



Article

Transcriptome and Physiological Analyses of Resistant and Susceptible Pepper (*Capsicum annuum*) to *Verticillium dahliae* Inoculum

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Abstract: Pepper (*Capsicum annuum*) is a globally important vegetable, and Verticillium wilt is an important disease affecting peppers and is caused by *Verticillium dahliae*, which can severely reduce yields. However, the molecular mechanisms underlying the responses of pepper to infection by *V. dahliae* are largely unknown. We performed physiological and transcriptome analysis using resistant and susceptible pepper cultivars inoculated with *V. dahliae*. Compared to the susceptible cultivar MS66, the resistant cultivar MS72 retained higher chlorophyll content and lower malondialdehyde content after inoculation. At 3 days after inoculation (DAI), compared with MS66, 534 differentially expressed genes (DEGs) were identified in MS72. At 5 DAI, 2392 DEGs were identified in MS72 compared with MS66. The DEGs in MS72 were mainly enriched in the cell wall and photosynthesis-related Gene Ontology terms, as well as in pathways such as cutin, suberin, wax biosynthesis, phenylpropanoid biosynthesis, and photosynthesis. Using weighted gene co-expression network analysis, 36 hub genes involved in the resistance response were identified, including the transcription factor *bHLH93* (Capana04g000815), defense-like protein 1 (MSTRG.5904), and miraculin-like (Capana10g002167). Our findings contribute to a more comprehensive understanding of the response mechanism of pepper to *V. dahliae* inoculation, providing new avenues for improving pepper resistance through breeding programs.

Keywords: pepper; Verticillium wilt; *Verticillium dahliae*; chlorophyll content; malondialdehyde concentration; differentially expressed genes; weighted gene co-expression network analysis; hub genes



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1. Introduction

Pepper (*Capsicum annuum*) is an annual or perennial plant of the Solanaceae family, native to Mexico and South America, and widely planted in tropical and subtropical regions worldwide [1]. Pepper is an economically important crop that is closely associated with human life. It not only serves as an essential fresh vegetable and seasoning in human diets, but is also an important industrial raw material in pharmaceutical, agricultural, and cosmetic industries [2,3]. Pepper is rich in vitamins, minerals, and antioxidants and has a unique flavor that can add color and taste to various dishes while providing health benefits [2,3]. Capsanthin and capsorubin extracted from pepper have important applications in the food, chemical, and agricultural industries [4]. According to data from the Food and Agriculture Organization of the United Nations (FAO) in 2022, China's pepper cultivation area of 759,817 ha exceeded one-third of the global pepper cultivation area (2,020,816 ha). However, the production of peppers is often affected by various abiotic and biotic constraints [5,6]. In particular, Verticillium wilt, caused by the fungus *Verticillium dahliae*, poses a serious threat to global pepper production [7–10].

Verticillium wilt is a disease caused by soil-borne pathogens, mainly including *V. dahliae*, *V. alboatrum*, *V. nigrescens*, *V. nubilum*, and *V. tricorpus*. Verticillium wilt was first reported in Sweden in 1918 [11]. Since then, Verticillium wilt has been observed and reported worldwide [10,12–14]. In 2014, Verticillium wilt was first detected in Gansu, China [15]. Verticillium wilt can affect over 400 plant species globally [16,17]. The *V. dahliae* in soil can infect peppers through root wounds. When the fungus enters the xylem vessels of the root, it rapidly reproduces and spreads along the xylem vessels to the aboveground parts of the peppers. As the disease progresses, symptoms such as leaf yellowing, progressive necrosis, leaf shedding, and delayed development appear, ultimately decreasing fruit yield and quality [8,13].

The prevention and control of *V. dahliae* is extremely difficult, as this fungus develops a resting structure known as microsclerotia, which can survive in soil for more than 14 years [18]. Presently, the prevention of pepper verticillium wilt is primarily dependent on chemical control methods; however, the environmental persistence and associated adverse effects of pesticides on human health remain a problem [19,20]. There are also ways of using beneficial bacteria for biological control, such as *Funneliformis mosseae* and *Bacillus siamensis*, to prevent and treat pepper Verticillium wilt caused by *V. dahliae* [21–23]; however, these methods have certain limitations, including unstable effectiveness and high costs. Generally, breeding cultivars that are resistant to *V. dahliae* in the pepper is considered to be the most effective control method [24,25]. Remarkably, certain pepper germplasms that are resistant to *V. dahliae* have been identified. Gurung et al. reported that eight pepper germplasms, including Grif 9073, PI 281396, PI 281397, PI 438666, PI 439292, PI 439297, PI 555616, and PI 594125, exhibited high resistance to *V. dahliae* VdCa59 and *V. dahliae* VdCf45 [9]. P.I. 215699 (a mixture of *C. baccatum* var. *microcarpum* and *C. annuum*), P.I. 535616 (*C. annuum*), and P.I. 555614 (*C. annuum*) also exhibit high resistance to *V. dahliae* Kleb [25]. Although multiple germplasms have been screened for resistance to *V. dahliae*, the resistance genes of these germplasms have not been cloned, and the resistance mechanism has not been elucidated.

The response of plants to pathogens is a complex process involving a series of biological reactions and physiological processes. In a study on the use of arbuscular mycorrhizal fungi (AMF) to prevent and control pepper Verticillium wilt, AMF were found to enhance pepper resistance by increasing the levels of new subtypes of acidic chitinase and superoxide dismutase (SOD), and by enhancing the activities of peroxidase and phenylalanine ammonia-lyase in peppers [26]. Another study revealed that AMF and salicylic acid (SA) increased resistance to Verticillium wilt by enhancing the activity of antioxidant enzymes such as SOD, ascorbate peroxidase, and catalase in chili plants [27]. In addition, studies have revealed that an increase in early phenolic compounds may be the reason why COA H (a formulation containing SA, soluble ammonium salts, and seawater extract from *Ascophyllum nodosum*) enhances the resistance of pepper seedlings to Verticillium wilt [28]. However, the molecular changes underlying these physiological responses of resistance to Verticillium wilt are unclear, and knowledge about the resistance mechanism of peppers to *V. dahliae* is limited.

In recent years, significant progress has been made using transcriptome technology to reveal the mechanisms underlying plant disease resistance. Using transcriptomics to study the response mechanisms of two different wheat varieties resistant to Fusarium head blight, Zhao et al. found that glutathione metabolism, phenylpropanoid biosynthesis, plant hormone signal transduction, and plant–pathogen interactions in the Kyoto Encyclopedia of Genes and Genomes (KEGG) were related to disease resistance. Additionally, five pathogenesis-related proteins were found to be involved in resistance regulation [29]. After inoculation with *Coniella diprodella*, transcriptome analysis of the leaves of the two grape varieties by Li et al. showed that the defense mechanisms of resistant varieties involved plant and pathogen interactions, sulfur transfer systems, and biosynthesis of sesquiterpenes and triterpenes, suberin, wax, and monoterpenes, and biosynthesis pathways of flavonoids and flavonols [30]. Transcriptome analysis showed that the phenylpropanoid biosynthesis

pathway was activated in cotton after inoculation with *V. dahliae*. Upon combining the results of this analysis with metabolomics data, five phenylpropane metabolites, caffeic acid, coniferyl alcohol, coniferin, scopoletin, and scopolin, were identified. In vitro experiments showed that they can significantly inhibit the growth of *V. dahliae*, indicating that they may play an important role in cotton's resistance to *V. dahliae* [31]. Recent studies have shown that the endophytic fungus *Gibellulopsis nigrescens* CEF08111 can significantly enhance pathways involved in plant–pathogen interactions, mitogen-activated protein kinase (MAPK) signaling, and plant hormone signal transduction, thereby improving cotton resistance to Verticillium wilt after 12 h of inoculation. The genes encoding calcium-dependent protein kinase (CDPK), flagellin sensing 2 (FLS2), resistance to *Pseudomonas syringae* pv. *maculicola* 1 (RPM1), and mitosis protein 2 (MYC2) may be involved in regulating resistance to cotton Verticillium wilt [32].

