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# Studying Gene Expression Changes in Infected Peach Leaves to Phytoplasma Using Transcriptome Data

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**Abstract**

**Introduction:** Phytoplasma disease significantly affects stone fruit trees worldwide, leading to symptoms such as witches' broom, decline, and eventual plant death. To explore its impact on peaches, we analyzed the transcriptomic profiles of diseased and healthy cherry plants. Several genes with differential expression were identified, most of which were involved in amino acid metabolism and secondary metabolite biosynthesis pathways, showing altered expression in phytoplasma-infected trees.

**Materials and Methods:** Total RNA was extracted from both infected and healthy peach leaves, and cDNA synthesis was performed for qRT-PCR analysis. Transcriptome sequencing data from infected cherry plants were collected and analyzed. Using Cytoscape, ten hub genes in the protein-protein interaction (PPI) network were identified, with five genes showing higher degrees of interaction. These hub genes were validated in peach plants using quantitative PCR (qRT-PCR).

**Results:** The study revealed increased expression of DNA-directed 5'-3' RNA polymerase activity in differentially expressed genes (DEGs) and hub genes, which were associated with disease occurrence, but no significant expression changes were observed in other selected genes in infected leaves. This study showed that the high expression of RNA polymerase genes in phytoplasma-infected cherry and peach plants depends on amino acid biosynthesis and secondary metabolite pathways.

**Conclusions:** This research provides a deeper understanding of the genetics and molecular processes in plants, which could enable researchers to improve traits in stone fruit trees in response to phytoplasma disease using advanced genome editing techniques. These findings could lead to the development of new, more resistant, and higher-yielding cultivars. The gene identified in this study serves as a promising biomarker for early detection of phytoplasma infection in stone fruits and offers insights for disease control strategies.

**Keywords:** Phytoplasma Infection, *Prunus persica*, Transcriptome Analysis, RT-qPCR

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## Introduction

Phytoplasmas are obligate pathogens that cause intensive yield losses in economically important crops, including stone fruit trees.[1] These pathogens are obligate vector-transmitted bacteria that colonize the phloem vessels of infected trees.[2] During the growing season, plants may experience premature flowering, followed by the early development of buds.[3] Phytoplasmas are among the most important limiting factors of several crop productions in Iran.[4] Stone fruit phytoplasma infection in Iran was detected in the 1990s with almond brooming disease in southern provinces.[5] Stone fruit phytoplasma damage in Iran varies by product type.[1] The exact amount of damage is not known because the diseased trees are removed from the production cycle.[6] In infected trees, the Phytoplasma pathogen affects various physiological, biochemical, and molecular processes in plants such as photosynthesis, hormone balance, and expression patterns.[7] Peach trees (*Prunus persica*) are vulnerable to this disease.[2,7] *Prunus persica* and *Prunus avium* naturally grow in Iran and are often exposed to phytoplasma diseases in their habitat.[8]

Phytoplasma diseases in stone fruit trees can spread rapidly within a given area and cause significant economic losses for farmers.[9] Since there are no effective treatments for phytoplasma diseases in stone fruit trees, controlling and managing these diseases is challenging.[10] These diseases are difficult to detect. Early due to their latent nature and ability to spread in the plant's phloem tissue. Additionally, because phytoplasmas are mainly spread by insect vectors, controlling these diseases requires targeted pesticides and pest management, which come with their challenges.[11]

Phytoplasma infections, in addition to causing visible damage, also result in significant molecular changes in the

host plants.[12] These changes involve modifications in hormonal regulation, gene expression patterns, and disruptions in photosynthesis, which ultimately lead to decreased resistance to other stress factors..[13] Research indicates that phytoplasma infections in stone fruit trees can lead to genetic changes in the plant's response to the disease, negatively impacting its resistance.[8] By identifying differentially expressed genes, this research clarifies the molecular mechanisms underlying the plant's response to phytoplasma infection.

High-throughput sequencing (RNA-Seq) is an essential component of gene expression research. Integrating and analyzing gene expression datasets can lead to meaningful insights and desired outcomes. In recent years, RNA-seq has become a powerful tool for exploring expression patterns of phytoplasma-host plant interactions.[14,15] Many studies have been conducted on the transcriptome analysis of trees infected with phytoplasma. For example, the transcriptomic analysis of *Paulownia* infected with phytoplasma causing "witches' broom" using RNA-Seq technique revealed that several genes exhibited significantly differential expression, confirming their role in biosynthetic pathways.[16] High-throughput RNA sequencing (RNA-Seq) was conducted to identify gene expression signatures linked to phytoplasma infection in Mexican lime trees. Ultimately, genes with differential expression in infected and healthy trees were compared, and their roles in metabolic and regulatory pathways were identified.[17] In jujube trees, the host's response to phytoplasma was studied at phenotypic, physiological, biochemical, and molecular levels using the RNA-Seq technique. Different genes were identified during the first and second stages of infection, which were involved in the biosynthesis of phytohormones, signal transduction, photosynthesis, and secondary metabolism.[18] In another study, DNA methylation patterns in response to phytoplasma infection in healthy and infected mulberry leaves were examined, and the significance of the identified genes in the mulberry's defense response to the disease was determined.[19] The transcriptome analysis in sweet cherry trees was performed to compare phytoplasma-infected and healthy trees. Ultimately, the phytoplasma infection led to the upregulation of a set of genes involved in metabolic and biosynthetic pathways, resulting in a change in the expression profile of the genes associated with them in the leaves of the phytoplasma-infected trees.[14] The transcriptome of healthy trees, compared to phytoplasma-infected trees in cranberry, identified several differentially expressed genes. These genes are crucial for the plant's metabolism and defense mechanisms.[20] One study involved comparing gene expression in symptomatic diseased leaves versus asymptomatic healthy leaves using RNA-Seq to understand how sweet cherry trees respond to phytoplasma infection and disease induction.[14] This study used RNA-Seq technology to thoroughly analyze published datasets of *P. avium*, a model stone fruit tree that is susceptible to phytoplasma disease. We analyzed transcriptomic data from infected and healthy cherry trees and validated differentially expressed genes (DEGs) in infected and healthy peach trees using RT-qPCR. The study aims to investigate changes in gene expression patterns in peaches affected by phytoplasma disease. This study aims to investigate changes in gene expression patterns in peach trees (*Prunus persica*) infected with phytoplasma disease. By identifying differentially expressed genes, this research can help clarify the molecular mechanisms behind the plant's response to phytoplasma infection. Ultimately, by using advanced data analysis, new molecular techniques, and applied studies in enhancing plant resistance to phytoplasma, this research aims to identify key defense genes and contribute to the development of innovative methods for disease management based on the identified genes.

