Elucidating Changes in Gene Expression in *Arabidopsis Thaliana* Resulting from Infection by *Pseudomonas syringae* pathovar *tomato DC3000*

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ABSTRACT

We report upregulation in genes related to both strengthening pathogen defenses and innate immune responses, as well as downregulation in genes related to growth development in *Arabidopsis thaliana* infected with *Pseudomonas syringae* pv *tomato DC3000*. Analyzing patterns in gene expression following infection of the model plant *A. thaliana* by *P. syringae* pv *tomato DC3000* can provide a basis for similar plant-pathogen interactions, as well as provide insight on the potential effects that a *P. syringae* outbreak would have on local agriculture. RNA-Seq data from Howard et al. (2013) was utilized for this study, alongside the *A. thaliana* TAIR10.1 reference genome and associated annotations from the NCBI database. MOCK and VIR samples at 1 hr, 6 hrs, and 12 hrs post-infection were analyzed. STAR was used to index the reference genome and align paired-reads to the reference genome in Bash. The resultant gene count data was uploaded into R Studio for count normalization using DeSeq2 and GO term enrichment using topGO. Analysis of the over-represented GO terms in *A. thaliana* following infection by *P. syringae* allowed for the elucidation of affected cellular mechanisms.

INTRODUCTION

Pseudomonas syringae is a model plant pathogen with over 50 host-specific variants that are commonly found in bodies of water and in soil (Monteil et al., 2014; Panstruga et al., 2022). This wide ecological spread of *P. syringae* poses a threat to local agriculture as a single infection has the potential to decimate crop fields (Monteil et al., 2014). *P. syringae* pathovar (pv) *tomato DC3000,* a model plant pathogen and one of the many host-specific variants of *P. syringae*, is able to infect the model plant *Arabidopsis thaliana* (Howard et al., 2013). This host-pathogen interaction is commonly studied as both organisms are fully-sequenced, and easy to maintain in a laboratory setting (Howard et al., 2013). Specifically, the changes in gene expression that arise following infection of *A. thaliana* by *P. syringae* pv *tomato DC3000* is commonly analyzed as this information may provide a basis for how the model plant adapts to pathogenic infection. Furthermore, as *P. syringae* shares many conserved-virulence genes with other plant pathogens, the results of such studies may provide insight on the model of infection employed by other plant pathogens (Xin et al., 2018). These studies may also provide insight into the cascading effects of a *P. syringae* outbreak in agricultural settings (Monteil et al., 2014; Panstruga et al., 2022).

The plant innate immune system has two major lines of defense which bolsters the immune response upon activation (Corwin et al., 2016). The primary line of defense is accomplished by transmembrane pattern recognition receptors (PRRs) which are responsible for recognizing pathogen-associated molecular patterns (PAMPs), damage-associated molecular patterns (DAMPs), and extracellular effector proteins produced by the infecting pathogen (Lolle et al., 2020). The secondary line of defense is accomplished by nod-like receptors (NLRs) which are responsible for detecting effector proteins released into cells during an infection (Lolle et al., 2020). Activation of these receptors results in a signalling cascade that induces the transcription of plant defense mechanisms (Corwin et al., 2016). As a result, resource allocation shifts towards defense-related processes and away from growth development (Lu et al., 2021). Howard et al. (2013) conducted a time course experiment on *A. thaliana* treated with *P. syringae* pv *tomato DC3000* and performed RNA-sequencing on samples collected at 1 hr, 6 hrs, and 12 hrs post-infection. Here, using the published RNA-seq results of Howard et al. (2013), we aim to elucidate which genes are affected in the downstream signalling cascade that results from the activation of PRRs and NLRs following infection by *P. syringae* pv *tomato DC3000* in *A. thaliana.* We hypothesize that genes related to the innate immune response and programmed cell death will be upregulated following pathogen-induced PRR and NLR signalling, while genes related to growth development will be downregulated. To accomplish this, the RNA-seq data published by Howard et al. (2013) was aligned to the indexed *A. thaliana* TAIR10.1 reference genome and a gene ontology (GO) term enrichment analysis was performed on the normalized gene counts.

RESULTS

Enriched GO terms from *A. thaliana* infected with *P. syringae* pv *tomato DC3000* (VIR) at 1 hr, 6 hrs, and 12 hrs, were compared to uninfected controls (MOCK) to determine if genes

related to innate immunity and programmed cell death are upregulated and if genes related to growth development are downregulated. Gene counts were obtained through STAR alignment to a reference genome. Gene counts were then normalized and analyzed for GO term enrichment.