To understand the molecular-level changes of different resistant materials in peppers after infection with *V. dahliae*, we conducted physiological and transcriptome analysis at three different time points after inoculation with *V. dahliae* in both resistant and susceptible cultivars. We identified the DEGs of these two cultivars and screened candidate genes for pepper resistance to *V. dahliae*. These results provide a foundation for understanding the response mechanism of peppers to *V. dahliae*, and offer valuable information for breeding disease-resistant pepper cultivars.

2. Materials and Methods

2.1. Plant Materials and Treatment

The pepper cultivars MS66 and MS72 used in this experiment were selected and provided by Maoming Maoshu Seed Industry Technology Co., Ltd. (Maoming, China). Our previous research has shown that MS66 is susceptible to *V. dahliae*, resulting in yellowing and wilting of leaves after inoculation, whereas MS72 is resistant to *V. dahliae*, with minimal leaf yellowing observed after inoculation. Peppers were sown on 5 October 2023, and seedlings were planted in a polyethylene film greenhouse (20–28 °C; natural sunlight; 11–12 h of sunlight per day) at Guangdong University of Petrochemical Technology. On 17 January 2024, leaves with similar shapes and sizes, no gaps, healthy physiological status, and no pests or diseases were selected for inoculation with *V. dahliae*. *V. dahliae* was grown on potato dextrose agar (PDA, Huankai, Guangzhou, China) dishes at 24 °C for 5 days, in the dark. A single colony was selected and incubated in sucrose sodium nitrate liquid medium (Huankai) at 24 °C for 5 days in the dark. Then, a suspension of 10^7 conidia/mL was prepared from the culture using distilled sterile water [33]. The petioles of detached leaves were placed in a spore suspension. The detached leaves were stored at a temperature of 24 ± 2 °C, with a day–night light cycle of 16/8 h [34,35]. Samples of 30 leaves with three replicates were collected before inoculation (day 0) and 3 and 5 days after inoculation (DAI), and labelled MS66-0, MS66-3, and MS66-5; and MS72-0, MS72-3, and MS72-5 for MS66 and MS72, respectively. Ten leaves from each sample period were frozen in liquid nitrogen and ground into fine powder for total RNA extraction. An additional 20 leaves were used to measure chlorophyll and malondialdehyde (MDA) content.

2.2. Biochemical Indices

Chlorophyll content was measured according to the methods outlined by Lichtenthaler et al. [36]. A uniformly sized sample of pepper leaves was obtained using a punching machine, and 0.1 g of the sample was weighed and soaked in 20 mL of 95% ethanol under dark conditions for 36 h. Afterward, 100 µL of the supernatant was taken and placed in an enzyme-linked immunosorbent assay (ELISA) plate. The light absorption values were measured at 665 nm and 649 nm using an ELISA reader (ReadMax 1200, Shanpu, Shanghai, China). The chlorophyll a (Ca) and chlorophyll b (Cb) contents were calculated as follows: $Ca = 13.95A_{665nm} - 6.88A_{649nm}$, $Cb = 24.96A_{649nm} - 7.32A_{665nm}$. MDA content was estimated using thiobarbituric acid (TBA) [37]. Leaf material (1 g) was homogenized in liquid nitrogen and homogenized in 5 mL 0.1% (w/v) TCA (Shanghai Yuanye Bio-Technology,

Shanghai, China) solution. The homogenate was centrifuged at $10,000 \times g$ for 30 min (4°C), and 0.5% TBA (Shanghai Yuanye Bio-Technology) was added to the obtained supernatant. The MDA concentration was measured spectrophotometrically at 532 nm. The analysis was performed in triplicate and displayed as mean $\pm$ SE. Statistical analysis was conducted using one-way ANOVA, and statistical significance ($p < 0.05$) was calculated using Duncan's test at the 5% level. Statistical analyses were performed using Sigmacalot11 (Systat, San Jose, CA, USA) and Excel software 2016 (Microsoft, Redmond, WA, USA).

2.3. RNA Extraction, Sequencing, and Data Analysis

Total RNA was extracted using TRIzol reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol. After extracting the total RNA, eukaryotic mRNA beads were enriched with oligo (dT). A NEBNext Ultra RNA Library Illumina Preparation Kit (NEB # 7530; New England Biolabs, Ipswich, MA, USA) was used for library preparation. The library was purified and amplified using polymerase chain reaction (PCR) after being connected to an Illumina sequencing adapter. The obtained cDNA library was sequenced by Gene Denovo Biotechnology Co. (Guangzhou, China) using an Illumina Novaseq 6000 (San Diego, CA, USA).

The raw sequence data reported in this study have been deposited in the Genome Sequence Archive (Genomics, Proteomics and Bioinformatics 2021) in the National Genomics Data Center (Nucleic Acids Res 2022), China National Center for Bioinformation/Beijing Institute of Genomics, Chinese Academy of Sciences (GSA: CRA018402), and are publicly accessible at <https://ngdc.cncb.ac.cn/gsa> (accessed on 16 August 2024) [38].

FASTP (version 0.18.0) [39] was used to filter raw data. Using Bowtie2, the ribosome reads were removed from the alignment, and the clean reads were compared with the chili genome solgenomics_ C. annuum _zunla [40]. The mapping readings for each sample were assembled using StringTie v1.3.1 [41] in a reference-based manner.

2.4. Differentially Expressed Gene Analysis and Weighted Gene Co-expression Network Analysis (WGCNA)

For each transcription region, RSEM [42] software was used to calculate the number of fragments read per kilobase transcript per million mappings (FPKM) to quantify its expression abundance and changes. Principal component analysis (PCA) and Pearson correlation coefficient analysis were performed on the gene expression levels of eighteen samples using OmicSmart (www.omicssmart.com) from Gene Denovo Biotechnology Co. (Guangzhou, China). DESeq2 (version 1.30.0) [43] was used to analyze the differential gene expression between the two groups. A false detection rate (FDR) < 0.05 and $|\log_2 \text{fold change (FC)}| > 1$ were used as screening criteria to obtain DEGs between the two samples. DEG sequences were compared with Gene Ontology (GO) databases (<http://www.geneontology.org/>, accessed on 10 March 2024) [44]. We also compared DEGs with the KEGG database using BLASTX and obtained the corresponding pathway annotation information [45].

Gene co-expression networks of DEGs for MS62 vs. MS72 at 3 DAI and 5 DAI were constructed using OmicSmart (<http://www.omicssmart.com>, accessed on 29 August 2024) [46]. Gene dendrograms were constructed using colors based on the correlations between the expression levels of genes, and were used to build clustering trees and divide the modules. In addition, correlations between the modules and samples were analyzed using WGCNA.

2.5. Validation of RNA-Seq Data by qRT-PCR

To verify the RNA-Seq results, nine genes were selected for qRT-PCR analysis using *CaActin* as an internal reference gene; specific primers were designed and are listed in Supplementary Table S1. The reactions were carried out in triplicate in a Vazyme FMR3 (Vazyme, Nanjing, China) system with AceQ Universal SYBR qPCR Master Mix (Vazyme). The reaction mixture consisted of 5 μL 2 $\times$ AceQ Universal SYBR qPCR Master Mix, 1.5 μL cDNA template, 0.4 μL each primer (10 $\mu\text{mol}/\mu\text{L}$), and 2.7 μL nuclease-free water. The

amplification program was 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. Melting curve analyses were performed at the end of the 40 cycles (95 °C for 5 s followed by a constant increase from 60 °C to 95 °C). Relative gene expression levels were normalized to that of *CaActin* and determined with the $2^{-\Delta\Delta CT}$ method [47]. SigmaPlot v.11 (Systat, San Jose, CA, USA) software was used for statistical analysis and data display.

3. Results

3.1. Disease and Biochemical Indices Assessment

Morphological changes on the leaves of the two pepper cultivars inoculated with *V. dahliae* were recorded at 3 and 5 DAI (Figure 1A). On the inoculated leaves of MS66, yellowing surrounded the leaf veins at 3 DAI. No yellowing was observed in the leaves of MS72. After 5 DAI, all of the MS66 leaves turned yellow and began to wilt, while MS72 leaves showed small yellow areas, and no signs of wilt (Figure 1A). Compared with the resistant MS72, the chlorophyll a and b contents of the leaves of MS66 decreased rapidly (Figure 1B,C), reaching a reduction of 72.1% and 75.4% at 5 DAI, respectively. The MDA concentration in the susceptible cultivar MS66 increased rapidly (Figure 1D). During the tested inoculation periods, the MS72 leaves retained high chlorophyll content and exhibited low peroxidation of membrane lipids, supporting the resistance to the used *V. dahliae* strain.

