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# Genomic and Transcriptomic Characterization of *Alternaria alternata* during Infection

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**Abstract:** Host-pathogen interactions are the result of the continuously evolving dynamics of the genomic interphases between pathogens and the host plants. Alternaria brown spot (ABS) caused by the pathogen *Alternaria alternata* is a serious threat to tangerine production. Although recent studies have made significant advances in the characterization of *A. alternata* virulence factors, a gap exists in the regulation of virulent genes throughout the course of *A. alternata* infection on host plants. To gain a better understanding of the dynamic defense transcriptome in *Alternaria alternata* during Infection, we performed a comparative transcriptome approach. After inoculation on citrus, we found that 2142, 1964, 2359 genes were up-regulated, and 1948, 1434, 1996 genes were down-regulated at 12 hours-post-inoculation (hpi), 24 hpi and 48 hpi, respectively. Among these genes, 1333 genes were up-regulated at three time points, and 1054 genes were down-regulated, indicating that most of the differentially expressed genes at the early stage of infection tended to remain differentially expressed at the later stage of infection. In addition to the genes that are known to be part of the infection network in plant-pathogen interactions, many novel genes related to plant-pathogen interaction were identified. Interestingly, our results indicate that *A. alternata* is able to rapidly alter its gene expression pattern during infection process, which is vital for the successful colonization of the pathogen. Moreover, this rapid alteration of gene expression is likely to be an adaptive mechanism, enabling the pathogen to quickly respond to any changes in the environment and adapt to the host's defense system. This ability to modify gene expression quickly in the face of environmental changes could play a critical role in the successful establishment of infection. RT-qPCR analysis confirmed that the expression pattern of nine randomly selected genes from the peroxisome pathway were consistent with the RNA-seq data. Our study provided a comprehensive study of the expression of genes during *A. alternata* infection of citrus, which may facilitate the understanding of host-plant interactions in *A. alternata*.

**Keywords:** *Alternaria alternata*; oxidative stress tolerance; host-pathogen interactions; transcriptome analysis; pathogenicity



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## 1. Introduction

Plants and pathogens have co-evolved over a long period of time, ensuing in a distinct "attack and defense" pattern [1–3]. The interactions between plants and pathogens are regulated by complicated regulatory networks [4], and understanding the interactions between plants and pathogens may be helpful in preventing plant diseases [5,6]. When a pathogenic agent is detected by the host plant, one of the earliest cellular responses is the rapid production of reactive oxygen species (ROS) [7,8], which includes hydrogen

peroxide, hydroxyl radicals, superoxide and singlet oxygen. ROS plays a crucial part in activating and adjusting plant defense, as well as integrating multiple stress response signal networks [9]. ROS acts as both toxic molecules, inhibiting and eliminating pathogens, and cellular signaling molecules, triggering a series of plant immune responses, such as pathogen-associated molecular pattern (PAMP)-induced immunity and effector-induced immunity (ETI). The extensive production of ROS eventually results in hypersensitive response (HR), in the form of programmed cell death (PCD) at the site of pathogen infection. This is accompanied by an accumulation of antioxidant enzymes, the synthesis of various phenolic compounds and phytoalexins [10], which helps to effectively restrict the invasion and spread of plant pathogens.

Plants have a wide array of defensive strategies against pathogen attack, such as HR-related necrosis, basal defense, induced systemic resistance, and pathogenesis-related proteins (PR-proteins). These mechanisms are essential for the survival of plants, and more research is required to comprehend how they collaborate to safeguard plants from pathogen attack. Hypersensitive response (HR) is an example of such defense mechanisms, which is famously characterized by the sudden death of cells at the site of infection [11]. The HR-related necrosis can help to restrain biotrophic or semi-biotrophic pathogens, but is unable to effectively combat necrotic pathogens that use dead organic matter as a source of sustenance. Therefore, plants have developed other strategies such as basal defense, induced systemic resistance, and PR-proteins to protect themselves against necrotic pathogens. Basal defense is triggered by multiple physical and chemical factors, such as light, temperature, and salinity. Induced systemic resistance is when a plant's immune system is activated and produces protective proteins when exposed to a certain pathogen. PR-proteins are a category of proteins generated by the plant to protect itself from pathogens.

Pathogens have evolved a series of sophisticated strategies to enable them to evade or circumvent the host's systemic defense response during infection, similar to the defense mechanisms evolved by plants to defend themselves from diseases [12]. These strategies include the production of toxins or chemicals that can disrupt the normal functioning of the immune system, the creation of molecules that are capable of inhibiting specific immune cells, the ability to enter host cells and modify their internal structure, and the capacity to hide from the host's immune system. Moreover, some pathogens have developed the ability to suppress the production of cytokines and other molecules that are essential for the host's immune response. Avirulent pathogens can be successfully detected by the plant's disease resistance mechanism, resulting in the biphasic accumulation of ROS. The initial phase is of a transient nature, followed by a sustained phase of increased intensity associated with disease resistance [13]. Nevertheless, the HR-related necrosis can only inhibit biotrophic or semi-biotrophic pathogens and is unable to effectively control necrotic pathogens that use dead organic matter as sources of nutrients [7].

Citrus is one of the most widely cultivated fruit crops in the world, having diversified more than eight million years ago in the Himalayan foothills of eastern Assam, northern Myanmar, and western Yunnan. Currently, the variety of citrus in the world is highly diversified, and the exact number of citrus varieties is unknown [14]. The citrus industry is invaluable, making a significant contribution to the supply of agricultural products, export of foreign exchange, and improved incomes for farmers, as well as boosting rural economic prosperity. However, citrus diseases are a major impediment to its development. These diseases can be divided into biotic or abiotic categories, and the most severe are citrus canker, citrus greening, citrus decline, citrus black spot, brown spot, and root rot. To effectively tackle these diseases, it is essential to utilize disease-resistant varieties and promote the use of advanced technologies such as biotechnology and precision agriculture. Moreover, further research should be conducted to better understand the citrus disease control, and the monitoring and early warning of citrus diseases should be intensified.

The genus *Alternaria* encompasses a wide range of necrotrophic fungi, which can inhabit a diverse range of hosts such as fruits, vegetables, cereals, seeds, and crop plants [15].

Among these, *A. alternata* is made up of at least seven distinct pathotypes, each of which produces host-specific toxins with distinct chemical compositions, resulting in serious diseases in different host plants [16]. In citrus, two pathotypes of *A. alternata* are mainly responsible for two distinct diseases, Alternaria brown spot (ABS) of tangerine and leaf spot of rough lemon [17,18]. Leaf spot of rough lemon is caused by the *A. alternata* rough lemon pathotype, which primarily infects rough lemon (*Citrus jambhiri* Lush) [19], whereas Alternaria brown spot is caused by the *A. alternata* tangerine pathotype, which primarily affects tangerine (*Citrus reticulata* Blanco), grapefruit, tangerine-grapefruit hybrids, and tangerine-orange hybrids, exhibiting as brown spots on the leaves and fruits with distinct yellow halos surrounding them [18]. The symptoms of Alternaria brown spot are easily identifiable, appearing as small, light brown spots with yellow halos on the leaves and fruits of citrus [20]. The severity of the disease can be increased by high humidity and spread by wind-blown conidia. Moreover, Alternaria brown spot is an important citrus disease, primarily affecting young leaves, shoots, and fruits, leading to significant losses in yields [21,22]. Furthermore, the pathogen produces a large number of conidia on young leaves and shoots, which can induce initial infection as well as multiple re-infections, resulting in serious losses in citrus orchards [23] (Figure 1).