## Materials and Methods

### *RNA-Seq Data Collection and Analysis*

RNA-seq datasets for phytoplasma disease in *Prunus avium* were obtained from NCBI SRA (https://www.ncbi.nlm.nih.gov/sra/SRX3323284). The data sets were related to healthy and infected leaves located at the top of 1-year-old shoots that displayed witch's broom symptoms. After downloading and decompressing the data sets, the integrity of the data was checked, and performed quality control with FastQC (version 2.11.5). The clean reads were mapped to the Prunus avium reference genome (https://www.ncbi.nlm.nih.gov/datasets/taxonomy/42229/) using HISAT v2-2.1.0. Assembly and quantification software, Cufflinks (version 2.2.1), and Samtools (version 1.9) were used for the file format conversion required during the alignment and quantification steps.

### *Differential Gene Expression Analysis*

Differential gene expression and transcript abundance were calculated using the Cufflinks program (1.3.0). The resulting lists of differentially expressed genes were filtered based on fold change $\geq 2$, fold change $\leq -2$, and q-value = 0.05. The selected differentially expressed genes were then clustered to identify common gene expression patterns. Additionally, analyze gene expression, raw expression data were obtained in the form of FPKM values. A threshold of FPKM > 5000 was applied to filter out genes with low expression levels, ensuring that only genes with significantly higher expression levels were included in further analyses. This cutoff was chosen to reduce noise and focus on genes with more reliable and biologically relevant expression levels. GO analyses were performed using AgriGO with a significance level cut-off of 0.05 (http://bioinformatics.sdstate.edu/go74/).[21]

### *Protein-Protein Interaction (PPI) Network Analyses*

To gain insights into the correlation among differentially

expressed genes (DEGs) at the protein level, a network of interactions between proteins, known as a PPI network, has been created. The network was constructed using data from The STRING database. The PPI network was visualized using Cytoscape, and the plug-in Cytohubba to identify the hub genes within the network. Key nodes in the PPI network were determined based on network centrality scores, and the hub genes were deduced accordingly. The PPI network's hub genes were analyzed topologically using Network Analyzer. Ten topological analysis methods were obtained through the cytoHubba plugin. Three centrality methods, degree, and closeness were employed to enhance sensitivity.[22]

### *Plant Material and Sampling*

Infected peach samples (Figure 1) were collected from naturally infected Peaches (crest cultivar) in Neka City, Mazandaran province, Iran. The leaves showing phytoplasma disease symptoms were used to detect phytoplasma by PCR amplification. Three leaf samples were carefully gathered from both healthy and phytoplasma-infected trees, setting the stage for essential subsequent experiments. The leaf samples were immediately frozen in liquid nitrogen and kept at −80 °C.

### *Investigating the Presence of Phytoplasma in Peaches*

In the DNA extraction procedure detailed by the Qiagen kit, phytoplasmas DNA was amplified using either single or nested PCR reactions. Four pairs of primers were used to obtain products as follows: PA2F/R are the primers for PCR, amplifying a product of 1187 bp, and nested NPA2F/R amplifying a product of 485 bp. PCRs with primer pairs PA2F/R and NPA2F/R were performed as previously described by Heinrich.[23] For PCR amplification with primer pairs PA2F/R and NPA2F/R, 100 ng of nucleic acid and 1.25 U Taq DNA polymerase were mixed with dNTPs (10 mM each), 10 pmol of each primer, 10x PCR buffer (Promega, Madison, WI, USA), $MgCl_2$ (25 mM, Promega), and 12 µl of sterile water. The parameters used for the 35-cycle PCR were denaturation at 94 °C for 30 s, annealing for 75 s at 60 °C, and primer extension for 90 s at 72 °C. For nested PCR amplification, the amplicons of the first PCR were diluted in a 1:40 ratio with distilled water. An automated thermocycler (Biorad DNA Thermal Cycler) was used for PCR amplification. The PCR was carried out three times for all samples to authenticate the amplification results. The DNA bands were then visualized by staining with ethidium bromide and a UV transilluminator. Subsequently, the inserts were sequenced bidirectionally by Microscence in Switzerland. The obtained sequences were assembled using Geneious (GENEIOUS, New Zealand), and were then utilized to search against the database using the NCBI by BLASTn (http://www.ncbi.nlm.nih.gov/).

**Figure 1.** Phytoplasma-infected Peach Trees with Symptoms of Infection Starting from one Branch, Leaf Rolling, Leaf Color Change, and Decay in Neka City, Mazandaran province of Iran.

### *Total RNA Extraction*

Total RNAs were extracted from fresh leaves of two phytoplasma-infected trees and two healthy peach trees (*P. persica*) respectively, during the vegetative stage, using a Macherey-Nagel Nucleospin RNA plant extraction kit with triazole. The RNA concentrations were first examined on a 1% agarose gel, and the concentrations were determined using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) with absorbance ratios of A260/280 and A260/230 nm.