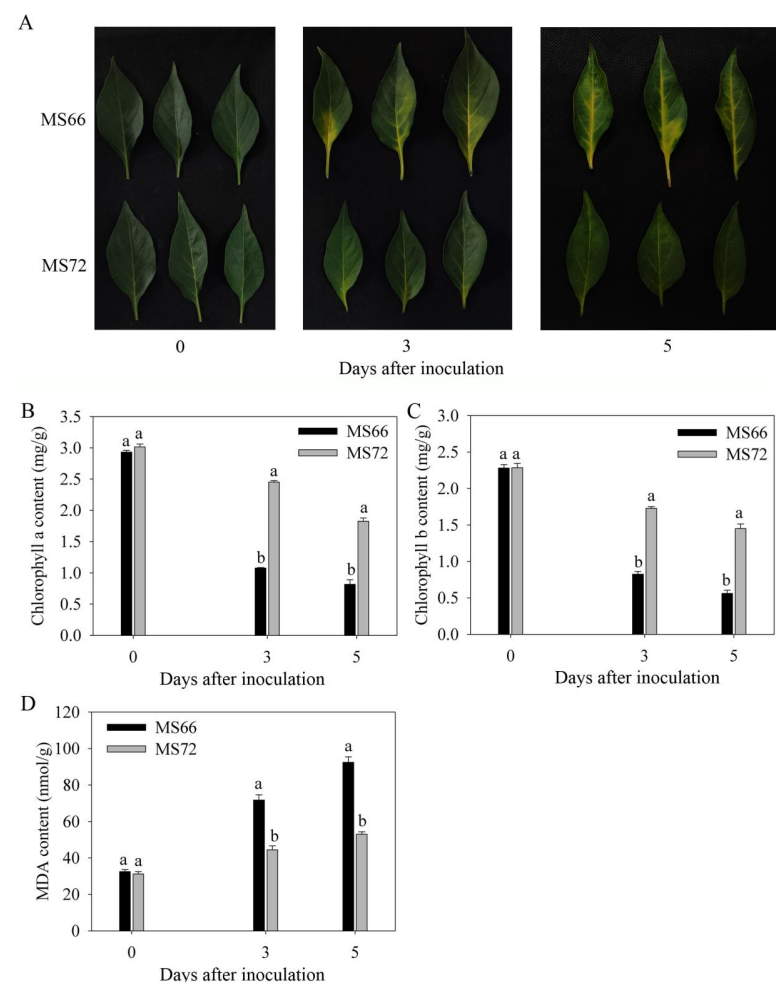


Figure 1. Interaction between *Verticillium dahliae* and MS66 (susceptible) and MS72 (resistant) pepper cultivars: (A) leaf phenotypes and symptom development; (B) chlorophyll a content; (C) chlorophyll b content; (D) malondialdehyde (MDA) content at 0, 3, and 5 days after inoculation (DAI). Histograms represent the mean ($n = 3$) $\pm$ SE. Values with the same letters are not significantly different according to Duncan's test ($p < 0.05$).

3.2. Sequencing Data Analysis

Through RNA sequencing, 111.9 billion raw reads were generated from 18 samples. After filtering, 111.0 billion high-quality clean reads were used for further analysis (Table 1). The average Q20 and Q30 values of clean data were 97.88% and 93.81%, respectively (Table 1). Additionally, the GC content of all samples exceeded 40% (Table 1). These results indicate that the sequencing quality is high and can be used for subsequent analysis. The PCA results show that three biological replicates can aggregate, forming six clear groups from 18 samples (Supplementary Figure S1). The Pearson correlation coefficient results showed a high correlation between biological replicates, which further demonstrated that the biological repeatability of sequencing data is high and scientific (Supplementary Figure S2). Collectively, the results show that the generated RNA-Seq sequencing data were reliable.

Table 1. Overview of the RNA sequencing data of 18 libraries. AF: After filter.

Sample	RawData (bp)	CleanData (bp)	AF_Q20 (%)	AF_Q30 (%)	AF_GC (%)
MS66-01 *	6,981,718,800	6,915,710,005	97.63%	93.34%	42.38%
MS66-02	6,258,249,000	6,197,996,540	97.49%	92.97%	42.63%
MS66-03	5,653,788,000	5,604,126,720	97.84%	93.71%	42.59%
MS66-31	5,674,388,700	5,629,439,592	98.05%	94.07%	42.13%
MS66-32	6,523,661,100	6,471,830,669	97.91%	93.82%	42.16%
MS66-33	6,125,935,800	6,079,521,125	98.06%	94.16%	42.21%
MS66-51	5,974,112,700	5,929,944,107	97.93%	93.83%	42.03%
MS66-52	6,492,510,300	6,429,441,859	98.05%	94.10%	42.01%
MS66-53	5,613,444,300	5,579,199,070	98.05%	94.11%	41.96%
MS72-01	5,662,599,600	5,608,834,245	97.56%	93.32%	42.72%
MS72-02	6,383,261,100	6,319,523,672	97.60%	93.37%	42.70%
MS72-03	6,431,895,000	6,377,583,237	97.71%	93.60%	42.70%
MS72-31	6,634,712,700	6,587,352,685	98.00%	94.00%	42.14%
MS72-32	6,406,942,800	6,364,254,446	98.11%	94.26%	42.22%
MS72-33	6,544,051,500	6,498,195,200	98.05%	94.20%	42.19%
MS72-51	6,177,454,500	6,125,703,488	97.94%	93.88%	42.09%
MS72-52	6,192,131,100	6,142,110,988	98.03%	94.11%	41.99%
MS72-53	6,225,490,200	6,168,686,984	97.89%	93.78%	41.89%

* The last digit of the sample name indicates biological duplication.

3.3. Transcriptome Profiles Analysis

3.3.1. DEGs of MS66 and MS72 Response to *V. dahliae* Inoculation

Upon inoculating the susceptible cultivar MS62 with *V. dahliae*, 5134 DEGs were identified at 3 DAI; 1916 of these genes were up-regulated, and 3218 down-regulated. At 5 DAI, 6909 genes were differentially expressed in MS66, with 2396 up-regulated and 4513 down-regulated. In the resistant cultivar MS72, 5073 genes were differentially expressed at 3 DAI, with 2204 up-regulated and 2869 down-regulated. After 5 days of inoculation, 4203 DEGs were identified in MS72, with 1848 up-regulated and 2335 down-regulated. Further analysis of the gene expression differences between two different cultivars after inoculation showed that, compared with susceptible cultivar MS62, 534 genes were differentially expressed in the resistant cultivar MS72 at 3 DAI, including 325 up-regulated and 209 down-regulated genes. At 5 DAI, a total of 2392 genes were differentially expressed in MS72 compared with MS66, with 1236 up-regulated and 1156 down-regulated genes (Figure 2).

