

Article

Metavirome Detection and Analysis of Viruses Present in Diseased Pumpkin in Shandong, China

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Abstract

Viral diseases pose a serious threat to pumpkin cultivation, which is an important cucurbitaceous vegetable crop. Recently, multi-virus mixed infections in plants have been continuously detected and reported. However, studies on mixed virus infections in pumpkins are limited. Through metavirome and polymerase chain reaction (PCR) analysis, we found that pumpkins exhibiting severe viral symptoms were co-infected with squash leaf curl China virus and tomato leaf curl New Delhi virus. Transcriptome analysis revealed that 2927 genes were upregulated, and 2273 were downregulated in virus-infected pumpkin plants, compared to the gene expression in healthy pumpkin plants. Cluster analysis showed that the expression of genes related to RNA silencing and the salicylic acid resistance pathway was higher in virus-infected pumpkin plants than in healthy pumpkin plants. Furthermore, quantitative real-time PCR confirmed that the expression pattern of genes related to RNA silencing and the salicylic acid resistance pathway aligned with the transcriptome sequencing results. Our findings provide a reference for investigating the mechanism of mixed infections by these two viruses to aid in the prevention and control of viral diseases in pumpkins.

Keywords: pumpkin; metavirome sequencing; squash leaf curl China virus; tomato leafcurl New Delhi virus; RNA silencing; salicylic acid resistance pathway

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1. Introduction

Pumpkin, a nutrient-rich vegetable crop, is widely cultivated worldwide owing to its economic benefits. Viral diseases are a prime cause affecting pumpkin yield and quality [1]. Several plant viruses infect pumpkins [2,3]. The phenomenon of mixed viral infection is common in plants, and the symptoms of viral diseases caused by different viral combinations are distinct [4–6]. Viral infections in pumpkin lead to symptoms such as leaf shrinkage, stunted growth, and reduced seed setting rates. However, there is limited information on the combination of viral infections that result in severe viral phenotypes in pumpkin plants, highlighting the need to evaluate mixed viral species.

Metavirome analysis is a new detection approach that integrates metatranscriptome theory with molecular biological techniques for identifying viruses [7,8]. Four viruses

linked to the yellowing disease of green Sichuan pepper (*Zanthoxylum armatum*) flowers have been successfully identified using metavirome analysis [9]. Additionally, viruses related to the paper mulberry (*Broussonetia papyrifera*) leaf curl disease have also been identified using this method [10]. The virus assemblage linked to flower yellowing disease in pepper trees has been analyzed using metagenomic sequencing [11]. Furthermore, metavirome detection has been used to explore the diversity of viruses in wild citrus, leading to the identification of 44 viral isolates [12]. However, the use of the metavirome detection technique in vegetable crops needs to be expanded.

Viral infections affect host resistance pathways. RNA silencing is a common defense mechanism in eukaryotes [13–15]. The jasmonic acid signaling pathway triggers the expression of OsAGO18 in response to rice stripe virus (RSV) infection and enhances the antiviral defense of rice by inducing RNA silencing [16]. The small RNA miR444 in rice responds to RSV infection by upregulating its expression, which alleviates the transcriptional suppression of OsRDR1, thus activating the OsRDR1-dependent antiviral RNA silencing pathway [17]. Overexpression of NtRDR1 and the presence of SlRDR1 can delay the accumulation of potato spindle tuber viroids within the host at the early stage of infection [18]. Salicylic acid (SA) plays an important role in plant antiviral defense. Barley stripe mosaic virus γ b protein interacts with NbTRXh1 to inhibit its reductase activity, thereby weakening NbTRXh1-mediated defense response through the SA pathway [19]. The interaction between the NIb protein of the turnip mosaic virus and host NPR1 disrupts the interaction between NPR1 and SUMO3, affects SUMOylation-dependent phosphorylation of NPR1, and inhibits NPR1-mediated resistance to viral infections through the SA pathway [20]. The 1a protein encoded by cucumber mosaic virus interacts with host NAC2 to affect its subcellular localization and stability, thereby inhibiting NAC2-mediated SA-dependent resistance [21].