Analysis of 1 hr VIR samples indicated an upregulation in a significant number of genes related to defense processes, such as "camalexin biosynthetic process", "defense response to fungus", "defense response to bacterium", "response to oxidative stress", "defense response to insect" (Table 1, Figure 1). Notably, camalexin is a key molecule in plant anti-fungal responses. Thus, although *P. syringae* pv *tomato DC3000* is not classified as a fungus or an insect, the response elicited by pathogenic infection may be similar to the anti-fungal response.

Downregulated genes were associated with the GO terms "chloroplast organization", "organelle localization", "polyol metabolic process", and "inositol metabolic process", which are correlated strongly with localization, as well as both biosynthetic and metabolic processes (Table 1, Figure 1). This suggests that growth-related genes were downregulated as the host focused on fighting infection. Interestingly, a portion of the GO terms were related to light response, such as "long-day photoperiodism, flowering", "photoinhibition", "response to far red light" (Table 1). As the ability for a plant to sense day and light ratios plays a crucial role in determining whether conditions are optimal for plant growth, downregulation of these genes suggests that the plant is placing less focus on growth development. Ultimately, at 1 hr post-infection, activation of the innate immune response resulted in an upregulation in genes useful in fighting infection from *P. syringae* pv *tomato DC3000* and a downregulation in genes related to growth development.

Table 1. The top 20 upregulated and downregulated GO terms at 1 hr post-infection of *A. thaliana* with *P. syringae* pv *tomato DC3000*.

Upregulated GO Terms	Downregulated GO Terms
circadian rhythm	chloroplast organization
camalexin biosynthetic process	long-day photoperiodism, flowering
indole metabolic process	polyol biosynthetic process
toxin catabolic process	organelle localization
starch catabolic process	inositol metabolic process
response to chitin	photoinhibition
defense response to bacterium	carbon utilization
response to carbohydrate	glycine catabolic process
defense response to fungus	response to temperature stimulus
response to oxidative stress	carbon fixation
response to bacterium	response to salicylic acid
copper ion homeostasis	inorganic anion transport
long-day photoperiodism, flowering	response to jasmonic acid
aging	response to cadmium ion
indole glucosinolate metabolic process	response to hydrogen peroxide
regulation of cicadian rhythm	response to gibberellin
glutamine family amino acid catabolic process	response to ethylene
cellular modified amino acid catabolic process	response to far red light
defense response to insect	photoperiodism
glutamate metabolic process	response to heat

