Phyton-
International Journal of
Experimental Botany

Tech Science Press

DOI: 10.32604/phyton.2022.023481

ARTICLE

Transcriptome Analysis of a Wild Eggplant Germplasm M239 in Response to *Verticillium dahliae* Infection

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Received: 28 April 2022 Accepted: 04 July 2022

ABSTRACT

In this study, wild eggplant germplasm No. M239, which is highly susceptible to *Verticillium* wilt, was used as the experimental material. The physiological and biochemical indices (SOD, PAL, MDA and soluble protein) of M239 roots were measured at different times (0, 12, 24, 36, 48, 60 and 72 h) post inoculation with *Verticillium dahliae*, and the key time points for the M239 response to *Verticillium* wilt infection were screened. Then, RNA-Seq technology was used to screen the differentially expressed genes (DEGs) in M239 roots at 0, 12 and 48 h post-inoculation (hpi). The transcriptional results of M239 were also compared with those resistance genes from some reported wild relative *Solanum* species (*S. sisymbriifolium* and *S. aculeatissimum*). Then some DEGs were chosen for validation by qRT–PCR. The results showed that 12 and 48 hpi were the turning points in the changes in all physiological and biochemical indices. A total of 6,783 DEGs were identified by RNA-Seq, including 6,141 DEGs (3,046 upregulated and 3,095 downregulated) at the M_12 h *vs*. M_0 h, 1,903 DEGs (792 upregulated and 1,111 downregulated) at M_48 h *vs*. M_12 h, and 1,261 DEGs that appeared simultaneously in both stages. KEGG enrichment analysis showed that there were 5 metabolic pathways enriched from DEGs, which were mostly related to primary metabolism, such as glycolysis, amino acid and ribosome biogenesis. Compared with the NCBI non-redundant protein (NR) database, one *Ve2* homologous gene and 8 PR protein-related genes were screened. Transcription factor analysis showed that there were a large number of DEGs, such as MYB, AP2-EREBP, bHLH, NAC and Orphans in the two stages. Compared with the reported *Verticillium* wilt-resistant wild eggplant species, it was found that there were fewer genes and enriched metabolic pathways in the M239 response to *Verticillium* wilt infection, and it also lacked the response of some known key resistance genes. These results proved that the above resistance genes and metabolic pathways played a key role in the wild eggplant response to *V. dahliae* infection.

KEYWORDS

Wild eggplant; *Verticillium* wilt; transcriptome sequencing; differentially expressed gene; KEGG pathway

Abbreviations

DEG	Differentially expressed gene
EVW	Eggplant *Verticillium* wilt
FPKM	Fragments per kilobase per million fragments

hpi	Hours post inoculation
GO	Gene Ontology
KEGG	Kyoto Encyclopedia of Genes and Genomes
MDA	Malondialdehyde
NCBI	National center for biotechnology information
NR	NCBI non-redundant protein
PAL	Phenylalanine ammonia-lyase
PR	Pathogenesis-related protein
qRT-PCR	Real-time quantitative PCR
SRA	Sequence Read Archive
SOD	Superoxide dismutase
TFs	Transcription factors

1 Introduction

Eggplant (*Solanum melongena* L.), a common vegetable crop, is native to Southeast Asia and India. Eggplant was introduced into China as early as the 4th~5th century Anno Domini (A.D.). It is generally believed that China is the second origin of eggplant. At present, China has the largest planting area of eggplant and the largest number of eggplant germplasm resources [1]. Eggplant *Verticillium* wilt (EVW) is a soil-borne vascular disease caused by *Verticillium* spp. Once it occurs, eggplant can be seriously reduced or can even fall out of production; thus, EVW has become one of the main diseases threatening eggplant production. According to some domestic reports, EVW in China is mainly caused by *Verticillium dahliae* [2].

In recent years, researchers have been trying to reveal the interaction mechanism between *V. dahliae* and different plants, such as *Arabidopsis* [3], tomato [4–6], cotton [7–10] and potato [11]. Meanwhile, some resistance genes to *Verticillium* wilt have been screened and identified in eggplant and its wild relatives, such as *SlVe* [12], *StVe* [13] and *StDAHP* [14]. Especially with the application of RNA-Seq technology [15–18], relevant data at the transcriptional level of plant *Verticillium* wilt have been obtained and utilized.

Many studies have shown that EVW-resistant materials mainly exist in the wild relatives *Solanum* species and semicultivated eggplant types [1,19]. There are rich wild eggplant resources in Yunnan Province and its surrounding areas of China [20]. However, research on *Verticillium* wilt resistance is mainly focused on several wild eggplant species, such as *S. torvum* [16], *S. aculeatissimum* [17], and *S. sisymbriifolium* [18], and there is little research on *Verticillium* wilt-susceptible materials in wild eggplants. The above wild eggplant resistance materials are not only distantly related to cultivated eggplant (i.e., widely incompatible with distant hybridization with cultivated eggplant), but also have great differences in their growth environments. These factors make it difficult to solve the disease problem in the actual production of eggplant. The study of wild eggplant materials close to cultivated eggplant can eliminate the huge interference caused by the differences in genetic background and growth environments between wild and cultivated eggplant to a certain extent and speed up the research and utilization of wild eggplant resources. Among them, a wild eggplant germplasm (No. V06B1239 of national vegetable medium-term data, M239) was collected from Dali City, Yunnan Province by the Institute of Horticultural Crops, Yunnan Academy of Agricultural Sciences in the 1990s. In our preliminary study [2], it was found that M239 was more susceptible to the disease than the cultivated eggplant after inoculation by *V. dahliae*, and its genetic background and morphology were close to those of the cultivated eggplants [21]. Therefore, it is speculated that M239 had a history of cultivation, but it was eliminated because of its poor disease resistance and gradually became wild.

Based on the previously screened *Verticillium* wilt highly susceptible material M239 [2], this study analysed its roots at different time points (0, 12 and 48 h) after M239 was inoculated with *V. dahliae* at the transcriptional level by the Illumina high-throughput sequencing method and established a transcriptome database of M239 in response to *V. dahliae* infection. The key pathways and resistance genes of plant resistance to *Verticillium* wilt were screened by comparative analysis with the reported resistant materials (*S. sisymbriifolium* and *S. aculeatissimum*, etc.) of wild eggplant species. These results are expected to fully reveal the transcriptome information characteristics of highly susceptible wild eggplant materials in response to *Verticillium* wilt, clarify the expression differences of transcriptional levels between highly resistant and highly susceptible materials of wild eggplants, provide a reference for studying eggplant *Verticillium* wilt resistance, screen resistant eggplant germplasm resources, and also lay a foundation for the screening and identification of *Verticillium* wilt resistance genes.

2 Materials and Methods

2.1 Tested Materials

The highly pathogenic strain QZ-S of *V. dahliae* and the highly susceptible material M239 (Fig. 1) screened in a preliminary experiment by our research group were used as the test materials [2]. The above test materials were all collected and preserved by the Institute of Horticultural Crops, Yunnan Academy of Agricultural Sciences.

