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Transcriptomic Response of *Arabidopsis thaliana* to *Pseudomonas syringae* Infection: An *In Silico* Approach

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ABSTRACT

Plant stresses caused by phytopathogenic bacteria are categorized into biotic stresses. The study aimed to perform a meta-analysis of *A. thaliana* transcriptomic data in response to infection by *P. syringae* and *P. syringae* pv. *maculicola*. The gene expression and transcription factors (TFs) of *A. thaliana* infected by the bacteria were investigated using published RNA-Seq data. Also, critical factors, including hub genes, protein-protein interaction (PPI), and micro RNAs (miRNAs), were analyzed. A total number of 22 biological pathways were significantly enriched with up-/down-regulated differentially expressed genes (DEGs) in the Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis. Also, 39 TFs of *A. thaliana* were altered during the bacterial infection. Moreover, 5034 DEGs were significantly different from non-stressed plants, of which 2291 and 2743 DEGs were up- and down-regulated, respectively. The expression of genes related to stress response, cellular process, metabolic process, and stimulus response was up-regulated in the bacteria-infected plant. In contrast, the down-regulation of genes involved in the cellular and biosynthesis processes was observed. Regarding molecular function, 412 genes associated with kinase, catalase, and oxidoreductase activities were up-regulated in the bacteria-infected plants, while down-regulation of hydrolase and transferase activity genes was observed. The PPI network showed 107 nodes and 189 edges. The most important hubs genes included MYC2, WRKY40, WRKY33, and other genes. Moreover, the total number of 41 miRNA families was determined during the *A. thaliana*-bacterium interaction. Infection of *A. thaliana* by *P. syringae* and *P. syringae* pv. *maculicola* induced the expression of some stress-responsive genes and pathways among which some defense-related hub genes were identified. The results provide a clearer understanding of the strategies applied to program defense pathways in bacterial infection of *A. thaliana*.

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Introduction

Plant stresses are generally categorized into two distinct groups including abiotic and biotic stresses (Umar *et al*., 2021). Plant pathogenic bacteria (PPB) are a considerable group of microorganisms that are responsible for biotic stress in a large number of plant species (Goto, 2012). *Pseudomonas syringae* van Hall is one of

the economically important species of PPB causing various diseases among cultivated and non-cultivated plants (Xin *et al*., 2018). It is a Gram-negative bacterium with rod-shaped cells and polar flagella that exists as more than 50 various pathovars (Arnold and Preston, 2019). Due to its ability to infect well-studied plant species e.g. Arabidopsis (*Arabidopsis thaliana* (L.) Heynh.), tobacco (*Nicotiana benthamiana* Domin), and tomato (*Solanum lycopersicum* L.), *P. syringae* has considered a remarkably important microorganism for biomolecular identification of the genomic aspects of plant-pathogen interactions(Mansfield *et al*., 2012). Additionally, there is an *A. thaliana*-infecting pathovar of *P. syringae*, viz. *maculicola*, which induces leaf spot symptoms on the infected plant (Takikawa and Takahashi, 2014).

Plant-pathogen interactions involve numerous biological and molecular pathways that lead to disease and/or resistance development in pathogen-challenged plants (Dodds and Rathjen, 2010). It has been demonstrated that bacterium-infected plants can survive the infection via different defensive pathways such as pathogen-associated molecular patterns (PAMP) (Zipfel and Robatzek, 2010), effector-mediated resistance (Pruitt *et al*., 2021) and gene silencing (Kong *et al*., 2022). Furthermore, there are a large number of studies reporting that microRNAs are involved in regulating gene expression to respond to both biotic and abiotic stresses (Zhang *et al*., 2023). Upon pathogen recognition, plants trigger diverse molecular pathways by which they exhibit resistance phenotype (Zhang and Zhou, 2010). This phenomenon requires a significant alternation in the expression of defense-related genes leading to resistance responses within the pathogen-challenged cell (Casassola *et al*., 2013; Yang *et al*., 2009). In contrast, PPB encounters the plant defense pathways through the secretion of pathogenicity-related molecules i.e., effectors suppressing the induced systemic resistance called ISR (Gangadharan *et al*., 2013).

