

Article

Comparative Analysis of the Expression Profiles of Pathogenesis-Related Genes in Tomato Systemically Infected with Tobacco Mosaic and Cucumber Mosaic Viruses

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Abstract: In this study, we used RT-qPCR to examine how PR genes were expressed in model tomato (*Solanum lycopersicum* L.) plants that had been infected with TMV or CMV. Under greenhouse conditions, the indirect ELISA data showed that both viruses were detected for the first time at 6 dpi. Then, the levels of accumulation increased very quickly, reaching a peak of 15 dpi. During the course of the study (1–15 dpi), the Delta C_T, NormFinder, BestKeeper, and GeNorm software tools revealed that the β -actin gene was the most informative reference gene in the virally infected tomato tissues. For both the TMV- and CMV-infected tomato plants, the transcriptional expression levels of most tested genes changed between activation and repression, especially in the first 12 dpi. Compared to mock-inoculated plants, the expression levels of *PR-1* were induced at all time intervals except at 8 dpi for CMV and at 6, 7, and 8 dpi for TMV infection. Conversely, the greater activation and accumulation of both viruses were associated with the greater up-regulation of *PR-2* at 8 dpi, with relative expression levels of 7.28- and 5.84-fold for TMV and CMV, respectively. The up-regulated expression of *PR-3*, *PR-4*, and *PR-7* was shown at 4 dpi. In contrast, the *PR-5* gene was inhibited in TMV at 1 dpi until 9 dpi, and the induction of this gene at 10 dpi increased by 1.72-fold, but *PR-5* was observed to up-regulate the expression of CMV at 1 dpi. This study provides the first valuable information on the comparative transcriptional levels of these tomato genes between TMV and CMV infections.

Keywords: tobacco mosaic virus; cucumber mosaic virus; tomato; pathogenesis-related proteins; RT-qPCR; gene expression



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1. Introduction

Plant viruses are the most important pathogens that cause serious crop production problems once they appear in the field [1,2]. Plant viruses are responsible for the world's most damaging agricultural diseases, which cost billions of dollars every year [3]. Tobacco mosaic virus (TMV)—genus Tobamovirus, family Virgaviridae—is one of the most common plant viruses that causes serious quality and crop production damage worldwide [4,5]. TMV is a single-stranded RNA virus that infects approximately 885 plant species from 65 families, mainly tobacco and tomato plants, as well as other members of the *Solanaceae* family [4,5]. TMV infection causes mosaic symptoms on the leaves as well as plant tissue yellowing. The virus has resulted in significant economic losses all over the world [6]. Cucumber mosaic virus (CMV)—genus Cucumovirus, family Bromoviridae—is one of the most damaging and economically essential plant viruses, causing serious crop quality and productivity problems worldwide [6]. It affects about 1200 species of plants in over 100 plant families around the world, including monocots and dicots [7]. CMV has three single-stranded

linear RNAs with a positive sense (RNA-1, RNA-2, and RNA-3) that code for four viral proteins [8].

When a pathogen attacks a plant, several signaling pathways are activated, leading to the production of secondary metabolites and the release of antimicrobial defense proteins. Pathogenesis-related (PR) proteins are a family of proteins that have been shown to accumulate in large amounts in plants following a pathogen infection. Since they were discovered in tobacco (*Nicotiana tabacum* L.) as a result of TMV infection, these proteins have been thoroughly studied and characterized [9]. Upon viral infection, the plant undergoes a series of gene expression changes, with several PR genes being induced as part of the defense response [9,10]. PR genes are involved in plant defense against biotic stressors, and a subset of these genes may be responsible for systemic acquired resistance to pathogens such as plant viruses [9]. Based on their functions, interactions with antibodies, amino acid sequences, number of molecules, and a few other factors, Linthorst and Van Loon [11] classified PR proteins into different categories. Because they are either extremely acidic or extremely basic, PR proteins are highly soluble and, as a result, reactive. At least 14 different protein families belong to the PR category. Most of these have known functions or activities: for example, PR-2 is a β -1,3-glucanase; PR-3, -4, -8, and -11 are different types of chitinase; PR-5 is a thaumatin-like protein; PR-6 is a proteinase inhibitor; PR-7 is an endoproteinase; PR-9 is a peroxidase; PR-10 is a ribonuclease; PR-12 is a defensin; PR-13 is a thionin; and PR-14 is a lipid-transfer protein [12]. There are often a large number of isoforms of each PR protein present in the diverse host plants.

Real-time quantitative polymerase chain reaction (RT-qPCR) has been shown to be a good way to measure gene expression in many situations, including those involving plant viruses [13]. This is because it is sensitive, accurate, and repeatable when it comes to detecting small amounts of RNA molecules. However, the results are only reliable if they have been normalized using a reference gene that is expressed the same way in all tissues and experimental conditions. Housekeeping genes are thus good candidates for universal internal controls. As a result, if an inappropriate reference gene is used, then the reliability and accuracy of RT-qPCR are compromised [14,15]. For the purpose of this study, we evaluated the stability of three potential housekeeping genes (*EF1- α* , *18S rRNA*, and *β -actin*) in a TMV and/or CMV tomato infection, grading their reliability as internal controls. Additionally, we examined how six tomato PR genes (*PR-1*, *PR-2*, *PR-3*, *PR-4*, *PR-5*, and *PR-7*) were expressed differently between TMV-infected, CMV-infected, and mock-inoculated samples every day for 15 days after infection (1–15 dpi) to compare and study how the plants reacted to the two viruses.

2. Materials and Methods

2.1. Sources of Tomato Seeds and Viruses

The Egyptian Agriculture Research center provided the virus-free tomato (*Solanum lycopersicum* L.) seeds of the Carmen F1 variety, which is susceptible to viral infection. In addition, two purified viral isolates (TMV; accession number MG264131 and CMV; accession number MN594112) were utilized as viral inoculums [16,17].

2.2. Greenhouse Experimental Design, Mechanical Inoculation and Sample Collection

The tomato seeds were sterilized in a controlled environment and then grown in sterile vermiculite pots. After 28 days of growth, the seedlings were moved to other pots. Each pot (20 cm) contained 3 kg of autoclave-sterilized sand and clay (1:1). After a week, each plant's top two true leaves were mechanically inoculated with 0.5 mL of 20 μ g/mL freshly prepared TMV or CMV [18]. The experiment was carried out over three treatments (Figure 1). The first treatment (mock) was the control treatment, in which tomato plants were inoculated with viral inoculation buffer. The second treatment was tomato plants inoculated with TMV. The third treatment was tomato plants inoculated with CMV. Each treatment consisted of five pots (five biological replicates), and each pot contained five tomato plants. All the plants were kept in the greenhouse at a temperature of 28/16 °C

day and night with a relative humidity of 65%. The CMV and TMV symptoms were observed on each day. The tomato leaves (not mechanically inoculated) were collected daily from 0 to 15 dpi and then subjected to further analyses (ELISA and RNA extraction). The independent biological replicate of each treatment was a pool of 15 tomato leaves that were collected from five plants, with three leaves per plant, grown in each pot. Each biological replicate was tested in three independent technical replicates for reliability.