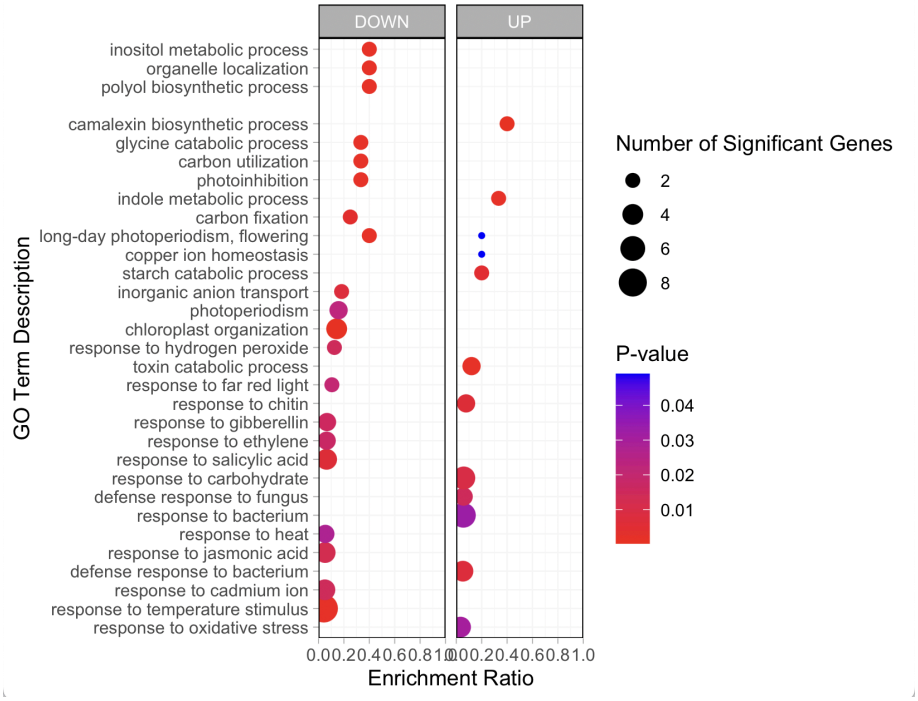

Figure 1. Graphical summary of biologically significant upregulated and downregulated GO terms at 1 hr post-infection of *A. thaliana* with *P. syringae* pv *tomato DC3000*. Larger circles

represent a greater number of significant genes for the given GO term. P-values are represented from a gradient of red (low) to blue (high).

Analysis of 6 hr VIR samples indicated an upregulation in the immune and defense pathways, as well as a downregulation in photosynthetic processes (Table 2, Figure 2). Upregulated genes were associated with plant stress and defense responses as shown through the GO terms: “ response to wounding”, “response to jasmonic acid”, “defense response to bacterium”, “response to fungus”, “carboxylic acid biosynthesis process”, “response to water deprivation”, and “response to external stimulus” (Figure 2). Notably, upregulated genes were associated with the “response to abscisic acid” which is expected as abscisic acid can enhance the defense response in early stage pathogenic infections by triggering stomatal closure on plant leaves (Alazem & Lin, 2017). This is a common defense mechanism triggered by PRR signalling in response to pathogen recognition as it is meant to limit the spread of pathogens which are able to enter and disseminate through the stomatal pores of a plant (Alazem & Lin, 2017). Upregulated genes were also associated with the GO terms “cytokinin-activated signaling pathway” and “oxylipin biosynthetic process”, which indicated further activation of immune pathways as compared to the 1 hr results (Table 2). Interestingly, many genes related to “response to water deprivation” were upregulated. Thus, it is possible that host responses to abiotic stressors and pathogens can result in similar changes in gene expression. It is also a possibility that the pathogenic response may dysregulate other pathways involved in plant homeostasis which indirectly contribute to plant water deprivation.

Downregulated genes were associated with GO terms related to light reactions, such as “photosynthesis”, “response to far red light”, “response to red light”, “photosynthetic electron transport in photosynthesis”, “photosynthesis, light harvesting”, “photoinhibition”,

“photosystem II assembly”, “photosynthesis, light reaction”, “red or far-red light signalling pathway” (Table 2). These results suggest that genes related to photosynthesis and perception of red light ratios are downregulated. Overall, at 6 hrs post-infection, efforts to photosynthesize appear to be decreased in order to prioritize defense and immune responses.

Table 2. The top 20 upregulated and downregulated GO terms at 6 hrs post-infection of *A. thaliana* with *P. syringae* pv *tomato DC3000*.

Upregulated GO Terms	Downregulated GO Terms
response to wounding	photosynthesis
cytokinin-activated signaling pathway	response to far red light
plant-type cell wall loosening	response to red light
salicylic acid metabolic process	response to blue light
gibberellic acid mediated signaling pathway	photosynthetic electron transport in photosynthesis
response to abscisic acid	photosynthesis, light harvesting
response to jasmonic acid	response to cold
response to gibberellin	circadian rhythm
response to external stimulus	photoinhibition
anatomical structure formation	photosystem II assembly
positive regulation of signal transduction	starch catabolic process
defense response to bacterium	de-etiolation
regulation of stomatal movement	photosynthesis, light reaction
jasmonic acid biosynthetic process	carbon utilization
oxylipin biosynthetic process	response to temperature stimulus
carboxylic acid biosynthetic process	chlorophyll biosynthetic process
response to fungus	cellular macromolecule catabolic process
response to water deprivation	anthocyanin-containing compound biosynthesis
chorismate metabolic process	starch biosynthetic process
response to red light	red or far-red light signaling pathway