Meta-analysis is an effective tool for exploring the genetic basis of plant-pathogen interactions, providing insights into future stress-associated responses within plant cells (Balan *et al*., 2018; Biniaz *et al*., 2022; Yang *et al*., 2021). Despite extensive research on *P. s syringae*, there remains a lack of comprehensive meta-analyses focusing on the transcriptional responses of *A.thaliana* under bacterial stress, which this study aims to address. Microarrays and RNA-Seq data are two main sources of transcriptome data that have been extensively used for meta-analysis (Bhargava *et al*., 2013). Meta-analysis would help the researchers to identify differentially expressed genes (DEGs) involved in plants under either biotic or abiotic stress (Biniaz *et al*., 2022; Yang *et al*., 2021). The results would help reveal how plants respond to pathogen invasion. Moreover, the determination of plant responses against pathogen infection would enable us to characterize the plant-pathogen interaction. Due to the large amount of transcriptome data generated for *A. thaliana*, these data can be used for performing meta-analysis to investigate stress-responsive genes (Jiang *et al*., 2017).

This study aimed to determine the common transcriptional regulation of *A. thaliana* plants under bacterial biotic stress caused by *P. syringae* and *P. syringae* pv. *maculicola*, using previously deposited RNA-Seq data. Critical factors including hub genes, pathways, gene series, and protein-protein interaction networks were identified by meta-analysis and functional enrichment analyses. The related DEGs associated with varied metabolic pathways (transcription factors (TFs) and miRNA families) were characterized. The results would give us a more clarified picture of the strategies applied to program defense pathways in the *A. thaliana*-bacteria interaction.

Materials and Methods

Input data

The response-related RNA-seq data for *A. thaliana*-bacteria interactions was recovered from ArrayExpress of the European Molecular Biology Laboratory - European Bioinformatics Institute (http://www.ebi.ac.uk/arrayexpress) (Table 1). The data sets were filtered for *A. thaliana*, to only include RNA-seq data. The adapter sequences and low-quality reads were deleted and adjusted using Phred quality scores (Ewing and Green, 1998). The quality of data was checked and located in the corresponding region of the *A. thaliana* genome. CLC

Genomics Workbench version 20 (QIAGEN, Germany) was used to investigate the expression profile of the genes. The counts per million (CPM) were applied for the normalization of the expression data.

Data analysis

The meta-analysis was conducted on an integrated dataset about DEGs in *A. thaliana*-bacteria interactions. Each dataset was classified into two groups including a stressed and non-stressed set. The SVA R package was used to alleviate the batch effect based on the empirical Bayes method. The DEGs participating in *A. thaliana*-bacteria interactions were determined by Fisher's method. The significance of the data was determined by measuring their *p*-values. The adjusted *p*-values of 0.01 and 0.05 (FDR <0.01) (Benjamini and Hochberg, 1995) were selected for subsequent analysis. The data was edited and analyzed by Bioconductor packages (http://www.bioconductor.org) including MetaMA. Figure 1 shows the workflow of the present study.

Table 1. Transcriptomic in-put data related to plant-pathogen interaction studies of *Arabidopsis thaliana* used for the present study

A Number	Pathogen Species	S Num	C Num	Organ	Reference
E-MTAB-4151	*Pseudomonas syringae* pv. *maculicola*	12	12	Leaf	(Bernsdorff *et al.*, 2016)
E-MTAB-4416	*Pseudomonas syringae*	3	3	Leaf	(Filichkin *et al*., 2015)
E-MTAB-4450	*Pseudomonas syringae*	12	6	Leaf	(Howard *et al.*, 2013)

A Num= Accession number; S Num= Samples number; C Num= Control number;

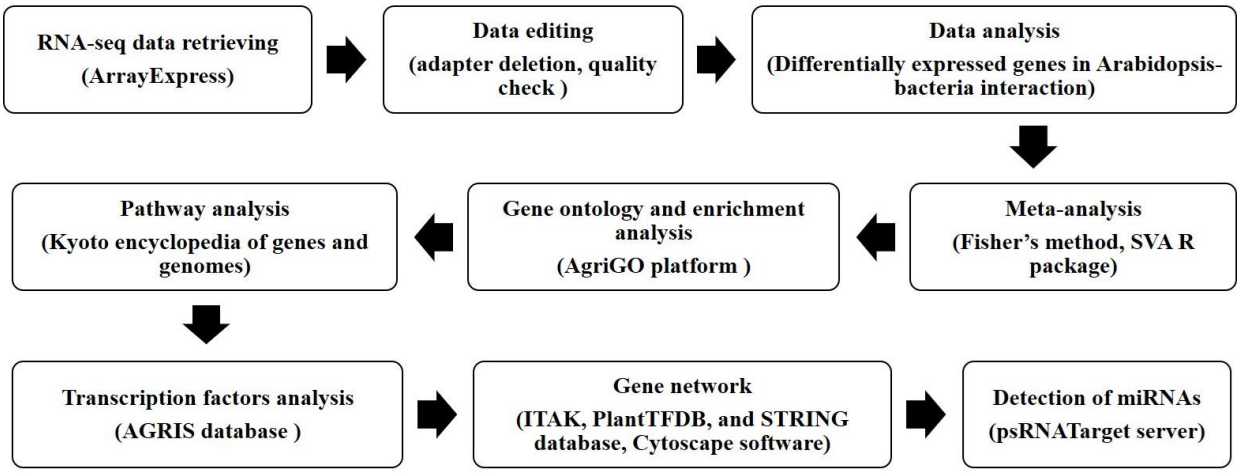


Fig. 1. The workflow of *in silico* analysis to reveal *Arabidopsis thaliana* responses to *Pseudomonas syringae* infection.

