Moin *et al*., 2016). Most importantly, previous transcriptome profiling research showed they were upregulated and maintained at a high expression level throughout the biotic infection stress to increase plant defenses (Al-Taweel *et al*., 2014). However, in disagreement with such results, the transcriptome analysis conducted in this research revealed the downregulation of the putative ribosomal protein L35 gene, which is required for pre-rRNA processing (Babiano and de la Cruz, 2010), in the accession GSE59721 experiment (Nobeokabouzu-komugi wheat genotype). Such alteration in expression may arise in response to neighboring cell death after fungal invasion and is usually transient, allowing a more rapid depletion of signal molecules and thus leading to a faster adaptation to the new stress conditions under fungal infection (Ludwig and Tenhaken, 2001).

Intriguingly, the glycerol-3-phosphate acyltransferase gene expression was significantly downregulated in the accession GSE12936 experiment. This gene is involved in the formation of cutin monomers. Subsequently, it facilitates their transport to the outer epidermal cell wall of the leaf to act as a barrier against pathogen attack and desiccation (Beisson *et al*., 2012). Nonetheless, cutin monomers released during a pathogen attack may conversely stimulate pathogen colonization by triggering the formation of appressoria, spore germination, and cutinase overexpression in the fungus (Wang *et al*., 2012). Consequently, cuticle-associated gene expressions are constantly altered in plants, which may show reduced expression levels under the influence of infection (Fawke *et al*., 2019).

From the Venn diagram analysis, eight significantly regulated genes overlapped differently between the five datasets. Four of the eight (sucrose-1-fructosyltransferase-like, aldo-keto reductase 3, aspartic proteinase nepenthesin-1-like, and glutathione S-transferase) were upregulated in response to *Z. tritici* infection (accession GSE169298), and two genes (mavicyanin-like and thaumatin-like) were upregulated in the GSE12936 and GSE13346 accession numbers experiments in response to *P. striiformis* and *B. graminis* infections, respectively. The reason behind such upregulation is probably related to the fact that the biological function of these six genes plays an important role in synergistically regulating the resistance of wheat plants' defense responses against a variety of plant pathogens. Such a hypothesis was backed by other research in which the mavicyanin-like protein, which belongs to the phytocyanin family of blue copper proteins, was the target of tae-miR408 (microRNA) regulation in wheat plants. This was found to play an essential role in the host plant's resistance to *P. striiformis* stripe rust, as this gene influences the expression of genes related to antioxidant defense (Feng *et al*., 2013).

Similarly, the aldo-keto reductase 3 gene encode an enzyme belonging to the aldo-keto reductase (AKR) family and is produced by wheat as a response to fungal infections. AKRs have been previously identified in the catabolizing of deoxynivalenol (DON). Deoxynivalenol is the most widely distributed mycotoxin worldwide and is primarily produced during infection and disease development in wheat with *F. graminearum* and *Z. tritici* fungal plant pathogens. The detoxification pathway of DON involves its epimerization, in which DON is catabolized into 3-keto-deoxynivalenol (3-keto-DON) and 3-*epi*-deoxynivalenol (3-*epi*-DON), which were revealed by a recent study to exhibit little to no toxicity compared to those of DON. (He *et al*., 2020). Furthermore, thaumatin-like proteins (TLPs), which are defined as pathogenesis-related protein family 5 (PR5) members, are widely distributed plant proteins that

are engaged in defense responses and have antifungal action against a variety of plant pathogens (Wang *et al*., 2020).

In the same context, several studies have shown that sucrose-1-fructosyltransferase-like, glutathione S-transferase, and aspartic proteinase nepenthesin-1-like are three widespread protein groups found to be overexpressed and can noticeably reduce disease symptoms and pathogen multiplication rates (Gullner *et al*., 2018; Janse *et al*., 2020; Bekalu *et al*., 2021). Several researchers have shown that fructan plays an integral part in plants' survival under abiotic and biotic stress conditions and that overexpression of sucrose 1-fructosyltransferase promotes fructan synthesis in plants. Fructans are known to be priming or defense stimulatory molecules that induce immunity against the fungal pathogen in several plant species (Janse *et al*., 2020). Also, plant glutathione S-transferases (GSTs) are multifunctional enzymes encoded by large gene families found by transcriptome investigations to be upregulated in the early phase of bacterial, fungal, and viral infections. The recognized roles of GSTs in disease resistance are their contribution to detoxifying toxic lipid hydroperoxides that accumulate during infections, reducing oxidative stress, and participating in intracellular hormone transport (Gullner *et al*., 2018). Finally, nepenthesins, which are categorized under the family of plant aspartic proteases, represent the only extracellular proteases of plant origin. A prior study revealed nepenthesin-1 aspartic protease as an effective activity inhibitor of fungal phytase, which is a type of phosphatase used as a pathogenesis factor to colonize host tissues during the infection (Bekalu *et al*., 2021).