### *cDNA Preparation*

Five µg of total RNA were used for cDNA library preparation. The cDNA was reverse-transcribed using the Denazist Asia cDNA synthesis kit (Denazist, Tehran).

### *Gene Expression*

Three plants from each treatment (uninfected and infected) were selected for RT-qPCR. Five hub genes related to differentially expressed genes in phytoplasma-infected trees compared to healthy trees (with q < 0.05 and high FPKM values) were selected for RNA-Seq data validation. For each of the five genes, gene-specific qRT-PCR primers were designed using Primer3Plus,[24] and a constitutively expressed actin gene was used as an internal control. The genes and the primers for the selected genes are listed in Table 1. The relative expression of each gene of interest was calculated with REST using the formula $2^{-\Delta\Delta CT}$ and expressed relative to Actin as the internal control.

**Table 1.** The Primer Sequences and the Housekeeping Gene (Actin) Sequences were Used in This Study

| Name | Primer sequences | Amplicon size |
|---|---|---|
| Actin | * AGCAGAGCGATTCCGTTGTCC<br>** CCTCCACTCAGCACTATGTTACCAT | 153 |
| PRUPE_1G143200 | * (F) ACCACCAGCTAAACGAGGCC<br>** (R) GGCTCGACACCACTCCTCTC | 134 |
| PRUPE_6G104700 | * (F) GTGATGTGGCCGTCTGGAAG<br>** (R) GCGCTAGACGCATTCTGACA | 178 |
| PRUPE_7G175200 | * (F) TGGATGCAGAGCGGTTGGAT<br>** (R) GGGCAAAGGGTCTGAATCGG | 113 |
| PRUPE_4G135700 | * (F) GAGGCAGTTCGTCGGTCACT<br>** (R) AGCACCTCATCATGGGACCG | 178 |
| PRUPE_7G146600 | * (F) ATAGACCGGACTTTGCCCCA<br>** (R) CTTCCCTCCCACCCATAGCA | 130 |

* Forward primer. ** Reverse primer

### *Validation of RNA-Seq Analysis by RT-qPCR*

RT-qPCR reactions were performed using the Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's directions. The reactions were run on the ABI 7500 Fast Real-Time Detection System (Applied Biosystems) with the following conditions: 95 °C for 10 min; and 35 cycles at 95 °C for 15 s and 60 °C for 1 min. There were three technical and two biological replicates for each sample.

### *Statistical Analysis*

The data with the normal distribution involved a one-way analysis of variance (ANOVA), followed by post-hoc Tukey's test. A significance level was set at $p<0.05$, conducted using SPSS software.

## Results

### *Analysis of Differentially Expressed Genes*

To identify key genes that are differentially expressed in phytoplasma-infected peach trees, approximately 108,792,204 single-end reads of 150 nucleotides in length were obtained using the Illumina HiSeq 2000 platform. After adapter removal and filtering of low-quality reads, 47,541,579 and 61,249,051 clean reads were obtained from the infected and healthy sample libraries, respectively.

The gene expression patterns of the plant libraries from both the diseased and healthy states were analyzed using RNA-Seq, resulting in expression data for approximately 26,811 genes in each RNA library. For each RNA library, a list of differentially Expressed genes (DEGs) was generated by filtering the expression data with threshold values: FPKM>5000 and *p*-value = 0.05 and fold change (-2, 2). Ultimately, 382 genes exhibited increased expression, while 83 genes showed decreased expression. These DEGs were used to create a PPI network in Cytoscape software (Figure 2 A). Gene hubs were identified through the overlap of the top ten genes obtained from three topological analysis methods: MCC, MNC, and DMNC. Among the five selected hub genes, three exhibited downregulation and two showed upregulation.

The top 10 DEGs of phytoplasma-infected trees were prioritized, respectively (Table. 2). They were identified by PPI network analysis and five hub genes based on degree calculation were identified by cytoHubba plugin (Figure 2 B). these genes were LOC110752314 (PRUPE_1G143200), LOC110755444 (PRUPE_6G104700), LOC110745289 (PRUPE_7G175200), LOC110768971 (PRUPE_4G135700), LOC110752959 (PRUPE_7G146600), LOC110753591 (PRUPE_1G356300), LOC110768318 (PRUPE_3G157000), LOC110763627 (PRUPE_3G150300), LOC110760411 (PRUPE_1G347000), and LOC110767393 (PRUPE_2G215600) (Figure 2 A). Among these genes, five genes were scrutinized based on their expression and biological significance (Figure 2 B, Table 3).

### *GO Function Enrichment Analysis of DEG*

The results of the GO analysis are mentioned in Table 3. Additionally, a GO enrichment analysis of DEGs in the most significant module was also conducted. The focus of this study was on the genes that are expressed in response to phytoplasma attacks. The study revealed that within these GO term responses, a gene encoding the transcript for the Helicase protein Mom1 (LOC110755444) and another gene

encoding the transcript for protein photoperiod-independent early-flowering1 (LOC110768971) were both significantly expressed in phytoplasma-infected leaves compared to healthy ones. This finding highlights the significant impact of phytoplasma infection on gene expression and emphasizes the importance of these genes in plant health. This finding highlights the significant effects of phytoplasma infection on gene expression and underscores the importance of these genes for plant health. There were also genes encoding transcript belonging to DNA-directed 5'-3' RNA polymerase activity that was up-regulated in (LOC110745289) phyto plasma infected leaves compared to healthy leaves. The gene encoding Histone-lysine N-methyltransferase protein (LOC 110752959) was downregulated, also the gene encoding Chromatin structure-remodeling complex protein SYD (LOC110752314) was downregulated as well. Therefore, genes associated with common GO terms exhibited different expression behaviors when attacked by phytoplasma. (Table 3).