Gene enrichment and functional analysis

The selected genes were investigated through meta-analysis. Enrichment analysis of Gene Ontology (GO) was carried out using pre-determined significant DEGs. AgriGO platform was used to perform enrichment analysis (Du *et al*., 2010). The corresponding data on GO was recovered according to GO terminologies for cellular components, biological processes, and molecular functions where the significant threshold of FDR < 0.05 was found. The pathway analysis of the Kyoto Encyclopedia of Genes and Genomes (KEGG) was applied to evaluate the significantly enriched pathways of the DEGs (http://david.abcc.ncifcrf.gov/). To detect the transcription factors (TFs) of the corresponding DEGs, the pre-identified TFs of *A. thaliana* were extracted from ITAK (http://itak.feilab.net/cgi-bin/itak/online_itak.cgi) and PlantTFDB (http://planttfdb.gao-lab.org/) databases. TF identification was conducted between the determined DEGs, and a list of Arabidopsis TFs was achieved from the AGRIS

database (https://agris-knowledgebase.org/AtTFDB/; 20 August 2024)

Gene network

First, TF identification analysis was performed by applying ITAK and PlantTFDB databases. Next, the network file was obtained through the STRING database (https://string-db.org/network/) with a confidence of 0.7 (Szklarczyk *et al*., 2015), and the network was drawn by Cytoscape software (ver. 3.10.1). The up/down regulated factors as well as those with the most interaction in this network were determined for the PPI analysis. The network analysis of PPI was conducted to reveal any predicted interactions among proteins with significantly different DEGs.

Detection of miRNAs

The identification of plant microRNAs associated with pathogen infection is required to study plant-pathogen interactions. Also, it is essential to reveal how plants react to pathogen invasion. Determination of potential and small RNAs can be performed using the psRNATarget server (http://plantgrn.noble.org/psRNATarget/). To this end, the parameters were set to default except in the case of maximum expectation which was set to 2.0 in this study.

Results

DEGs determination

The RNA-seq data recovered from three corresponding studies including 27 samples of *A. thaliana-Pseudomonas syringae* interaction was used to detect pathogen-responsive genes in the host plant. Additionally, a total number of 21 samples from control (mock-inoculated) samples were used to collect their corresponding RNA-seq data (Table 1). A total number of 5034 DEGs were found to be significantly different from those in the mock-inoculated plants. Among these, 2291 and 2743 DEGs were found to be up-and down-regulated, respectively (Supplementary file). A total number of 55 genes involved in the *A. thaliana*-bacteria interaction were detected among the up-regulated DEGs. These involve WRKY DNA-binding protein 25 (*WRKY25*) (AT2G30250), WRKY family transcription factor (*WRKY22*) (AT4G01250), and WRKY DNA-binding protein 33 (*WRKY33*) (AT2G38470). Moreover, 736 and 106 genes involved in membrane and stress response were found to be up-regulated in *A. thaliana* (Supplementary file). Ubiquitin-associated (*UBA*) (AT4G24690), stress-inducible protein (*HOP3*) (AT4G12400), and heat shock factor 4 (HSF4) (AT4G36990) are of stress-responsive genes up-regulated in *A. thaliana*-infected plants. In contrast, 789 and 39 genes participating in membrane and photosynthesis were detected among down-regulated DEGs in *A. thaliana* (Supplementary file).

GO enrichment analysis

GO analysis was carried out for the determination of DEG functions during *A. thaliana*-bacteria interaction. The expression of several genes associated with cellular components, molecular functions, and biological processes was found to be altered in the *A. thaliana*-bacteria interaction (Fig. 2, Supplementary file). The expression of genes related to stress response (GO: 0006950), cellular process (GO: 0009987), metabolic process (GO: 0008152) and stimulus-response (GO: 0050896) were up-regulated in the bacteria-infected plant. In contrast, down-regulation of genes involved in the cellular process (GO: 0009987) and biosynthesis process (GO: 0009058) was observed. In the case of molecular function, kinase, catalase, and oxidoreductase activity (GO: 0016301, GO: 0003824, and GO: 0016491, respectively) genes were up-regulated within the bacteria-infected plant while down-regulation of hydrolase and transferase activity (GO: 0016787 and GO: 0016740, respectively) genes were found (Fig. 2). The cellular component genes such as cell (GO: 0005623), cytoplasm (GO: 0005737), and membrane (GO: 0016020) were up-regulated during the bacteria-infected plant, while intracellular (GO: 0005622), chloroplast (GO: 0009507), and organelle (GO: 0043226) genes were down-regulated (Fig. 2).