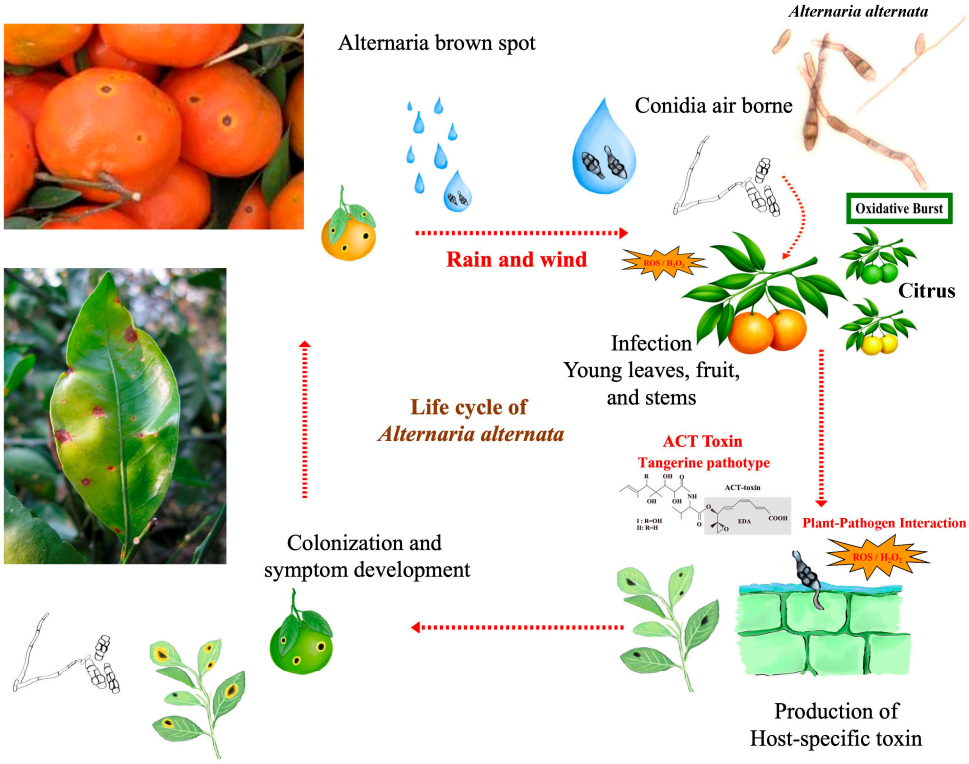


**Figure 1.** Life cycle of *Alternaria alternata*, the causal agent of citrus Alternaria brown spot.

Despite the fact that recent studies have made significant progress in the characteristics of individual *A. alternata* pathogenicity factors such as ROS detoxication regulator GPx3 [24], Glr1, Tlr1, Tsa1 [25], ACT toxin regulator ACTR [26], cysteine regulator MetR [27], MetB, MetC, MetX [28], ROS stress regulator Yap1 [29], as well as its genome with ACT toxin gene clusters [30,31], the gene expression profile of *A. alternata* during infection, as well as its physiological and metabolic changes have been only sparsely studied. To this end, we performed comparative transcriptome analysis to evaluate the gene expression patterns of *A. alternata* during interaction with the host plant. In particular, RNA-Seq was employed to examine the early stages of transcriptome analysis of *A. alternata* 12-, 24-, and 48 h post-inoculation (hpi), compared with uninoculated controls. Our results revealed a comprehensive list of differentially expressed genes of *A. alternata* during infection of citrus, which may shed light on the events occurring early in the infection process.

Furthermore, the changes in the metabolic response of *A. alternata* during infection were further investigated, which may expand our understanding of key genes and pathways in plant-necrotrophic pathogen interactions. Therefore, this work provides new insights into A. atlernata pathogenesis, and offers a descriptive model for the transcriptome along the continuum of citrus leave infection.

## 2. Materials and Methods

### *2.1. Fungal Strains and Culture Conditions*

*A. alternata* wild-type (WT) strain Z7, isolated from diseased citrus leaves in Wenzhou, Zhejiang Province, China, was used as the tested strain in this study [21]. The isolate was stored in 20% glycerol solutions at −80 °C until further use. It was then grown on PDA medium (200 g of potato, 20 g of glucose, 20 g of agar powder, 1 L of purified water) at 25 °C for 3 days before use. The inoculum of *A. alternata* Z7 was prepared by incubating the isolate on PDB medium (potato dextrose broth, consisting of 200 g of potato and 20 g of dextrose per liter of deionized water, providing a nutrient base and a carbohydrate source respectively) for 2 days.

### *2.2. Plant Preparation and Inoculation Experiment*

*A. alternata* Z7 strain was grown in liquid PDB at 25 °C in a shaker incubator for two days. The mycelium harvested from the PDB medium was delicately placed on detached tangerine leaves for inoculation. Subsequently, the inoculated leaves were kept in a moistened plastic box at room temperature for lesion development. To guarantee the precision of the experiment, at least 20 leaves were utilized and all experiments were repeated twice. A negative control was prepared using the pure culture of *A. alternata* Z7. Samples gathered from the fungal isolate developed in PDB medium and on citrus leaves at 12-, 24-, and 48 h post-inoculaton (hpi) were subjected to RNA-Seq analysis. Moreover, to compare *A. alternata* in oxidative stress (hydrogen peroxide), RNA-Seq data of *A. alternata* treated with $H_2O_2$ were utilized in comparative transcriptome analysis [31].

### *2.3. RNA Extraction, Library Preparation, and Illumina Sequencing*

For RNA preparation, the mycelium of each treatment was quickly frozen in liquid nitrogen and ground into a fine powder with an RNase-free mortar and pestle that had been pre-cooled with liquid nitrogen. To evaluate the expression levels of each gene, RNA-Seq was conducted with two biological replicates of each sample. The libraries were prepared using the IlluminaTruSeq RNA Sample Preparation Kit and the sequencing was performed using the Illumina Hiseq 2000 platform, which generated 50 base-pair single-end reads.

### *2.4. Genome Annotation and Functional Analysis*

The *A. alternata* Z7 genome (http://www.zjudata.com/alternaria/blast.php, accessed on 10 March 2022) [30]. were BLASTp searched against the Eukaryotic Orthologous Groups (KOG) database, downloaded from the NCBI website (ftp://ftp.ncbi.nih.gov/pub/COG/KOG/kyva, accessed on 10 March 2022), and assigned to functional categories based on an e-value cutoff of $1 \times 10^{-5}$. To gain further insights into gene function, the coding regions of the *A. alternata* Z7 genome were annotated by emapper version 2.0.1b, using the emapper DB v2.0 databases (http://eggnog5.embl.de, accessed on 10 March 2022), including Pfam, Uniprot, GO, Nr, KEGG, Interproscan, eggNOG, and Refseq [33]. To visualize the annotation information, Circos diagram was used to generate circular genome maps, which display the chromosomal locations, secondary metabolite gene clusters, shared differentially expressed genes during infection, and GC content [34].

### *2.5. Transcriptome Analysis*

The *A. alternata* Z7 genome was obtained from the citrus-associated Alternaira genomic database (http://www.zjudata.com/alternaria/blast.php, accessed on 10 March 2022) [30] and the NCBI GenBank (https://www.ncbi.nlm.nih.gov/genome, accessed on 10 March

2022), and served as a reference genome for mapping the Illumina reads [30,31]. Raw reads were then processed with Trimmomatic v0.36 [35] to remove adaptor sequences and low-quality bases. Subsequently, the cleaned reads were mapped to the reference genome using Hisat2 v2.1.0 with default parameters [36]. The mapped reads were converted into a count matrix containing gene and sample information using htseq-count [37]. The differential gene expression analysis in RNA-Seq data was conducted using the R package DESeq2, which provides functions to normalize count data, estimate variance-mean dependence in count data, and test for differential expression based on the negative binomial distribution [38]. Genes with an absolute value log2 (folding change) greater than 1 and a false discovery rate (FDR) less than 0.05 relative to the inoculum grown on PDB medium were identified as differentially expressed genes (DEGs). Subsequently, the R clusterProfiler package was employed to uncover enriched biological terms and pathways including Gene Ontology (GO) and KEGG pathway from gene expression data. The main function of the R clusterProfiler package supports a wide range of enrichment analysis methods, including Fisher's exact test and hypergeometric test. The R clusterProfiler package also provides tools for visualizing gene annotations and a comprehensive suite of functions for analyzing gene lists, gene sets, pathways, and gene ontology (GO) terms [39]. The gene expression of different SM gene clusters were visualized using R package ComplexHeatmap [40]. The ACT toxin gene cluster of different infection stage was visualized using R package genoplotR [41].

### *2.6. RT-qPCR Analysis*

To confirm the transcriptome data obtained from RNA-Seq, quantitative reverse transcription PCR (qRT-PCR) was conducted using the Applied Biosystems QuantStudio™ 6 Flex qPCR platform. *A. alternata* strain Z7 was cultivated in liquid PDB medium on a rotary shaker at 26 °C in the dark for two days. Then, the mycelium was inoculated onto citrus leaves for 12, 24, and 48 h and the inoculated mycelium was immediately ground into a fine powder in liquid nitrogen for RNA extraction. A list of all the primers used for RT-qPCR analysis can be found in the Supplementary Table S1.

## 3. Results

### *3.1. Genomic Characterization of Alternaria alternata Z7*

To identify the orthologous and paralogous proteins of *A. alternata* Z7, the proteome was mapped to the KOG database, a eukaryote-specific version of the Clusters of Orthologous Groups (COG) tool. The statistical diagram of the number of annotated genes in KOG database showed that a total of 5934 genes were assigned to 25 classes of KOGs, including "general function prediction only" (1224, 20.63%), "posttranslational modification, protein turnover, chaperones" (564, 9.50%), "signal transduction mechanisms" (420, 7.08%), "secondary metabolites biosynthesis, transport and catabolism" (372, 6.27%), "energy production and conversion" (365, 6.15%), "carbohydrate transport and metabolism" (362, 6.10%), "lipid transport and metabolism" (359, 6.05%), "function unknown" (350, 5.90%), "translation, ribosomal structure and biogenesis" (338, 5.70%), "amino acid transport and metabolism" (323, 5.44%), "intracellular trafficking, secretion, and vesicular transport" (299, 5.04%), "transcription" (273, 4.60%), "RNA processing and modification" (229, 3.86%), "replication, recombination and repair" (215, 3.62%), "cell cycle control, cell division, chromosome partitioning" (194, 3.27%), "inorganic ion transport and metabolism" (174, 2.93%), "cytoskeleton" (121, 2.04%), "chromatin structure and dynamics" (117, 1.97%), "coenzyme transport and metabolism" (106, 1.79%), "cell wall/membrane/envelope biogenesis" (90, 1.52%), "nucleotide transport and metabolism" (90, 1.52%), "defense mechanisms" (60, 1.01%), "nuclear structure" (28, 0.47%), "extracellular structures" (12, 0.20%), "cell motility" (6, 0.10%) (Figure 2, Supplementary Table S2).