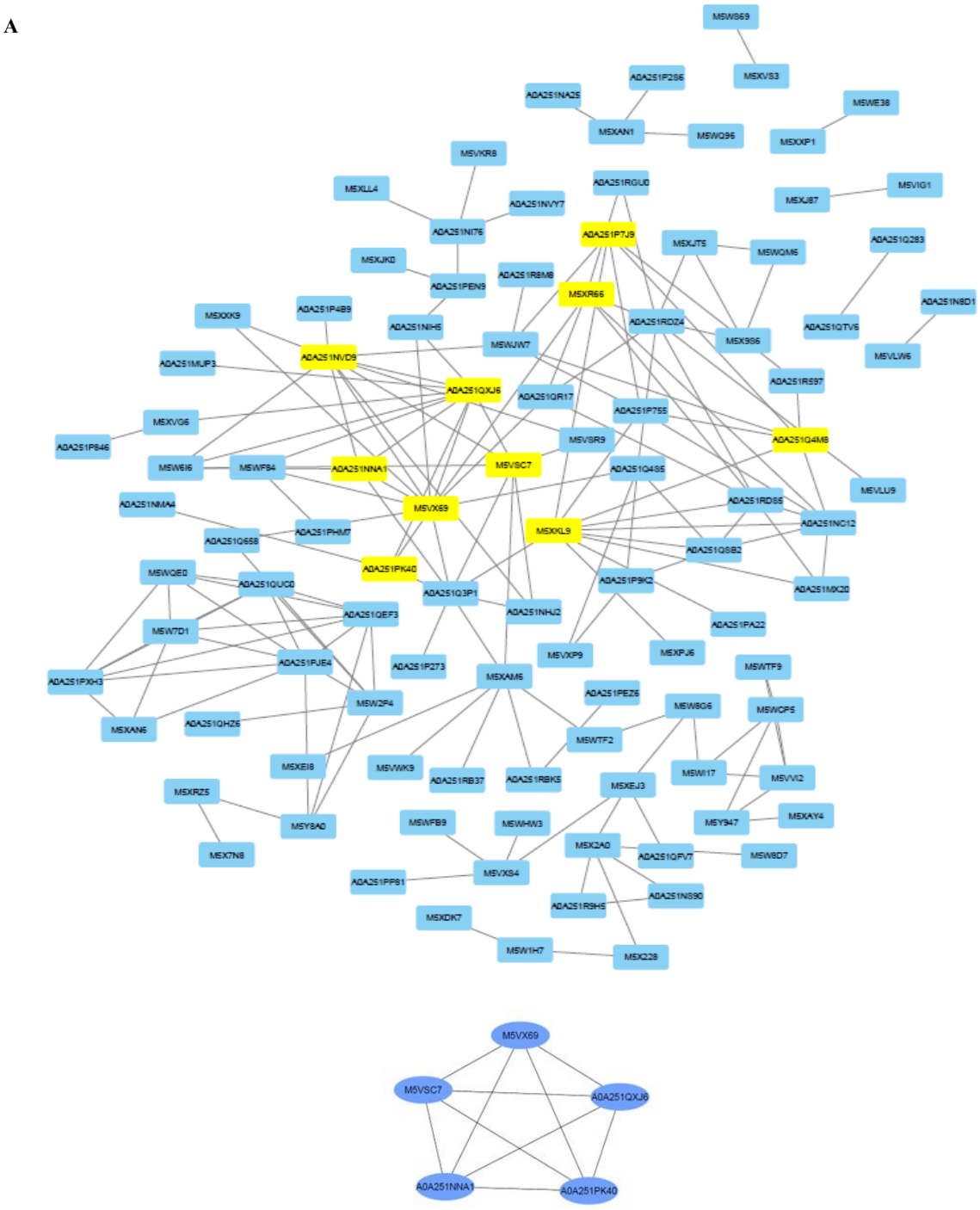


**Figure 2. A**) Protein-Protein Interaction (PPI) Network was Constructed Based on Connections with Hub Genes. **B)** The top ten genes were identified by cytoHubba.

**Table.2**: Top Ten up- and Down-regulated DEGs in Phytoplasma Infected Cherry Leaves by RNA-Seq ($p$-value≤0.05). Gene expression values were compared to Healthy leaves. (*) five selected Hub genes

| Gene name | Ncbi accession number | String ID | Fold change | FDR (p-value) |
|---|---|---|---|---|
| **Up-regulated gene** | | | | |
| LOC110745289* | PRUPE_7G175200 | A0A251QXJ6 | 290.9545455 | 0 |
| LOC110768971* | PRUPE_4G135700 | A0A251PK40 | 124 | 1.728E-53 |
| LOC110753591 | PRUPE_1G356300 | M5XXK9 | 112.75 | 2.257E-190 |
| LOC110768318 | PRUPE_3G157000 | A0A251PWT3 | 68.6666 | 1.658E-43 |
| LOC110767393 | PRUPE_2G215600 | A0A251QJE8 | 65.7777 | 3.193E-122 |
| LOC18776654 | PRUPE_5G043100 | M5WCP5 | 57 | 8.928E-13 |
| LOC18785400 | PRUPE_2G114500 | A0A251QEF3 | 52.833 | 7.975E-129 |
| LOC109949513 | PRUPE_6G133800 | A0A251NSM7 | 52 | 1.094E-11 |
| LOC18774325 | PRUPE_6G018900 | A0A251NIT8 | 48 | 8.151E-11 |
| LOC18785441 | PRUPE_2G137300 | A0A251QFN7 | 46.5 | 8.814E-20 |
| **Down regulated gene** | | | | |
| LOC110752314* | PRUPE_1G143200 | A0A251QXJ6 | -10582 | 2.59E-15 |
| LOC110752959* | PRUPE_7G146600 | M5VX69 | -3631 | 0 |
| LOC110763627 | PRUPE_3G150300 | A0A251Q3P1 | -2676 | 6.41E-15 |
| LOC110755444* | PRUPE_6G104700 | A0A251NNA1 | -2000 | 1.64E-12 |
| LOC110760411 | PRUPE_1G347000 | M5XVG6 | -1812 | 7.39E-12 |
| LOC18789916 | PRUPE_1G019400 | A0A251QSB2 | -1803.5 | 0 |
| LOC18773121 | PRUPE_6G192500 | A0A251NVD9 | -819.5 | 0 |
| LOC18783199 | PRUPE_3G104300 | M5XAM6 | -677.333 | 6.56E-16 |
| LOC18792788 | PRUPE_1G233200 | M5XKL9 | -665 | 7.044E-15 |
| LOC18776331 | PRUPE_5G057400 | A0A251P4B9 | -661 | 6.41E-08 |