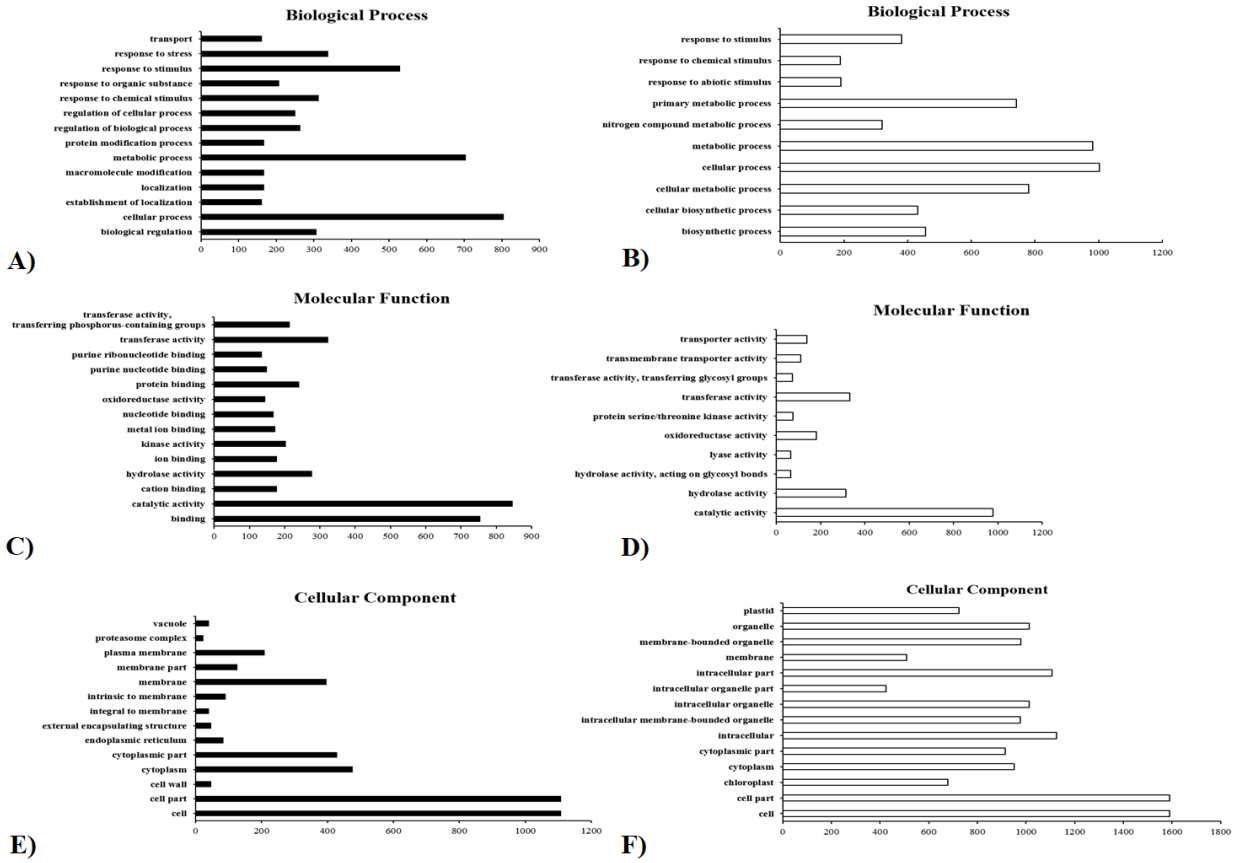


Fig. 2. Gene ontology enrichment analysis of the differentially expressed genes of *Arabidopsis thaliana* in response to *Pseudomonas syringae* infection: A and B) The enriched genes were arrayed into three groups based on gene function: biological process; C and D) Molecular function; and E and F) Cellular components; Up- and down-regulated genes were presented in black and white color bars, respectively.