Pumpkin virus disease causes considerable economic losses every year, and effective control measures have not been identified to date. In production practice, damage can only be reduced by isolating the source of infection, disrupting the transmission pathways, and improving the resistance of pumpkin plants. Among these strategies, in-depth understanding and regulation of pumpkin resistance pathways and their improvement are the most economical and effective ways to control pumpkin virus disease. Therefore, in this study, we aimed to identify viral species in pumpkin plants exhibiting severe viral-disease phenotypes during field production. Additionally, we assessed the plant resistance pathways to provide a reference for antiviral research in pumpkin cultivation.

2. Materials and Methods

2.1. Plant Materials

Pumpkin plants were obtained from an experimental field at the Shandong Academy of Agricultural Sciences, Jinan, Shandong Province, China. Ten each of normal (MS) and virus-infected (SS) pumpkin plants grown for 30 days in the field were collected. Leaves of MS plants were normally expanded, and leaf color and plant height were normal (Figure 1a and Figure S1a). Leaves of SS plants were severely yellow and curly, and plants were dwarf (Figure 1b and Figure S1b). The second and third expanded leaves of the upper parts of MS and SS plants were collected, packed into three parts, frozen in liquid nitrogen, and transported to Novogene Co., Ltd. (Beijing, China) for metavirome sequencing.



Figure 1. Phenotypic comparison of pumpkin plants in the field: (a) normal (MS) plant phenotype; (b) virus-infected (SS) plant phenotype.

2.2. Total RNA Extraction and cDNA Synthesis

Leaves (1 g) were ground in a mortar using liquid nitrogen; the powder was collected into a 2 mL microcentrifuge tube, and 1 mL TriQuick Reagent (R1100; Solarbio, Beijing, China) was added, shaken, and homogenized. After centrifugation at 12,000 rpm for 10 min, 400 μ L supernatant was taken into a fresh microcentrifuge tube, and 200 μ L chloroform was added and mixed. The mixture was kept at room temperature for 3 min and centrifuged at 12,000 rpm for 10 min, and a 400- μ L aliquot collected from the upper layer was mixed with 200 μ L isopropanol. After allowing it to stand for 10 min, the mixture was centrifuged at 12,000 rpm for 10 min to precipitate RNA. The RNA pellet was washed with 75% ethanol and dissolved in RNase-free deionized water. cDNA was synthesized by reverse transcription using HiScript III All-in-one RT SuperMix Perfect for qPCR (R333; Vazyme, Nanjing, China) according to the manufacturer's instructions.

2.3. Transcriptome Sequencing and Analysis

Ribosomal RNA was removed from total RNA using a TIANSeq rRNA Depletion Kit (NR101-T7; TIANGEN, Beijing, China); RNA was broken into approximately 250–300 bp fragments; and cDNA was obtained by reverse transcription. After end repair, A tail, adaptor ligation, fragment size selection, and amplification by polymerase chain reaction (PCR), a library was prepared for quality control and sequencing. Fastp (<https://github.com/OpenGene/fastp>, 26 November 2025) was used to preprocess the raw sequencing data to obtain clean data for subsequent analyses [22]. The reference genome (NCBI RefSeq assembly: GCF_002738365.1) was aligned using HISAT2; gene expression was analyzed; and read counts of each gene were obtained using HTSeq-count [23–25]. R was used to perform principal component analysis and gene mapping to evaluate sample

biological duplication. Differentially expressed genes were analyzed using DESeq2, and genes that met the $\text{Padj} \leq 0.05$ and $|\log_2(\text{fold change})| \geq 1.0$ threshold were defined as differentially expressed genes [26].