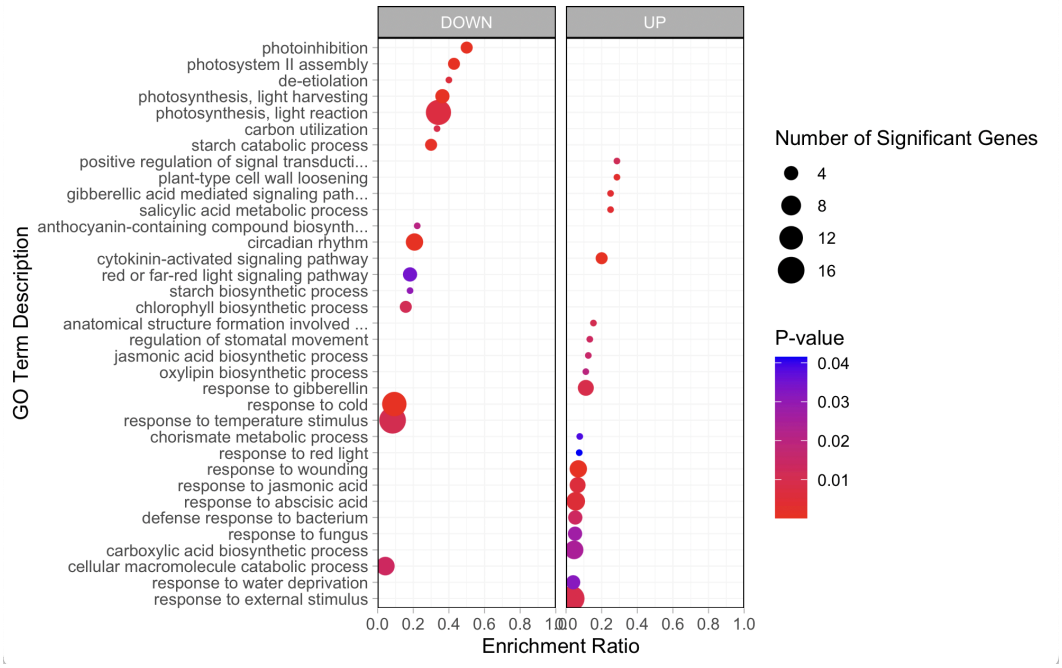

Figure 2. Graphical summary of biologically significant upregulated and downregulated GO terms at 6 hrs post-infection of *A. thaliana* with *P. syringae* pv *tomato DC3000*. Larger circles represent a greater number of significant genes for the given GO term. P-values are represented from a gradient of red (low) to blue (high).

Analysis of 12 hr VIR samples indicated an upregulation in immune and defense responses, as well as a downregulation in metabolic and catabolic processes (Table 3, Figure 3). At 12 hrs post-infection, compared to 1 hr and 6 hrs post-infection, genes involved in immune processes are more significantly upregulated, such as those relating to “oxylipin process”, “ jasmonic acid signaling pathway”, and “regulation of immune response” (Figure 3). Additionally, a significant number of genes related to stress response continue to be upregulated, as indicated by the GO terms “response to other organism”, “response to osmotic stress”, “response to salt stress”, “and response to fungus” (Figure 3). Interestingly, the GO term “multicellular organism development” was associated with upregulated genes at 12 hrs post-infection which deviates from what is expected since this GO term is related to development (Table 3). However, this GO term is described as the development from an

initial condition to a later condition, which suggests that the host is attempting to grow new leaves as a different strategy to overcoming infection (Hill et al., 2010). Another interesting GO term associated with upregulated genes is “negative regulation of abscisic acid” (Table 3). Previously, we saw at 6 hours that upregulated genes were associated with the “response to abscisic acid” (Table 2). As such, this comparison between the 6 hrs and 12 hrs post-infection timepoints indicate that the plant has shifted away from abscisic acid-related immune responses. This is expected since abscisic acid can impede the plant’s defenses by antagonizing other defense-related hormone pathways, such as salicylic acid synthesis, once the bacterial infection is established in plant tissues (Alazem & Lin, 2017). Thus, the upregulation of genes related to the “negative regulation of abscisic acid” may indicate that the *P. syringae* infection is established in the plant at 12 hrs post-infection and abscisic acid is no longer aiding in plant defense.