**Table 3.** NCBI Accession Number of nt Sequence Obtained by Searching the GenBank nt Database for the Corresponding Annotation Delimited to *Prunus persica*. (http://www.geneontology.org/; accessed 2 January 2022)

| Gene ID | String interaction database in *prunus persica* | Description | RNA-Seq Expression status |
|---|---|---|---|
| Actin | EMJ 05561 | structural constituent of the cytoskeleton | |
| LOC110752314 (Gene 1) | PRUPE_1G143200 | Chromatin structure remodeling complex protein SYD<br>ATP-dependent activity<br>ATP-binding<br>DNA binding<br>Histone binding<br>Positive regulation of transcription by RNA polymerase I<br>System development | Down-regulated |
| LOC110755444 (Gene 2) | PRUPE_6G104700 | Helicase protein MOM1<br>heterochromatin formation | Down-regulated |
| LOC110745289 (Gene 3) | PRUPE_7G175200 | DNA-directed 5'-3'RNA polymerase activity<br>RNA polymerase II, core complex<br>DNA binding<br>Metal ion binding<br>Transcription by RNA polymerase II | Up-regulated |
| LOC110768971(Gene 4) | PRUPE_4G135700 | -Protein photoperiod-independent early-flowering1<br>ATP binding<br>Histone binding<br>SWr1 complex | Up-regulated |
| LOC110752959 (Gene 5) | PRUPE_7G146600 | Histone-lysine-N-methyltransferase SUVR5<br>Zinc ion binding<br>Chromosome<br>Nucleus | Down-regulated |

**Table 4.** Comparison of Expression of Selected Hub Genes in Cherry and Peach Leaves Infected with Phytoplasma

| Gene ID | Fold change in cherry (RNA-seq analysis) $p$-value<0.05 | Fold change in peach (Real-time PCR) $p$-value<0.05 |
|---|---|---|
| LOC110752314 (Gene 1) | -2.9853 downregulated | 1.051868 not change to control |
| LOC110755444 (Gene 2) | -2.45578 downregulated | 1.022185 not change to control |
| LOC110745289 (Gene 3) | 3.67345 upregulated | 1/0001535 up-regulated |
| LOC110768971(Gene 4) | 2.75081 upregulated | 1.228395 not change to control |
| LOC110752959 (Gene 5) | 5.6432 upregulated | 1.319699 not change to control |

All (DEGs) were analyzed using GO data and categorized into three functional groups: Biological Process, Molecular Function, and Cellular Component. A significant number of genes in the molecular function category of 26 GO terms were linked with "organic cyclic compound binding"(GO:0097159) "heterocyclic compound binding" (GO:1901363) and "binding"(GO:0005488) The cellular component function category containing 13 GO terms showed that "cellular anatomical entity"(GO:0110165) and "intracellular anatomical structure"(GO:0043231) had the highest proportion. In terms of 24 GO biological processes, the most enriched terms were "cellular process"(GO:0009987)

"metabolic process"(GO:0008152) and "Organic substance metabolic process"(GO:0071704) (Figure 3). Most of the differentially expressed genes encode proteins involved in amino acid biosynthesis pathways and secondary metabolite biosynthesis.

### *Analysis of Field Survey*

All infected peach plant leaves used in the experiment belonged to the same cultivar (crest), as determined by PCR. The PCR assay confirmed the presence of phytoplasma in the infected peach leaf samples. At the same time, uninfected plants tested negative (Figure 4). The obtained sequence showed 98% similarity with the phytoplasma 16S rRNA gene sequence.