2.4. Verification of Known Viral Expression

After comparing the effective data with the host reference genome (NCBI RefSeq assembly: GCF_002738365.1), the host gene was removed, and the non-host sequence was used to annotate viral sequence information. Using DIAMOND (<https://github.com/bbuchfink/diamond/>, 28 October 2025) [27], the spliced sequences were compared with the database GenBank (<https://ftp.ncbi.nih.gov/genbank/>, 28/10/2025), and viral sequences with ≥ 90 identity were screened as a known virus annotated in the GenBank database. Similarly, after comparison with NR (<https://ftp.ncbi.nlm.nih.gov/blast/db/>, 28 October 2025), Refseq virus (<https://ftp.ncbi.nlm.nih.gov/refseq/>, 28 October 2025), and CDD (<https://ftp.ncbi.nlm.nih.gov/pub/mmdb/cdd/>, 28 October 2025) databases, the viruses screened from the four databases were combined as known viruses for subsequent analysis. The information of species classification annotated by each database was extracted as the species annotation of known viruses. The fragments per kilobase million (FPKM) method was used to calculate the relative abundance of each gene, and the relative-abundance table of species was obtained.

2.5. PCR and Quantitative Real-Time PCR (qRT-PCR)

cDNA obtained from plant total RNA was amplified by PCR using a 2× Taq Master Mix (P112; Vazyme) according to the manufacturer's instructions. PCR products were electrophoresed on 1% agarose gel at 80 V for 40 min, and gels were observed using UV light to analyze the products. qRT-PCR was performed using a ChamQ SYBR Color qPCR Master Mix (Vazyme, Nanjing, China) on a real-time PCR system (CFX96; Bio-Rad Laboratories). The thermal cycles comprised pre-denaturation at 95 °C for 3 min and 40 cycles of denaturation at 95 °C for 20 s, annealing at 60 °C, and extension at 72 °C for 15 s. Melting-curve analysis was also conducted. EF1α and actin were selected as internal reference genes. The primer sequences and other instructions are shown in Table S1.

2.6. Statistical Analysis

All experiments were repeated at least thrice. Data are presented as mean ± SD. Statistical analysis was performed using t-test for significant difference using GraphPad Prism v.7.0.

3. Results

3.1. Metavirome Sequencing and Analysis of Viral Gene Expression

Transcriptome sequencing was conducted on three MS and three SS leaves, and the filtered raw data of 5.17 G (MS-1), 5.23 G (MS-2), 5.53 G (MS-3), 4.87 G (SS-1), 5.54 G (SS-2), and 6.47 G (SS-3) were obtained (Figure 2a,b). The data were compared with the host reference genome (NCBI RefSeq assembly: GCF_002738365.1), and 0.37 G (MS-1), 0.38 G (MS-2), 0.41 G (MS-3), 0.8 G (SS-1), 0.91 G (SS-2), and 1.08 G (SS-3) non-host sequences were obtained (Table S2). The non-host sequences were compared with the viral reference sequences; viral gene expression was analyzed (Table S3–S8), and the FPKM density distribution map was obtained by conversion and correction (Figure 2c,d). The quantities of non-host sequences differed significantly between the two groups, yet the viral gene expression within each group was consistent, which was suitable for subsequent analysis of viral species.