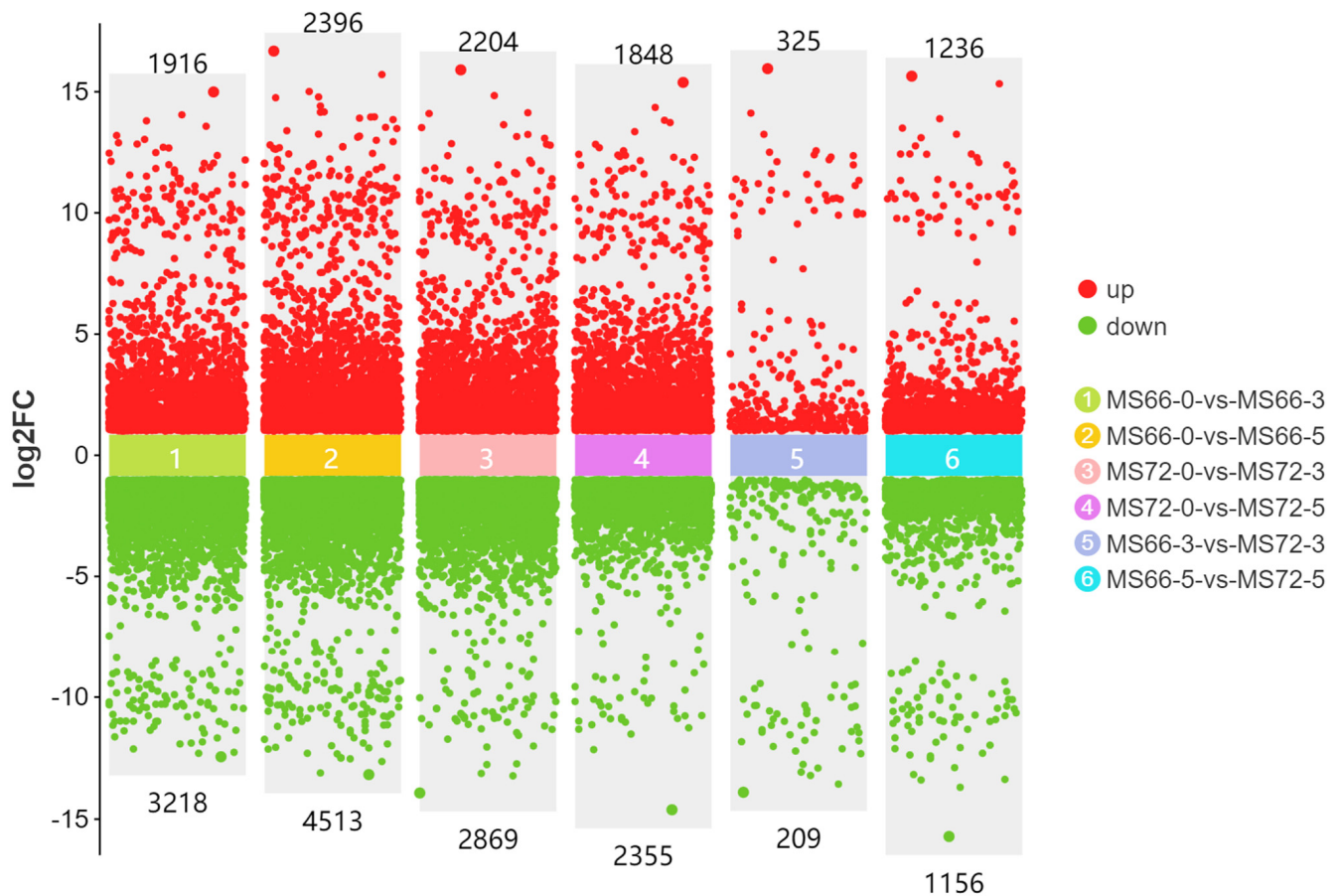


Figure 2. Number of differentially expressed genes (DEGs) in six comparative combinations of MS66 and MS72. Red dots indicate up-regulated DEGs, while green dots indicate down-regulated DEGs. The digits above the red dots and below the green dots represent the number of up-regulated DEGs and down-regulated DEGs, respectively.

3.3.2. GO Analyses of DEGs

The DEGs of the susceptible cultivar MS62 were mainly enriched in GO items related to chloroplast structure and kinases at 3 DAI, including photosynthetic membrane (GO: 0034357) and kinase activity (GO: 0004672) (Supplementary Figure S2, Table S2). At 5 DAI, the DEGs of MS62 were mainly enriched in GO items related to chloroplast structure (GO: 0009507) and photosynthesis (GO: 0015979) (Supplementary Figure S3, Table S2). Surprisingly, in the susceptible cultivar MS66, most DEGs in significantly enriched GO terms were down-regulated (Supplementary Figures S2 and S3).

In resistant cultivar MS72, the DEGs at 3 DAI were mainly enriched in GO items related to chloroplast structure and redox reactions, including photosynthetic membrane (GO:0034357), protein kinase activity (GO:0004672), and phosphorylation (GO:0006468) (Supplementary Figure S3, Table S2). At 5 DAI, the DEGs of MS72 were mainly enriched in GO items related to thylakoid (GO:0009579), photosynthetic membrane (GO:0034357), and cell wall organization (GO:0071555) (Supplementary Figure S3, Table S2).

At 3 DAI, the DEGs of MS72 compared with MS66 were significantly enriched in stress response and cell wall synthesis-related ontologies, such as the cell wall macromolecular metabolic process (GO:0044036) and response to oxidative stress (GO:0006979) (Figure 3A, Table S2). In contrast, GO terms significantly enriched for chloroplast structure and photosynthesis-related processes, including photosynthesis (GO:0015979) and photosynthetic membranes (GO:0034357), were observed in MS72 compared with MS66 at 5 DAI. In addition, compared with MS66, most DEGs of MS72 at 3 and 5 DAI were up-regulated in significantly enriched GO terms (Figure 3A, Table S2).

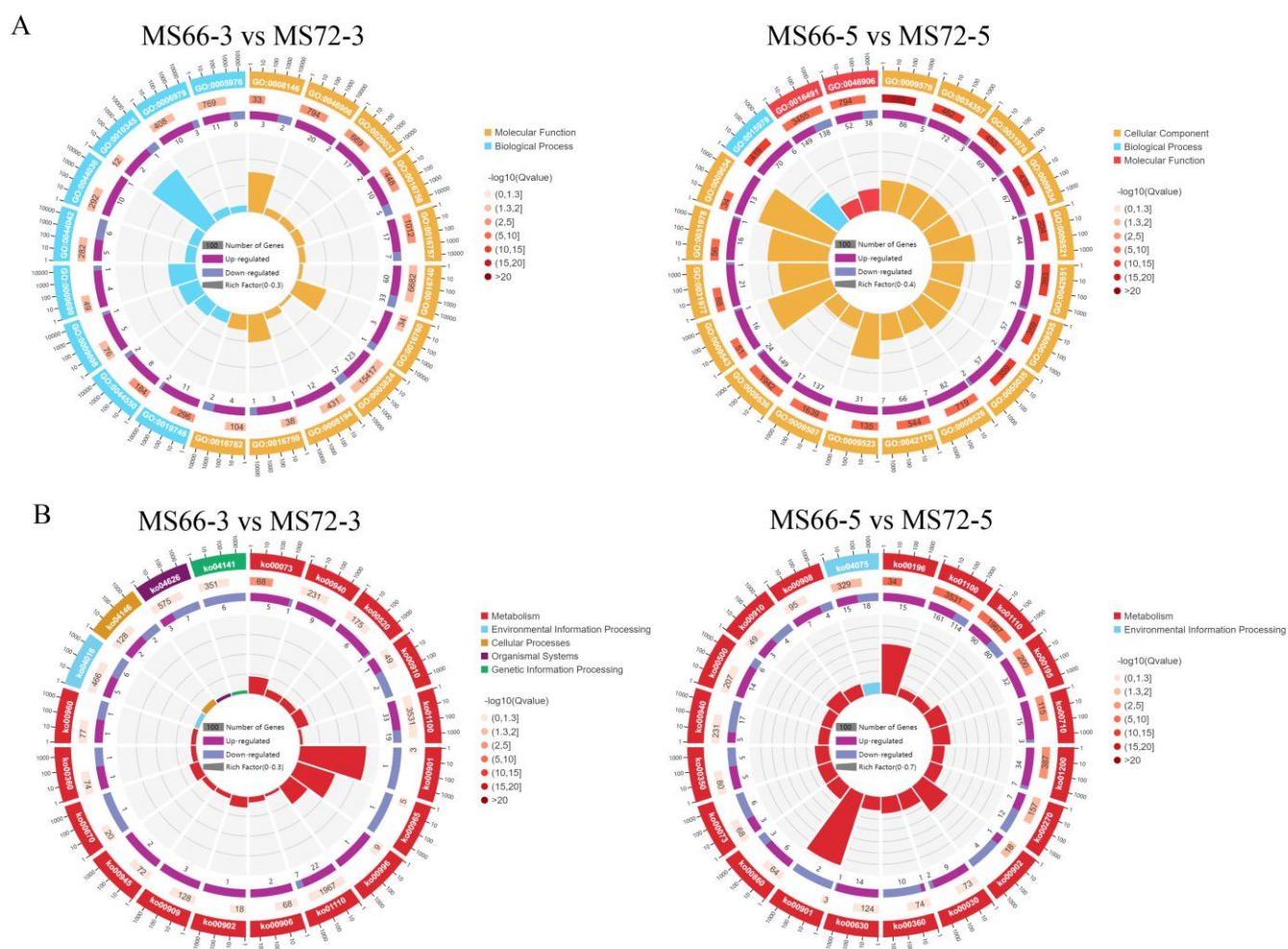


Figure 3. Top twenty enriched GO annotations (A) and KEGG annotations (B) for differentially expressed genes in MS66-3 vs. MS72-3 and MS66-5 vs. MS72-5 groups.