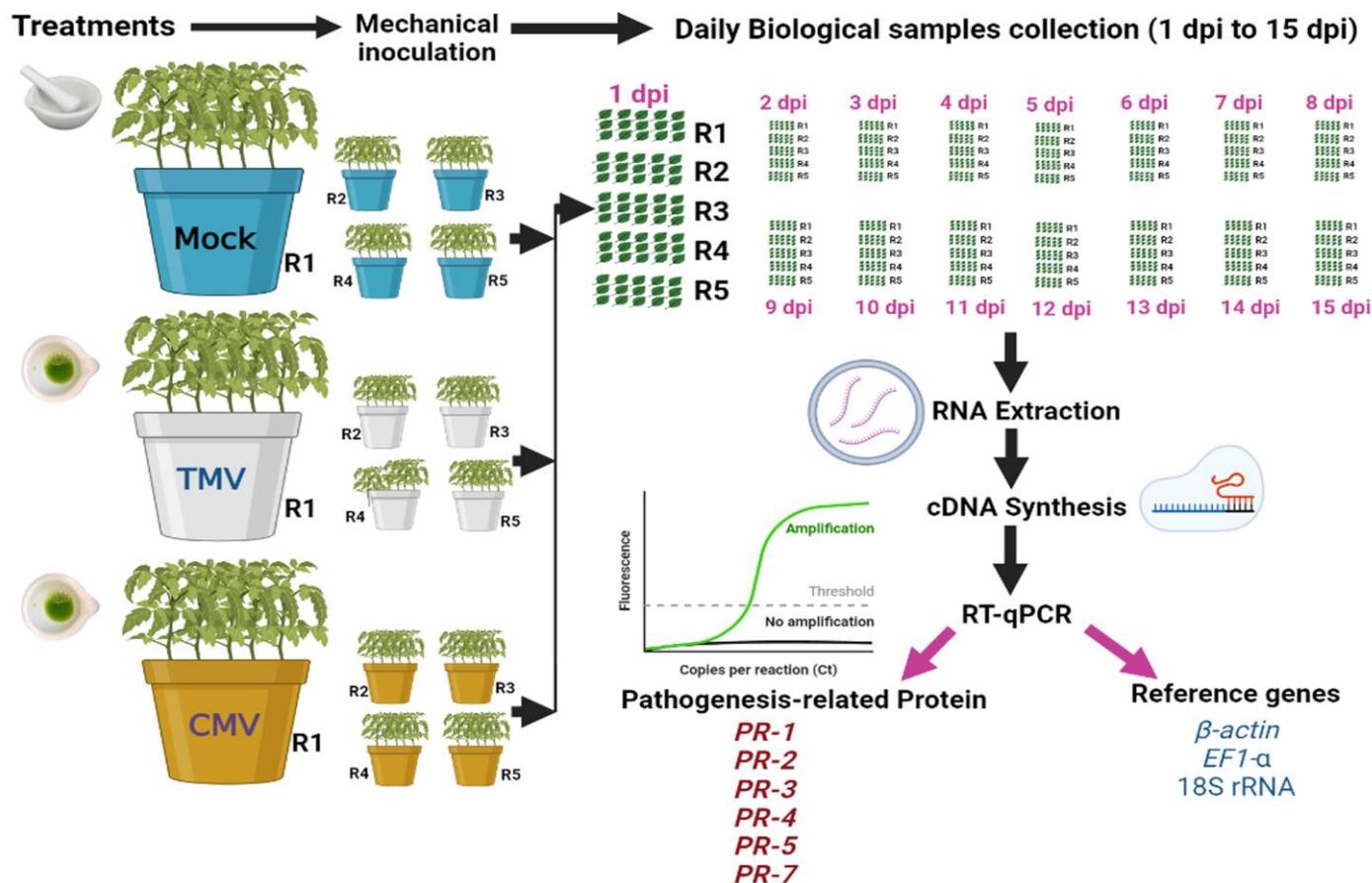


Figure 1. A schematic diagram summarizing the experimental study.

2.3. Detection of TMV or CMV by Enzyme-Linked Immunosorbent Assay

The indirect enzyme-linked immunosorbent assay (I-ELISA) was used to detect the viral infection in the collected samples as previously described [19]. An absorbance at 405 nm was used as the unit of measurement for the ELISA results. If the absorbance value of the sample was double the threshold value of the healthy control samples, then it was said that there were viruses in the sample.

2.4. RNA Extraction and cDNA Synthesis

Total RNA was isolated from tomato leaves (100 mg fresh weight) using the RNeasy Plant Mini Kit (QIAGEN, Hilden, Germany), as recommended by the supplier. UV-Vis spectroscopy was used to determine the purity and quantity of the isolated RNA (EMCLAB Instruments GmbH, Duisburg, Germany). The RNA integrity was assessed by visualizing the quality of the isolated RNA via 1.2% agarose gel electrophoresis. For each sample, 1 µg of DNase-I-treated RNA was used as a template for cDNA synthesis using a reverse transcriptase enzyme (M-MuLV Reverse Transcriptase, Biolabs, New England, Ipswich, MA, USA) with oligo (dT) and random hexamer primers. The RT-PCR reaction was carried out in 20 µL and programmed at 42 °C for 1 h, followed by a deactivation step at 80 °C for 5 min. The cDNA was then stored at −20 °C until used in RT-qPCR.

2.5. Normalization and Standardization of the Housekeeping Genes

Three housekeeping genes were tested during the post-viral infection time course to identify the most stably expressed reference gene(s) in virally infected tomato plants: β -actin, elongation factor 1- α (EF1- α), and the 18S rRNA. The data were analyzed using four different programs: the Delta C_T method (<https://toptipbio.com/delta-delta-ct-qpcr>; accessed on 1 January 2023), NormFinder (<https://moma.dk/normfinder-software>; accessed on 2 January 2023), BestKeeper (<https://www.gene-quantification.de/bestkeeper.html>; accessed on 2 January 2023), and GeNorm (<https://genorm.cmgg>; accessed on 3 January 2023).