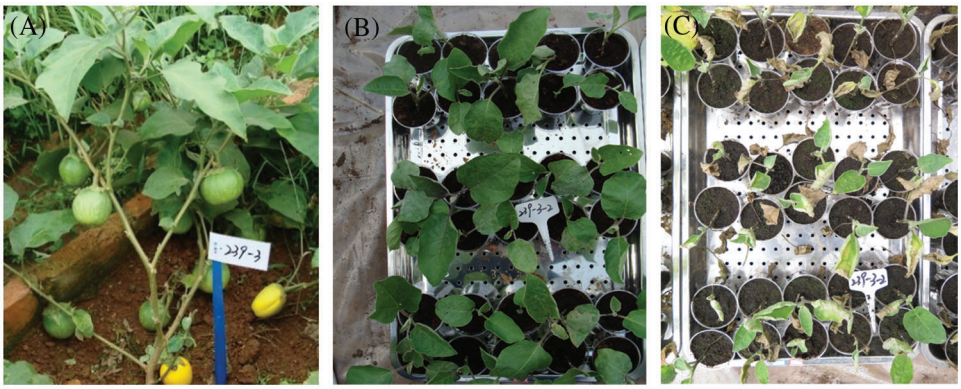

Figure 1: Wild eggplant M239, a highly susceptible material to *Verticillium* wilt. (A) the whole plant; (B) normal seedlings; (C) infected seedlings

2.2 Determination of Physiological Indices in M239 after Inoculation with V. dahliae

Key measures for the sowing and growing of M239 seedlings were used as described by the method of Wu et al. [2]. At the plant's four true leaves stage, the *Verticillium* wilt pathogen was artificially inoculated according to the method of Wu et al. [18]. Plants at 0, 12, 24, 36, 48, 60 and 72 h post-inoculation (hpi) were selected, and the roots of 5~10 plants with consistent growth were taken. After liquid nitrogen treatment, they were immediately stored at −80°C for the next physiological and biochemical index determination and transcriptome sequencing. The plants at 0 hpi were used as the control (CK), and each treatment was repeated three times.

The physiological indices were measured with a kit from the Nanjing Jiancheng Bioengineering Institute. Total SOD enzyme activity was determined by an A001-1 kit (product number, the same below), PAL enzyme activity was determined by an A137-1 kit, MDA content was determined by an A003-1 kit, and soluble protein content was determined by an A145-1 kit. A UV1600 ultraviolet spectrophotometer (Shanghai Yoke Instrument Co., Ltd., China) was used to measure the absorbance

value. The specific enzyme activity determination and calculations were performed according to the instructions of the corresponding kit.

2.3 Sequencing and Transcriptome Analysis of M239 after Inoculation with V. dahliae

According to the results of physiological and biochemical indices, the roots of M239 at the key time points (0, 12, and 48 hpi) after inoculation with *V. dahliae* were selected for transcriptome sequencing and analysis. The experiment was divided into three groups with three biological repetitions in each group.

2.3.1 Extraction and Quality Detection of Total RNA

Total RNA from each sample was extracted using an RNAout 1.0 kit (Beijing Tianenze Gene Technology Co., Ltd., China). A 1% agarose gel was used to detect the quality of RNA, and its purity was detected by NanoDrop (Implen, USA). The RNA concentration was measured by a Qubit RNA analysis kit and a Qubit 2.0 fluorometer (Life Technologies, USA). RNA integrity was evaluated using an RNA Nano 6000 analysis kit and an Agilent 2100 biological analyser (Agilent Technologies, USA). The RNA was then used for the next step of database construction.

2.3.2 Transcriptome Sequencing, Data Assembly and Functional Annotation

The construction and sequencing of the cDNA library were completed by Beijing Novogene Bioinformatics Technology Co., Ltd., China. A cDNA library was successfully constructed, and high-throughput sequencing was carried out using the Illumina sequencing platform (Illumina HiSeq 2000). The raw data (or raw reads) generated by sequencing were obtained after removing the connector, poly-N and low-quality reads ($Q < 20$). Subsequent analysis was based on the above high-quality clean reads.

Compared with the eggplant reference genome [22], unigenes were obtained by applying HISAT software (v2.0.4), and the unmatched sequences were assembled with Cufflinks software (v2.2.1). Then new transcripts were predicted by comparing the output results of Cufflinks with known gene models. The assembly, sequencing and splicing were all completed by Beijing Novogene Bioinformatics Technology Co., Ltd., China.

2.3.3 Identification of Differentially Expressed Genes (DEGs)

The reads per kilobase of exon model per million mapped reads (FPKM) value was used to quantify the gene expression level. DEGs were screened by the DESeq R software package (v1.10.1). For the experiments with biological duplication, DESeq eliminated the biological variation. Therefore, the threshold standard of DEGs set by transcriptome data was padj < 0.05 in this study. KEGG enrichment analysis of DEGs was carried out by KOBAS software [23]. A corrected p value (q value) <0.05 was used as the standard for enrichment and screening. The BLAST procedure in NCBI was used for sequence alignment and searching homologous genes.

2.4 qRT–PCR Validation

RNA from M239 roots was reverse transcribed into cDNA by a kit (*Easyscript*®First Strand cDNA Synthesis SuperMax, Trans, Beijing, China), and *18S* RNA from eggplant was used as a reference gene. Twenty DEGs were randomly selected, and the transcriptome data were verified by qRT–PCR. The specific primer sequences are shown in Table 1. The qRT–PCR procedure was performed according to the kit instructions (*Transstart*® Tip Green qPCR SuperMax, Trans, Beijing, China) and run on an Applied Biosystems 7500 real-time fluorescence quantitative PCR (USA). Relative gene expression levels were calculated using the $2^{-\Delta\Delta CT}$ method [24].

2.5 Data Statistics and Analysis

The software programs Excel 2010 and SPSS 19.0 were used for data processing and analysis, and the figures were drawn in Sigmaplot 10.0 software.