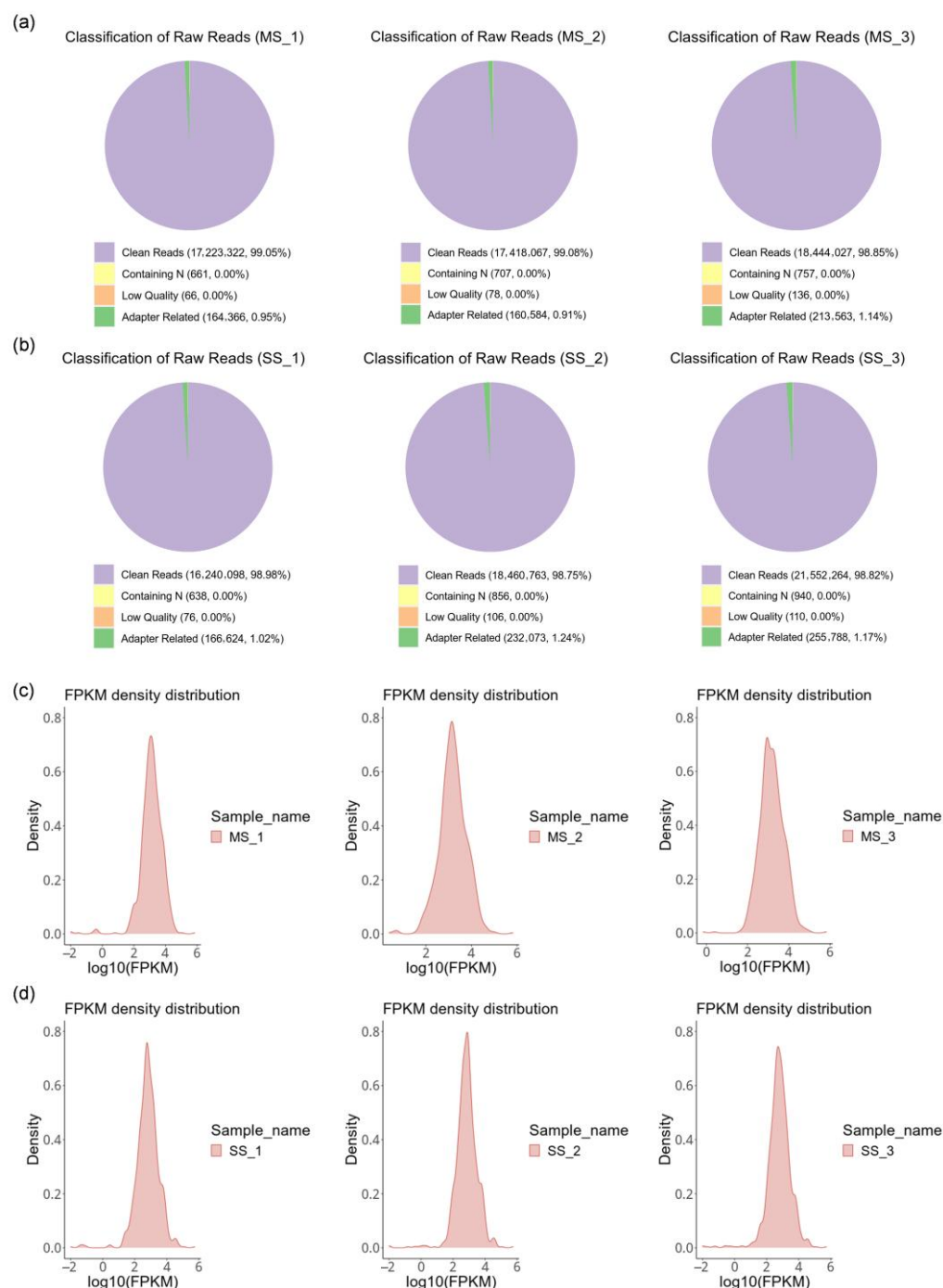


Figure 2. Metavirome sequencing and analysis of viral gene expression: (a) metavirome sequencing data filtering ratio diagram of normal (MS) plants; (b) metavirome sequencing data filtering ratio diagram of virus-infected (SS) plants; (c) density distribution of viral gene expression (FPKM) in normal (MS) plants; (d) density distribution of viral gene expression (FPKM) in virus-infected (SS) plants.