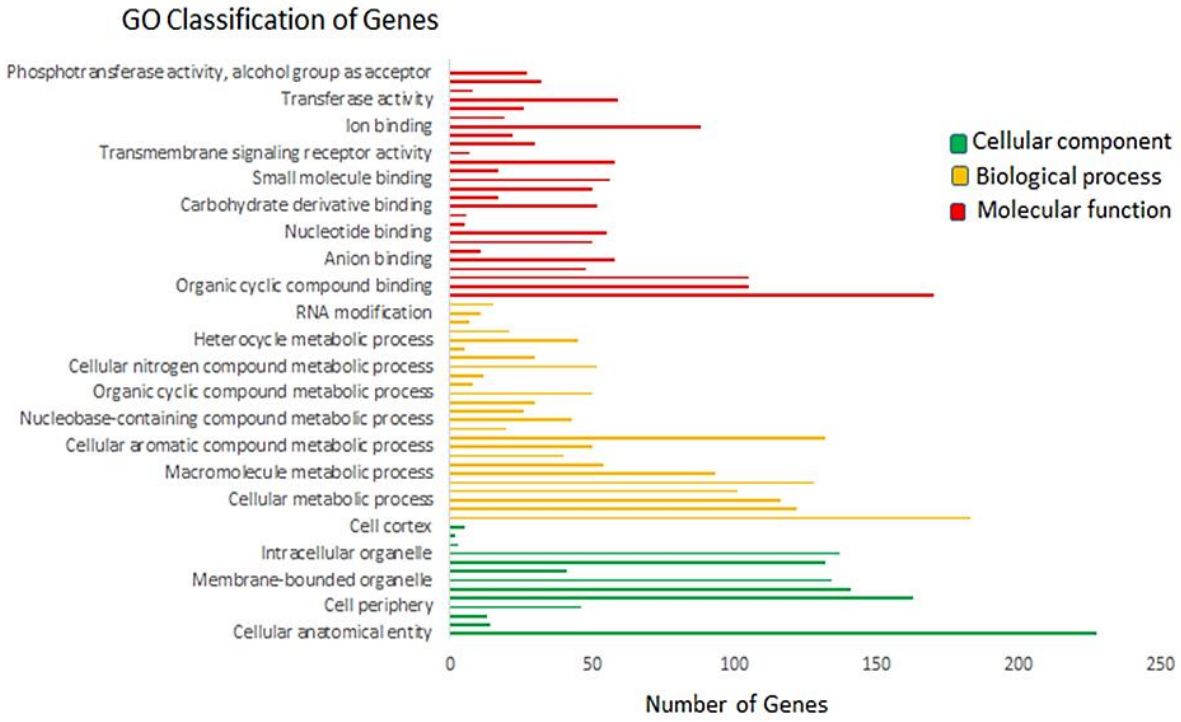


**Figure 3**. Gene ONTOLOGY (GO) Enrichment was Performed on Differentially Expressed Genes (DEGs) in Phytoplasma-Infected Peach Leaves Compared to Healthy Ones. DEGs were classified under three GO categories: Biological Processes (green), Cellular Components (blue), and Molecular Functions (red).

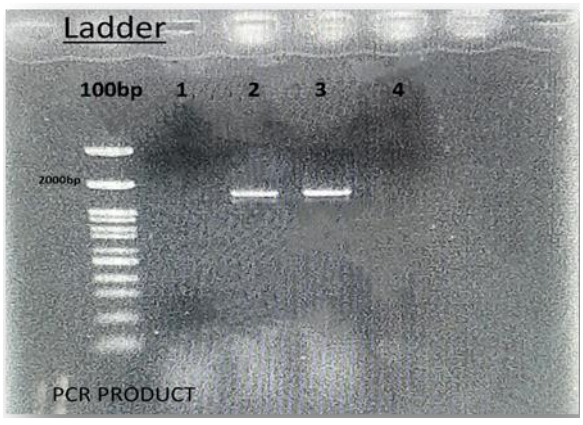


**Figure 4.** PCR Products Were Obtained from Healthy and Infected Peach Leaves Using Primer Pairs PA2F/R. The amplified products from the healthy leaves numbered 1 and 4, while those from the phytoplasma-infected leaves were 2 and 3. The amplified products were then recovered and sequenced at Micro Science (Swiss).

### *Validation of Selected DEGs Using qRT-PCR*

The results related to qPCR (Figure 5) showed a significant increase in the LOC110745289 gene and no significant change in other genes ($p$<0.05). Cherry RNA-seq expression data were used and additional data to identify the genes with different expressions in infected peach trees. To ensure accuracy,

melting curves for each primer pair were conducted, and observed one peak, indicating successful amplification, in every case. In gene 1 (LOC110752314), there was no significant difference in the expression between healthy and phytoplasma-infected peach plants after the appearance of the disease. No significant difference was observed in gene 2 (LOC110755444). In gene 3 (LOC110745289), after the development of the disease, a significant increase in this gene expression was observed in diseased trees. In gene 4 (LOC110768971), no significant difference was observed in healthy and diseased plants. In gene 5 (LOC110752959), no significant decrease was observed in peach trees.