Table 1: Primer sequences for qRT-PCR in this study

Primer name	Primer sequence (5′-3′)	Primer name	Primer sequence (5′-3′)
18sRNA-F	TAGTTGGACTTTGGGATGGC	18sRNA-R	AGAGCGTAGGCTTGCTTTGA
Sme2.5_00057.1_g00033.1-F	CAAGTTGGTGTCGGCCCTAT	Sme2.5_00057.1_g00033.1-R	ATCGACCCACATCTTGACGG
Sme2.5_01795.1_g00002.1-F	TCGGTGGTTGACTTCACGAG	Sme2.5_01795.1_g00002.1-R	ATCGTTCCCGACTTGTGTCC
Sme2.5_02344.1_g00010.1-F	CTGCACTGGTTTCCTTTGCC	Sme2.5_02344.1_g00010.1-R	GTGTGAGCATCCGAACATGC
Sme2.5_02676.1_g00002.1-F	AAGAACGGATCCATCTCCGC	Sme2.5_02676.1_g00002.1-R	CACATCAAAGCGAGAACCGT
Sme2.5_03889.1_g00006.1-F	GTACTTCCCGACGAATGCCT	Sme2.5_03889.1_g00006.1-R	GCATCATTTGCAGCTCCGTC
Sme2.5_04615.1_g00004.1-F	ACAACAACAACAACCGGTGC	Sme2.5_04615.1_g00004.1-R	CTCTGAGCGAACCGACAACT
Sme2.5_05154.1_g00001.1-F	TTGTTGGTCTTGGCGGACTT	Sme2.5_05154.1_g00001.1-R	GCCCAATTGCCTCGTCTTTC
Sme2.5_12415.1_g00003.1-F	ACCAACCGATGTGCTAGTGG	Sme2.5_12415.1_g00003.1-R	CGTTGTCCGACATAGTTGCC
Sme2.5_03951.1_g00001.1-F	CGACTCGGACGAAATGTTCC	Sme2.5_03951.1_g00001.1-R	ATGGCCTACGCCTAACACCT
Sme2.5_02673.1_g00006.1-F	GCGTAACGGACCAAGCTAGT	Sme2.5_02673.1_g00006.1-R	CCAGTTGAGAATCTGCGGGT
Sme2.5_00112.1_g00012.1-F	TGGCCTGAAATGAACCTCCC	Sme2.5_00112.1_g00012.1-R	TCGGATGCGATAAACGTGCT
Sme2.5_00351.1_g00007.1-F	CTCCATGGTGGCGTGATGAT	Sme2.5_00351.1_g00007.1-R	CCCTCGCTCTTAGCTTCACC
Sme2.5_00563.1_g00016.1-F	CTGTCGCGTCTCTACTCCAT	Sme2.5_00563.1_g00016.1-R	TGACGGCATGTCCACCAAAT
Sme2.5_01193.1_g00010.1-F	TGGCTCGGTATTGCTGGATG	Sme2.5_01193.1_g00010.1-R	GGTCCACCAACCAAAAGAACAG
Sme2.5_02584.1_g00008.1-F	GAACCCTCACGTTTGGAGGT	Sme2.5_02584.1_g00008.1-R	CAAGGCCAACGACACCAATG
Sme2.5_02838.1_g00002.1-F	TCCAAGTAGCCCAAGTGGTG	Sme2.5_02838.1_g00002.1-R	TGCTGTTCTTGAACTCCGCT
Sme2.5_17321.1_g00001.1-F	CAACCAAGTTGTCGGATGGC	Sme2.5_17321.1_g00001.1-R	AGGTGCACCATCCATGCTAA
Sme2.5_24598.1_g00001.1-F	TGCCCATTTGCAGAGGGAAT	Sme2.5_24598.1_g00001.1-R	ACACCGATCCATCACATCCC
Sme2.5_07159.1_g00004.1-F	TCGGCATGACCAAAGCAGAT	Sme2.5_07159.1_g00004.1-R	CTCAGCTACGAGATACGCGG

3 Results

3.1 Physiological Response of M239 within 72 h after Inoculation with V. dahliae

Four physiological and biochemical indices (SOD, PAL, MDA, and soluble protein) were measured within 72 h after M239 was inoculated with *V. dahliae* to study the physiological response mechanism of M239 inoculated with *V. dahliae* and screen the accurate sampling and analysis time for the next transcriptome sequencing.

As shown in Fig. 2, it can be seen that within 72 h after M239 was inoculated with *V. dahliae*, all physiological and biochemical indices had changed significantly compared with CK (0 hpi). Among them, SOD activity increased rapidly after inoculation and began to decrease after reaching a peak at 12 hpi. PAL activity increased rapidly after inoculation, began to change slowly after 6 hpi, reached the highest value at 12 hpi, then began to decrease rapidly, and reached a low point at 48 hpi. The change in MDA content was not obvious, and there were only two small peaks and valleys at 12 and 48 hpi, respectively. The content of soluble protein first increased, then decreased and finally increased after inoculation, there were two peaks at 6 and 48 hpi and an inflection point at 24 hpi.

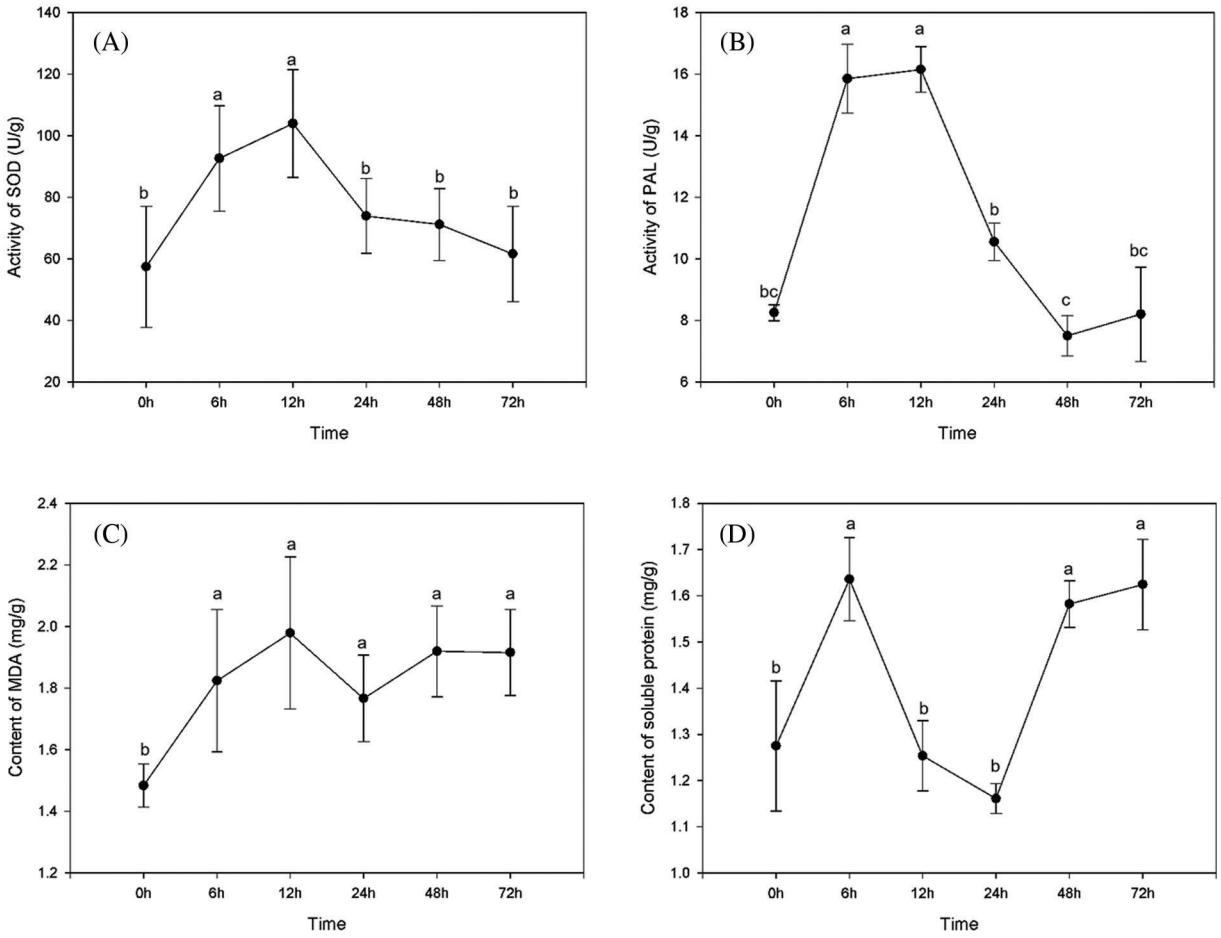

Figure 2: Changes in physiological and biochemical indices in M239 within 72 h post-inoculation with *V. dahliae*. (A) SOD; (B) PAL; (C) MDA; (D) Soluble protein. Each point on the graph represents the average of 3 replicates, and the vertical lines on each point are standard deviations. Different lowercase letters show significant level at 0.05

The above results showed that the physiological and biochemical characteristics of M239 after inoculation with *V. dahliae* changed to varying degrees. In addition, 12 and 48 hpi appeared as inflection points in many indices, suggesting that they were two important plant disease interaction time points. Therefore, 12 and 48 hpi were used as the key time points for subsequent transcriptome sequencing.