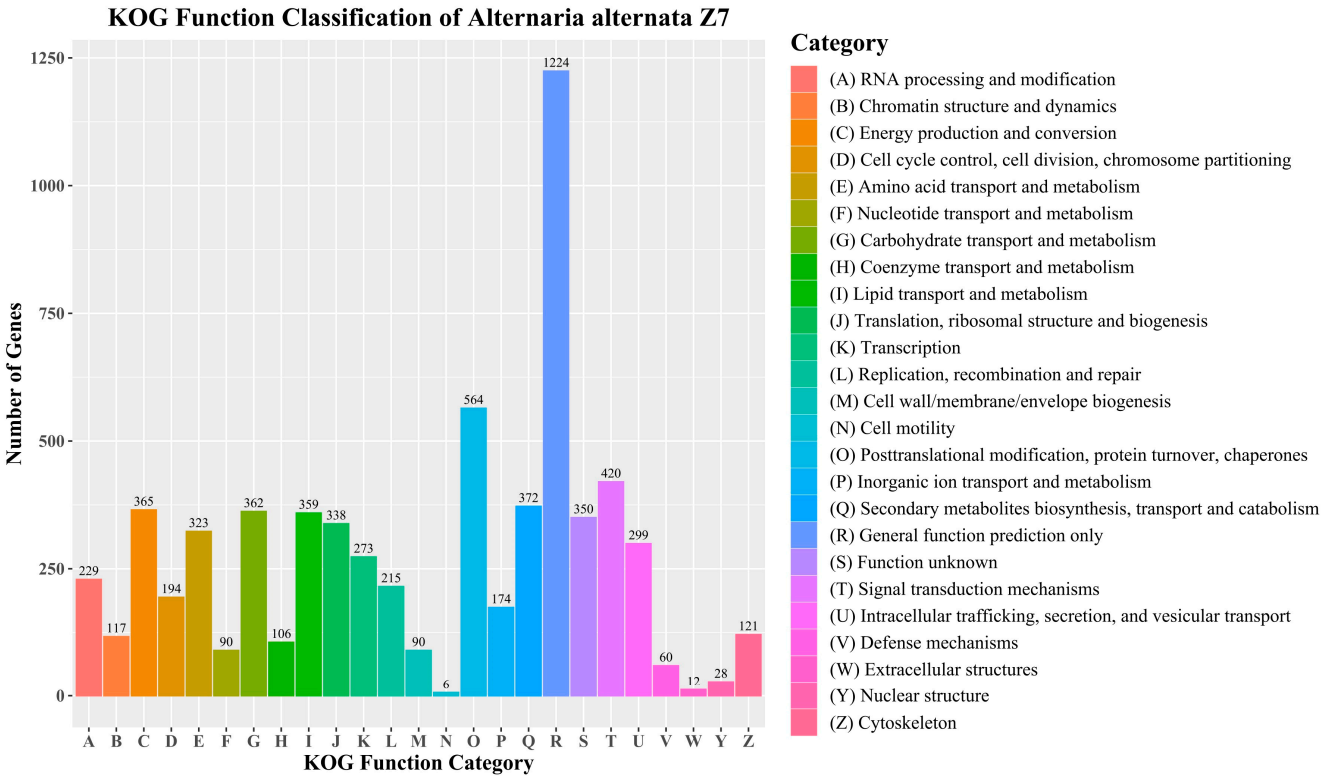


**Figure 2.** The x-axis of the KOG annotation of the global proteome in *Alternaria alternata* Z7 displays the KOG function classification of the consensus sequence, while the y-axis indicates the exact number of genes present in each functional class.

### *3.2. Gene Expression Patterns of A. alternata during Infection*

RNA-Seq was employed to conduct time-course transcriptome profiling in order to identify differentially expressed genes involved in *A. alternata* infection of citrus. Mycelium was collected from liquid PDB medium immediately prior to inoculation (time 0) and at 12-, 24-, and 48 h post-inoculation. Ultimately, 4090 DEGs were identified in *A. alternata* at 12 h after inoculation of fungus on citrus, comprising 2142 up-regulated DEGs and 1948 down-regulated DEGs (Figure 3A,D, Supplementary Table S3). In contrast, 3398 DEGs, comprising 1964 up-regulated genes and 1434 down-regulated genes were obtained in *A. alternata* after 24 h of inoculation (Figure 3B,E, Supplementary Table S4). Moreover, at 48 h of inoculation, 4355 DEGs with 2359 up-regulated and 1996 down-regulated genes were identified in fungal infected samples (Figure 3C,F, Supplementary Table S5). Our result demonstrated that a large portion of genes were differentially expressed in *A. alternata* during the infection stages. To systematically compare DEGs in each treatment, we performed a comparative transcriptomic analysis of DEGs, and found that 1333 up-regulated (43.6%) and 1054 down-regulated overlapping genes (42.3%) were present among *A. alternata* inoculated on citrus leaves for 12, 24, and 48 h, suggesting that *A. alternata* induces a large number of genes at the infection stage, thereby triggering a transcriptional response with many shared differentially expressed genes. Moreover, when comparing the gene expression patterns of *A. alternata* during the infection stages and oxidative stress, we discovered that there were 127 up-regulated (3.5%) and 163 down-regulated (6.1%) overlapping genes between WT + citrus and WT + $H_2O_2$, demonstrating that both the treatment of oxidative stress and plant-pathogen interaction can significantly down-regulate many genes (Figure 3G,H).

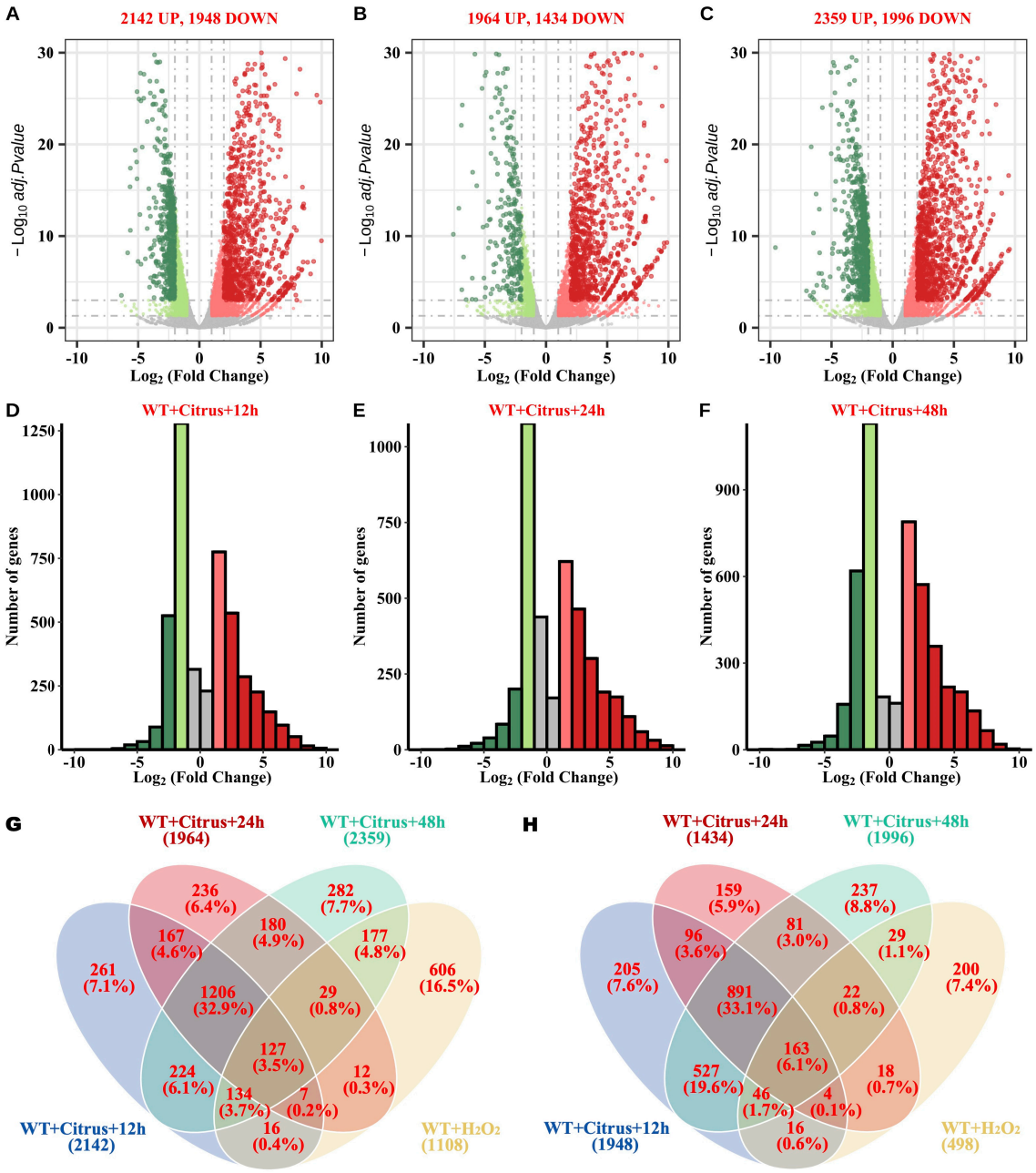


**Figure 3.** The global gene expression pattern of *Alternaria alternata* Z7 during infection in transcriptome profiles is studied. (**A**–**F**) The gene expression pattern of *A. alternata* during infection. (**A**–**C**) Volcano plots of the gene expression pattern of *A. alternata.* inoculated on citrus leaves at 12-, 24-, and 48 h post-inoculation (dpi). The y-axis of the volcano plots corresponds to the mean expression value of log10 [adjust p-value, also known as false discovery rate (FDR)], and the x-axis displays the log2(fold change value). The red dots represent the significantly differentially expressed transcripts [false discovery rate (FDR) <0.05] up-regulated under the indicated treatments compared to *A. alternata* Z7. The green dots symbolize the significantly differentially expressed transcripts (FDR < 0.05) down-regulated under the indicated treatments compared to *A. alternata* Z7. The grey dots show the transcripts whose expression levels did not reach statistical significance (FDR > 0.05) or the absolute value of log2(fold change value) was smaller than 1. (**D**–**F**) Histogram plots of the gene expression pattern of *A. alternata* Z7 inoculated on citrus leaves at 12-, 24-, and 48 h post-inoculation (dpi). (**G**) Venn diagram describing the overlaps among up-regulated genes in *A. alternata* Z7 inoculated on citrus leaves and treated with $H_2O_2$, while (**H**) is a Venn diagram illustrating the overlaps among down-regulated genes in *A. alternata* Z7 inoculated on citrus leaves and treated with $H_2O_2$.