2.6. RT-qPCR Assay

Six different PR genes (*PR-1*, *PR-2*, *PR-3*, *PR-4*, *PR-5*, and *PR-7*) were tested in this investigation. Table 1 displays the primer sequences. A total of 20 μ L of a combination containing 1 μ L of each primer at 10 pmol/ μ L, 1 μ L of template cDNA, 10 μ L of 2xSYBR Green PCR Master Mix, and 7 μ L of nuclease-free water were used for the RT-qPCR reaction. Three independent replicates of each sample were run on the Rotor-Gene 6000 (QIAGEN, ABI System, Miami, FL, USA). The thermal cycling amplification technique included a 10 min denaturation at 95 °C, then 40 cycles of 15 s denaturation at the same temperature, 30 s annealing at 60 °C, and 30 s extension at 72 °C. After 40 cycles, melting curves were obtained to filter out any non-specific products. The qPCR efficiency was measured for each gene and was between 93% and 100% for all genes. The melting curve study at temperatures between 55 and 95 °C showed only one amplified product for all genes.

Table 1. Nucleotide sequences of PR gene primers used in this study.

Gene Name	Specific Class	Accession Number	Nucleotide Sequence (5'-3')	Reference
<i>PR-1</i>	Pathogenesis related protein-1	AJ011520	Forward: CCAAGACTATCTTGGCGGTT Reverse: GAACCTAAGCCACGATACCA	[20]
<i>PR-2</i>	β -1, 3-glucanases	M80604	Forward: TATAGCCGTTGGAAACGAAG Reverse: CAACTTGCCATCACATTCTG	[21]
<i>PR-3</i>	Chitinase	AF043248	Forward: ACTGGAGGATGGGCTTCAGCA Reverse: TGGATGGGGCCTCGTCCGAA	[22]
<i>PR-4</i>	Chitin-binding proteins Classes I (hevein-like domain)	X67244F	Forward: GACAACAATGCGGTCTCAAGG Reverse: AGCATGTTTCTGGAATCAGGCTG	[23]
<i>PR-5</i>	Thaumatococcus-like protein	Y17275	Forward: ATGGGGTAAACCACCAAACA Reverse: GTTAGTTGGGCCGAAAGACA	[24]
<i>PR-7</i>	Endoproteinase	BT013707	Forward: AGGCAGGATTTGCTGGTATGATGCT Reverse: ATACGCATCCTTCTGTCCCATTCGA	[25]
<i>EF1-α</i>	Housekeeping gene	AB061263	Forward: ATTGGAAATGGATATGCTCCA Reverse: TCCTTACCTGAACGCCTGTCA	[25]
18S rRNA	Housekeeping gene	X51576	Forward: GGGCATTTCGTATTTCATAGTCAGA Reverse: GTTCTTGATTAATGAAAACATCCT	

2.7. RT-qPCR Data Analysis

To figure out the relative expression ratio, the method created by Livak and Schmittgen [26] was used. With the use of an automated threshold analysis using the Applied Biosystems Integrated (ABI) system, we were able to calculate the C_T (threshold of cycle) value for each

gene. The equations show the mathematical model of the relative expression ratio to be obtained, where:

$$\Delta C_{T \text{ target}} = (C_{T \text{ target}} - C_{T \text{ reference}}), \Delta C_{T \text{ control}} = (C_{T \text{ control}} - C_{T \text{ reference}})$$

The equations show the mathematical model of the relative gene expression level ratio to be obtained, where:

$$\Delta\Delta C_{T \text{ expression}} = (\Delta C_{T \text{ target}} - \Delta C_{T \text{ control}}), \text{ accordingly } 2^{-\Delta\Delta C_{T \text{ expression}}} \text{ algorithm.}$$

2.8. Statistical Analysis

Using the CoStat program, we performed a one-way analysis of variance (ANOVA) on the relative expression of three biological replicates for each time, and we used post hoc Tukey's honest differences (HSD) to distinguish between means at the $p \leq 0.05$ level. The relative expression levels with the most statistically significant differences were plotted, and the standard deviation (\pm SD) is shown as a column bar. If the value was more than one, then it meant that gene expression was being turned up (up-regulated), and if it was less than one, then it meant that expression was being turned down (down-regulated).

3. Results and Discussion

3.1. Symptoms Development and Virus Detection

Under greenhouse conditions, both TMV- and CMV-infected tomato plants displayed multiple morphological changes. Similar to previous reports [27,28], the development of systemic mosaic with chlorosis symptoms on TMV tomato leaves was reported at 11 dpi. On the other hand, early CMV symptoms were noticed at 13 dpi. At 14 dpi, all the TMV-inoculated plants showed severe TMV-like symptoms such as mosaic, leaf deformation, and thickness on some tomato leaves (Figure 2B). On the other hand, at 16 dpi, all of the plants that had been infected with CMV showed severe symptoms such as mottling, mosaic, yellowing, and flecks of dead tissue (Figure 2C). The viral infections had a significant effect on leaf morphogenesis, which changed the shape of the leaves and slowed the growth of the whole plant [29,30]. An I-ELISA assay (Table 2) revealed that the first detection of viral infections was at 6 dpi for both TMV and CMV infection. Then, there was a dramatic increase in the accumulation levels, reaching a maximum of 15 dpi. No virus was detected in the mock-inoculated samples. The obtained results support previous studies, which showed that a viral infection might be detected in the early stages of an infected potato plant or other differential hosts [31,32].

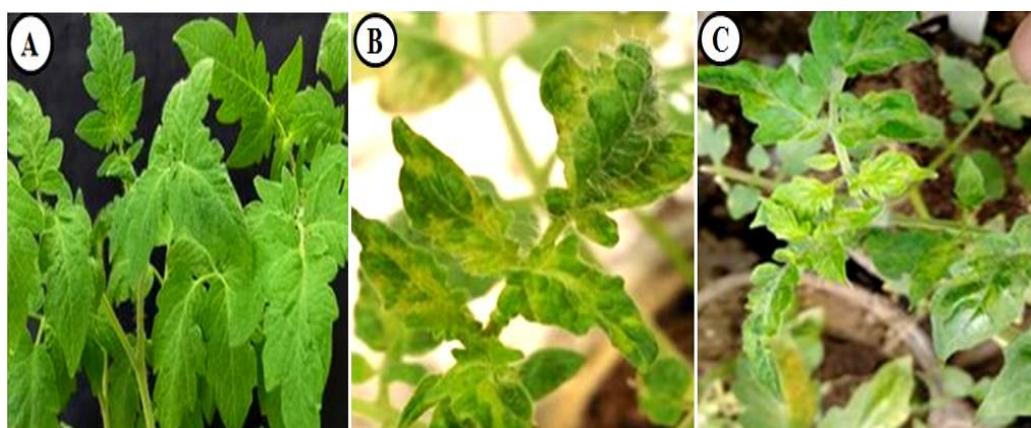


Figure 2. Overview of tomato plants showing TMV and CMV symptoms under greenhouse conditions at 14 days post-viral infection. (A) Tomato control plants; (B) TMV-infected tomato plants; (C) CMV-infected tomato plants.