3.2 Transcriptional Response of M239 Inoculated with V. dahliae

3.2.1 Transcriptome Sequencing and Splicing

The output of the sequencing data is shown in Table 2. An average of 51, 343, 125 clean reads from 9 databases were obtained by transcriptome sequencing of M239 roots at 0, 12 and 48 h post-inoculation with *V. dahliae*, and its average comparison rate with the eggplant reference genome was 89.61% (Table 3). The raw sequencing data have been stored in the SRA (Sequence Read Archive) database (https://www.ncbi.nlm.nih.gov/sra/PRJNA495025), and the accession number is PRJNA495025.

Table 2: Output quality of transcriptome sequencing data for M239

Sample name	Raw reads	Clean reads	Clean bases	Error rate (%)	Q20 (%)	Q30 (%)	GC content (%)
M_0 h_1	55875066	53219188	7.98G	0.02	96.54	91.72	42.58
M_0 h_2	57373012	54306754	8.15G	0.02	96.21	91.08	42.48
M_0 h_3	52275602	49706942	7.46G	0.02	96.50	91.66	42.59
M_12 h_1	49361046	46921340	7.04G	0.02	96.54	91.70	42.54
M_12 h_2	55920920	53242872	7.99G	0.02	96.61	91.88	42.43
M_12 h_3	57561612	54271070	8.14G	0.02	96.67	91.99	42.44
M_48 h_1	58614700	55064358	8.26G	0.02	96.63	91.89	42.99
M_48 h_2	49660980	47207738	7.08G	0.02	96.45	91.54	42.68
M_48 h_3	51135286	48147864	7.22G	0.02	97.07	92.69	42.55

Table 3: Comparison between M239 transcriptome sequencing reads and the eggplant reference genome

Sample name	M_0 h_1	M_0 h_2	M_0 h_3	M_12 h_1	M_12 h_2	M_12 h_3	M_48 h_1	M_48 h_2	M_48 h_3
Total reads	53219188	54306754	49706942	46921340	53242872	54271070	55064358	47207738	48147864
Total mapped	47481759 (89.22%)	49015285 (90.26%)	43343093 (87.2%)	42570447 (90.73%)	48338132 (90.79%)	49385823 (91%)	48760486 (88.55%)	42017988 (89.01%)	43191748 (89.71%)
Multiple mapped	1551136 (2.91%)	1573194 (2.9%)	1345502 (2.71%)	1380268 (2.94%)	1647803 (3.09%)	1602907 (2.95%)	1821912 (3.31%)	1509203 (3.2%)	1453338 (3.02%)
Uniquely mapped	45930623 (86.3%)	47442091 (87.36%)	41997591 (84.49%)	41190179 (87.79%)	46690329 (87.69%)	47782916 (88.04%)	46938574 (85.24%)	40508785 (85.81%)	41738410 (86.69%)
Reads map to '+'	22960549 (43.14%)	23718342 (43.67%)	20995881 (42.24%)	20591360 (43.88%)	23346298 (43.85%)	23889948 (44.02%)	23463334 (42.61%)	20247568 (42.89%)	20863882 (43.33%)
Reads map to '−'	22970074 (43.16%)	23723749 (43.68%)	21001710 (42.25%)	20598819 (43.9%)	23344031 (43.84%)	23892968 (44.03%)	23475240 (42.63%)	20261217 (42.92%)	20874528 (43.36%)
Non-splice reads	30438284 (57.19%)	31176449 (57.41%)	27292769 (54.91%)	26008674 (55.43%)	29685115 (55.75%)	30466210 (56.14%)	29330442 (53.27%)	25680935 (54.4%)	26320555 (54.67%)
Splice reads	15492339 (29.11%)	16265642 (29.95%)	14704822 (29.58%)	15181505 (32.36%)	17005214 (31.94%)	17316706 (31.91%)	17608132 (31.98%)	14827850 (31.41%)	15417855 (32.02%)

3.2.2 *Identification of DEGs*

Fig. 3 shows that there were 6,141 DEGs in M239 at 12 hpi compared with 0 hpi (the first stage, M_12 h *vs*. M_0 h, the same below), of which 3,046 were upregulated and 3,095 were downregulated. Compared with 12 hpi (the second stage, M_48 h *vs*. M_12 h, the same below), there were 1,903 DEGs at 48 hpi, of which 792 were upregulated and 1,111 were downregulated. A total of 1,261 DEGs appeared at the same time in both stages. Compared with 0 hpi (M_48 h *vs*. M_0 h, the same below), there were 3,329 DEGs, of which 1,272 were upregulated and 2,057 were downregulated.

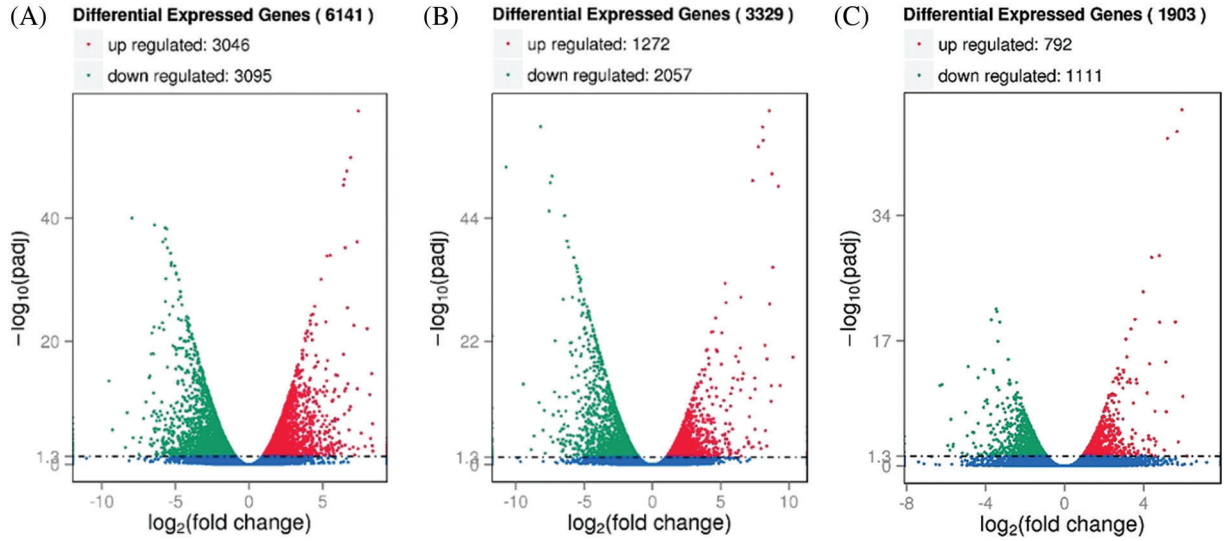

Figure 3: Volcano map of differentially expressed genes (DEGs) in M239. (A) M_12 h *vs*. M_0 h; (B) M_48 h *vs*. M_0 h; (C) M_48 h *vs*. M_12 h

3.2.3 *KEGG Enrichment Pathway Analysis of DEGs*

After M239 was inoculated with *V. dahliae*, the KEGG enrichment pathways (q value < 0.05) of DEGs were less and more related to the primary metabolism, such as glycolysis, amino acid and ribosome production. Compared with 0 hpi (CK), only one pathway, "ribosome", was enriched at 12 hpi (Fig. 4A), and only one pathway, "glycolysis/gluconeogenesis", was enriched at 48 hpi (Fig. 4B). Compared with 12 hpi, three pathways, namely, "circadian rhythm-plant", "brassinosteroid biosynthesis", and "ribosome biogenesis in eukaryotes", were enriched at 48 hpi (Fig. 4C).