A downregulation in GO terms related to metabolic and catabolic processes was observed, as shown through the GO terms “pentose-phosphate shunt”, “pentose metabolic process”, “glucose catabolic process”, “alcohol catabolic process”, “malate metabolic process”, “cellular carbohydrate catabolic process” (Table 3). These metabolic and catabolic pathways are generally related to energy production where a high energy output is associated with plant development (Hashida et al., 2009). Notably, genes associated with the “Nicotinamide metabolic process” are also downregulated which further impacts plant energy production as nicotinamide is a component of NAD, an important coenzyme used in ATP production during processes such as oxidative phosphorylation (Hashida et al., 2009). As such, a downregulation in these genes indicates that the host may be decreasing energy generation, likely to focus on fighting infection. Alongside a downregulation in metabolic processes, there is evidence of impacts on plant cell growth which is exhibited by the downregulation of

genes related to the “regulation of meristem growth”, “root hair elongation”, “cell morphogenesis”, and “cell wall macromolecule catabolic process”. A downregulation in cell morphogenesis genes may affect cell proliferation and differentiation, thereby impairing plant growth (De Lorenzo et al., 2019). Plant growth may also be impacted through the observed downregulation of genes related to cell wall macromolecule catabolic processes by preventing dynamic cell wall activity (De Lorenzo et al., 2019). During cell division, the cell wall is remodelled and undergoes multiple changes as it must be expanded and broken down to accommodate changes in cell shape and size (De Lorenzo et al., 2019). Therefore, in order for cell division and proliferation to occur, cell wall catabolic processes must be active. Thus, the observed downregulation of genes related to the catabolism of the cell wall may prevent cell division and consequently result in a negative impact on plant growth and regeneration. Surprisingly, genes associated with the “activation of innate immune response” were downregulated (Table 3). This indicates that the expression of genes relating to immune response activation is no longer necessary, thereby suggesting that the immune response had already been initiated by 12 hrs post-infection. Generally, at 12 hrs post-infection, genes related to immune processes are strongly upregulated while those related to energy production are downregulated.

Table 3. The top 20 upregulated and downregulated GO terms at 12 hrs post-infection of *A. thaliana* with *P. syringae* pv *tomato DC3000*.

Upregulated GO Terms	Downregulated GO Terms
response to wounding	pentose-phosphate shunt
response to external stimulus	pentose metabolic process
response to abscisic acid	nicotinamide metabolic process
jasmonic acid biosynthetic process	glucose catabolic process
response to jasmonic acid	alcohol catabolic process
response to water deprivation	regulation of meristem growth
oxylipin biosynthetic process	malate metabolic process
response to desiccation	cell wall macromolecule catabolic process
negative regulation of abscisic acid	base-excision repair
response to fungus	cellular carbohydrate catabolic process
jasmonic acid mediated signaling pathway	carbon fixation
response to osmotic stress	activation of innate immune response
fatty acid biosynthetic process	cellular component morphogenesis
oxylipin metabolic process	root hair elongation
regulation of immune response	cell morphogenesis
multicellular organism development	response to glucose
response to other organism	recognition of pollen
response to chitin	gravitropism
response to salt stress	transition metal ion transport
regulation of DNA-templated transcription	pollen-pistil interaction