The Circos diagram analysis also illustrated the genome location of the genes with expressions of DEGs during infection, as well as the location, length, and expression level of the genes related to the biosynthesis of secondary metabolites (Figure 4).

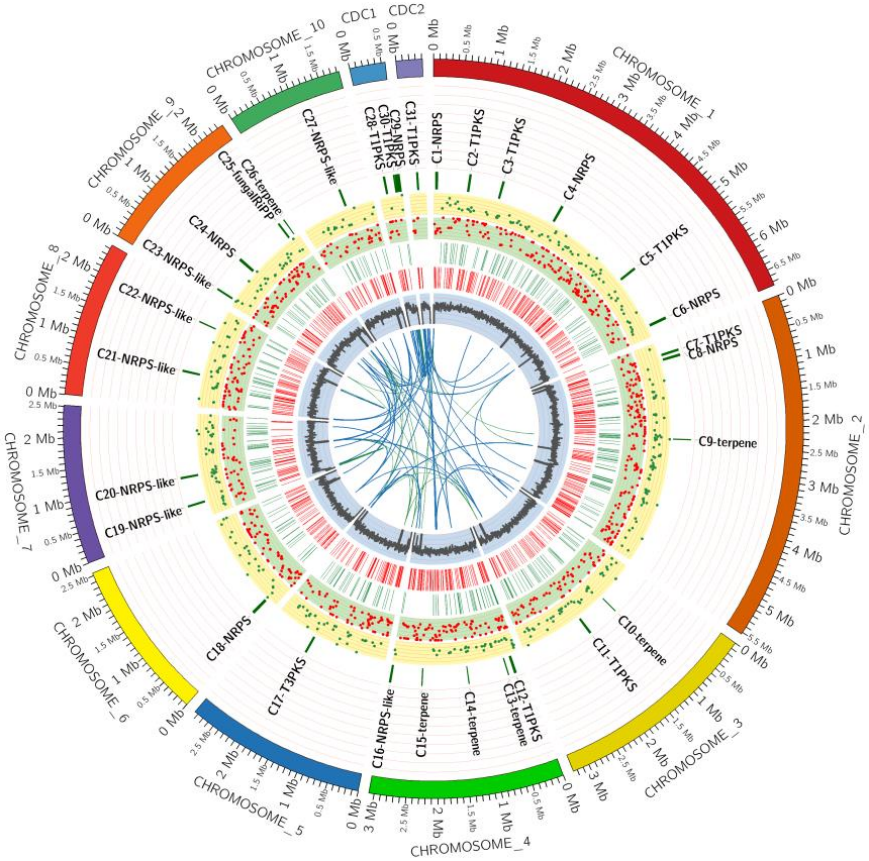


**Figure 4.** Circos diagram is an effective visual tool for displaying the common differences in gene expression in *Alternaria alternata* inoculated on a host plant compared to uninoculated treatment at 12, 24, and 48 h post-inoculation. Each concentric circle, starting from the outermost layer and moving toward the center, represents chromosomal locations, secondary metabolite gene clusters, differentially expressed genes (DEGs) that are commonly up-regulated or down-regulated in the three infection stages, and GC content. At the very center, gene duplications are represented. Notably, red dots signify transcripts that have been significantly up-regulated (FDR < 0.05) in *A. alternata* inoculated on a host plant, while green dots signify transcripts that have been significantly down-regulated (FDR < 0.05).

### *3.3. Multiple Critical Genes in GO Categories Were Affected in A. alternata during Infection*

The results of the gene ontology (GO) enrichment analysis of the up-regulated and down-regulated genes from *A. alternata* infection stages (12 h, 24 h, and 48 h) indicate that specific transcriptional regulation is involved in the infection process (Figure 5). The majority of up-regulated genes belong to the biological process (BP), including "transmembrane transport", "small molecule catabolic process", "potassium ion transport", "potassium ion transmembrane transport", "polysaccharide metabolic process", "polysaccharide catabolic process", "pectin metabolic process", "pectin catabolic process", "oxidation-reduction process", "organic acid catabolic process", "monocarboxylic acid catabolic process", "galacturonan metabolic process", "fatty acid catabolic process", "carboxylic acid catabolic process", "carbohydrate metabolic process", "carbohydrate catabolic process", "antibiotic catabolic process"; cellular component (CC), including "integral component of membrane", "extracellular region"; molecular function (MF), including "transporter activity", "transmembrane transporter activity", "potassium ion transmembrane transporter activity", "pectate lyase

activity", "oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen", "oxidoreductase activity, acting on CH-OH group of donors", "oxidoreductase activity", "monooxygenase activity", "metallocarboxypeptidase activity", "iron ion binding", "hydrolase activity, hydrolyzing O-glycosyl compounds", "hydrolase activity, acting on glycosyl bonds", "heme binding", "glucosidase activity", "flavin adenine dinucleotide binding", "carbohydrate binding", "FAD binding", "ABC-type transmembrane transporter activity". This suggests that *A. alternata* is able to induce specific transcriptional regulation in order to effectively meet its infection needs. The GO categories of the three infection stages displayed similar patterns, indicating that the infection process was consistent throughout the time course. This further emphasizes the significance of specific transcriptional regulation in the *A. alternata* infection process.

After assigning DEGs to the GO category, most down-regulated genes belong to the biological process (BP), including "translation", "small molecule catabolic process", "ribosome biogenesis", "ribonucleoprotein complex biogenesis", "rRNA processing", "oxidation-reduction process", "organonitrogen compound biosynthetic process", "organic acid catabolic process", "ncRNA processing", "ncRNA metabolic process", "gene expression", "cofactor metabolic process", "cellular nitrogen compound metabolic process", "cellular nitrogen compound biosynthetic process", "cellular biosynthetic process", "cellular amino acid metabolic process", "cellular amino acid catabolic process", "cellular amino acid biosynthetic process", "cellular amide metabolic process", "carboxylic acid catabolic process", "amide biosynthetic process", "alpha-amino acid metabolic process"; cellular component (CC), including "ribosome", "ribosomal subunit", "ribonucleoprotein complex", "preribosome, large subunit precursor", "preribosome", "organelle lumen", "organelle envelope lumen", "nucleolus", "non-membrane-bounded organelle", "mitochondrion", "mitochondrial protein complex", "mitochondrial intermembrane space", "membrane-enclosed lumen", "large ribosomal subunit", "intracellular organelle lumen", "intracellular non-membrane-bounded organelle", "cytosolic ribosome", "cytosolic large ribosomal subunit"; molecular function (MF), including "unfolded protein binding", "structural molecule activity", "structural constituent of ribosome", "snoRNA binding", "rRNA binding", "organic cyclic compound binding", "nucleic acid binding", "lyase activity", "heterocyclic compound binding", "cofactor binding", "catalytic activity, acting on a tRNA", "RNA binding", "2 iron, 2 sulfur cluster binding" (Supplementary Table S6). It is expected that, when the pathogen is separated from the medium, the process of metabolism needs to be reduced to maintain the successful infection of the host plant.

The GO analysis offers invaluable insights into the pathogenesis of plant-pathogen interaction by pinpointing the relevant genes and their functions. The results from the GO analysis point to a pivotal role of transporter activity and binding in the pathogenesis of plant-pathogen interaction. This corroborates the critical role of transporter activity and binding in the pathogenesis, as these functions can provide nourishment and enable the transition from vegetative growth stage to infection stage in plant-pathogen interaction. Notably, although the up-regulated genes and down-regulated genes were nearly equal, genes enriched in GO enrichment terms were mainly down-regulated, indicating that *A. alternata* metabolism is inhibited during infection. This implies that the transcriptional response of *A. alternata* during citrus infestation is persistent and significant. Thus, our results further demonstrate that *A. alternata* is involved in the citrus brown spot infection process and plays an essential role in the regulation of citrus metabolism. The results suggest that transporter activity and binding exert a crucial role in the pathogenesis, whereas the down-regulated genes within the biological process group may be linked to the diminished metabolism.


**Figure 5.** Comparative Gene Ontology (GO) analysis was conducted on the differentially expressed genes (DEGs) between the transcriptome of *Alternaria alternata* and *A. alternata* inoculated on citrus leaves for 12-, 24-, and 48 h, common (differentially expressed genes that are commonly up-regulated or down-regulated in the three infection stages) and *A. alternata* under oxidative stress (WT + $H_2O_2$). The results were organized into three main GO categories (biological processes, cellular components, and molecular functions), with the count representing the number of DEGs annotated in each GO category. The x-axis indicated the different mutants, while the y-axis indicated the GO term. The color was used to denote the -log10(q value) of each GO category.