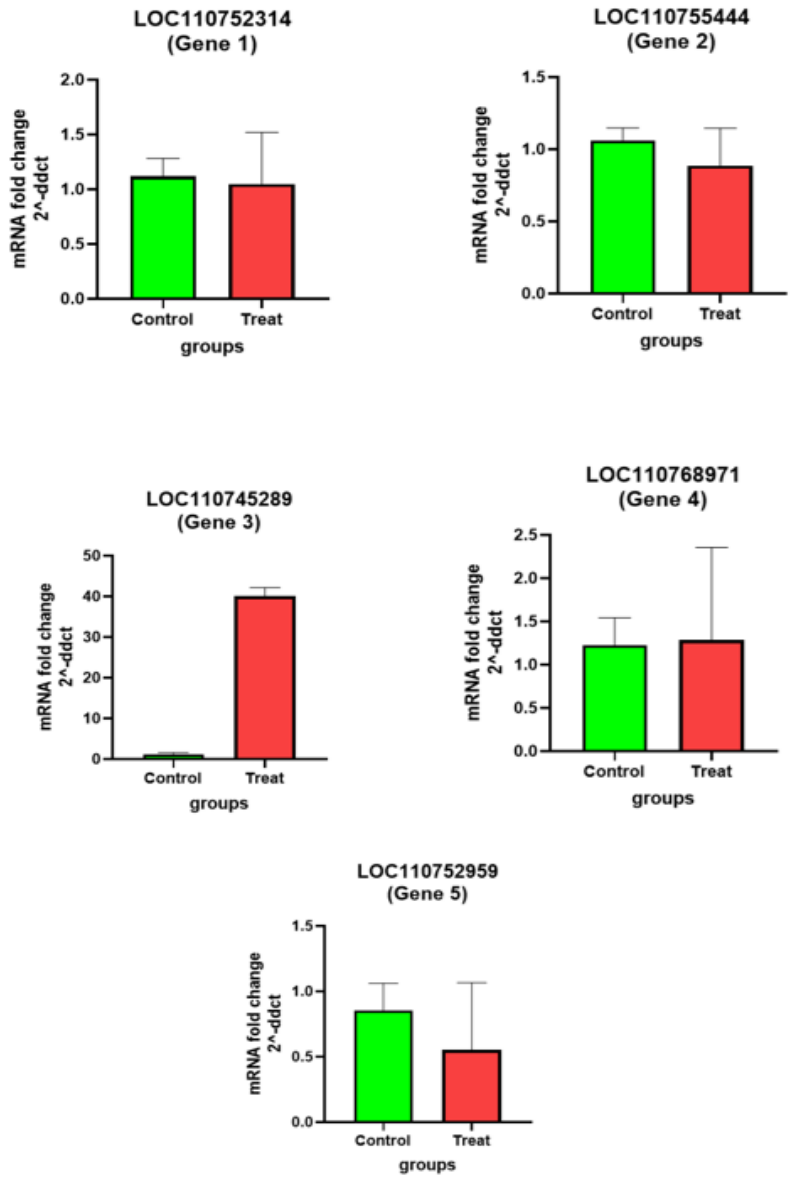


**Figure 5.** The Following Text Describes Bar Plots that Show the Expression of Candidate Genes in the Leaves of a Peach Selection, Both Healthy (control-green) and Infected (treat-orange). The data is shown as MNE relative to Actin. The MNE value for each sample was calculated from 3 biological replications with 2 repeats per replication. The asterisk denotes statistically significant differences with a significance level of $p \leq 0.05$.

## Discussion

Phytoplasmas cause various symptoms in host plants, such as yellowing, phyllody, and witches' broom. Studies have shown that the symptoms caused by the phytoplasma pathogen result from changes in the gene expression of infected plants compared to healthy ones.[25] Although phytoplasmas are classified into various taxonomic groups, some general conclusions can be drawn about their interactions with host plants. Based on existing information, analysis of phytoplasma gene expression patterns has shown that they use transcription factors to change their gene expression and host changes.[26] This study aimed to identify a common set of transcriptional changes that occur in Peach phytoplasma-infected leaves. The results indicate differences in the number of up-and down-regulated DEGs under disease stress, which can be used to profile gene expression.

Based on the ontology analysis, the genes examined in the biosynthetic pathways of amino acids and secondary metabolites are closely linked to known defense pathways in plants. Some amino acids play key roles in defense responses, acting as precursors for PR proteins and defensive enzymes. Additionally, amino acids like glutamate and tryptophan play important roles in defense signaling, contributing to the regulation of hormonal pathways such as salicylic acid and jasmonic acid, which are involved in responses to diseases. On the other hand, secondary metabolites are often crucial in plant defense against pathogens and pests. Some of the most important include antibiotics, alkaloids, flavonoids, and terpenoids, which may be produced through the biosynthetic pathways of amino acids and other metabolic precursors.

About biosynthesis of amino acids genes, the gene LOC110745289 investigated in this study play important roles in amino acid biosynthesis pathways. RNA polymerase indirectly and directly influences the pathways of amino acid biosynthesis. Many genes responsible for producing enzymes involved in amino acid biosynthetic pathways are transcribed with the help of RNA polymerase. Amino acid biosynthesis plays a crucial role in cellular defense responses.[27] In this study, the hub gene LOC110745289 in cherry and peach trees was associated with increased expression. Based on ontological analysis, this gene plays a significant role in RNA polymerase activities. Amino acids not only serve as structural building blocks for proteins in cells but also act as important precursors for secondary metabolites and signaling molecules that are involved in responses to environmental stresses, such as pathogens, environmental stressors, and cellular damage.[28] All organisms use DNA-dependent RNA polymerases to transfer genetic information from genomic DNA to RNA and to synthesize all the RNA needed for cellular functions.[29] A study that investigated the regulation of RNA polymerase subunit genes in olive trees revealed that these genes are spatially and temporally regulated by biotic and abiotic stresses.[30] The study showed that when trees are infected with phytoplasma, they overexpress this gene to synthesize the RNA required for proper cell function. Cells may need to increase RNA production in response to threats such as viral or bacterial infections. As a result, this is achieved by enhancing the activity of DNA-directed RNA polymerase, leading to faster transcription of genes that are essential for combating infections or responding to biological stress. Peach and cherry trees respond to disease stress by upregulating the expression of the LOC110745289 gene, which helps them successfully adapt to new conditions.

About Biosynthesis of secondary metabolites, all the genes studied, except for LOC110768971, had a direct role in the biosynthesis of secondary metabolites. However, Only the gene LOC110745289 has been expressed in both cherry and peach trees. RNA polymerase plays a fundamental role in gene transcription and can be directly involved in the biosynthesis of secondary metabolites.[30] Many of the genes involved in the biosynthetic pathways of secondary metabolites, such as terpenoids, flavonoids, alkaloids, and other metabolites, require transcription into mRNA by RNA polymerase. Therefore, RNA polymerase indirectly and directly influences the biosynthesis of secondary metabolites. In the transcript analysis of cherry trees, the gene LOC110752314 showed a decrease in expression, while its expression did not significantly change in the infected peach trees. The function of this gene, is associated with hydrolase and helicase activity, aiding in ATP and histone binding. Helicases act as potential molecules for abiotic stress tolerance in plants. DNA and RNA helicases strongly impact the plant's defense mechanisms, protecting it from pathogen infections and other stress factors.[31] Additionally, hydrolases are involved in the biosynthesis and regulation of glycans, mobilization of bioenergy, plant defense, symbiosis, signaling, secondary metabolism, and glycolipid metabolism.[32] The reduced expression of helicase genes can impair a plant's ability to unwind double-stranded DNA, thereby affecting the crucial process of DNA replication and repair. This leads to the accumulation of genetic damage and reduces the plant's ability to withstand environmental stress factors.[31] The reduction or alteration of helicase gene expression could be one of the plant's responses to stressful conditions.