3.3.3. Pathway Enrichment Analyses of DEGs

In MS66-0 vs. MS66-3, MS66-0 vs. MS66-5, and MS72-0 vs. MS72-3 DEG comparisons, significantly enriched pathways were observed to be related to signaling pathways, including the MAPK signaling pathway in plants (ko04016), plant–pathogen interactions (ko04626), and plant hormone signal transduction (ko04075) (Supplementary Figure S3, Table S3). In the MS72-0 vs. MS72-5 comparison, the top 20 significantly enriched pathways were primarily related to metabolic pathways and photosynthesis, including phenylpropanoid biosynthesis (ko00940), stilbenoid, diarylheptanoid, and gingerol biosynthesis (ko00945), starch and sucrose metabolism (ko00500), and photosynthesis (ko00195) (Supplementary Figure S3, Table S3). Compared with the susceptible cultivar MS66, DEGs in the resistant cultivar MS72 at 3 DAI were significantly enriched in pathways such as cutin, suberin, and wax biosynthesis (ko00073) (Figure 3B, Table S2). At 5 DAI, DEGs in MS72 were significantly enriched in phenylpropanoid biosynthesis (ko00940), starch and sucrose metabolism (ko00500), photosynthesis (ko00195), and plant hormone signal transduction (ko04075) compared with MS66 (Figure 3B, Table S3).

3.3.4. Resistance Signaling Related Pathways Analysis to DEGs

We further analyzed the expression characteristics of signal pathways in DEGs. In the plant–pathogen interaction pathway, eight genes (Capana02g002878, Capana02g003359, Capana02g003630, Capana10g001171, Capana11g000029, Capana11g000407, Capana11g000420, Capana11g000436, and Capana11g000436) encoding calcium-binding proteins (CML),

two WRKY transcription factors (Capana01g000167 and Capana09g001251), and mitogen-activated protein kinase 3-like (*MPK3*, Capana06g002562) in the susceptible cultivar MS66 were significantly up-regulated at 5 DAI. Conversely, in the resistant cultivar MS72, respiratory burst oxidase homologous protein B (*RbohB*, Capana08g001513), pathogenesis-related protein 1A-like (*PRB1*, Capana08g002191), and two LRR receptor-like serine/threonine protein kinases (*EFR*, MSTRG.19679 and MSTRG.4848) were up-regulated at 5 DAI (Figure 4A, Table S4).

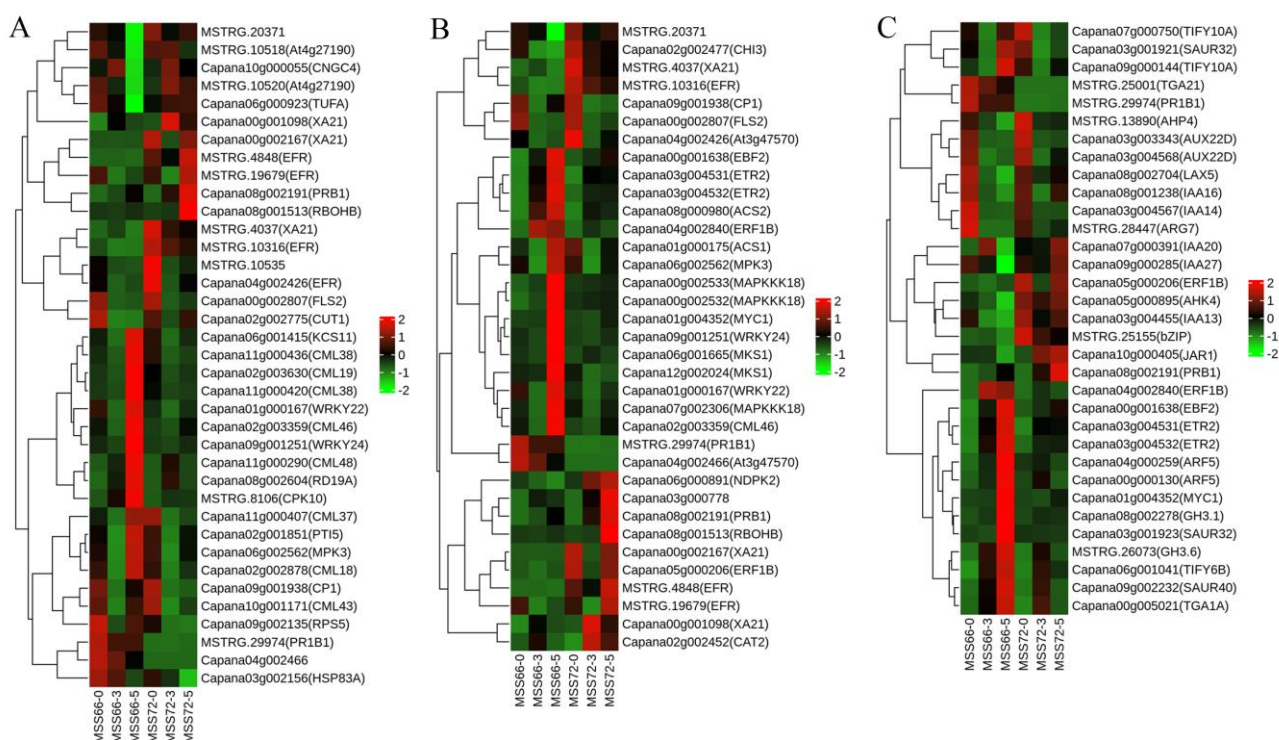


Figure 4. Heat map of DEGs in three resistance signaling-related pathways. (A) Plant–pathogen interaction; (B) MAPK signaling pathway—plant; (C) plant hormone signal transduction. The row Z-scores of the fragments per kilobase of transcript per million mapped fragments data are shown.

In the MAPK signaling pathway, three mitogen-activated protein kinase kinase kinase 2-like (Capana00g002532, Capana00g002533, and Capana07g002306), *MPK3* (Capana06g002562), two ethylene receptor 2 genes (*ETR2*, Capana03g004531 and Capana03g004532), two WRKY transcription factors (Capana01g000167 and Capana09g001251), and transcription factor MYC2-like (Capana01g004352) were significantly up-regulated at 5 DAI in the susceptible cultivar MS66. In contrast, in the resistant cultivar MS72, catalase isozyme 2 (Capana02g002452), *RbohB* (Capana08g001513), *PRB1* (Capana08g002191), and two *EFR* (MSTRG.19679 and MSTRG.4848) were up-regulated at 5 DAI (Figure 4B, Table S4).

Sixteen DEGs were up-regulated in the susceptible cultivar MS66 after inoculation; among these DEGs, two *CaETR2* (Capana03g004531 and Capana03g004532) and one ethylene-responsive transcription factor 1B-like (ERF1B, Capana04g002840) belong to the ethylene signaling pathway (Figure 4C, Table S4). In addition, seven DEGs belonged to the auxin signaling pathway (Figure 4C, Table S4), four DEGs, including two auxin-responsive protein (IAA, Capana07g000391, Capana09g000285) belonging to the auxin signaling pathway, jasmonic acid-amido synthetase JAR1-like (JAR1, Capana10g000405) belonging to the JA signaling pathway, and pathogenesis-related protein 1A-like (PR1, Capana08g002191) belonging to the SA signaling pathway, were up-regulated in the resistant cultivar MS72 after inoculation (Figure 4C, Table S4).

3.3.5. Key Gene Modules Screened by WGCNA

A gene clustering tree was constructed based on the correlation between gene expression levels, and the gene modules were divided according to the clustering relationship between the genes, yielding 16 stable expression modules (Figure 5A,B). Among these modules, the blue module contained the most genes, with 523 genes, whereas the grey module contained only two genes (Figure 5B).