### *3.4. Multiple Critical Genes Pathways Were Affected in A. alternata during Infection*

We perform a comparative KEGG pathway analysis to explore the important metabolic pathways that were influenced during the infection stage (Figure 6). We mapped the differentially expressed genes (DEGs) to the reference pathways in KEGG to investigate the interaction between *A. alternata* and citrus. The results of the KEGG pathway analysis revealed that *A. alternata* inoculated on citrus leaves can significantly up-regulate many genes involved in several critical metabolic pathways, including the ABC transporters (ko02010), amino sugar and nucleotide sugar metabolism (ko00520), arginine and proline metabolism (ko00330), beta-alanine metabolism (ko00410), cyanoamino acid metabolism (ko00460), enzymes with EC numbers (ko99980), fatty acid degradation (ko00071), fructose and mannose metabolism (ko00051), glycerolipid metabolism (ko00561), glycolysis/gluconeogenesis (ko00010), other glycan degradation (ko00511), pentose and glucuronate interconversions (ko00040), peroxisome (ko04146), phenylalanine metabolism (ko00360), pyruvate metabolism (ko00620), starch and sucrose metabolism (ko00500), transporters (ko02000), tryptophan metabolism (ko00380), tyrosine metabolism (ko00350), valine, leucine and isoleucine degradation (ko00280). Furthermore, KEGG pathway enrichment analysis indicated that the down-regulated genes of *A. alternata* during infection stages were significantly associated with the pathways involved in the DNA replication proteins (ko03032), RNA polymerase (ko03020), amino acid related enzymes (ko01007), aminoacyl-tRNA biosynthesis (ko00970), mitochondrial biogenesis (ko03029), one carbon pool by folate (ko00670), oxidative phosphorylation (ko00190), phenylalanine, tyrosine and tryptophan biosynthesis (ko00400), pyrimidine metabolism (ko00240), ribosome (ko03010), ribosome biogenesis (ko03009), ribosome biogenesis in eukaryotes (ko03008), transfer RNA biogenesis (ko03016), translation factors (ko03012), indicating that *A. alternata* inoculated on citrus leaves can extensively affect many genes enriched in multiple critical metabolic pathways (Supplementary Table S7).

By examining the expression of genes enriched in the KEGG pathway, we found that the genes related to ROS production, such as those in the oxidative phosphorylation pathway, were down-regulated in all infection stages. Notably, 23 genes in the peroxisome pathway of *A. alternata* were up-regulated and 8 genes were down-regulated after inoculating on citrus leaves for 12 h. Furthermore, 47 genes were down-regulated, while a single gene, AALT_g4460 (PMA1, plasma membrane ATPase, OWY51174) was up-regulated in the oxidative phosphorylation pathway. Therefore, the results showed that oxidative phosphorylation was triggered after 12 h of *A. alternata* infection on citrus leaves.

Intriguingly, KEGG enrichment analysis revealed that *A. alternata* under oxidative stress significantly up-regulated the expression of many genes enriched in the ascorbate and aldarate metabolism (ko00053), cysteine and methionine metabolism (ko00270), glutathione metabolism (ko00480), glycerolipid metabolism (ko00561), lysine biosynthesis (ko00300), nucleotide excision repair (ko03420), pentose phosphate pathway (ko00030), phenylalanine, tyrosine and tryptophan biosynthesis (ko00400), pyruvate metabolism (ko00620), selenocompound metabolism (ko00450), sulfur metabolism (ko00920), tryptophan metabolism (ko00380). KEGG enrichment analysis also revealed that *A. alternata* under oxidative stress significantly down-regulated the expression of many genes enriched in the alanine, aspartate and glutamate metabolism (ko00250), cell cycle - yeast (ko04111), chaperones and folding catalysts (ko03110), chromosome and associated proteins (ko03036), meiosis (ko04113), ribosome biogenesis (ko03009), ribosome biogenesis in eukaryotes (ko03008). Notably, glutathione metabolism is an essential pathway for the removal of reactive oxygen species (ROS), and further research is needed to fully understand its role in ROS detoxification [25].

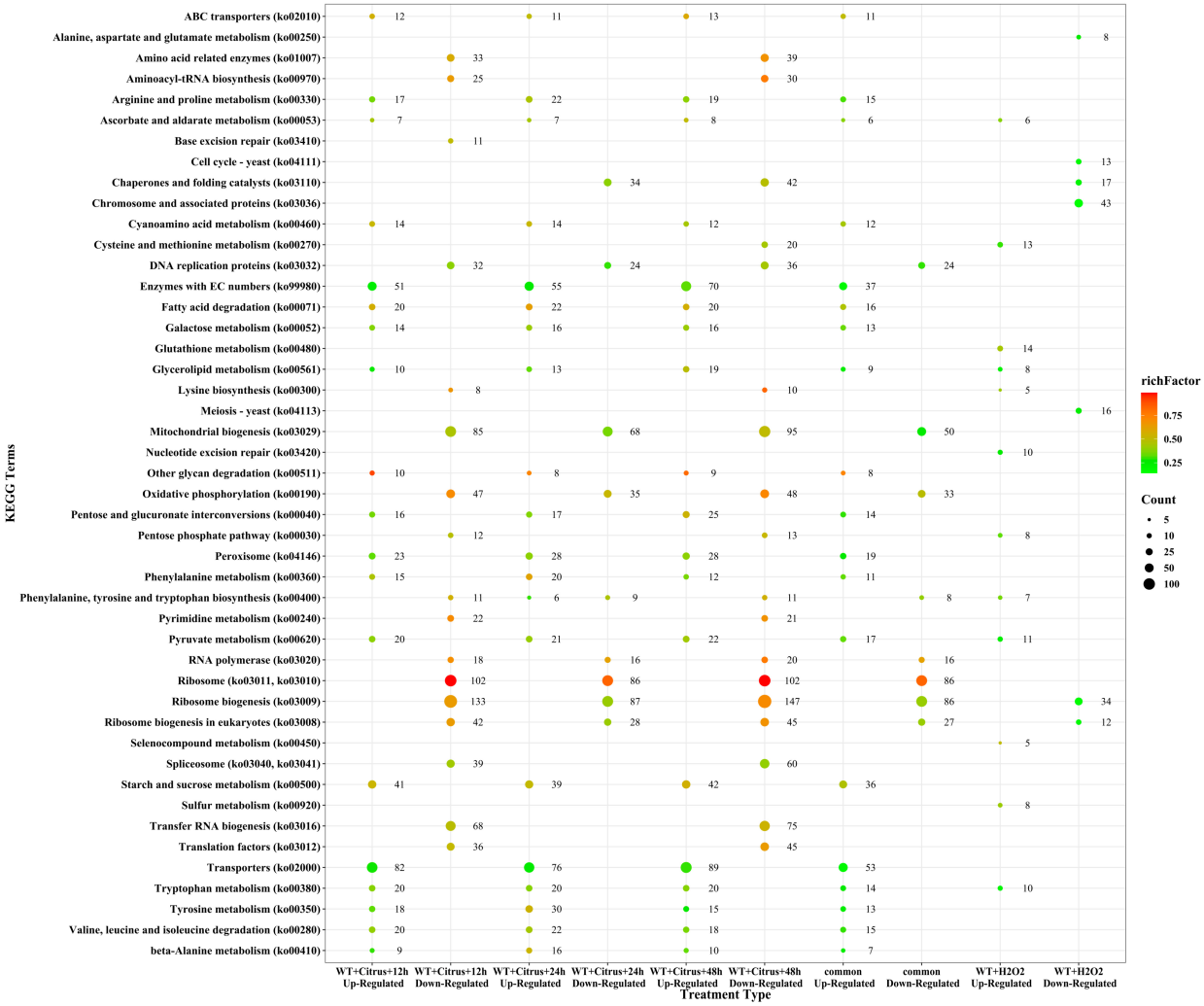


**Figure 6.** Comparative KEGG pathways analysis was conducted on the differentially expressed genes (DEGs) between the transcriptome of *Alternaria alternata* and *A. alternata* inoculated on citrus leaves at 12 h, 24 h, 48 h, common (differentially expressed genes that are commonly up-regulated or down-regulated in the three infection stages), and *A. alternata* under oxidative stress (WT + $H_2O_2$). The x-axis of the graph indicates different mutants while the y-axis displays the KEGG pathway. The count, which is represented in the graph, stands for the number of differentially expressed genes enriched in the pathway. Moreover, the color in the graph reflects the enrichment factor of each pathway.

### *3.5. Multiple Critical SM Gene Clusters Were Regulated in A. alternata during Infection*

*A. alternata* produces a variety of secondary metabolites with important ecological functions that enable them to adapt to various environments. These molecules have been exploited in host plant and medicinal applications, such as antibiotics and anti-infectives. To explore the regulatory role of secondary metabolites gene clusters during infection, we examined the transcriptional responses of 30 biosynthetic gene clusters in *A. alternata*, as predicted by antiSMASH 5.0 [42]. Overall, the results showed that *A. alternata* exhibited comprehensive regulation during infection. The gene expression of cluster 20 NRPS like, cluster 23 NRPS like, cluster 7 T1PKS, cluster 27 NRPS like, cluster 6 NRPS (dimethyloprogen), cluster 18 NRPS, cluster 19 NRPS like (phomopsins), cluster 1 NRPS, cluster 28 T1PKS (mellein), cluster 10 terpene, cluster 24 NRPS, and cluster 12 T1PKS were strongly up-regulated, while the transcript level of cluster 20 NRPS like, cluster 27 NRPS like, cluster 6 NRPS (dimethyloprogen), cluster 18 NRPS, cluster 3 T1PKS (melanin), cluster 19 NRPS like (Phomopsins), cluster 29 NRPS (ACT Toxin I), cluster 16 NRPS like, cluster 21 NRPS like, and cluster 11 T1PKS were significantly down-regulated in *A. alternata* during infection stages (Figure 7A).