3.2.4 *Screening of Resistance Genes to Verticillium Wilt*

Verticillium Wilt Resistance Gene (Ve) and Pathogenesis-Related Protein (PR) Genes

In M239 (Table 4), one gene was compared as *Ve2* (sme2.5_02673.1_g00006.1) by the NR (non-redundant protein sequence) database. After BLAST through NCBI, it was found that the identified gene was the most similar (similarity 98.2%) to the sequence of the *Verticillium* wilt resistance gene *SaVe2* in *S. aethiopicum* (accession number: AY598746.1). At the same time, eight *PR* genes were screened from the DEGs of M239. Among them, four genes encoded PR1 proteins, two genes encoded PR5 proteins, and two genes encoded STH-2 and PRB1 proteins, respectively.

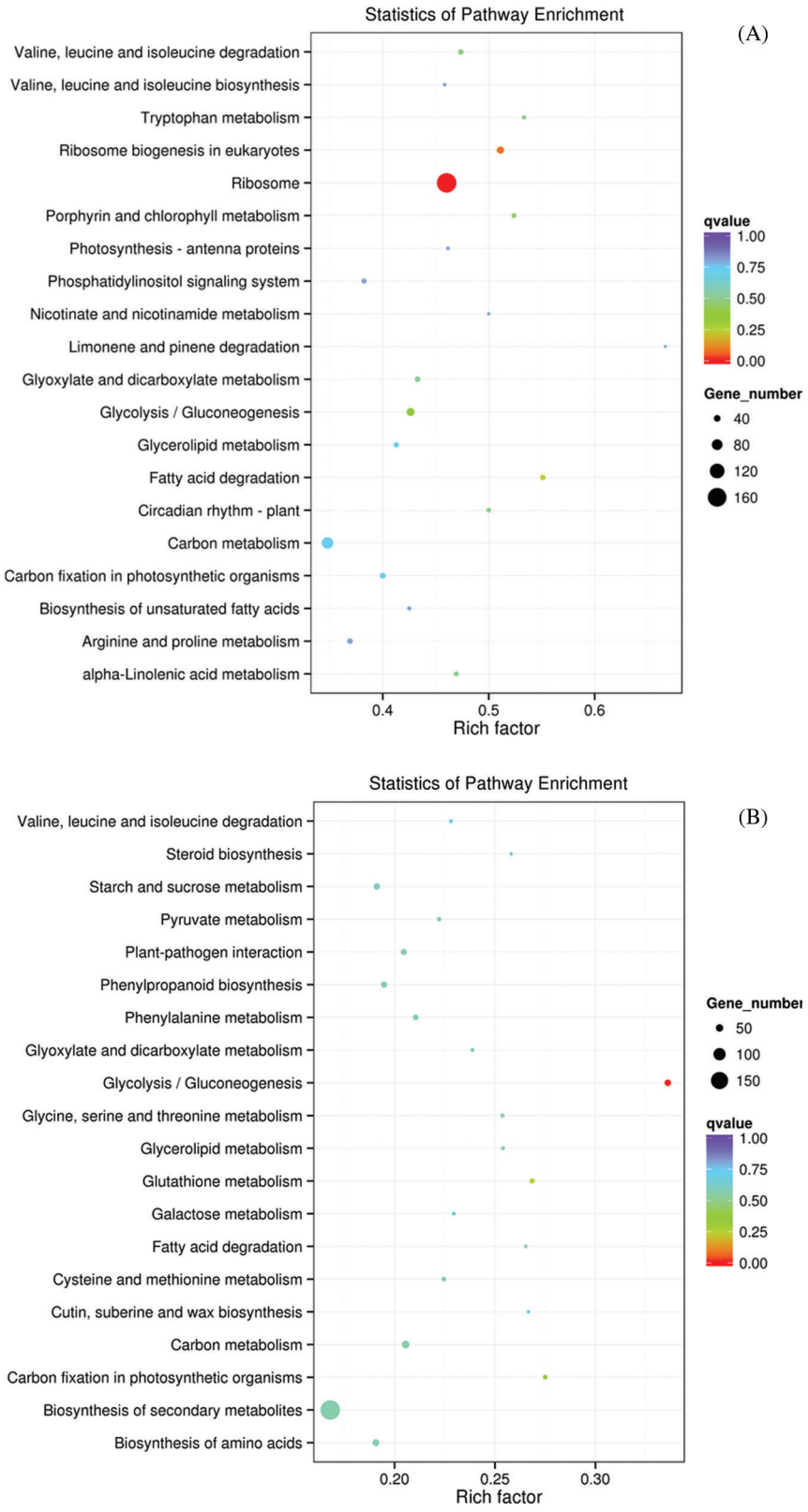

Figure 4: (Continued)

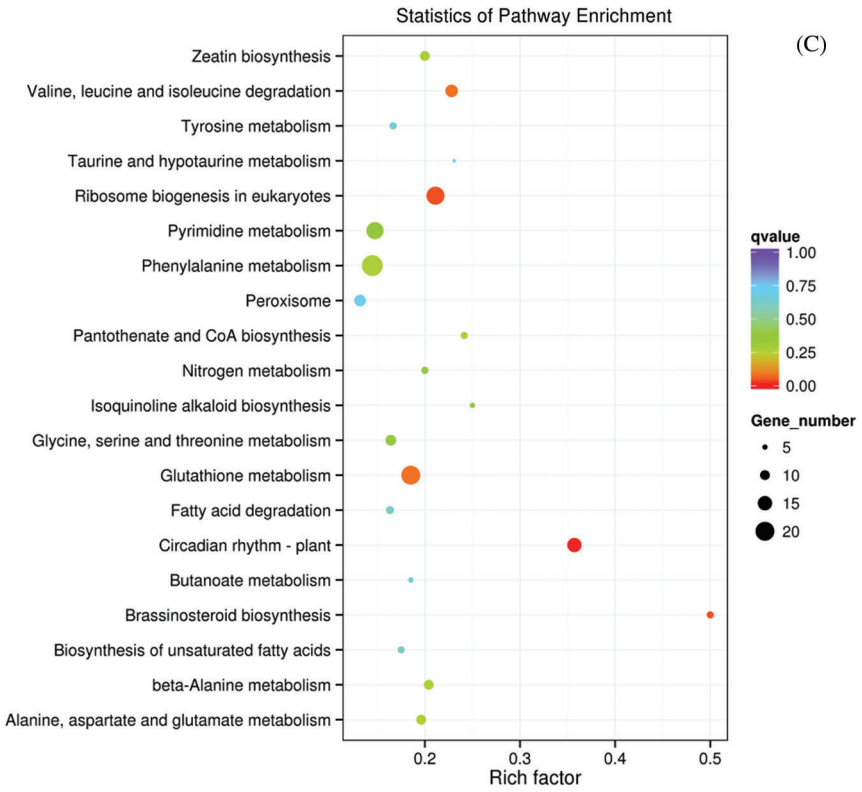

Figure 4: Scatter diagram of KEGG enrichment pathways in M239. (A) M_12 h *vs*. M_0 h; (B) M_48 h *vs*. M_0 h; (C) M_48 h *vs*. M_12 h