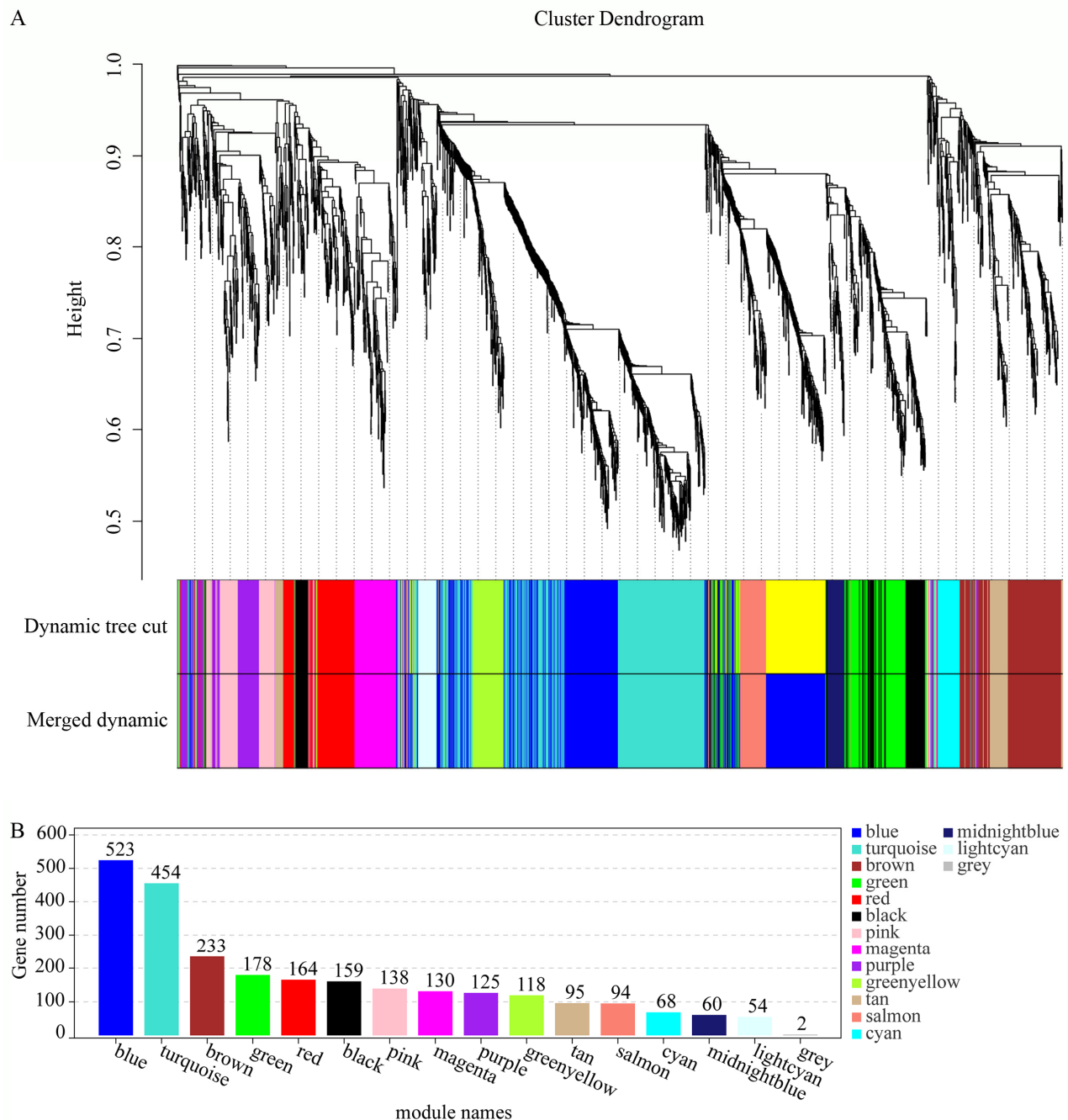


Figure 5. Key gene modules screened by WGCNA. (A) Module-level clustering diagram of DEGs based on WGCNA analysis. “Dynamic Tree Cut” refers to the module division based on clustering results. “Merged Dynamic” represents the division of merged modules with expression patterns similar to module similarity. (B) Number of genes in each module, with modules named after colors.

The module-sample expression pattern heatmap showed that the genes in the pink and purple modules of the resistant cultivar MS72 were significantly up-regulated after inoculation, whereas the genes of the susceptible cultivar MS66 did not show significant changes after inoculation (Figure 6). This indicates that these up-regulated genes may be involved in regulating disease resistance.

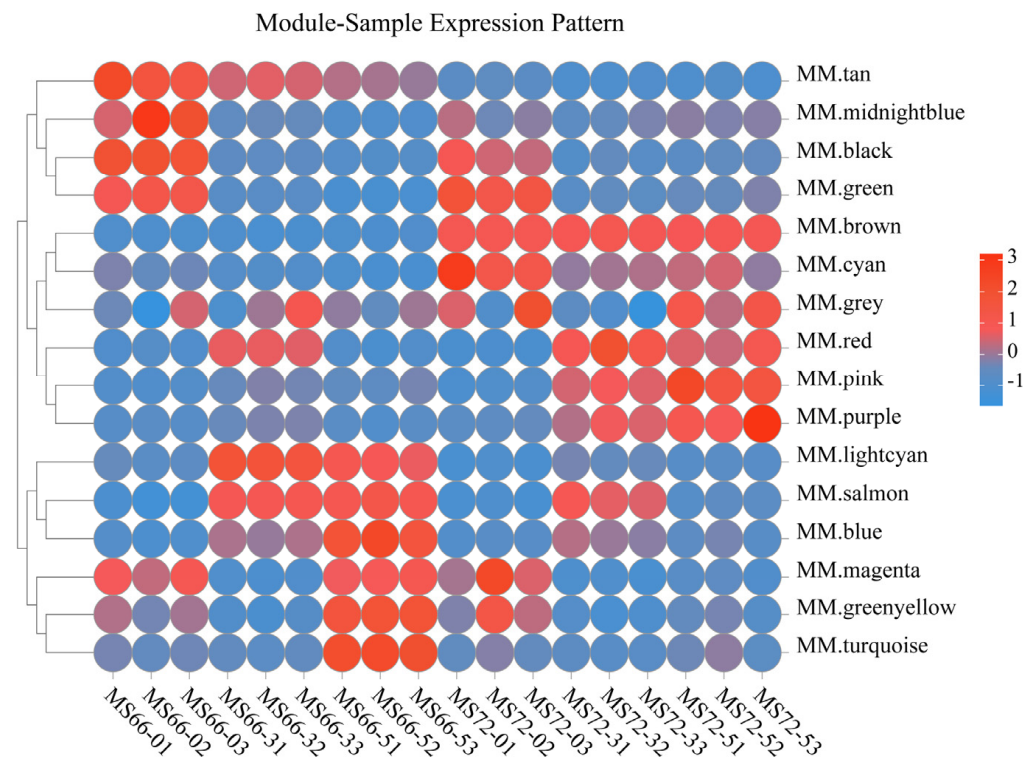


Figure 6. Expression pattern of each gene module in different samples. The x-axis represents different samples, and the y-axis represents different modules. The red color in the figure indicates high expression level, while the blue color indicates low expression level.

3.3.6. Hub Gene Analysis of Key Modules by WGCNA

In the pink module, six genes were located in the core position based on gene connectivity values and were selected as hub genes (Figure 7A, Table S5), including zeatin O-glucosyltransferase-like isoform X1 (MSTRG.32287), UPF0481 protein At3g02645 (Capana05g000620), GTP-binding protein-like (Capana09g002310), hypothetical protein BC332_17964 (Capana07g000070), wound-induced proteinase inhibitor 2-like (Capana03g001467), and Pin-II type proteinase inhibitor 7 (Capana03g001469). Among them, wound-induced protein inhibitor 2-like (Capana03g001467) and Pin-II type protein inhibitor 7 (Capana03g001469) both belong to the PIN-II protein family and exhibit fungicide-like effects [48].