Furthermore, the results showed that *A. alternata* can significantly up-regulate 2 genes in cluster29 NRPS (ACT Toxin I), including AALT_g11737 (putative oxidoreductase ucpA, OWY54909), AALT_g11748 (metalloprotease, OWY54920), and down-regulate three genes in cluster29 NRPS (ACT Toxin I), including AALT_g11751 (ACTT5, acyl-CoA synthetase, OWY54923), AALT_g11753 (hypothetical protein, OWY54925), AALT_g11758 (Aft11-1, OWY54930) during infection stages (Figure 7B).

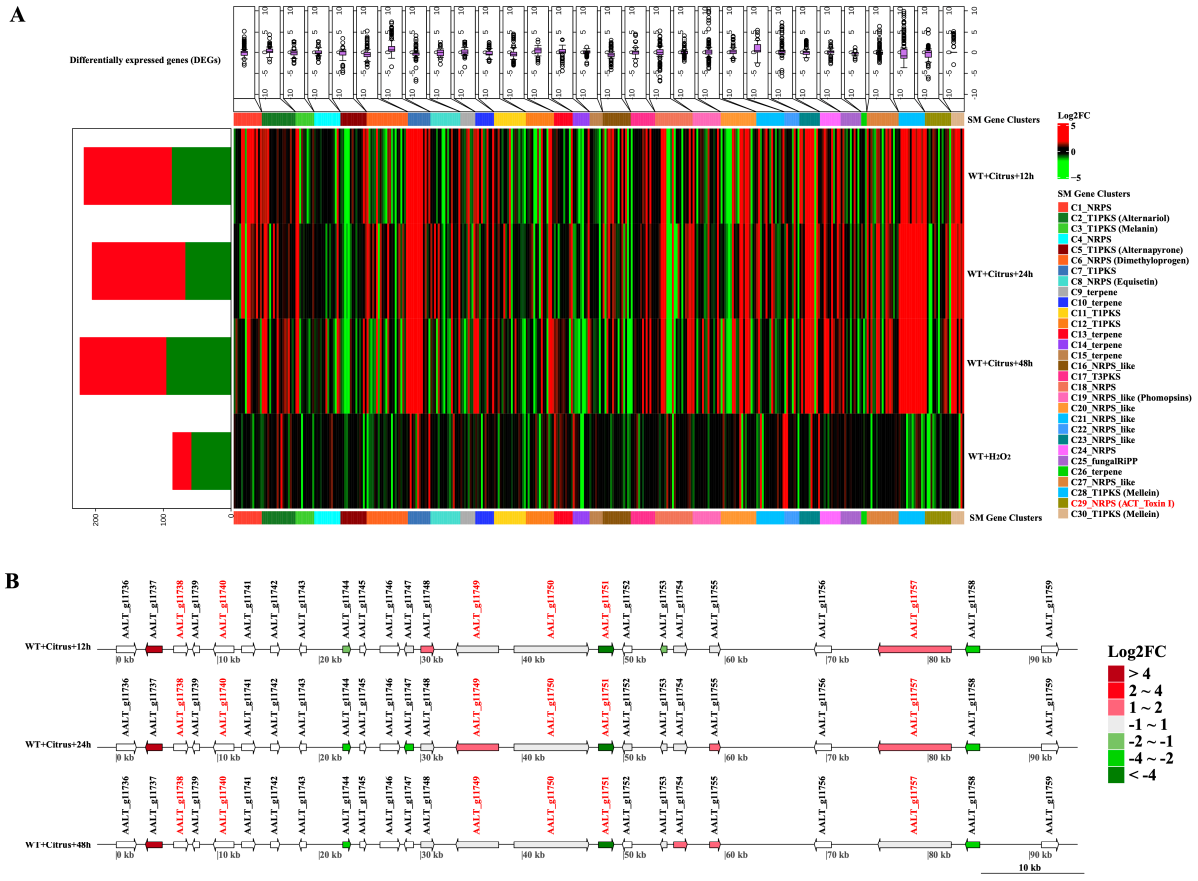


**Figure 7.** Multiple critical SM gene clusters were regulated in *Alternaria alternata* during infection. (**A**) The gene expression pattern of SM gene clusters in *A. alternata* when it was inoculated on citrus leaves for 12 h, 24 h, 48 h and *A. alternata* under oxidative stress ($H_2O_2$) was compared to that of *A. alternata* under the growth stage. The genes were represented in different colors to indicate their differential expression levels in the different treatments compared to the *A. alternata* under the growth stage. These included polyketide synthases (pks), nonribosomal peptide synthetases (nrps), Type1 (t1), putative biosynthetic types (cf_putative) and terpene synthetase (terpene). (**B**) The gene expression pattern of the ACT toxin gene cluster of *A. alternata* inoculated on citrus leaves for 12, 24, and 48 h was also compared to that of *A. alternata* under the growth stage. The text in red represents the core gene of the secondary metabolite gene cluster.

### *3.6. Multiple Critical Genes Related to Plant-Pathogen Interaction Were Affected in Alternaria alternata during Infection*

The results demonstrated that the transcript levels of 68 genes related to plant-pathogen interaction were up-regulated in *A. alternata* during infection stages (Table 1). We also found that the gene expression of 41 genes involved in plant-pathogen interaction was down-regulated in *A. alternata* inoculated on citrus leaves, indicating that certain genes are activated or suppressed during infection, and that the regulation of these genes is likely to be involved in the onset and development of disease. Furthermore, the findings suggest that regulating the expression of these genes may be a potential strategy for controlling plant-pathogen interaction. Therefore, this study has provided valuable information about

the molecular mechanisms of plant-pathogen interaction in *A. alternata* and may provide new ideas for the development of effective control strategies. These data may provide an important insight into the molecular mechanisms of plant-pathogen interaction in *A. alternata* during disease development stages.