Table 2. Indirect ELISA detection of TMV or CMV in a 1:10 dilution of sap extracted from inoculated tomato plants collected at different time intervals (1:15 dpi).

Days Post-Inoculation (dpi)	Absorbance ELISA Values at 405 nm			
	TMV		CMV	
	ELISA Value	Result	ELISA Value	Result
1	0.212 ± 0.024	-	0.208 ± 0.035	-
2	0.223 ± 0.037	-	0.217 ± 0.029	-
3	0.243 ± 0.049	-	0.255 ± 0.041	-
4	0.267 ± 0.019	-	0.287 ± 0.052	-
5	0.347 ± 0.027	-	0.315 ± 0.063	-
6	0.424 ± 0.036	+	0.417 ± 0.043	+
7	0.517 ± 0.044	+	0.491 ± 0.079	+
8	0.580 ± 0.025	+	0.538 ± 0.095	+
9	0.654 ± 0.068	+	0.576 ± 0.082	+
10	0.750 ± 0.024	+	0.638 ± 0.076	+
11	0.808 ± 0.043	+	0.781 ± 0.097	+
12	0.926 ± 0.079	+	0.856 ± 0.081	+
13	0.997 ± 0.087	+	0.894 ± 0.099	+
14	1.174 ± 0.089	+	0.975 ± 0.098	+
15	1.561 ± 0.103	+	1.269 ± 0.111	+
Mock-inoculated plants (healthy)	0.187 ± 0.011	-	0.187 ± 0.011	-

3.2. Determination and Standardization of the Housekeeping Genes

In order to provide an overview of the relative abundance of the three reference (housekeeping) genes in tomatoes, we determined cycle threshold (C_T) values for all of these genes under both TMV and CMV infections (Supplementary Figure S1). The C_T values for *EF1- α* and *18S rRNA* greatly fluctuated, with a difference between the largest and smallest values of about 60% for *18S rRNA* and 48% for *EF1- α* . Even though there was little *β -actin* C_T fluctuation observed, the difference between the largest and smallest values did not exceed 4%, and it had the lowest standard error of all samples (Figures 3 and 4). This provided the necessary evidence that *β -actin* could be used appropriately as an internal control in the downstream gene expression analyses of TMV and/or CMV tomato infection. The stability of the housekeeping genes was demonstrated in four distinct ways (Figures 3 and 4). Based on the data analysis, it was determined that actin exhibited the highest level of gene expression stability.

The obtained results are consistent with those of Mascia et al. [25], who reported that *β -actin* was one of the four least stable genes throughout virally infected tomato tissues. In RT-qPCR assays, the two housekeeping genes (*18S rRNA* and *EF1- α*) are typically used as reference genes [33], and *18S rRNA* has been shown to be suitable for normalization in cereals infected with the barley yellow dwarf virus [34]. Even though *EF1- α* was sequentially rated by the GeNorm analysis, the performance of *EF1- α* and *18S rRNA* was inadequate [35]. Additionally, our findings are in line with previous research in virus-infected tomatoes, which found highly varied levels of expression for both *18S rRNA* and *EF1- α* [25]. On the other hand, the incorrect use of invalidated reference genes might introduce bias into the research and lead to data misinterpretation [36]. Nevertheless, precise transcript normalization using stably expressed reference genes at diverse biological and physiological states is required for an accurate target gene expression analysis [37]. The data for the melt curve analysis and standard curves developed for each potential reference gene were included in the study, as well as a set of primers constructed for the ten candidate genes. In the future, this information can be used to choose one reference gene for use in normalizing RT-qPCR data related to tomato–virus interactions [38].

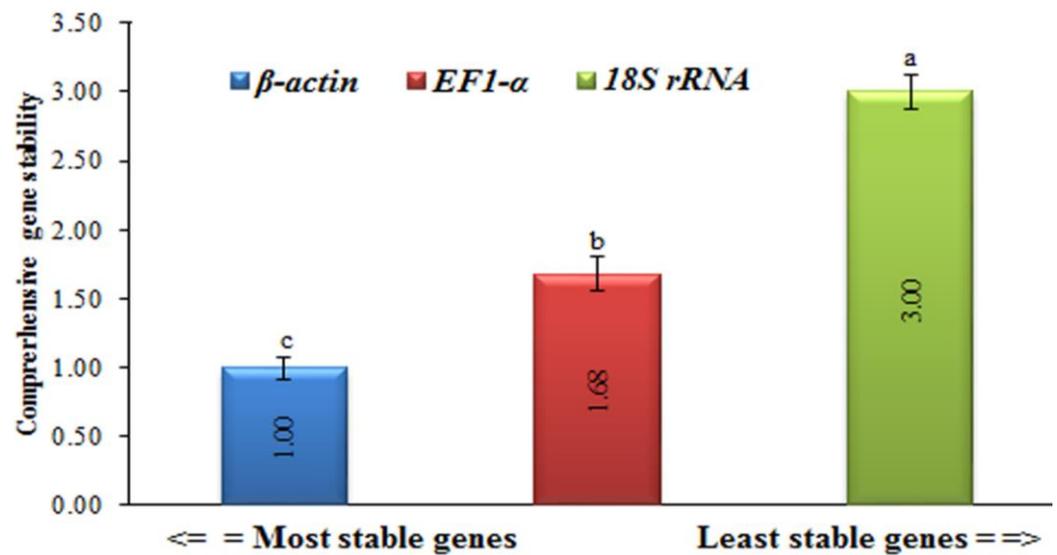


Figure 3. The four different methods used to evaluate the gene expression stability levels of house-keeping genes in healthy, TMV-, and CMV-infected tomato plants. The Delta C_T method, NormFinder, and BestKeeper (as is harbored by β -actin) indicates more stable gene expression. Average gene stability values (M value) across all treatment groups using GeNorm revealed more stable β -actin and EF1- α genes. The values in the columns represent the means of five independent biological samples, while the error bars show the standard deviation (SD). According to Tukey’s HSD test ($p \leq 0.05$), the values in the columns labeled with the same letter (a, b, and c) do not differ statistically.