In the purple module, 29 genes were located in the core position and were selected as hub genes (Figure 7B). These hub genes included transcription factor bHLH93-like (*bHLH93*, Capana04g000815). In addition, many of these hub genes are involved in plant resistance regulation, such as flower-specific defensin-like (*D1*, MSTRG.5910, MSTRG.24407, MSTRG.36069, and MSTRG.5904), serine protease inhibitor 1-like (*SPI*, Capana03g001482), miraculin-like (*MLP*, Capana06g000841 and Capana10g002167), chitin-binding lectin 1-like (*CBLs*, Capana03g000778), pectinesterase 2.2 (*PME2.2*, MSTRG.2104), and cellulose synthase-like protein G1 (*CSLG1*, Capana07g001101) (Figure 7B, Table S5).

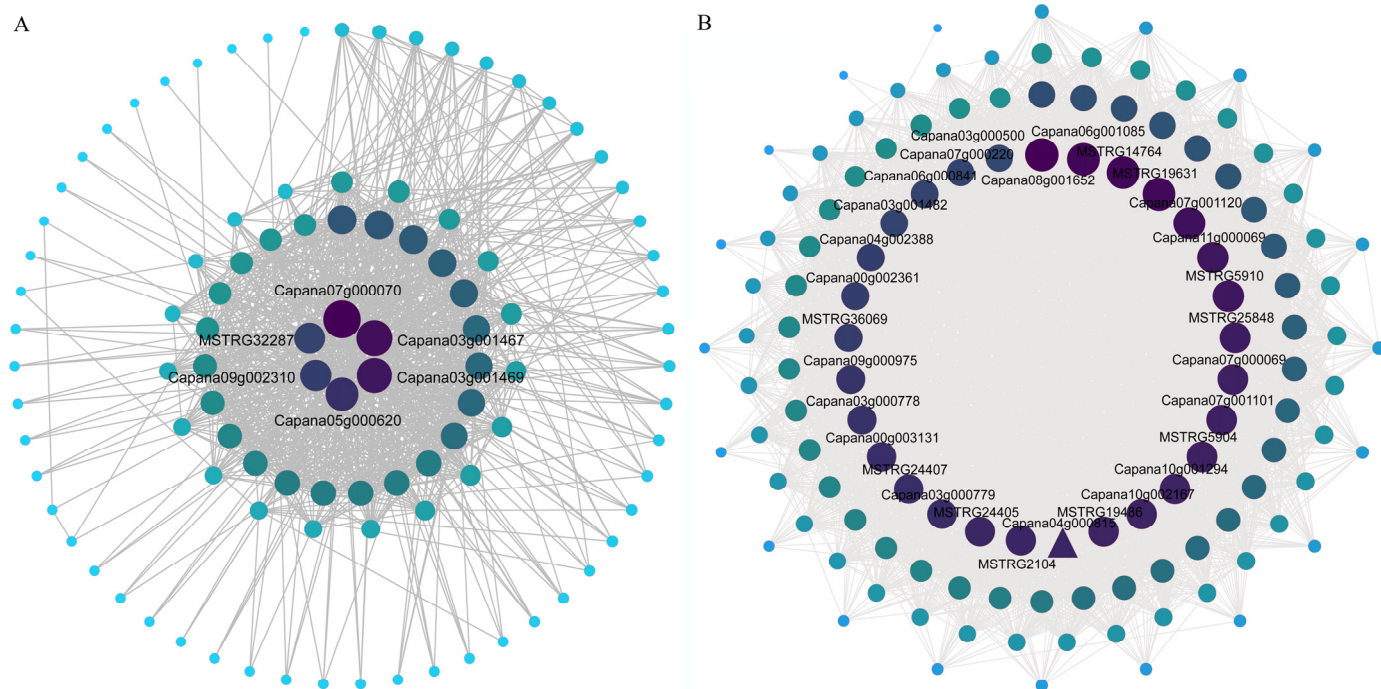


Figure 7. Gene interaction network analysis in the pink (A) and purple (B) modules. Different colors display connectivity abundance, with purple indicating high connectivity, blue indicating low connectivity, and triangles representing transcription factors.

3.4. Validation of RNA-Seq Data by Quantitative Real-Time (qRT)-PCR

The qRT-PCR and RNA-Seq results of nine hub genes showed similar expression profiles, with a Pearson correlation coefficient of 0.9281 ($p < 0.0001$) between the RNA-Seq and qRT-PCR data, confirming the reliability of the RNA-Seq data (Figure 8).

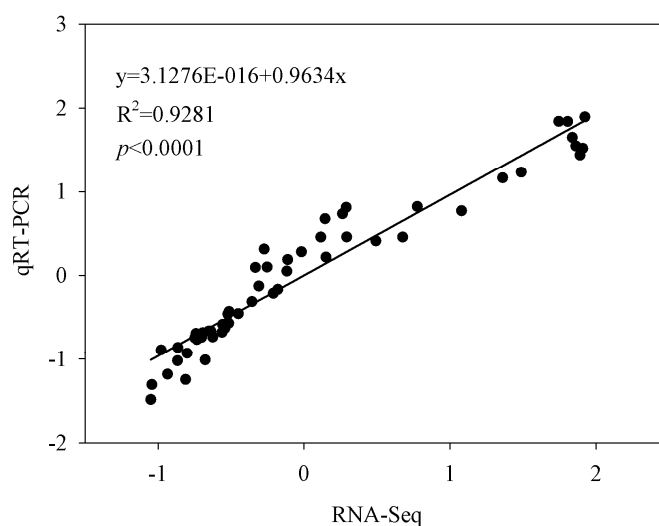


Figure 8. Validation of the RNA-Seq gene expression data by quantitative reverse transcription PCR (qRT-PCR). Nine hub genes from the RNA-Seq data were used for qRT-PCR assays. The row Z-scores of the fragments per kilobase of transcript per million mapped fragments and qRT-PCR data are shown.

4. Discussion

Pepper is an important global crop, serving not only as a key vegetable, but also as a vital industrial raw material for the pharmaceutical, agricultural, and cosmetics indus-

tries [2,3]. Verticillium wilt is a serious disease that causes yellowing of pepper leaves and significantly decreases pepper yield [8,13]. Although research has been conducted in recent years on pepper resistance to Verticillium wilt [26–28], and resistant cultivars have been developed [24,25], the gene regulatory network underlying the molecular mechanisms that confer resistance has not been explored. Furthermore, the key resistance genes and their regulatory mechanisms remain unclear, which is not conducive to breeding resistant pepper cultivars. Numerous studies have shown that the differential expression and regulation of resistance-related genes play important roles in plant responses to various pathogens [29–32]. Our research demonstrates that infection with *V. dahliae* causes lipid peroxidation of cell membranes in MS66, leading to a rapid decrease in chlorophyll content, whereas MS72 maintains high chlorophyll levels and low MDA content, indicating its resistance to *V. dahliae*. Subsequently, we used RNA-Seq to analyze the transcriptomes of resistant (MS72) and susceptible (MS66) cultivars at different time points after inoculation with *V. dahliae*. In MS66, DEGs at 3 DAI and 5 DAI were mainly enriched in GO terms related to chloroplast structure, kinase signaling, and photosynthesis, with most being down-regulated. In the resistant cultivar MS72, DEGs were mainly enriched in the chloroplast structure, kinase signaling, cell wall components, and antioxidant enzymes after inoculation. Further analysis of gene expression differences between the two cultivars at the same time points showed that DEGs were mainly enriched in the GO terms related to cell wall synthesis, reactive oxygen species stress, and photosynthesis, with most of these genes up-regulated in the resistant cultivar MS72. The GO analysis results of DEGs are consistent with the observed physiological changes, indicating that these genes are key genes in response to *V. dahliae* infection in different resistant peppers.