**Table 1.** The genes of *Alternaria alternata* related to plant-pathogen interaction.

| Gene ID | Protein ID | WT + 12 h | WT + 24 h | WT + 48 h | WT + $H_2O_2$ | Annotation |
|---|---|---|---|---|---|---|
| AALT_g8702 | OWY49743.1 | −3.59 | −4.57 | −6.59 | −1.16 | CBM18, carbohydrate binding module family 18 |
| AALT_g9592 | OWY42369.1 | −4.42 | −2.14 | −4.00 | −1.84 | FTR1, iron permease FTR1 family |
| AALT_g6701 | OWY55098.1 | −3.51 | −2.61 | −3.97 | 2.05 | ERG3, sterol desaturase family |
| AALT_g260 | OWY42744.1 | −3.48 | −2.28 | −4.23 | −0.61 | ARG1, arginosuccinate synthase |
| AALT_g3024 | OWY52659.1 | −3.34 | −3.98 | −2.27 | −0.73 | Glycoside hydrolase family 28 protein |
| AALT_g3675 | OWY44810.1 | −3.51 | −2.48 | −3.18 | −0.01 | Jacalin like lectin domain |
| AALT_g5000 | OWY57849.1 | −2.27 | −2.82 | −3.32 | −0.67 | bZIP transcription factor |
| AALT_g7369 | OWY48574.1 | −3.45 | −1.70 | −2.93 | −0.01 | GPP1, haloacid dehalogenase like hydrolase |
| AALT_g7743 | OWY50139.1 | −2.77 | −1.99 | −3.17 | 0.13 | ERG10, belongs to the thiolase family |
| AALT_g7699 | OWY48045.1 | −2.59 | −2.33 | −2.94 | 1.28 | ERG11, belongs to the cytochrome P450 family |
| AALT_g2814 | OWY52449.1 | −2.12 | −2.37 | −3.15 | 0.09 | CCP1, belongs to the peroxidase family |
| AALT_g4788 | OWY57637.1 | −2.57 | −1.79 | −3.04 | −0.94 | URA5, phosphoribosyl transferase domain |
| AALT_g3969 | OWY45104.1 | −3.08 | −1.11 | −3.03 | −0.57 | ARG4, argininosuccinate lyase C terminal |
| AALT_g1579 | OWY41826.1 | −2.39 | −2.42 | −2.19 | 0.65 | Major facilitator superfamily |
| AALT_g5087 | OWY57936.1 | −1.88 | −2.12 | −2.96 | −0.21 | ARO4, stereospecific condensation of PEP |
| AALT_g9214 | OWY57068.1 | −2.36 | −1.77 | −2.69 | −0.31 | cyp1, PPIases accelerate the folding of proteins. |
| AALT_g9127 | OWY51854.1 | −2.14 | −1.43 | −2.80 | 0.26 | CDC7, protein tyrosine kinase |
| AALT_g3183 | OWY52818.1 | −2.08 | −1.48 | −2.47 | 0.14 | Mitochondrial PGP phosphatase |
| AALT_g6591 | OWY47556.1 | 2.20 | 2.79 | 1.29 | −0.24 | Belongs to the thiolase family |
| AALT_g8160 | OWY48788.1 | 2.41 | 2.18 | 2.03 | 1.04 | cytochrome P450 |
| AALT_g1296 | OWY41543.1 | 1.87 | 2.25 | 2.57 | 0.03 | Belongs to the glycosyl hydrolase 31 family |
| AALT_g6581 | OWY47546.1 | 2.25 | 2.11 | 2.34 | −0.06 | Homeodomain |
| AALT_g5268 | OWY58117.1 | 2.21 | 2.39 | 2.11 | −1.10 | CHS3, chitin synthase |
| AALT_g374 | OWY42858.1 | 2.53 | 1.58 | 2.95 | 0.55 | NCR1, Niemann Pick C1 N terminus |
| AALT_g4774 | OWY57623.1 | 2.55 | 2.39 | 2.27 | 0.03 | MaoC like domain |
| AALT_g4259 | OWY50973.1 | 1.92 | 2.22 | 3.20 | −0.44 | cyp3, PPIases accelerate the folding of proteins. |
| AALT_g8336 | OWY45374.1 | 2.39 | 1.42 | 3.68 | −0.20 | Alcohol dehydrogenase GroES like domain |
| AALT_g6866 | OWY55263.1 | 1.73 | 3.09 | 2.74 | −0.59 | Enoyl (acyl carrier protein) reductase |
| AALT_g65 | OWY42549.1 | 2.49 | 2.42 | 2.92 | −0.35 | Zinc finger transcription factor |
| AALT_g12014 | OWY45860.1 | 2.92 | 1.32 | 3.68 | −2.07 | Fungal specific transcription factor domain |
| AALT_g3883 | OWY45018.1 | 3.15 | 2.49 | 2.30 | −0.12 | Fungal specific transcription factor domain |
| AALT_g11132 | OWY55775.1 | 2.04 | 3.38 | 2.73 | 0.62 | Chitin binding domain |
| AALT_g6936 | OWY55333.1 | 2.50 | 3.71 | 2.34 | −0.13 | the iron ascorbate dependent oxidoreductase family |
| AALT_g10705 | OWY51536.1 | 3.70 | 2.70 | 2.63 | −2.75 | plyE, polysaccharide lyase family 3 protein |
| AALT_g5513 | OWY43764.1 | 3.71 | 2.74 | 3.90 | −0.41 | $C_2H_2$ type zinc finger |
| AALT_g6798 | OWY55195.1 | 3.27 | 3.49 | 4.22 | 0.17 | Basic region leucine zipper |
| AALT_g2197 | OWY46214.1 | 3.90 | 3.36 | 3.78 | −0.42 | Glycosyl hydrolase family 61 |
| AALT_g9490 | OWY49079.1 | 2.97 | 4.35 | 4.33 | 0.92 | CDR1, belongs to the ABC transporter superfamily |
| AALT_g1240 | OWY41487.1 | 4.00 | 4.29 | 3.56 | −0.31 | ENA2, belongs to the cation transport ATPase |
| AALT_g1011 | OWY41258.1 | 4.03 | 3.97 | 3.95 | −0.05 | $C_2H_2$ type zinc finger |
| AALT_g5353 | OWY43604.1 | 2.88 | 4.67 | 4.50 | −0.44 | Belongs to the thiolase family |
| AALT_g5423 | OWY43674.1 | 3.63 | 3.60 | 4.86 | 0.75 | scytalone dehydratase |
| AALT_g2730 | OWY46747.1 | 4.64 | 4.64 | 3.16 | 1.81 | polyketide synthase |
| AALT_g9423 | OWY49012.1 | 3.16 | 3.20 | 6.11 | 0.54 | Polysaccharide lyase family 1 protein |
| AALT_g6502 | OWY47467.1 | 3.17 | 4.67 | 4.71 | −0.63 | Polysaccharide lyase family 3 protein |
| AALT_g9572 | OWY42349.1 | 6.23 | 2.92 | 4.05 | −1.50 | brlA, zinc finger |
| AALT_g4000 | OWY45135.1 | 4.02 | 4.97 | 4.72 | −0.20 | ICL1, the isocitrate lyase PEP mutase superfamily. |
| AALT_g8883 | OWY54340.1 | 5.14 | 3.98 | 4.75 | −0.66 | the glycosyl hydrolase 11 (cellulase G) family |
| AALT_g1567 | OWY41814.1 | 3.53 | 6.08 | 4.49 | 0.36 | FAD binding domain |
| AALT_g3819 | OWY44954.1 | 3.84 | 4.78 | 6.69 | −0.37 | Polysaccharide lyase family 3 protein |
| AALT_g6230 | OWY47195.1 | 4.38 | 4.75 | 6.50 | 0.06 | the ABC transporter superfamily. ABCG family |
| AALT_g600 | OWY43084.1 | 4.57 | 5.71 | 5.45 | −0.14 | CDR1, the ABC transporter superfamily |
| AALT_g9863 | OWY52019.1 | 7.15 | 5.42 | 4.93 | −0.98 | EXG2, the glycosyl hydrolase 5 (cellulase A) family |
| AALT_g11942 | OWY51608.1 | 4.65 | 6.50 | 6.64 | 0.00 | KR domain |
| AALT_g2054 | OWY46071.1 | 5.91 | 6.53 | 6.60 | −1.02 | ENA2, the cation transport ATPase family |
| AALT_g9836 | OWY51992.1 | 5.81 | 5.87 | 8.22 | 0.39 | Pectate lyase |
| AALT_g9484 | OWY49073.1 | 5.23 | 6.91 | 8.24 | −0.41 | Polysaccharide lyase family 3 protein |
| AALT_g11182 | OWY53848.1 | 6.33 | 8.90 | 6.87 | −2.50 | SnoaL like domain |
| AALT_g11588 | OWY44559.1 | 7.27 | 8.13 | 7.22 | 0.96 | GMC oxidoreductase |
| AALT_g8391 | OWY45429.1 | 7.29 | 8.33 | 7.63 | 0.67 | Ricin b lectin |
| AALT_g3371 | OWY53006.1 | 9.60 | 8.07 | 7.13 | −0.04 | Fungal trichothecene efflux pump (TRI12) |

### *3.7. Multiple Critical Genes Related to Peroxisome Were Activated in Alternaria alternata during Infection*

Peroxisomes are small, membrane-enclosed organelles with at least 50 different enzymes involved in a variety of metabolic reactions, including several aspects of energy metabolism. Our RNA-seq analysis showed that at least 19 genes in the peroxisome pathway were up-regulated in *A. alternata* when inoculated on tangerine leaves, suggesting that *A. alternata* can rapidly respond to the host environment by regulating the expression of genes related to the peroxisome pathway. To validate these findings, qPCR was performed on nine randomly selected genes from the peroxisome pathway. The expression pattern of each gene, as quantitated in the qRT-PCR, was found to be consistent with the RNA-seq data (Figure 8). This suggests that peroxisome plays an essential role in the metabolic reaction of *A. alternata* during infection. The increased expression of the peroxisome pathway implies that peroxisome could be involved in the metabolic reactions of *A. alternata* during infection. The qRT-PCR results further validate the accuracy of the RNA-Seq data. Nonetheless, further experiments are needed to determine the precise role of peroxisome in the metabolic reaction of *A. alternata* during infection.

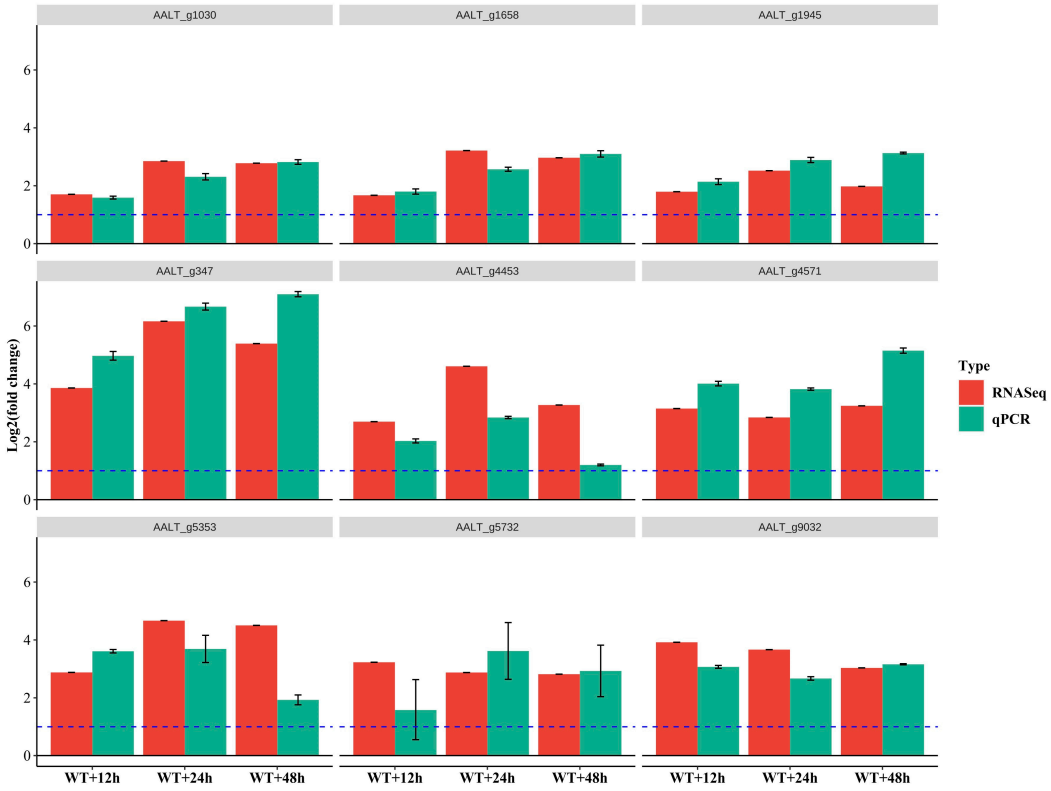


**Figure 8.** The expression of the gene related to the peroxisome pathway in *Alternaria alternata* during infection stages was investigated. For this purpose, mycelia of *A. alternata* Z7 were inoculated on tangerine leaves and harvested at 12-, 24-, and 48 h post-inoculation. The blue dotted line in the figure represents Log2(FC) = 1.