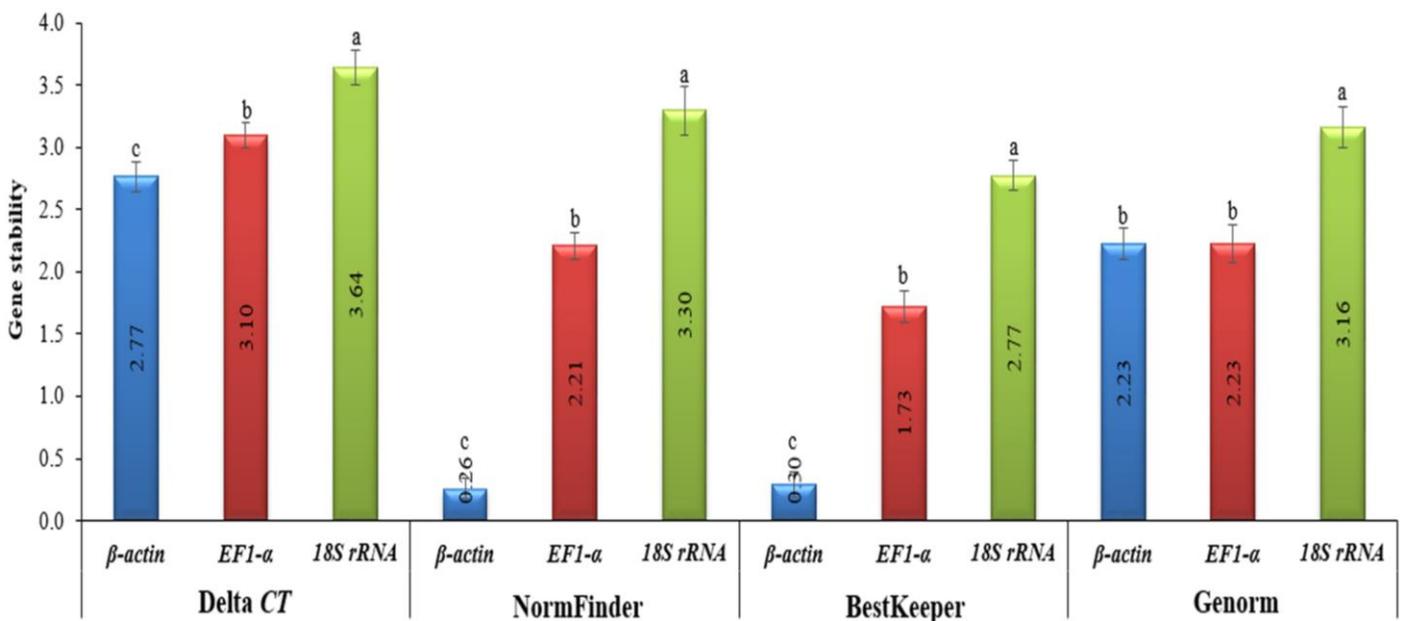


Figure 4. Comprehensive gene expression stability levels of three housekeeping genes (β -actin, EF1- α , and 18S rRNA) in healthy, TMV-, and CMV-infected leaf tissue samples of tomato plants. Statistically, there is no significant change between the means of the columns that start with the same letter.

3.3. RT-qPCR Analysis of Pathogenesis-Related (PR) Gene Expressions

There have been a number of studies that point to pathogenesis-related proteins (PR) as the proteins that are responsible for systemic acquired resistance (SAR), as well as the proteins that are effective at halting the progression, multiplication, or spread of pathogens [27,35,39]. In the current study, both TMV and CMV infections induced the expression of PR-1 at almost identical time intervals (Figure 5). The highest transcriptional levels of PR-1 were observed in TMV-infected tomato plants at 1, 2, 4, 5, 9, and 15 dpi, with

levels 2.93-fold, 2.92-fold, 2.09-fold, 4.79-fold, and 2.30-fold, and 1.99-fold, respectively, higher than the control. On the other hand, the higher gene expression of CMV-infected tomato plants was shown at 1, 2, 4, 6, 7, 10, and 11 dpi, with the relative expression being 2.80-fold, 3.37-fold, 4.35-fold, 2.50-fold, 4.84-fold, 2.10-fold, and 3.44-fold, respectively, greater than the control. These results agree with those of ElMorsi et al. [35], who reported that the *PR-1* gene was quickly induced or up-regulated during the early stages of an onion plant with IYSV infection, and it was continuously expressed in onion tissues. Additionally, similar results here agree with Cutt et al. [40], who found that *PR-1* proteins in tobacco were associated with viral resistance. Similar results indicated that the induction of *PR-1* at 2 dpi appeared to reflect the early activation of SA biosynthesis in PVY-infected potato tissues [41]. On the other hand, the *PR-1* gene expression decreased at 6, 7, and 8 dpi (0.81-fold, 0.99-fold, and 0.90-fold, respectively) in the TMV-infected tomato plants compared with the CMV-infected tomato plants, where the down-regulation of the gene expression occurred at 8 dpi only (Figure 5). These data suggest that the tomato *PR-1* is not sufficient for resistance to TMV or CMV. Plants synthesize different *PR-1* proteins and have different ways of fighting off various plant pathogens [42,43]. Many studies have found that the increased expression of *PR-1* in transgenic plants is linked to increased resistance to bacteria, fungi, and oomycetes, but not to viruses [44,45]. According to the findings of this study, the relative gene expression of *PR-1* may be associated with a response to viral infection.