Table 4: Expression profiles of *Ve* and *PR* genes in M239

Gene name	Gene ID	Gene definition	FPKM value		
			0 hpi	12 hpi	48 hpi
Ve2	Sme2.5_02673.1_g00006.1	verticillium wilt disease resistance protein *Ve2*	8.69	4.17	4.10
PR1	Sme2.5_12415.1_g00001.1	pathogenesis-related protein 1	431.78	10.90	106.36
PR1	Sme2.5_04458.1_g00001.1	pathogenesis-related protein 1	9.814	0.188	5.35
PR5	Sme2.5_00015.1_g00029.1	pathogenesis-related protein 5	1.89	15.46	5.76
PR1	Sme2.5_00096.1_g00005.1	pathogenesis-related protein PR-1	0.087	1.84	2.49
STH-2	Sme2.5_06695.1_g00001.1	pathogenesis-related protein STH-2-like	3.90	1.21	1.84
PR5	Sme2.5_00171.1_g00010.1	pathogenesis-related protein 5	41.86	3.24	38.60
PR1	Sme2.5_12415.1_g00003.1	pathogenesis-related protein 1	47.05	0.85	12.88
PRB1	Sme2.5_01013.1_g00013.1	pathogenesis-related protein PRB1-3	0.04	0.44	2.28

Transcription Factors (TFs)

Through the comparison of DEGs, we found that M239 had 537 and 277 *TFs* in the first stage (Fig. 5A) and the second stage (Fig. 5B), which were classified into 58 and 50 gene families, respectively. After inoculation, MYB, AP2-EREBP, WRKY, bHLH, NAC, and Orphans were the most enriched gene families (Tables S1 and S2). The main *TFs* in the two stages were relatively similar, and MYB, AP2-EREBP, bHLH, NAC, and Orphans appeared in large numbers in the two stages.

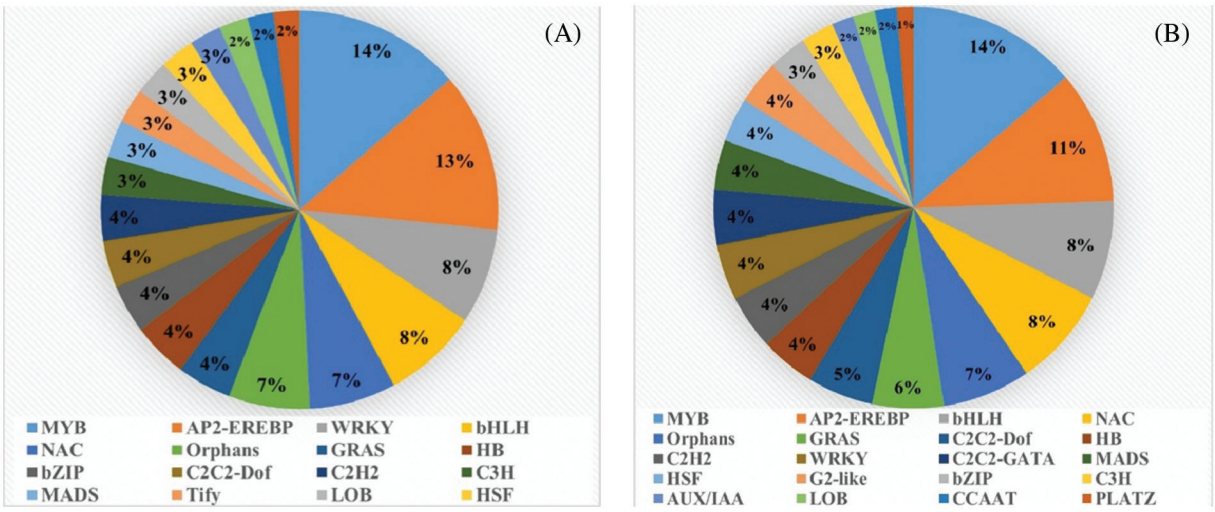

Figure 5: Classification of transcription factors in the differentially expressed genes (DEGs). (A) M_12 h *vs*. M_0 h, (B) M_48 h *vs*. M_12 h

3.3 qRT-PCR Validation

The validation results showed that some gene expression trends of M239 identified by qRT–PCR were consistent with those in the transcriptome data (Fig. 6), indicating that the results of this transcriptome sequencing are accurate and reliable and can be used for further analysis. Importantly, these validated genes belonged to three KEGG pathways (Table 5), namely, “plant hormone signal transduction”, “plant-pathogen interaction”, and “phenylpropanoid biosynthesis”, which are important pathways in response to biotic stress. Meanwhile, some candidate genes (*PR1*, *Ve2*, *HSP90*) were validated (Table 5).

4 Discussion

Molecular research on plant resistance to *Verticillium* wilt has been carried out in tomato [25], potato [26] and tobacco [27]. However, the process of plant response to *Verticillium* wilt infection involves many factors and multiple resistance genes, and the information provided by these studies is insufficient to clarify the molecular mechanism of eggplant defence against *V. dahliae* infection. In addition, the current research mainly focuses on *Verticillium* wilt-resistant materials, such as *S. torvum*, *S. sisymbriifolium*, and other wild eggplant species, and there has been little research on *Verticillium* wilt susceptible materials. The significant differences in genetic background and growth environment between the wild and cultivated eggplants have greatly interfered with related research at the transcriptional level. Screening wild eggplant materials with similar growth environments and similar phenotypes *vs*. the cultivated eggplant can reduce the difference between them to a certain extent and build a bridge between the wild and cultivated eggplants.