3.2. Statistics and Verification of Known Virus Expression

The viral species were annotated according to the reference sequence, and the 10 top species with the highest relative expression in each sample were selected to draw a statistical diagram of their relative expression (Figure 3a and Table S9). Among them, squash leaf curl China virus (SLCCNV) and tomato leaf curl New Delhi virus (ToLCNDV) were the main viruses in leaves of SS plants (Figure 3a). However, leaves of MS plants did not contain these two viruses (Figure 3a). The results of cluster analysis were similar to those of relative expression of species (Figure 3b and Table S10). Although cucurbit chlorotic

yellow virus (CCYV) was detected, its expression and proportion were minimal (Figure 3b and Table S10). PCR analysis confirmed the presence of SLCCNV and ToLCNDV in the leaves of SS plants, whereas CCYV was not detected (Figure 3c–e). The few signals of CCYV may be misreading caused by background noise. The above viruses were not detected in leaves of MS plants by PCR (Figure 3c–e). These results indicate that the diseased pumpkin plants were co-infected with SLCCNV and ToLCNDV.

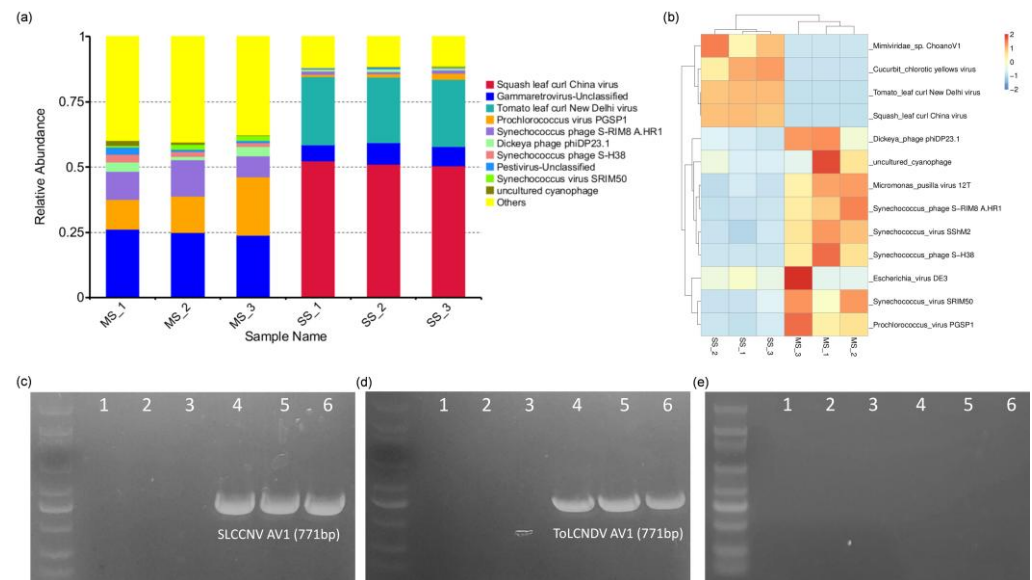


Figure 3. Statistics and verification of known viral expression: (a) statistical diagram of relative expression of viral species in two groups of pumpkin plants; normal (MS) and virus-infected (SS); (b) cluster diagram of viral species expression in two groups of pumpkin plants; (c–e) detection of three pumpkin viruses by PCR (lanes 1–3, MS plant samples; lanes 4–6, SS plant samples; the leftmost lane, marker).

3.3. Upregulation of Resistance Pathway-Related Genes in Response to Mixed Infection by SLCCNV and ToLCNDV

We compared and analyzed the data of two groups of pumpkin transcriptome (Figure 4a,b, and Table S11) and found 5200 differentially expressed genes, of which 2927 were upregulated, and 2273 were downregulated in the SS group, compared to the expression of genes in the control group (Table S12). Cluster analysis of differentially expressed genes in RNA silencing and SA signaling pathways showed that most of them detected in these two resistance pathways were upregulated in SS plants (Figure 4a,b). qRT-PCR analysis revealed that the expression of six RNA silencing pathway-related genes in SS plants was significantly upregulated ($p < 0.001$) than that of these genes in MS plants (Figure 5a). The expression of seven SA signaling pathway-related genes was detected. Among them, the expression of LOC111436010 ($p < 0.05$) and LOC111457839 ($p < 0.001$) was significantly downregulated, and that of other genes was significantly upregulated ($p < 0.001$; Figure 5b). The trend of differential gene regulation detected by qRT-PCR was consistent with the trend of transcriptome sequencing results. The above results indicate that most of the genes in the RNA silencing and SA signaling pathways in SS plants are upregulated in response to virus infection.