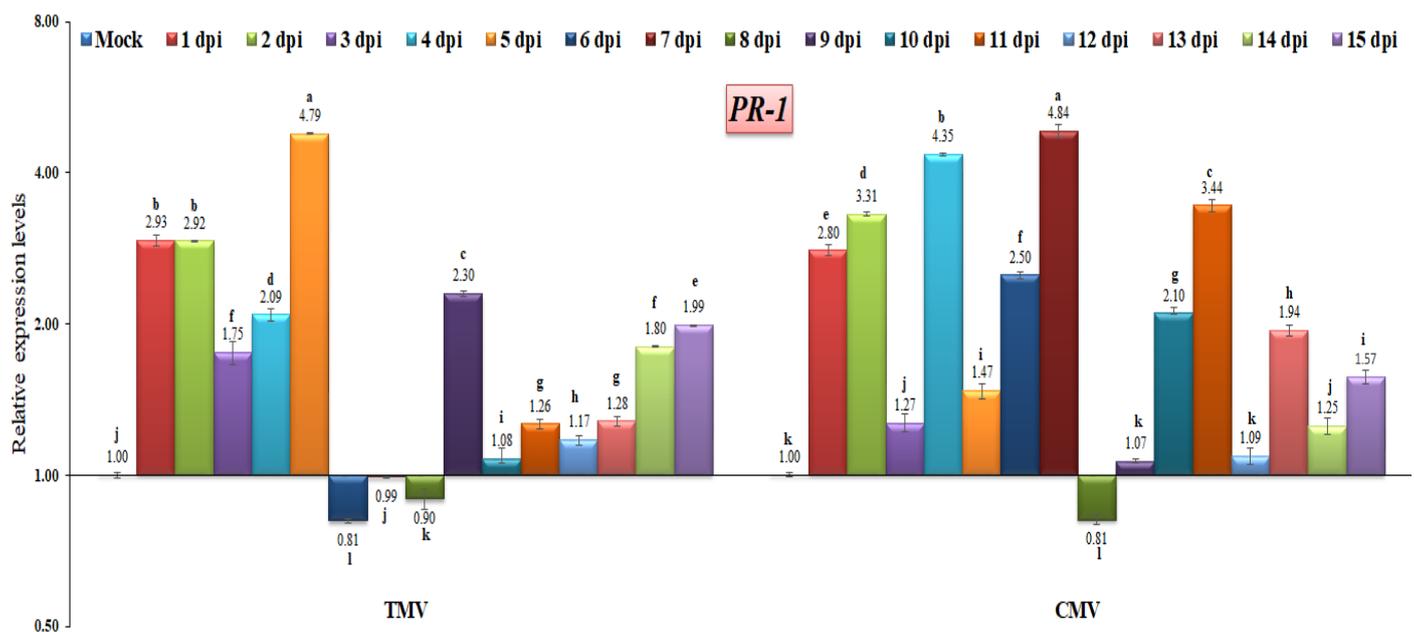


Figure 5. Comparison between the relative transcriptional levels of the tomato *PR-1* gene in TMV- and CMV-infected tissues compared to control at 1 to 15 dpi. The columns show the mean value from five biological replicates, and the bars represent the standard deviation (SD). Means were distinguished using Tukey's HSD test at $p \leq 0.05$ levels, and small letters are used to represent differences. Columns of data showing different letters are significant.

The *PR-2* gene in tomato plants infected with TMV or CMV showed significant transcriptional levels at 8 dpi, with levels of 7.28-fold and 5.84-fold, respectively, as compared to uninfected tomato plants. The expression level was down-regulated at the first three days of TMV infection, with relative expression level changes of 0.7-fold, 0.97-fold, and 0.81-fold, respectively. On the other hand, compared to the control, the CMV-infected tomato plants showed up-regulation at all days post-infection (Figure 6). Therefore, we hypothesized that the *PR-2* gene was related to viral infection. The *PR-2* gene, which codes for a 1,3-glucanase, plays a role in the cell-to-cell spread of virus movement complexes by limiting callose accumulation around the plasmodesmata [46]. TMV is thought to increase

tobacco activation for *PR-2* genes, allowing it to move more easily into cells [47]. Our findings are in agreement with the previous studies that reported a clear induction of *PR-2* during viral infections in Arabidopsis, tobacco, potato, onion and tomato plants [35,48,49]. Furthermore, lowering tobacco *PR-2* reduces susceptibility to viral infection [50]. Moreover, the reduction in tobacco *PR-2* expression decreased viral infection susceptibility, whereas overexpression increased potato virus Y movement across cells [51,52]. Although *PR-1* expression is the most commonly utilized as a marker for resistance response after viral infection, it has been demonstrated that *PR-2* is elevated in response to viral infection in tobacco leaves [53].

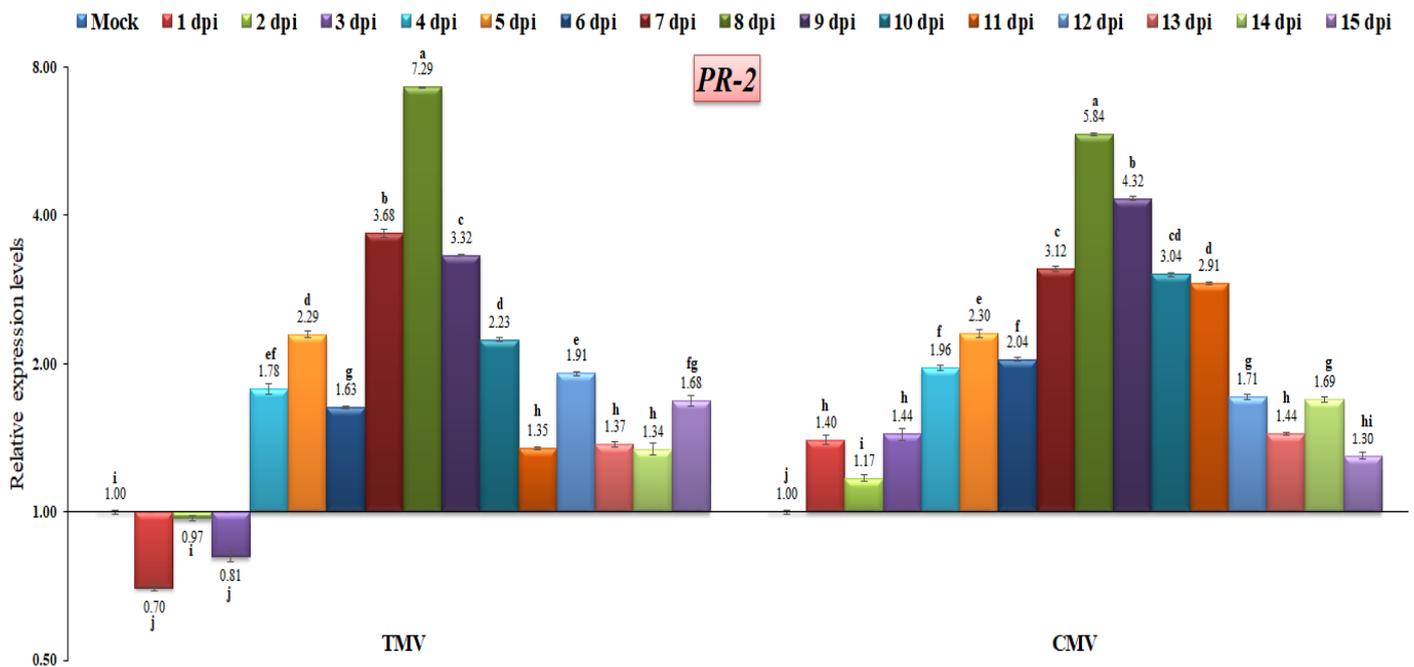


Figure 6. Comparison between the relative transcriptional levels of the tomato *PR-2* gene in TMV- and CMV-infected tissues compared to control at 1 to 15 dpi. The columns show the mean value from five biological replicates, and the bars represent the standard deviation (SD). Means were distinguished using Tukey's HSD test at $p \leq 0.05$ levels, and small letters are used to represent differences. Columns of data showing different letters are significant.