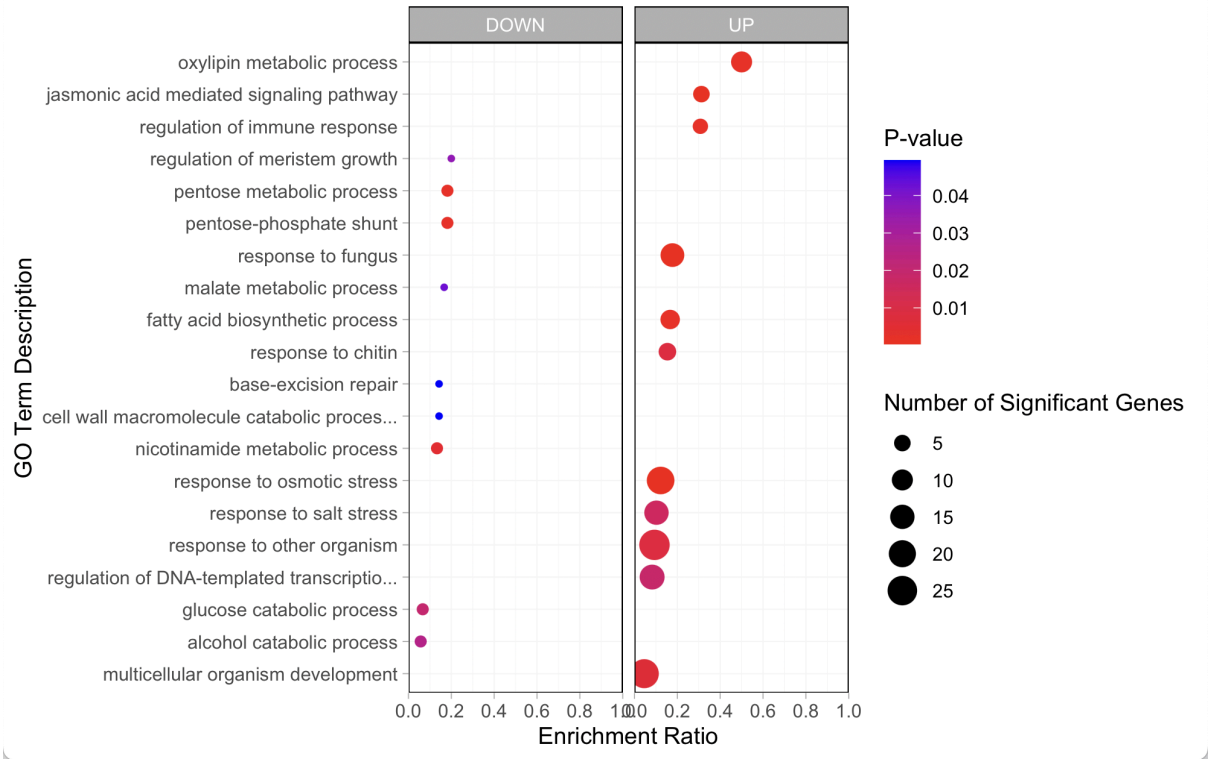

Figure 3. Graphical summary of biologically significant upregulated and downregulated GO

terms at 12 hrs post-infection of *A. thaliana* with *P. syringae* pv *tomato DC3000*. Larger circles represent a greater number of significant genes for the given GO term. P-values are represented from a gradient of red (low) to blue (high).

DISCUSSION

This study aimed to test if genes related to both the innate immune response and programmed cell death were upregulated and if genes related to growth development were downregulated at 1 hr, 6 hrs, and 12 hrs post-infection of *A. thaliana* with *P. syringae* pv *tomato DC3000.* Generally, at all time points, genes related to defense and immune response were upregulated, whereas various processes related to growth and metabolism were downregulated (Table 1, Table 2, Table 3). However, no GO terms associated with programmed cell death were found. As programmed cell death to fight infection tends to occur at later stages of infection, the absence of GO terms associated with programmed cell death may be attributed to the lack of samples analyzed at later points along the infection time course (Pitsili, 2020). Since immune response genes were most greatly upregulated at 6 hrs and 12 hrs, this suggests that changes in gene expression occur to recruit the immune response between 1 hr and 6 hrs of infection. Biologically, this upregulation likely aligns with the time at which the damage caused by *P. syringae* pv *tomato DC3000* is sufficient to trigger an immune response in *A. thaliana*. The observed delay in the activation of the innate immune response may be due to the additional physical barriers employed by plants which include waxy coatings, rigid cell walls, and anti-microbial enzymes (Nürnberger, 2004). These additional defense mechanisms likely result in a higher threshold of infection that is required to recruit the innate immune response as compared to mammals (Nürnberger, 2004).

The findings of this study indicate that downregulated processes aligned closely to both metabolic and catabolic processes, as well as light reaction processes. Similarly to the effect of other pathogen species on differential expression, infection by *P. syringae* pv *tomato DC3000* resulted in the downregulation of genes related to growth and development (Lu et al., 2021). Additionally, downregulation of photosynthetic processes has previously been implicated as early as 3 hours after infection with *P. syringae* pv *tomato DC3000* (Bonfig, 2006). Thus, correlating closely with our results which indicated a downregulation of photosynthesis at 6 hours of infection. Biologically, this suggests that energy generation for growth is decreased in favour of processes to fight infection.

While these results are generally congruent with the original hypothesis, the quality of the data obtained from Howard et al. (2013) limits the conclusions that may be drawn from the study. PCA plots of each timepoint do not show distinct clustering of the MOCK and VIR groups which suggests that batch effects may be present within the samples (Figure S1). This limits the validity and generalisability of the results obtained due to the effects conferred by changes in sample handling methods. Thus, a repeated data set to confirm these findings would serve to provide greater validity to the findings presented. Additionally, since programmed cell death does not appear to be recruited until later stages of infection, future studies should aim to monitor infection until senescence or close to senescence to elucidate at which point genes related to programmed cell death may be differentially expressed. Agriculturally, this would inform researchers about the time course of pathogenic infection, with an emphasis on the late stages of pathogenic infection in crops. Furthermore, these studies would allow for a greater understanding of the point at which infection is more difficult to eradicate and is affecting crop yield.