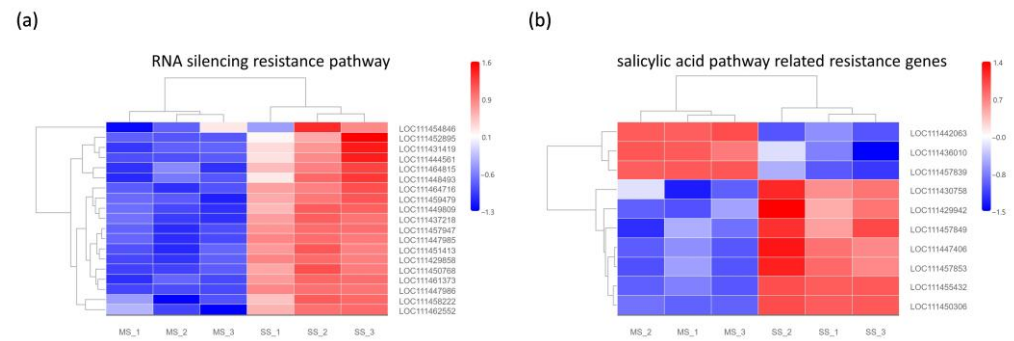


Figure 4. Resistance pathway genes were upregulated in response to mixed infection by squash leaf curl China virus (SLCCNV) and tomato leaf curl New Delhi virus (ToLCNDV): (a) cluster thermogram of differentially expressed genes in RNA silencing pathway; (b) cluster thermogram of differentially expressed genes in SA signaling pathway.

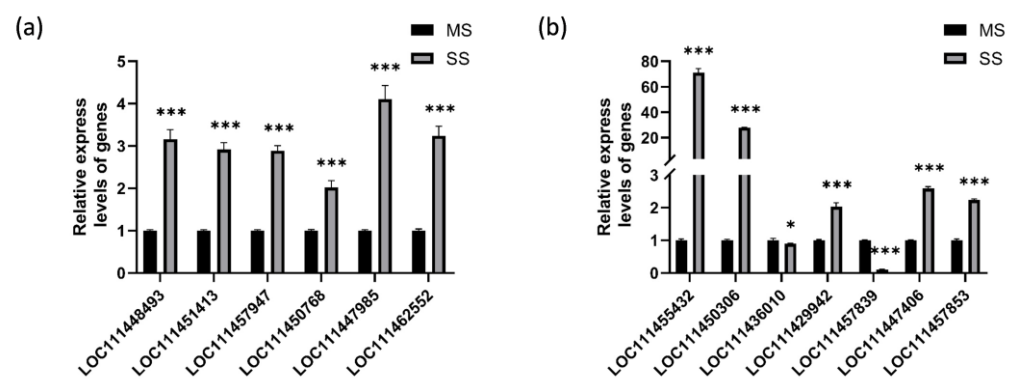


Figure 5. qRT-PCR validation. (a) Relative expression of RNA silencing pathway-related genes in normal (MS) and virus-infected (SS) plants detected by qRT-PCR. (b) Relative expression of SA signaling pathway-related genes in MS and SS plants detected by qRT-PCR. All experiments were conducted thrice. The bar represents SD. * $p < 0.05$, *** $p < 0.001$.

4. Discussion

SLCCNV and ToLCNDV are geminiviruses, which primarily infect pumpkin plants and can be transmitted by *Bemisia tabaci* [28–30]. SLCCNV-encoded AC5 inhibits host RNA silencing to promote viral infection [31]. The three proteins, AV2, AC2, and AC4, of ToLCNDV inhibit plant RNA silencing and are closely related to the pathogenicity of ToLCNDV [32,33]. Through metaviromics, we identified a mixed infection by SLCCNV and ToLCNDV. In susceptible pumpkin plants, the SLCCNV fragments were approximately twice as numerous as those of ToLCNDV fragments.