The gene LOC110755444 is a hub gene that exhibited decreased expression in the transcriptome analysis of cherry tree leaves, while no significant change in expression was noted in the leaves of peach trees. Due to the lack of annotation for this gene, its exact function has not yet been fully determined, but helicase activity and heterochromatin formation have been proposed for this gene. The differences in the gene expression patterns between cherry and peach trees can be attributed to factors such as variations in plant species, developmental stages, and environmental conditions. Heterochromatin, in addition to its role in gene silencing, is also involved in DNA repair.[33] Phytoplasma infections can affect a plant's DNA repair system, resulting in changes to heterochromatin structure and an increase in genetic damage. These changes could lead to alterations in the plant's biological processes, including protein production and metabolism.[34] In the reduction of this gene expression in cherry trees and the lack of significant expression changes in peach trees, various factors are involved, including environ mental and biological stressors, genetic factors, epigenetic regulation, internal factors such as hormones, and even the deficiency of certain nutrients.[35]

In this study, the hub gene LOC110768971 showed increased expression in cherry trees, while no significant change was observed in peach trees. This gene is involved in delaying flowering. In sesame plants infected with the

phytoplasma pathogen, which was associated with flower abnormalities, flowering-delay genes, including PIE1, showed increased expression.[34] Studies on phytoplasma-infected cherry trees have shown that abnormalities were also observed in the flowers during phytoplasma infection. Therefore, it can be concluded that the increased expression of this gene in cherry trees is associated with the occurrence of phyllody in infected trees.[36] Given that no specific changes were observed in the flowers of phytoplasma-infected peach trees, the lack of change in gene expression in peach trees is justifiable. According to the findings, the hub gene LOC110752959, which encodes the Histone-lysine N-methyltransferase SUVR5 isoform X2 protein, was examined. This enzyme plays a significant role in regulating gene expression and various biological processes. Histone methyl ation, including H3K27me3, can influence the regulation of genes associated with responses to environmental stress and pathogen attacks.[37] Plants use complex mechanisms, including epigenetic changes at the chromatin level, to defend against pathogen attacks (bacteria, fungi, and viruses), for example, some defense genes activated in response to pathogens are affected by histone methylation.[38] Enzymes like SUVR5 can activate or repress defense-related genes by methylating histones, which causes structural changes in the chromatin.[38] Additionally, plants typically secrete various hormones (such as salicylic acid, gibberellic acid, and auxin) in response to damage or pathogen threats. These hormones can directly and indirectly influence the activity of defense genes. Enzymes like SUVR5 may play a role in regulating these networks through epigenetic modifications.[39] SUVR5 may also play a role in responding to other biotic stresses, including pests and plant competitors.[38] This enzyme may indirectly regulate defense gene expression and help plants adapt to these threats.[39]

In a transcriptomic study of Paulownia trees infected with phytoplasma, this gene exhibited heightened expression.[40] However, in a transcriptomic study of phytoplasma-infected cherry leaves, the expression of this gene was reduced, which could have significant implications for the plant's survival under stress. As we know, histone methylation genes can either activate or repress gene expression depending on the location and intensity of changes.[39] Therefore, the decrease in the expression of this gene in cherry trees and the lack of change in peach trees may be due to differences in the severity of pathogen infection and even differences in the sampling time.

## Conclusion

The results of RNA-Seq analysis highlighted the changing nature of the interaction between cherry phytoplasma and disease at the transcriptional level. Real-time PCR confirmed the expression of one of the studied genes. However, there may be variation due to species/genotype, culture type, and stress factors.[18] Also the difference in expression levels between the RNA-Seq results and RT-qPCR may be due to the bioinformatics process used in the RNA-Seq analysis. This process involves several factors that can affect the reproducibility of quantitative expression profiles, including alignment choices and estimation of transcript expression.[40] It is possible that other factors influenced this difference in results. One possible factor is that the transcriptome is constantly changing. Additionally, the RNA-Seq analysis used a mixture of biological samples, which were tested separately in the RT-qPCR assays.[41] Cherry and peach trees are both in the Rosaceae family and have the same species, so they have many genes in common. On the other hand, due to the lack of cherry plant gene annotation, bioinformatics studies often rely on peach species genes for protein network analysis. In this study, using cherry tree data, we investigated the expression of some hub genes in peach species, considering that these two trees have different phenology in terms of growth and the time of infection of these trees with the phytoplasma pathogen also is a different. It seems that this is due to the dynamic nature of the plant transcriptome and the timing of infection with the specific pathogen.[42] By identifying key genes, gene editing techniques such as CRISPR-Cas9 or RNA interference (RNAi) can be employed to investigate the effects of reducing or increasing the expression of these genes on the resistance of plants to phytoplasma.[43] These methods can help clarify the precise role of each gene in the plant's response to the disease.[44] In this study, gene 3 has shown increased expression in response to phytoplasma in both cherry and peach trees. In future studies, the levels of associated proteins for this gene can be measured using techniques like Western Blot or ELISA to establish a correlation between gene expression changes and protein levels. Ultimately, the results of this research can serve as a foundation for developing phyto plasma-resistant cultivars in peach trees and other plants. With the use of molecular and genetic information, it would be possible to propagate disease-resistant plants, leading to more sustainable and resilient agricultural practices.

## Authors' Contributions

All authors contributed to the study's conception and design. AZT contributed to the investigation, writing the original draft, data curation, and formal analysis. SN contributed to supervision, funding acquisition, methodology, project administration, resources, writing review & editing. MT contributed to conceptualization, software writing review & editing. HHP contributed to Validation, investigation, visualization, Writing review & editing. All participating authors read, commented, and approved the current version of the manuscript.

## Conflict of Interest Disclosures

The authors declare that they have no conflicts of interest.

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