CONCLUSION

The processes affected by changes in gene regulation from infection by *P. syringae* on *A. thaliana* are important to analyze because these results may provide insight on other pathogenic models of infection. The findings of the present study suggest that genes related to both the innate immune response and defense response are upregulated while genes related to growth development are downregulated in *A. thaliana* infected with *P. syringae* pv *tomato DC3000*. However, the samples used in this analysis may be subject to batch effects as indicated by the quality check performed on the dataset. Thus, it is recommended that further studies are performed on additional datasets to confirm the findings of this analysis.

As *P. syringae* shares many virulence genes with other plant-pathogens, these findings may suggest that other similar pathogenic models of infection will also result in an upregulation in genes related to both the innate immune response and pathogen defenses, as well as a downregulation in growth development related genes (Xin et al., 2013). Furthermore, these results indicate that, in the event of a *P. syringae* outbreak in agricultural settings, it is likely that crop yields will decrease due to the reduced expression of genes related to growth development.

METHODS

Sourcing the RNA-Sequencing Data, Reference Genome and Associated Annotations

Raw RNA-sequencing read data was obtained from data published by Howard et al. (2013) from the NCBI Sequence Read Archive (SRA, accession SRP010938). Analyses were performed using the control (MOCK) and virulent *Pseudomonas*-infected experimental (VIR) samples for 1 hr, 6 hrs, and 12 hrs post-infection. No analyses were performed on the

non-virulent *Pseudomonas*-infected experimental (AVIR) samples. The *Arabidopsis thaliana* TAIR10.1 reference genome and reference genome annotations were downloaded from the NCBI Database. The Bourne Again Shell (Bash) command line interpreter was used to index the reference genome and align reads to the reference genome.

Indexing the Reference Genome

The fast RNA-sequence read mapper, Spliced Transcript Alignment to a Reference (STAR), was used to index the *A. thaliana* reference genome with a thread count of 20 and an overhang of 100 bases.

Aligning to the Reference Genome

Read pairs were identified for both MOCK and VIR samples at each timepoint. STAR was then used to align the paired RNA-seq read data to the reference genome with a thread count of 16. Gene counts were generated using the *-quantMode GeneCounts* option in STAR.

Loading Packages in RStudio

The BiocManager, tidyverse, pheatmap, RColorBrewer, and dplyr packages were installed from CRAN. The DESeq2 and topGO packages were installed from Bioconductor. Following installation, all packages were loaded.

Count Normalization using DESeq2 & Quality Assessment

R Studio was used to compile gene expression counts data files into a dataframe, then transformed into a matrix. A metadata file whose rownames match the name and order of the data frame matrix was then created. The *DESeqDataSetFromMatrix* function was then used

to transform the data into the shape of a *SummarizedExperiment*. Each VIR gene count file for a given time point was assigned to its corresponding reference MOCK gene count file using the *relevel* function. DESeq2 was then run on the dataset. Log transformations were performed on the resultant 1hr, 6hr, and 12hr datasets and both a PCA plot and distance matrix were generated for each.

Data Manipulation for Gene Ontology Term Enrichment

The resultant 1hr, 6hr, and 12hr processed gene expression counts data was filtered to remove any rows with missing data. Further filtering was performed to isolate differentially expressed genes by removing any results with an adjusted *p*-value > 0.05.

Gene Ontology Term Enrichment using TopGO

A list of enriched gene ontology (GO) terms in the gene lists from the RNA-seq data was obtained from data published by Howard et al. (2013). This list was reverse-mapped such that each GO term was associated with multiple gene IDs which was ill-suited for the purposes of our project. Thus, the file was manipulated such that each gene ID was associated with multiple GO terms.