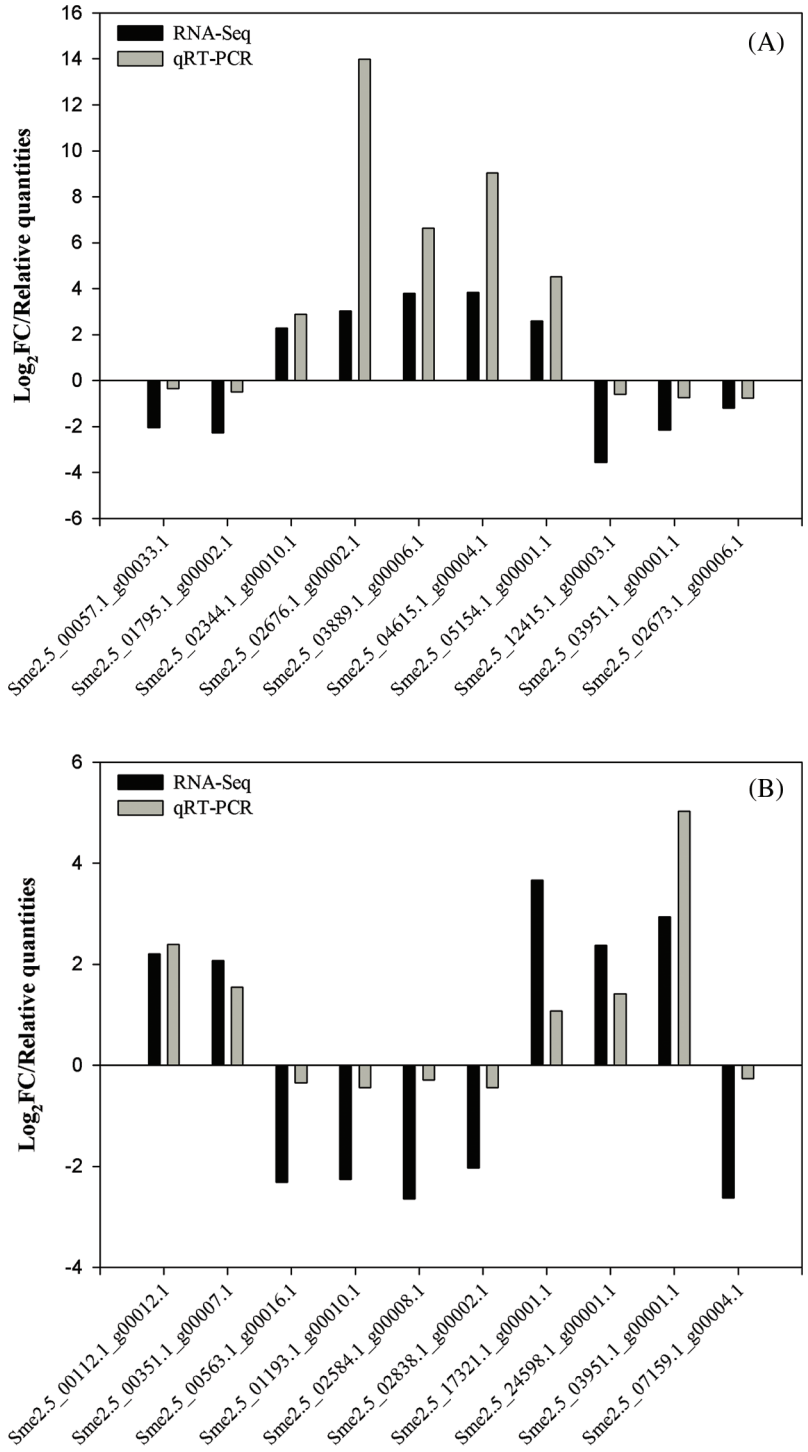

Figure 6: qRT –PCR validation for some differentially expressed genes (DEGs). (A) M_12 h *vs*. M_0 h; (B) M_48 h *vs*. M_12 h

Table 5: 20 DEGs identified genes and their KEGG pathways for qRT-PCR validation

Gene name	Gene ID	Gene definition	KEGG pathway
PR1	Sme2.5_00057.1_g00033.1	pathogenesis-related protein 1	PHST
MYC2	Sme2.5_01795.1_g00002.1	transcription factor MYC2	PHST
ETR4	Sme2.5_02344.1_g00010.1	ethylene receptor homolog precursor	PHST
CaCML	Sme2.5_02676.1_g00002.1	calcium-binding protein CML25	PPI
EBF1/2	Sme2.5_03889.1_g00006.1	EIN3-binding F-box protein 1	PHST
PYL	Sme2.5_04615.1_g00004.1	abscisic acid receptor PYL4-like	PHST
8-HGO	Sme2.5_05154.1_g00001.1	8-hydroxygeraniol dehydrogenase	PB
P4	Sme2.5_12415.1_g00003.1	pathogenesis-related leaf protein 4 precursor	PPI
ERF1/2	Sme2.5_03951.1_g00001.1	ethylene-responsive transcription factor 1B-like	PHST
Ve2	Sme2.5_02673.1_g00006.1	verticillium wilt disease resistance protein Ve2	PPI
SILYK13	Sme2.5_00112.1_g00012.1	LysM receptor-like kinase Lyk13 precusor	PPI
NHO1	Sme2.5_00351.1_g00007.1	glycerol kinase	PPI
AUX1	Sme2.5_00563.1_g00016.1	LAX2 protein	PHST
POD11	Sme2.5_01193.1_g00010.1	peroxidase 11	PB
8-HGO	Sme2.5_02584.1_g00008.1	8-hydroxygeraniol dehydrogenase	PB
CYCD3C2	Sme2.5_02838.1_g00002.1	D type cyclin-2	PHST
IAA19	Sme2.5_17321.1_g00001.1	IAA 19	PHST
POD47	Sme2.5_24598.1_g00001.1	peroxidase 47	PB
ERF1/2	Sme2.5_03951.1_g00001.1	ethylene-responsive transcription factor 1B-like	PHST
HSP90	Sme2.5_07159.1_g00004.1	heat shock protein 90	PPI

Note: PHST, Plant hormone signal transduction; PPI, Plant-pathogen interaction; PB, Phenylpropanoid biosynthesis.

In this study, we used M239, a material highly susceptible to *Verticillium* wilt, as the transcriptome material to analyse the DEGs, KEGG pathways and resistance related genes in its roots at different times within 48 h post-inoculation with *V. dahliae*, and compared the results with the reported disease-resistant materials *S. aculeatissimum* [17] and *S. sisymbriifolium* [18].

4.1 Analysis of DEGs and Their KEGG Enrichment Pathways

In their long-term coevolution with pathogens, plants have developed a series of disease resistance responses to inhibit the infection of pathogens. The resistance response is mainly composed of two innate immunity pathways, namely, PTI (pattern-associated molecular patterns, PAMP-triggered immunity) and ETI (effector-triggered immunity) [28]. From the analysis results of DEGs in this study, it can be seen that DEGs in each stage of M239 were mainly downregulated genes. In the study of Zhou et al. [17], 11,696 DEGs were upregulated and 5,949 were downregulated at 72 hpi. In a previous transcriptome study on *S. sisymbriifolium* inoculated with *V. dahliae* [18], there were significantly more upregulated (4,137) than downregulated DEGs (2,922). After the resistant wild eggplant material was inoculated with *V. dahliae*, more genes participated in the response, which showed that more genes were significantly upregulated, so as to prevent the invasion of pathogens. After the susceptible material M239 was inoculated with *V. dahliae*, due to its innate immune ability, some genes participated in the resistance

response and showed differential expression. However, compared with the resistant material, M239 lacked the positive response of some genes, showing that most genes were downregulated.

In addition, in the plant disease resistance response, secondary metabolites can be used as biochemical barriers to prevent pathogen infection and as signal substances participating in disease resistance [29]. In the KEGG enrichment analysis of DEGs in this study, it was found that only a few metabolic pathways in M239 reached the enrichment level, among which ribosom-related metabolic pathways such as "ribosome", "ribosome biogenesis in eukaryotes", "glycolysis/gluconeogenesis", and "ribosome biogenesis in eukaryotes" were the most enriched. In the resistant materials of *S. aculeatissimum* [17], "metabolic pathways", "biosynthesis of secondary metabolites", "plant pathogen interaction", and "plant hormone signal transmission" were the four most enriched metabolic pathways. In *S. sisymbriifolium* [18], the most enriched pathways were "plant hormone signal transduction", "phenylpropanoid biosynthesis", "plant pathogen interaction", and "taurine and taurine metabolism".

Through the comparison of the above transcriptome data, it was found that after *V. dahliae* inoculation, many metabolic pathways were enriched in the *Verticillium* wilt-resistant materials *S. aculeatissimum* and *S. sisymbriifolium*; these mainly included the secondary metabolic pathways related to disease resistance, such as plant disease interaction, plant hormone signal transduction and secondary metabolite synthesis. In the susceptible material M239, the enriched metabolic pathways were increasingly less related to primary metabolism, such as sugar metabolism, and amino acid and ribosome formation.