The *PR-3* family of proteins includes chitinases, which are responsible for catalyzing the hydrolysis of chitin [39,54]. Chitinases are mainly involved in the organism's defense against pathogen attack in both animals and plants [55]. The present study showed that *PR-3* gene expression was rapidly induced following the TMV infection of tomato plants, increasing from 1.45-fold at 1 dpi to 4-fold at 8 dpi (Figure 7). Conversely, *PR-3* was down-regulated at 11 and 12 dpi, with a relative expression level about 0.53-fold lower than the control (Figure 7). On the other hand, *PR-3* was induced in most of the CMV-infected tomato plants, with the highest relative expression levels being 4.90-, 4.07-, and 4.26-fold higher than the control at 4, 9, and 14 dpi, respectively (Figure 7). These results are the same as those of Abdelkhalek et al. [56], who found that *PR-3* accumulated in the first few days after a viral infection and became more active and higher until it reached its highest level at 4 days post-infection, which was 4.64-fold higher than in the control group. Additionally, the present findings agree with those of earlier research on the activation of chitinases in response to viral infections [57,58]. It has also been discovered that the infection caused by a pathogen boosts the naturally occurring production of enzymes that hydrolyze chitin [55,59–61]. On the other hand, it was seen that *Setaria viridis* at 10 days post-viral infection had a substantial reduction in *PR-3* expression [62]. Such a down-

regulation or decreased *PR-3* expression level may be due to viral suppressor activity or cellular damage [56].

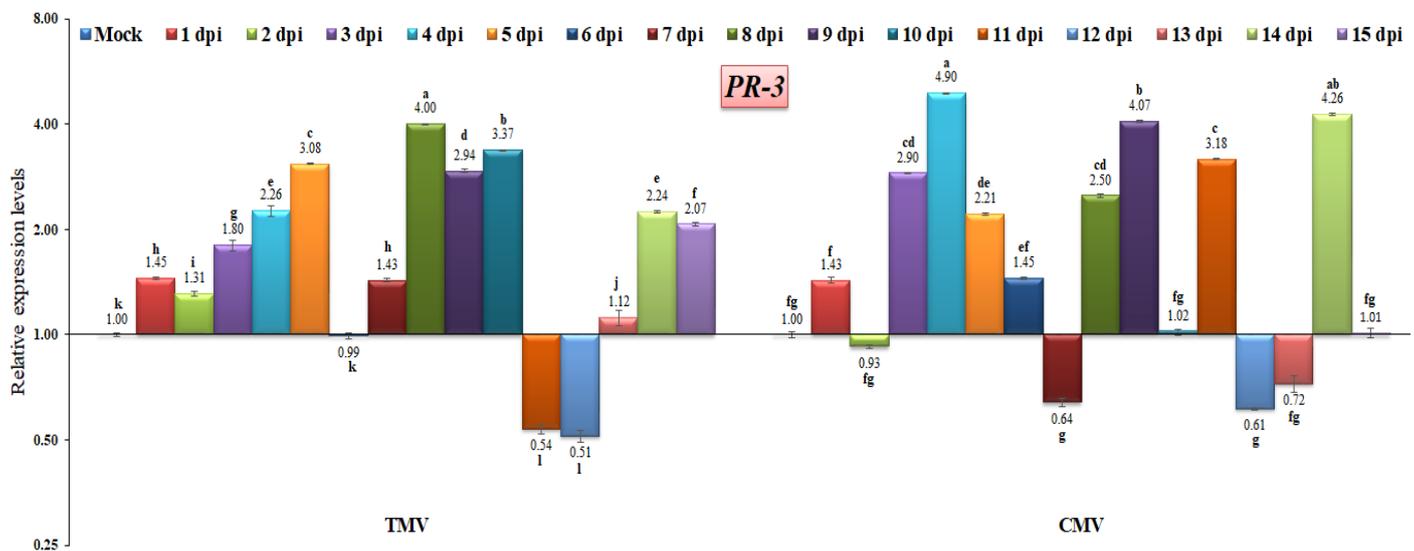


Figure 7. Comparison between the relative transcriptional levels of the tomato *PR-3* gene in TMV- and CMV-infected tissues compared to control at 1 to 15 dpi. The columns show the mean value from five biological replicates, and the bars represent the standard deviation (SD). Means were distinguished using Tukey's HSD test at $p \leq 0.05$ levels, and small letters are used to represent differences. Columns of data showing different letters are significant.

The *PR-4* protein family is classified as chitinases [39] and can be linked with a chitin-binding domain, also known as the hevein-like domain. This relationship distinguishes *PR-4* classes I (presence of a hevein-like domain) and II (absence of a hevein-like domain) [63,64]. However, other reports have shown its RNase activity [65] or nuclease activity [66]. In this study, the results revealed that *PR-4* (class I) was induced after three days of both viral infection and then increased, reaching maximum expression levels of 4.62-fold at 8 dpi in TMV-infected plants and 2.26-fold at 4 dpi in CMV-infected plants when compared to the control (Figure 8). Compared to the TMV-infected plant profile, the CMV-infected plants exhibited lower levels of *PR-4* expression, either through down-regulation at 1, 2, 6, and 7 dpi or up-regulation at 3, 4, 5, 8, 9, 10, 14, and 15 dpi (Figure 8). Thus, the expression profile of *PR-4* may depend on the type of viral infection. The outcomes obtained are consistent with those that Wang et al. [67] reported. They stated that the TMV-infection-induced tobacco plants expressed *PR-4* at moderate levels. It was reported that the increased overexpression of the *PR-4* gene in *Vitis vinifera* led to improved resistance to infection by powdery mildew [68]. As a result, we hypothesized that *PR-4* might not be associated with TMV or CMV resistance.