KEGG pathway analysis

When *A. thaliana* plants were simultaneously infected by *P. syringae* and *P. syringae* pv. *maculicola*, a total number of 22 biological pathways were significantly enriched with up-/down-regulated DEGs in KEGG analysis. Eighteen DEGs including pentose phosphate pathway, biosynthesis of secondary metabolites, metabolic pathways, pentose and glucuronate interconversions, glycine, serine and thereonine metabolism, fatty acid metabolism, fatty acid elongation, carbon metabolism, carbon fixation in photosynthetic organisms, biosynthesis of amino acids, photosynthesis-antenna proteins, metabolic pathway, glycolysis/gluconeogenesis, glyoxylate and dicarboxylate metabolism, prophyrin and chlorophyll metabolism, valine, leucine and isoleucine biosynthesis (Fig. 3). However, five DEGs were found to be down-regulated which include proteosome, protein processing in the endoplasmic reticulum, plant-pathogen interaction, endocytosis, and biosynthesis of amino acids (Fig. 3).

Elucidation of TFs

The TF activity in *A. thaliana* plants under co-infection of *P. syringae* and *P. syringae* pv. *maculicola* was assayed. The results demonstrated that the total number of 39 specific *A. thaliana*-originated TFs were altered in *A. thaliana*-bacteria interaction (Fig. 4). Of these, TFs including ABI3VP1, Alfin-like, AP2-EREBP, BBR/BPC, bHLH, bZIP, BZR, C2C2-CO-like, C2C2-Dof, C2C2-Gata, C2H2, C3H, CPP, G2-like, GRAS, Homeobox, HSF, MYB, NAC, RAV, SBP, TCP, Trihelix and WRKY were up-regulated. In contrast, down-regulation of AP2-EREBP, ARF, ARID, ARR-B, bHLH, bZIP, BZR, C2C2-CO-like, C2C2-Dof, C2C2-Gata, C2C2-YABBY, C2H2, C3H, CAMTA, CCAAT-HAP2, CPP, EIL, G2-like, GRAS, GRF, Homeobox, HRT, MADS, MYB, Orphan,

PHD, REM, SBP, TCP, Trihelix, Whirly, WRKY and ZF-HD TFs was observed (Fig. 4).

Protein interactions of TFs

To reveal the interactions of infection-responsive genes induced in *A. thaliana*, protein-protein interaction (PPI) networks were drawn according to the identified DEGs. The PPI network showed 107 nodes and 189 edges (Fig. 5). The most important hubs were MYC2, WRKY40, WRKY33, TIFY10A, ZAT10, ZAT12, DREB2A, TIFY9, MYB15 and JAZ7. Also, CCA1, ERF094, Atmyb2, WRKY70, LHY, PIF4, NAC072, KAN3 and ERF2 genes were other important hub genes. It should be noted that all these transcription factors are among the identified modules

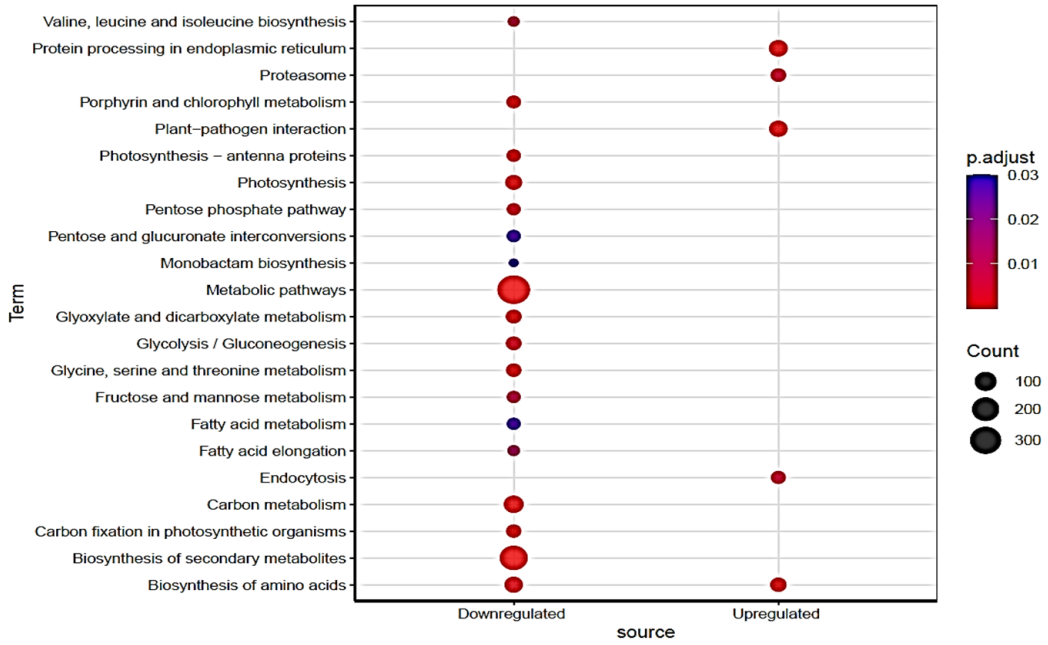


Fig. 3. The pathway mapping of the differentially expressed genes (DEGs) from the KEGG analysis in *Arabidopsis thaliana* infected by *Pseudomonas syringae* (FDR < 0.05). The vertical axis shows the name of the enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway. The color of the pathways is based on the FDR, respectively. The gene count of each DEG was presented according to the circle scale.