Surprisingly, all the previously mentioned overlapping genes (apart from thaumatin-like), which were significantly upregulated against *Z. tritici* in the GSE169298 dataset, were downregulated significantly against *F. graminearum* in the GSE59721 Nobeokabouzu-komugi wheat genotype experiment. Moreover, the chitinase-encoding CLP gene in *Triticum aestivum*, which is supposed to play a potential role in antifungal activity, was downregulated in the same two datasets in response to the fungal attack. This may be explained based on the fact that during the process of infection, pathogens secrete effector proteins into the plant cells to manipulate plant physiology and inhibit host defense responses (Wang *et al*., 2022). *Fusarium graminearum* is a hemibiotroph pathogen that combines both features of biotrophic and necrotrophic pathogens, including the possession of a broad range of effectors. Unfortunately, till now, only a few of them have been identified during *F. graminearum* infection in wheat (Duba *et al*., 2018).

Lastly, flavin-containing monooxygenase 1 was found to be downregulated in *Fusarium graminearum* infection of both wheat genotypes, Sumai 3 and Nobeokabouzu-komugi. Flavin-containing monooxygenase 1 has multiple functions; among the most widely recognized are auxin and specialized metabolite biosynthesis (Sun and Bakkeren, 2024). Occasionally, processes related to metabolism and development are downregulated during microbial infection, possibly due to a reallocation toward secondary metabolic pathways and defense mechanisms. For example, jasmonates, a well-known inducer of plant defense mechanisms, can simultaneously suppress growth-related hormones and metabolites (Savchenko *et al*., 2019). Hence, it is very likely that enhanced pathogen-induced mediated defenses in both wheat genotypes, Sumai 3 and Nobeokabouzu-komugi, led to downregulation of biosynthesis-related processes.

Protein-protein interaction analysis

The prediction of probable interactions (PPI network) between the significantly differentially expressed genes-encoded proteins revealed that, in all wheat genotypes, interactions between enzymes involved in defense response to fungus based on the adjusted p-value of the logFC of the total genes in the analyzed datasets (Supplementary Materials), four genes (chitinase 1, chitinase 3, xylanase inhibitor TAXI-I, and xylanase inhibitor TAXI-III) were significantly upregulated. The crucial role of chitinase enzymes in fungal disease management has been associated with their catalytic ability to degrade chitin, the principal constituent of the fungal cell wall, without any adverse side effects on the host plant (Jalil *et al*., 2015; Kumar *et al*., 2018). In tobacco and tomato plants, chitinase inhibitors such as Avr4 from *Cladosporium fulvum* bind to chitin in fungal cell walls, preventing its degradation by plant chitinases. Consequently, plants reinforce the immune response against fungal pathogens (van den Burg *et al*., 2006). Furthermore, due to the high specificity of chitinases toward chitin, several studies explored the potential for developing transgenic disease-resistant plants (Fahmy *et al*., 2018).

Xylanases produced by fungal pathogens contribute to pathogenesis with their capacity to degrade xylan contained in the plant cell wall. They can also induce

cell death in the host tissues independent of their enzymatic activity (Noda *et al*., 2010). Plants have developed the ability to inhibit microbial xylanases, but not those derived from other plants, as a means of disease resistance. The inaugural xylanase inhibitor, which inhibits microbial xylanases and hence impedes the degradation of plant xylan, was identified in wheat extracts of *Triticum aestivum* after its isolation and characterization (McLauchlan *et al*., 1999). The transcriptome results from this research indicate that the protein expression of xylanase inhibitors is significantly induced during biotic stress conditions. This hypothesis is supported by other studies in which the upregulated gene expression of xylanase inhibitors in plants had a dramatic inhibiting role against various classes of fungal xylanases. Additionally, TAXI-I and TAXI-III xylanase inhibitors have shown delaying effects on FHB symptoms, making them ideal candidates as fungal stress-resistance proteins (Moscetti *et al*., 2013; Liu *et al*., 2021; Lin *et al*., 2024).