KEGG results showed that the DEGs in both cultivars were enriched in three signaling pathways: plant–pathogen interaction, MAPK signaling, and plant hormone signaling transduction. These findings align with those of previous research studies, which showed that inoculation with *V. dahliae* in plants such as cotton [49] and tomato [50] can lead to differential expression in three signaling pathways. There were significant differences in the expression patterns of the three signaling pathway genes between the two resistant pepper cultivars. Ca^{2+} influx is an early key response in PAMP signal transduction mediated by the channel protein CNGC [51]. Ca^{2+} can activate CDPKs, which are intracellular calcium signaling response proteins that play an important role in plant responses to abiotic stress and pathogens [52,53]. In this study, we found that *CaCNGC* and *CaCPK* expressions were up-regulated in the resistant cultivar MS72 compared to MS66. Simultaneously, the expression of the downstream respiratory burst oxidase homologous protein B (*CaRbohB*) was up-regulated. Mutating respiratory burst oxidase homologs in rice can reduce resistance to leaf blight in mutant rice [54], whereas transient expression of tomato *SlRbohB* in tobacco enhances resistance to *Botrytis cinerea* [55]. This suggests that the up-regulation of *CaRbohB* expression may be related to the resistance of MS72 to *V. dahliae*. WRKY is one of the largest transcription factor families in plants, widely involved in plant responses to biotic, abiotic, and hormonal stress. Among them, WRKY22 and WRKY24 are key transcription factors in plant–pathogen interactions and MAPK signaling pathways that can induce downstream signaling pathways to promote defense responses against pathogens and pests [56,57]. Interestingly, *CaWRKY22* and *CaWRKY24* expression were down-regulated in the resistant cultivar MS72. Research on sweet oranges has shown that *CsWRKY22* negatively regulates resistance to ulcer disease, and its overexpression promotes susceptibility to ulcer disease in Wanjincheng orange, whereas RNAi-mediated inhibition of *CsWRKY22* expression enhances resistance to ulcer disease [58].

Plant hormones play important regulatory roles in plant defense responses [59]. In this study, we found that most DEGs in the ethylene signaling pathway of the susceptible cultivar MS66 were significantly up-regulated, indicating that ethylene signaling may be involved in the regulation of yellowing and senescence of MS66 pepper leaves (Figure 4C, Table S4). Two *CaIAA*, one *CaJAR1*, and one *CaPR1* were up-regulated in the resistant cultivar MS72 after inoculation (Figure 4C, Table S4). IAA proteins play an important

role in the auxin signaling pathway and are widely involved in plant development and responses to abiotic and biotic stresses. Notably, the overexpression of MeAux/IAA in tobacco can enhance resistance to *Xanthomonas axonopodis* pv. *manihotis* (Xan), whereas silencing MeAux/IAA confers sensitivity [60]. JA signal-deficient mutant *jar1* was found to be more susceptible to *Plasmodiophora brassicae* in *Arabidopsis* [61]. In cotton, the overexpression of *GhMYB36* can up-regulate the expression of *GhPR1*, thereby enhancing *V. dahlia* resistance. The down-regulation of *GhPR1* gene expression through VIGS makes plants more susceptible to *V. dahlia* infection and exhibits a more severe wilting phenotype compared to control plants [62]. This result indicates that the *CaIAA*, one *CaJAR1*, and one *CaPR1* may be involved in regulating resistance to *V. dahliae* of pepper.

Specific plant proteins are often activated under pathological conditions and are crucial for combating diseases. We used WGCNA to screen 35 hub genes in the pink and purple modules, including five *CaD1* genes (MSTRG.5910, MSTRG.5904, MSTRG.24405, MSTRG.24407, and MSTRG.36069) encoding plant defensins. Plant defensins are small alkaline peptides that function as protein translation inhibitors, alpha-amylase and protease inhibitors, or ion channel blockers [63,64]. Their most significant activity is their ability to inhibit the growth of various fungi and yeasts [63–65]. We also identified two *CaMLP* genes whose expressions were up-regulated in MS72 through WGCNA. MLPs are members of the Kunitz trypsin inhibitor family that play a role in protecting plants from pathogen invasion [66,67]. Overexpression of *StMLP1* in potatoes enhances their resistance to *Pseudomonas aeruginosa* [68]. Many plants and animals have CBLs, which can interfere with the synthesis and/or deposition of chitin in fungal cell walls. Therefore, most CBL compounds exhibit antifungal activity against plant pathogens [69–71], with the cell wall being the first line of defense against external pathogens [72]. A *CaCBL* (Capana03g000778) was screened as a hub gene and significantly up-regulated in MS72, indicating its potential involvement in resistance to *V. dahliae*. GO analysis revealed that the DEGs of the disease-resistant cultivar MS72 were enriched in cell wall-associated ontology. We further screened three hub genes, *CaCSLG1*, *CaPME2.2*, and *CaDIR21*, which are involved in cellulose modification and lignin accumulation, similar to known genes such as *HvCslD2* and *TaDIR13*. Barley *HvCslD2* encodes a specific cellulose-modifying enzyme, and silencing *HvCslD2* significantly reduced pathogen-induced cellulose deposition in the papillae, which increases susceptibility to powdery mildew [73]. Overexpression of the wheat *TaDIR13* in tobacco increases the total lignin accumulation, and exhibits strong resistance to *Phytophthora syringae* and *P. parasitica* [74]. The hub gene *CabHLH93* in resistant cultivars was significantly up-regulated after inoculation with *V. dahliae*. The bHLH superfamily is a transcription factor family in the helix loop helix (HLH) region [75] and is involved in plant biotic and abiotic stress responses [76,77]. In tobacco, the bHLH transcription factor GhPAS1 confers resistance to cotton Verticillium wilt by regulating specific members of the ERF and genes involved in lignin biosynthesis and plant defense [77]. In tomatoes, the effector RipI of *Fusarium* wilt induces host defense responses by interacting with the *bHLH93* transcription factor, thereby enhancing resistance to bacterial wilt disease [78]. In summary, these hub genes may be key genes that confer resistance to Verticillium wilt in MS72. However, this study only investigated the disease resistance regulatory network of leaves, and it is still unclear whether the selected hub genes play a role in regulating resistance throughout the entire plant. We are still exploring the functional mechanism of the hub genes revealed in this study to elucidate the molecular basis of the observed resistance to Verticillium wilt in MS72 pepper plants and provide ideas for breeding new cultivars resistant to Verticillium wilt. In addition, the research results offer potential strategies for breeding Verticillium wilt-resistant pepper varieties, such as overexpression of the *CabHLH93* gene in pepper, which could be a key method for breeding resistant cultivars.

5. Conclusions

Our research results indicate that inoculation with *V. dahliae* leads to a rapid decrease in chlorophyll content and an increase in MDA content in the susceptible cultivar MS66,

ultimately resulting in leaf yellowing and wilting, whereas the resistant cultivar MS72 maintains high chlorophyll content and exhibits low peroxidation of membrane lipids. RNA-Seq analysis showed that, compared with MS66, DEGs in MS72 were mainly enriched in the GO terms related to cell wall, antioxidant, and photosynthesis, as well as in pathways such as cutin, suberin, wax biosynthesis, phenylpropanoid biosynthesis, and photosynthesis. DEGs related to the ethylene signaling pathway were up-regulated in MS66, whereas two *CaIAA*, one *CaJAR1*, and one *CaPR1* of plant hormone signaling pathways were up-regulated in MS72 after inoculation. Simultaneously, 35 hub genes were identified in up-regulated genes of MS72 through WGCNA. The key genes identified in this study may serve as a reference for improving pepper resistance through breeding programs. Ultimately, these findings may aid the development of robust disease-resistant crop cultivars that provide economic benefits and contribute to sustainable agriculture.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/horticulturae10111160/s1>, Figure S1: Principal component analysis (PCA) of eighteen samples. Figure S2: Pearson correlation coefficient heatmap of eighteen samples. Figure S3: Top twenty significantly enriched GO terms of DEGs in MS66-0 vs. MS66-3, MS66-0 vs. MS66-5, MS72-0 vs. MS72-3, and MS72-0 vs. MS72-5 groups. Figure S4: DEGs enriched in KEGG pathways in MS66-0 vs. MS66-3, MS66-0 vs. MS66-5, MS72-0 vs. MS72-3, and MS72-0 vs. MS72-5 groups. Table S1: qRT-PCR primers used for validation of RNA-Seq data. Table S2: GO Information of DEGs. Table S3: KEGG pathway information of DEGs. Table S4: Information on three signaling pathways. Table S5: Hub genes of the pink and purple module.

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Conflicts of Interest: Author Qiucheng Qiu and Qidi Sun were employed by the company (Maoming Maoshu Seed Industry Technology Co., Ltd). The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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