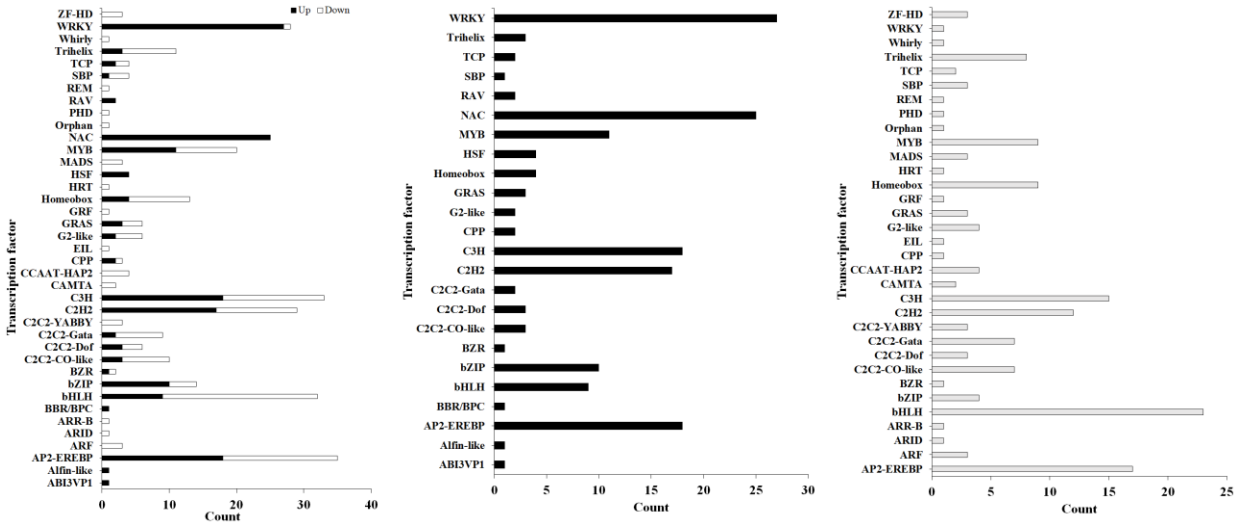
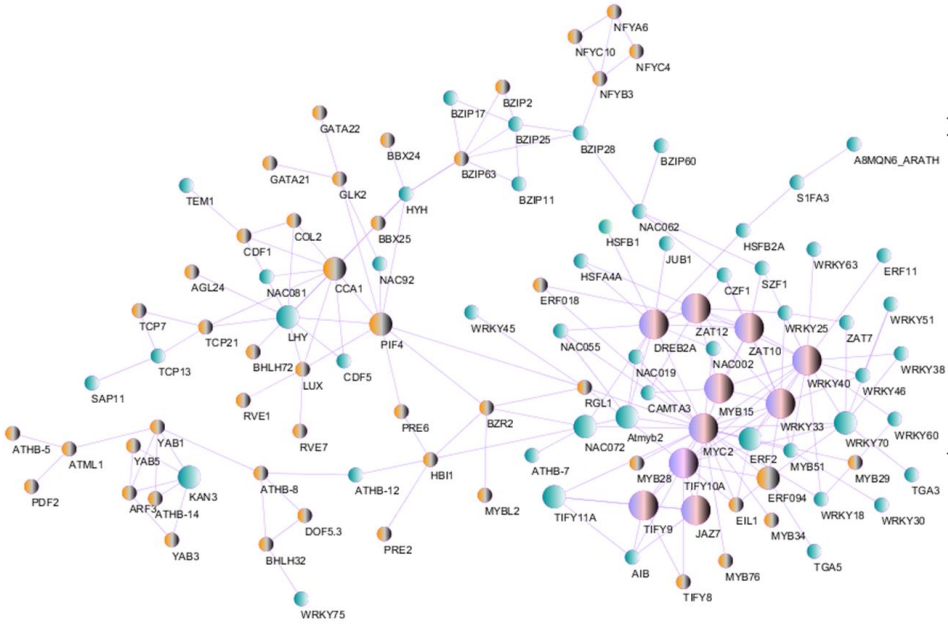


Fig. 4. Transcription factors of *Arabidopsis thaliana* altered in response to the infection of *Pseudomonas syringae* isolates, presenting the direction of change in gene activity: up-regulated (black); down-regulated (white).