The resultant file was loaded into R Studio and a character vector was created that contained the names of all the gene IDs in the mapping file. Biologically relevant upregulated genes were isolated by filtering the 1hr, 6hr, and 12hr processed gene expression counts data for a log2 Fold Change >= 1. Biologically relevant downregulated genes were isolated by filtering the 1hr, 6hr, and 12hr processed gene expression counts data for a log2 Fold Change <= -1. Gene IDs were then extracted from the upregulated and downregulated datasets to create a

list of the upregulated and downregulated genes for each timepoint. Using TopGO, GO term enrichment was then performed on the list of upregulated and downregulated genes for each timepoint. Fisher's exact test with the weight01 algorithm was used to confirm the enrichment of a given gene. A summary of the top 20 GO terms for the upregulated and downregulated genes for each timepoint was then obtained.

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SUPPLEMENTARY DATA

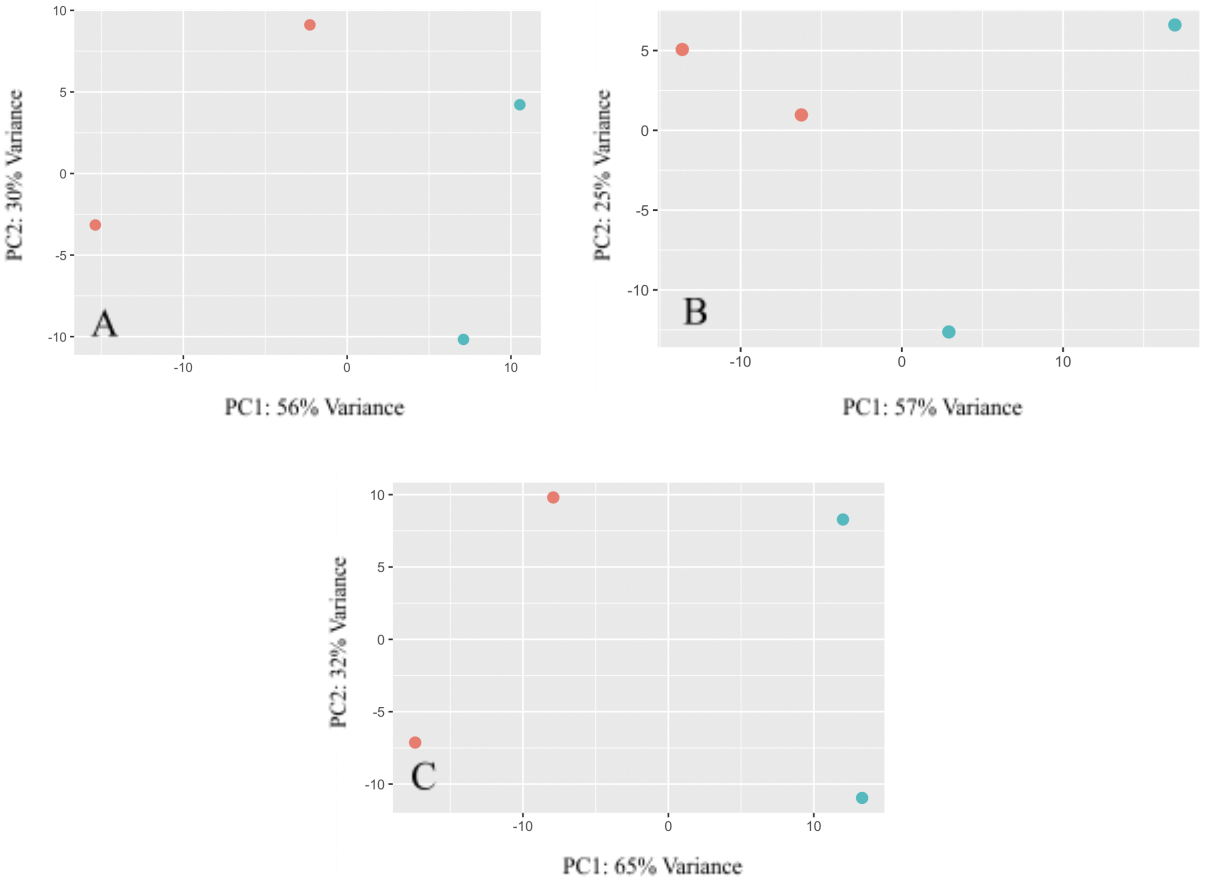

Figure S1. PCA plots of control (orange) and infection (blue) at 1 hr (A), 6 hr (B), and 12 hr (C) of A. thaliana with *P. syringae* pv *tomato DC3000.*

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