Enhancing the natural resistance of pumpkins is the most economical and effective strategy for managing viral diseases in pumpkins. Utilizing RNA silencing can enhance the resistance of plant hosts to geminiviruses such as ToLCNDV [34,35]. Double-stranded RNA designed based on the AC2 sequence of ToLCNDV can reduce ToLCNDV content in *Nicotiana benthamiana* leaves and roots [36]. Exogenous application of double-stranded RNA targeting the ToLCNDV DNA-A chain reduces ToLCNDV accumulation in zucchini [37]. In this study, a mixed infection of SLCCNV and ToLCNDV triggered the activation of genes related to the RNA silencing pathway in pumpkin. Plant viruses are constantly evolving through natural selection, so the resistance pathway of plants induced by viruses is not enough to resist the infection of plant viruses. Additional exogenous double-stranded RNA spraying may help to reduce the damage of viral diseases by improving RNA silencing resistance in plants.

The SA signaling and RNA-silencing pathways synergistically confer antiviral resistance. SA treatment can induce the expression of multiple RNA silencing pathway-related genes in plants and enhance their resistance to RNA viruses [38]. SA can enhance the activity or stability of RNA-dependent RNA polymerase and improve the resistance of *N. benthamiana* to tobacco mosaic virus [39]. SA increases the expression of RNA-silencing pathway-related genes and enhances the resistance of Arabidopsis to bamboo mosaic virus [40]. We noticed that infection of SLCCNV and ToLCNDV increased the expression of SA signaling and RNA-silencing pathway-related genes in pumpkin. In our future research, we aim to focus on the genes in the SA signaling pathway that respond strongly to viral infections. Our findings also revealed that the level of gene up-regulation induced by virus infection in the SA signaling pathway was higher than that in the RNA silencing pathway, although the consistency of induced expression in SA signaling pathway genes by virus infection was lower than that of RNA silencing pathway genes. The down-regulation of a small number of SA signaling pathway genes during viral infection may balance this more responsive pathway, aligning with the long-term coexistence of viruses and plant hosts. Further investigation is needed to determine if there is a deeper connection between the SA resistance and RNA silencing pathways.

The present study identified a mixed infection combination of SLCCNV and ToLCNDV, which may be the direct cause of severe symptoms of pumpkin virus disease. Since no single virus-infected pumpkin plants were found in the field for comparison, it remains uncertain whether SLCCNV or ToLCNDV alone can cause similar symptoms. The mixed infection induced the expression of genes related to RNA silencing and SA signaling pathways. The phenomenon of virus-induced plant host resistance pathway gene expression is universal [16,19]. Although the intensity of induced expression of host resistance pathway genes varies with different virus infections, manipulating the resistance pathway to resist virus infection holds significant potential for application. Overall, this study provides a reference for further investigation into the mechanisms of mixed viral invasions and antiviral strategies in pumpkin cultivation.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/v18020232/s1>, Figure S1: Phenotypic comparison of pumpkin plants in field (high angle); Table S1: primer sequences and usage instructions; Table S2: Datastat; Table S3: MS_1.Readcount_FPKM; Table S4: MS_2.Readcount_FPKM; Table S5: MS_3.Readcount_FPKM; Table S6: SS_1.Readcount_FPKM; Table S7: SS_2.Readcount_FPKM; Table S8: SS_3.Readcount_FPKM; Table S9: table.s10.tran; Table S10: cluster.s; Table S11: SSvsMS_deg; Table S12: SSvsMS_deg_all.

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Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

SLCCNV	squash leaf curl China virus
ToLCNDV	tomato leaf curl New Delhi virus
SA	salicylic acid

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