Rank	Name	Gene ID	Score	up/down
1	MYC2	AT1G32640	94	up
2	WRKY40	AT1G80840	66	up
3	WRKY33	AT2G38470	64	up
4	TIFY10A	AT1G19180	46	up
5	ZAT10	AT1G27730	44	up
6	ZAT12	AT5G59820	38	up
7	DREB2A	AT5G05410	29	up
8	TIFY9	AT5G13220	26	up
8	MYB15	AT3G23250	26	up
10	JAZ7	AT2G34600	24	up
11	CCA1	AT2G46830	22	down
12	ERF094	AT1G06160	19	down
13	Atmyb2	AT2G47190	18	up
14	WRKY70	AT3G56400	17	up
14	LHY	AT1G01060	17	up
16	PIF4	AT2G43010	13	down
17	NAC072	AT4G27410	12	up
17	KAN3	AT4G17695	12	up
19	ERF2	AT5G47220	8	up

Fig. 5. Protein-protein interaction network remarking critical hub genes in *Arabidopsis thaliana -Pseudomonas syringae* interaction. The table shows critical hub genes according to their importance in the network.

miRNA assay

To identify DEGs-related potential miRNAs, the high-specific psRNATarget algorithm was used to compute the input data according to the penalty score selection (2 being highly stringent). A total number of 41 miRNA families was determined (Fig. 6).

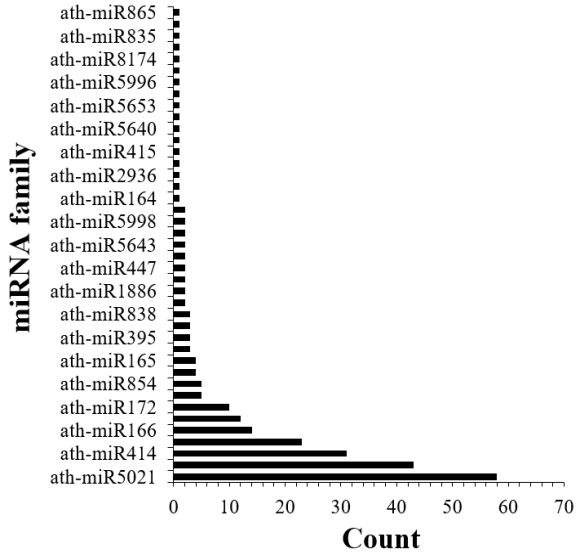


Fig. 6. The differentially expressed genes-related micro RNAs were revealed by the computational algorithm psRNATarget server. The filtering was performed based on a highly stringent penalty score.

Discussion

Transcriptional analysis of the *A. thaliana* has been demonstrated following infection with the *P. syringae* pv. *Tomato* (Lewis *et al*., 2015). The genome-wide expression changes following infection with *Pseudomonas syringae* pv *tomato* DC3000 showed the suppression of chloroplast-associated genes and the genes involved in chromatin assembly and induction of ubiquitin-related genes (Lewis *et al*., 2015). In this study, the gene expression of *A. thaliana* infected by *P. syringae* and *P. syringae* pv. *maculicola* was evaluated.

DEGs analysis results showed that a few genes involved in plant-pathogen interaction were up-regulated which include, *WRKY25*, *WRKY22*, and *WRKY33*. Recently, the WRKY transcription factor gene family (i.e., *WRKY25* and *WRKY22*) have been found to act as a defense-responsive gene in *A. thaliana* plants challenged by *P. syringae* pv. *Tomato* (Ramos *et al*., 2023). Interestingly, (Zheng *et al*., 2006) demonstrated that *A. thaliana WRKY33* is a critical factor for resistance against two pathogenic fungi (*Botrytis cinerea* and *Alternaria brassicicola*). Although the defensive response of *WRKY33*-mutant *A. thaliana* against *P. syringae* was not adversely affected, the ectopic expression of *WRKY33* led to an increased level of host susceptibility to the