Unexpectedly, the defensin-like protein 1 gene expression level showed strong suppression with logFC between > -4 and > -8 (Supplementary Materials) in response to *Fusarium graminearum*. The reason for that is currently unclear. However, the effectors produced by pathogenic fungi are most likely involved in its suppression, as previously explained by Duba *et al*. (2018). In certain species or strains of fungi, downregulation of defensin-like protein 1 might occur to redirect energy toward other defense mechanisms (Tesfaye *et al*., 2013).

The predicted network suggests a synchronized defense response that aligns with known plant pathways to chitin-containing and xylan-degrading pathogens, potentially reflecting an immune mechanism activated in response to fungal threats. The interactions are as follows: The plant cell recognizes fungal infection through pathogen-associated molecular patterns like β-glucans from the fungal cell wall. In response to fungal infection, β-glucanase is induced as part of the triggered immunity; this enzyme degrades the fungal cell wall by breaking down β-glucans and making it more susceptible to further degradation or attack by other defense molecules. The degradation products (β-glucan oligomers) can stimulate further defense responses (Balasubramanian *et al*., 2012). The next step is the induction of chitinase-1 and chitinase-3, which address the chitin-containing aspects of the pathogen's structure, with the first identifying and degrading the pathogen's chitin and the second modulating the immune response and inflammation to promote tissue healing and balance. Simultaneously, upregulated xylanase inhibitors TAXI-I and TAXI-III regulate or inhibit xylanases produced by pathogens before targeting plant cell walls. In addition, the fungal xylanase recognition pathway in plants triggers programmed cell death at the infection site and the production of ROS and other immune responses (Ron and Avni, 2004).

In cases of active biotic stress response and upregulation of defensin-like protein-1, it will complete the action of chitinases and xylanase inhibitors by disrupting microbial membranes and killing the invading microorganisms directly. Together, these proteins form a complex immune response that protects the host from pathogens and ensures that the fungal pathogen is attacked structurally by degrading the cell wall and functionally by disrupting the membrane (Jones and Dangl, 2006).

CONCLUSION

Building on the RNA sequence data from previous research and the comprehensively conducted transcriptome analysis in this work, we drew different gene expression profiles of *Triticum aestivum* genotypes infected with different species of fungi. We identified and compared the potential functions of these genes. This comparison effectively narrowed down the key genes and defense pathways involved in the wheat response to fungal infection and identified some unique, significantly expressed genes specific to wheat genotypes. Our results identified several candidate genes that may enhance the resistance of wheat varieties to fungal infection; however, many remain unidentified and could serve as intriguing candidates for further investigation.

AUTHOR CONTRIBUTIONS

Conceptualization and implementing of the methodology, R.M.A. and A.M.H; analyzing and interpreting the data, R.M.A., A.S.A., M.R.A., M.S.A., M.Y.Y., M.M.M., N.N.M., and A.M.H; original draft preparation, R.M.A., A.S.A., M.R.A., M.S.A., M.Y.Y., M.M.M., N.N.M., and A.M.H; reviewing and editing final version prior to journal submission, R.M.A. and A.M.H. All authors have read and agreed to the published version of the manuscript.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

ETHICS APPROVAL

Not applicable.

DATA AVAILABILITY STATEMENT

All data is available in the manuscript and the supplementary material. The output data and the analysis of the output data associated with this study are openly available on: https://drive.google.com/drive/folders/1lfA5nNjrxuSvkzOl7xxuj7wHXECKQceh

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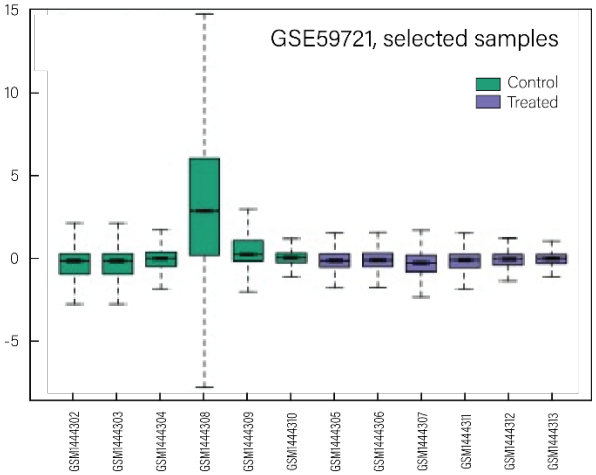

Supplementary Figure. Box plot shows the distribution value between the control and treated samples, representing the analysis for the accession number GSE 59721-Nobeokabouzu-komugi genotype before excluding sample number GSM1444308 from the analysis. Sample SM1444308 had a median-centered value that deviated and had to be removed from the data set.