It is known that plant disease interactions are one of the most important pathways in the defence response of plants to pathogens. Several studies have shown that many genes of the plant disease interaction pathway are involved in the interaction process between plants and *V. dahliae* [18,30–32]. In addition, plant hormones are important signalling molecules that regulate the development process of plants; they regulate plants in various biotic and abiotic stress responses by forming signal networks [33]. Studies have shown that jasmonic acid (JA)-mediated disease resistance signal transduction plays a key role in the interaction between cotton and *V. dahliae* [34,35]. Lignin and phytoalexin produced by the phenylpropanoid metabolic pathway also play key roles in the process of plant antibacterial activity. Lignin can enhance the ability of the host cell wall to resist pathogen penetration, while phytoalexin has a direct toxic effect on pathogens [36]. In this study, M239 may be unable to resist the further invasion of pathogens, and finally become susceptible, due to the lack of response of the above key metabolic pathways. The above results proved that plant-pathogen interaction, plant hormone signal transduction and secondary metabolite synthesis are the key disease resistance pathways in the interaction between eggplant and *V. dahliae*. In addition, the brassinolide synthesis pathway was significantly enriched at 12~48 h after M239 was inoculated with *V. dahliae*, indicating that this pathway may play an important role in plant innate immune resistance.

4.2 Ve Genes

Plant resistance genes can mainly prevent pathogen invasion in plants, i.e., R genes, which include NB-LRR (nucleus-binding leucine rich repeat) genes, RLK (receptor-like kinases) genes, and RLP (receptor-like protein) genes [37,38]. Among them, plant receptor-like proteins (RLPs), as a class of cell surface receptors, widely exist in higher plants and play important roles in regulating plant growth and development and the stress response [39]. NB-LRR proteins can directly or indirectly recognize pathogen-specific effectors and stimulate specific immune responses [40,41].

Ve genes encode leucine-rich repeat proteins as cell surface receptors, namely, eLRR-RLPs (leucine-rich repeat receptor-like proteins), which are the most reported important genes related to *Verticillium* wilt resistance [4–6,9,11–13]. The *Ve* gene was first isolated and cloned from tomato (*S. lycopersicum*) and can effectively resist *V. dahliae* race 1. This gene locus includes two members, *Ve1* and *Ve2* [5]. Previous

studies have shown that *Ve1* rather than *Ve2* plays a major role in resistance to *V. dahliae* race 1 in tomato [4–6]. In potato, *Ve1* and *Ve2* were both found to have *Verticillium* wilt resistance [42,43]. In addition, three homologous Ve genes, *mVe1*, *Vr1*, and *Gbve1*, were proven to play major roles in resistance to *Verticillium* wilt in mint, lettuce, and cotton, respectively [9,44,45]. However, it is not clear whether *Ve1* or *Ve2* is the main resistance gene in the resistance process of eggplant *Verticillium* wilt.

In this study, there was only a *Ve2* homologous gene in M239. However, the *Ve1* and *Ve2* genes were both transcriptionally expressed in the resistant materials *S. torvum* [13] and *S. sisymbriifolium* [18]. Therefore, compared with *Ve2*, *Ve1* may play a key role in wild eggplant *Verticillium* wilt resistance, though this speculation still needs further verification.

4.3 PR Proteins

Plant pathogenesis-related proteins (PRs) are a class of proteins produced by plants induced by biotic or abiotic stress and are related to the response of plants to stress. Studies have shown that PR1, PR2, PR5 and PR10 play a wide range of roles in biotic stress [46–48] and are often used as indicator genes in plant disease resistance response and systemic acquired resistance (SAR) [49–51]. Among them, PR1 has been proven to have antiviral diffusion functions, limiting fungal invasion and protecting plants against stress [52]. PR5, also known as thaumatin-like protein, can destroy the integrity of the fungal cell wall by changing the permeability of the cell membrane and then inhibit fungal growth [53,54]. PR10 is a ribozyme, and studies have shown that it protects plants against fungi [48,55].

In this study, the differential expression of *PR1* and *PR5* was detected in M239. However, in the transcriptome sequencing of *S. aculeatissimum* [17] and *S. sisymbriifolium* [18], *PR1*, *PR5* and *PR10* were found to be simultaneously differentially expressed, and *PR10* showed a significant upwards trend within 48 h after *S. sisymbriifolium* was inoculated with *V. dahliae*. The PR1, PR5 and PR10 proteins may all have certain functions in the interaction between the wild eggplant and *V. dahliae*, and the PR10 protein may play a more critical role in this process.

4.4 TFs

TFs, as important upstream regulatory proteins, play important roles in plant pathogen interactions [56]. Among them, bZIP, NAC, MYB, AP2-EREBPE and WRKY are common transcription factor families in plants. These transcription factors usually act as positive or negative regulatory factors in plant hormone-mediated disease resistance and stress signal regulation [3,57]. WRKY transcription factors are one of the largest transcription factor families in higher plants and play an important role in regulating the plant response to stress [31,58]. In addition, bHLH transcription factors often combine with the MBY family to form complexes and regulate the downstream expression of target genes together [59].

In this study, after M239 was inoculated with *V. dahliae*, six TF families MYB, AP2-EREBP, WRK, bHLH, NAC, and Orphans, were all detected at different time periods. In *S. sisymbriifolium*, MYB, AP2-EREBP, Orphan, and bHLH were the top six TF families with the largest numbers, indicating that these four types of transcription factors may play a basic regulatory role in the interaction between the wild eggplant and *V. dahliae*. In addition, the WRKY family had more DEGs within 48 h after *S. sisymbriifolium* was inoculated with *V. dahliae*, while in M239, WRKY genes were differentially expressed only at the initial stage of infection (0~12 h post-inoculation). It is suggested that WRKY transcription factors may also play an important role in transcriptional regulation of wild eggplant resistance to *Verticillium* wilt.

5 Conclusion

In this study, we screened and analyzed DEGs, KEGG enrichment pathways and key resistance genes in the roots of M239, a highly susceptible material to *Verticillium* wilt, within 48 h after inoculation with

V. dahliae by transcriptome sequencing, and compared the results with the reportedly resistant wild eggplant materials (*S. aculeatissimum* and *S. sisymbriifolium*). It was found that in the process of responding to *V. dahliae*, the susceptible material M239 had fewer genes and metabolic pathways involved in the defence response, lacked the responses of some known key disease resistance genes and metabolic pathways, failed to form an effective defence network, and ultimately became susceptible to infection.

The above results are helpful to better understand the interaction process between the wild eggplant and *V. dahliae*, and provide some ideas for further understanding the molecular mechanism of plant resistance to *Verticillium* wilt. However, further research is needed due to the complex diversity of plant *Verticillium* wilt resistance mechanisms, and the significant difference in the genetic backgrounds of different materials.

Authorship: Liyan Wu and Guanghui Du designed the experiments. Jie Cheng, Yaju Gong, Rui Bao, Zhibin Li and Min Gui conducted the experiments and analyzed the data. Liyan Wu and Guanghui Du wrote the manuscript.

Availability of Data and Materials: The raw sequencing data in this study were deposited at the NCBI Sequence Read Archive (SRA) Database (Accession No. PRJNA495025).

Funding Statement: This work was supported by the National Natural Science Foundation of China (31960594), the Yunnan Fundamental Research Projects (202201AT070074, 2019FB059), and the Yunnan Technical Innovation Talent Training Project (202205AD160029).

Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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Appendix

Table S1: Transcription factors at M_12 h *vs*. M_0 h in M239

Table S2: Transcription factors at M_48 h *vs*. M_12 h in M239