pathogenic bacterium (Zheng *et al*., 2006). In the case of stress-responsive genes upregulated during *P. syringae* infection in *A. thaliana*, two genes were found to be up-regulated including *UBA, HOP3,* and *HSF4.* Similarly, it has been observed that the ubiquitin pathway mediated by UBA genes is essential for *A. thaliana* immunity against bacterial disease caused by *P. syringae* (Goritschnig *et al*., 2007). Moreover, a study conducted by Fernández-Bautista *et al*. (2017) has reported that *HOP3* is one of the regulating elements required for pathogen-responsive pathways in *A. thaliana* plants. *HSF4,* referred to as *HSF4* or *TBF1*, is another up-regulated gene of *A. thaliana* in response to bacterial attack which has been found to play a role in systemic acquired resistance (SAR) (Fernández-Bautista *et al*., 2017). Herein, the up-regulation of *JAZ7* as a hub gene was identified in *A. thaliana* during the bacterial infection. Supportively, the defensive role of this gene in *A. thaliana* has been demonstrated against different pathogens such as *P. syringae* DC3000 (Zhang *et al*., 2018), *Fusarium oxysporum* (Thatcher *et al*., 2016) and *B. cinerea* (Hanif *et al*., 2018). The gene *MYC2* was one of the up-regulated hub genes *P. syringae* infection. This gene has been reported to play a role in abscisic acid (ABA) (Abe *et al*., 2003) and jasmonic acid (JA) (Boter *et al*., 2004) signaling pathways. Also, *Atmyb2* and *NAC072* which have been shown to act in the ABA signaling pathway (Abe *et al*., 2003; Li *et al*., 2016), were up-regulated in the bacteria-infected *A. thalian.* Due to the role of ABA and JA in the plant defense system (Bari and Jones, 2009), up-regulation of *MYC2, Atmyb2,* and *NAC072* might be associated with defense response in *A. thaliana* infected by *P. syringae.* Similarly, two homologs of TIFY genes including *TIFY10A* and *TIFY9* were found to be up-regulated in *A. thaliana*-bacteria interaction. It has been demonstrated that overexpression of TIFY genes is involved in rice growth promotion by acting in the JA signaling pathway (Hakata *et al*., 2017) suggesting the defense-related role of *TIFY10A* and *TIFY9* in *A. thaliana* against *P. syringae.* Interestingly, some hub genes have been identified to be up-regulated during bacterial infection of *A. thaliana* which are abiotic stress-responsive genes including *ZAT10* (Nguyen *et al*., 2016), *ZAT12* (Davletova *et al*., 2005), *DREB2A* (Sakuma *et al*., 2006) and *ERF094* (Cheng *et al*., 2013). Moreover, a few growth- and development-responsive genes were found to be up-regulated during the bacterial infection which include *KAN3* (Hawker and Bowman, 2004), *PIF4* (Xu and Zhu, 2021), *CCA1* and *LHY* (Mizoguchi *et al*., 2002). Further experiments are required to validate the possible role of these genes in *A. thaliana* against bacterial infection. Taken together, it seems that a variety of defense-responsive genes have been up-regulated in *A. thaliana* plants challenged by the bacterial pathogen. Oxidoreductase activity was found to be an up-regulated molecular function during *P. syringae* infection in *A. thaliana.* Accordingly, it has been shown that oxidoreductase is activated during defense response in *A. thaliana* plants (Somssich *et al*., 1996).

KEGG pathway analyses showed that a variety of host responses to the bacterial challenge have been triggered. These responses including the metabolic pathways and their metabolites have been found to interact with each other shaping the host-pathogen interaction (Bednarek, 2012). It has been demonstrated that plant disease agents produce metabolites that change the metabolism of sugars, fatty acids, and proteins (Misra *et al*., 2016). These compounds can be involved in certain defense-related responses of the host during bacterial infection (Hartmann, 2008; Piasecka *et al*., 2015). The biosynthesis of antimicrobial compounds is one of the induced responses to pathogen infection (Ahuja *et al*., 2012). Similarly, several genes involved in the metabolism of secondary metabolites were detected by DEG analysis. The secondary metabolites have been considered a large group of compounds participating in plant defense against pathogens (Zaynab *et al*., 2018). Herein, a total number of 39 photosynthesis-related genes were found to be down-regulated during the bacterial infection in the *A. thaliana* plant. Previous studies have shown that photosynthesis is likely to reduce as a result of pathogen infection (Berger *et al*., 2004). This reduction might be because the host plant applies other physiological pathways to respond to the pathogenic invasion (Bobik and Burch-Smith, 2015). Additionally, it has been shown that the combination of photosynthesis-related proteins,

reactive oxygen species (ROS), and phytohormones is essential for the defense of plants. The downregulation of genes involved in photosynthesis may be associated with the inhibition of peroxidase and catalase, resulting in an accumulation of H_2O_2. The elevated levels of H_2O_2, salicylic acid, and the proteins PR1 and PR5 may initiate the hypersensitive response (Hu *et al*., 2020). Taken together, it seems that the *A. thaliana* plant significantly modulates its pathogen-associated responses through the regulation of gene expression during the infection caused by *P. syringae* and *P. syringae* pv. *maculicola.*

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Conflict of Interest

The authors declare no conflict of interest.

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