## 4. Discussion

Determining the gene expression pattern of pathogens during host infection is a fundamental research question in plant pathology [43,44]. To address this, we characterized the transcriptome of *A. alternata* inoculated on citrus leaves at 12-, 24-, and 48 h intervals post-inoculation. Our comparative transcriptome analysis revealed that the gene expression patterns at the three infection stages were significantly distinct from the control. Moreover, the gene expression patterns of the pathogen in different infection stages were found to be quite similar. Subsequently, we performed a series of functional analyses of *A. alternata* during infection stages, aiming to compare the similarities and differences in gene expression.

Our results suggest that *A. alternata* can swiftly modify its gene expression pattern in the course of infection, which is essential for the successful infection of the pathogen. It appears that *A. alternata* is capable of rapidly responding to environmental changes, such as nutrient availability, by adjusting its gene expression pattern. This suggests that *A. alternata* is a highly adaptive pathogen and is likely to be successful in establishing itself in a wide range of environmental conditions [18,25]. This knowledge can be utilized to further comprehend the pathogenic mechanism of the fungus and to develop effective strategies to control it.

Previous studies have conducted a transcriptome analysis that has uncovered the worldwide effects of $H_2O_2$ on gene expression, offering fresh insights into the probable pathways that the phytopathogen *A. alternata* utilizes to deal with oxidative stress [31]. The outcomes of this study demonstrated that a significant number of transcriptionally regulated genes in *A. alternata* are markedly changed during the infection of citrus. The up-regulation of genes related to transporters, amino acid metabolism, peroxisome, steroid biosynthesis, and oxidative phosphorylation pathways implies that *A. alternata* metabolism is considerably modified during the infection process. Moreover, the noteworthy up-regulation of certain metabolic process categories, such as hydrolase activity, hydrolyzing O-glycosyl compounds, transmembrane transporter activity, oxidoreductase activity, and iron ion binding, demonstrates that these genes could take part in controlling the interaction between *A. alternata* and its host plants. To further the understanding of the precise roles of the up-regulated genes in the infection process and to identify potential targets for controlling the spread of *A. alternata* infections, more studies are necessary.

Surprisingly, our research revealed that *A. alternata* can modulate its biological and physiological processes by adjusting its RNA metabolism, which is beneficial for the infection process. In addition, we observed that many genes related to transmembrane transporter activity, such as those in the major facilitator superfamily (MFS) transporter family, were significantly up-regulated in *A. alternata* during the infection stage compared to the control. Furthermore, pathways relevant to antioxidation, such as "oxidoreductase activity" and "redox process", were identified as being of importance in the *A. alternata*. Significantly, many of these key functional genes were also profoundly affected during oxidative stresses, indicating that *A. alternata* can be influenced by various reactive oxygen species (ROS) and host stresses [32]. In fact, it is well established that the invasion of pathogens can trigger the release of ROS, and cause extensive damage to proteins, cell membrane lipids, and the genetic material DNA. Extensive studies have demonstrated that the disruption of genes related to the ROS scavenging pathway can result in decreased pathogenicity on host plants, while the inactivation of ROS stress regulators can lead to increased sensitivity to oxidative stresses, and thus reduced virulence in host plants [29,45–47].

The findings from this study suggest that *A. alternata* can adjust its biological and physiological processes in order to effectively adapt to the host environment [48]. By up-regulating the expression of genes associated with the antioxidation pathway, *A. alternata* can improve its protection against the oxidative stress induced by the host. This enhanced protection facilitates the pathogen's growth and infection of the host. Furthermore, it appears that by inhibiting the expression of ROS stress regulators, *A. alternata* is able to increase its virulence on the host. Moreover, this study provides evidence that *A. alternata* has the capacity to regulate its biological and physiological processes in order to enhance its adaptation to the environment. The findings demonstrate the significance of the antioxidation pathway and its role in allowing the pathogen to infect the host [49]. Additionally, it seems that the suppression of ROS stress regulators can lead to amplified virulence in *A. alternata*. This knowledge could be utilized to design approaches to control the propagation of this pathogen.

The ability to withstand various stresses is essential for the survival and successful infection of *A. alternata*, allowing the pathogen to conquer host-generated reactive oxygen species (ROS) [18,50]. Subsequently, KEGG pathway enrichment analysis demonstrated the particular relevance of oxidative respiration-related pathways to this investigation. Upon analyzing the expression of genes in the pathways, it was observed that the genes related

to ROS generation were significantly upregulated, while those linked to ROS degradation were significantly downregulated, in accordance with *A. alternata*'s transition from aerobic to anaerobic conditions. This may suggest that *A. alternata* actively attempts to minimize the effect of ROS on itself during infection. Therefore, it is clear that ROS plays a critical role in the *A. alternata* infection process. Consequently, an increase in ROS levels in citrus may potentially help decrease the prevalence of citrus brown spot. In addition, the potential of *A. alternata* to withstand stress is evidently vital in the survival and infection process, which was further confirmed by KEGG pathway enrichment analysis. Furthermore, the expression of genes related to ROS generation were significantly upregulated, while those linked to ROS degradation were significantly downregulated, implying that *A. alternata* is actively counteracting the impact of ROS on itself during infection. Thus, it can be hypothesized that increasing ROS levels in citrus may assist in reducing the incidence of citrus brown spot caused by *A. alternata*. For a more comprehensive understanding of how *A. alternata* responds to oxidative stress and how this knowledge can be applied to control the fungal pathogen, further research is needed.

Secondary metabolites have been demonstrated to be of great importance in the pathogenesis of *A. alternata*, and many related genes to secondary metabolism have yet to be characterized [51,52]. Accordingly, this study sought to investigate the effect of secondary metabolic gene expression during *A. alternata* infection in citrus. Our results revealed that two typeIPKs metabolic gene clusters (C7 T1PKS and C28 T1PKS (Mellein)) had significantly up-regulated DEGs. These gene clusters are responsible for the synthesis of polyketides which generate a set of secondary metabolites found in fungi. The increased expression of the T1PKS gene cluster implies that *A. alternata* may have been secreting a large amount of mycotoxins to infect citrus. This indicates that secondary metabolic gene expression is involved in the infection process of *A. alternata* in citrus and that polyketides may be playing a role in defense or intercellular transport. Consequently, further research is required to gain a better understanding of the role that secondary metabolic gene expression plays in *A. alternata* infection. Specifically, further investigations should focus on the expression of other secondary metabolic genes and the role they play in the infection process. Additionally, it would be beneficial to analyze the effects of different environmental and physiological conditions on the expression of these genes, as well as explore the downstream effects of gene expression, such as the production of mycotoxins [26].

## 5. Conclusions

The present study provides novel insight into the expression pattern of essential metabolic processes of *A. alternata*, which was found to be significantly affected at the transcriptional level upon infecting citrus and ROS being a critical factor in the development of citrus brown spot. These results deepen our understanding of the interaction between *A. alternata* and citrus. Nevertheless, to gain a more complete view of this interaction, further studies should be conducted to explore the role of secondary metabolic gene expression during *A. alternata* infection, the effects of different environmental and physiological conditions on gene expression, as well as the expression of these genes in the *A. alternata*-infected citrus fruits to assess the potential contribution of secondary metabolites to resistance or susceptibility to the disease.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy13030809/s1, Table S1: list of all the primers used for RT-qPCR analysis; Table S2: The KOG annotation of *Alternaria alternata* Z7 in this study; Table S3: The differentially expressed genes (DEGs) of wildtype during 12 h infection; Table S4: The differentially expressed genes (DEGs) of wildtype during 24 h infection; Table S5: The differentially expressed genes (DEGs) of wildtype during 48 h infection; Table S6: The gene expression of different GO and its annotation in this study; Table S7: The gene expression of different KEGG pathway and its annotation in this study; Table S8: Protein sequence in the present study.

**Author Contributions:** Conceptualization, Y.G. and C.J.; methodology, Y.G. and L.L.; software, Y.G.; validation, Y.G.; formal analysis, Y.G.; investigation, Y.G. and M.W.; resources, Y.G. and X.S.; data curation, Y.G., M.W., H.M. and X.S.; writing—original draft preparation, Y.G., Q.N., J.K., X.L., X.Z. and Y.C.; writing—review and editing, Y.G., Q.N., X.L., N.S. and C.J.; visualization, Y.G.; supervision, Y.G.; project administration, Y.G., H.L. and C.J.; funding acquisition, Y.G., H.L. and C.J. All authors have read and agreed to the published version of the manuscript.

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**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** All the RNA-Seq Illumina data have been deposited at NCBI under the BioProject accession no. PRJNA895027. All the Protein sequences involved in this study can be found in Supplementary Table S8.

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**Conflicts of Interest:** The authors declare no conflict of interest.

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