Concerning *PR-5*, it encodes a thaumatin-like protein, is present in cell vacuoles, and has antifungal activities [69]. *PR-5* is activated by the host plant against a wide range of pathogens, and it is thought to be a basal resistance protein [9,67]. In the current study, the *PR-5* gene exhibited down-regulation at almost all time intervals after TMV infection, except at 10, 13, 14, and 15 dpi, with relative expression levels that were 1.72-, 2.15-, 1.32-, and 1.15-fold higher than the control, respectively (Figure 8). In the current study, the *PR-5* gene had a low expression level at 1 to 9 dpi with a range of 0.25- to 0.96-fold in TMV-infected tomato plants (Figure 9). However, the highest expression level of *PR-5* was observed at 13, 14 and 15 dpi, with values of 2.15-fold, 1.32-fold, and 1.15-fold, respectively. Compared to the TMV-infected tomato plants, the CMV-infected tomato plants showed *PR-5* expression at 1 dpi, which was 2.11-fold higher than the control (Figure 9). Additionally, the *PR-5* gene reached its highest level of expression after it increased by 4.34-fold at 4 dpi. After that, the gene expression decreased and fell from 0.67-fold to 0.47-fold at 5, 6, 7, 8,

and 9 dpi. We suggest that in this study, the viral infection switched off the *PR-5* gene expression in this time period, which then highly increased to 4.85 at 10 dpi (Figure 9). It was reported that the virus infection had a moderate effect on *PR-5* expression [35]. The up-regulation of *PR-5* induces resistance in tomatoes, resulting in delayed symptom appearance and tomato spotted wilt virus accumulation [70]. The overexpression of *PR-5* in tobacco-vein-banding-mosaic-virus-infected tobacco plants and beet-severe-curly-top-virus-infected *Arabidopsis* has also been observed [71,72]. Similar results were reported by Wang et al. [73], who noticed that infection with the turnip crinkle virus resulted in an increase in *PR-5* expression. In addition, the rice dwarf virus also leads to the activation of the *PR-5* gene, suggesting that the *PR-5* domain might bind a polypeptide ligand and raising the possibility that mature *PR-5* proteins may also interact with polypeptides [74].

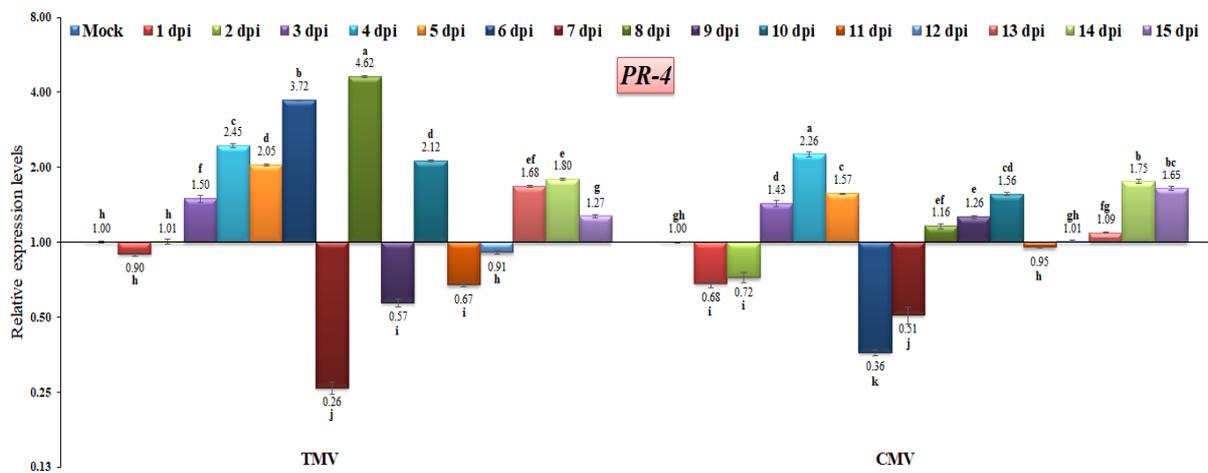


Figure 8. Comparison between the relative transcriptional levels of the tomato *PR-4* gene in TMV- and CMV-infected tissues compared to control at 1 to 15 dpi. The columns show the mean value from five biological replicates, and the bars represent the standard deviation (SD). Means were distinguished using Tukey’s HSD test at $p \leq 0.05$ levels, and small letters are used to represent differences. Columns of data showing different letters are significant.

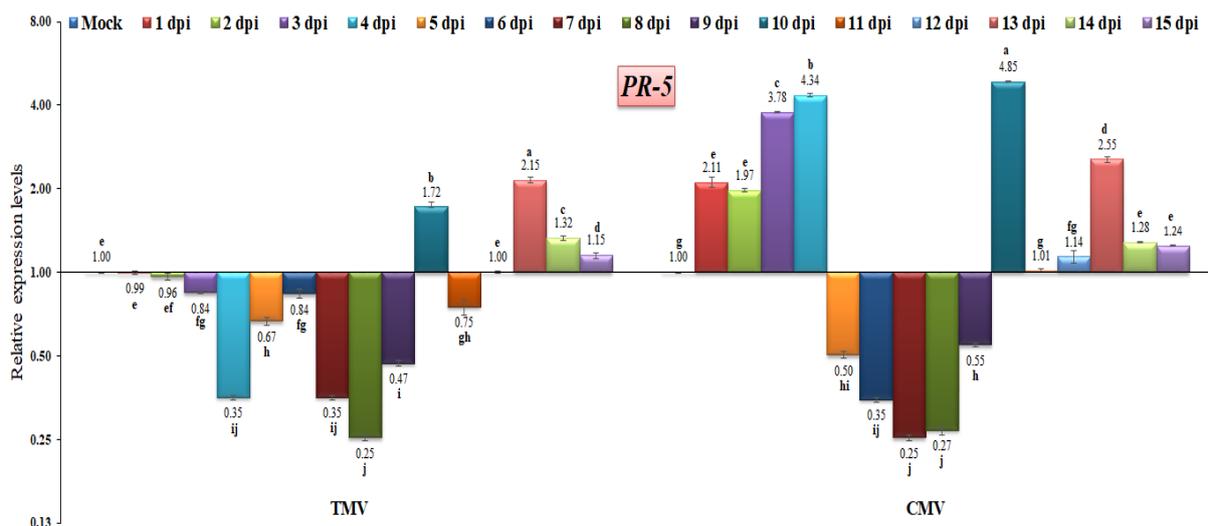


Figure 9. Comparison between the relative transcriptional levels of the tomato *PR-5* gene in TMV- and CMV-infected tissues compared to control at 1 to 15 dpi. The columns show the mean value from five biological replicates, and the bars represent the standard deviation (SD). Means were distinguished using Tukey’s HSD test at $p \leq 0.05$ levels, and small letters are used to represent differences. Columns of data showing different letters are significant.

PR-7, which codes for plant proteases, is known to be an important part of defensive response proteins and to help break down the cell walls of microorganisms [39,75]. TMV was shown to shut down *PR-7* gene expression at 1 dpi by 0.77-fold compared to the control (Figure 10). These two viruses (TMV and CMV) were up-regulated at 2 dpi and had almost the same expression levels at this point (1.77-fold and 1.65-fold, respectively). At 14 and 15 dpi, TMV-infected tomato plants showed that *PR-7* was building up and expressing itself much more than before (Figure 10). However, in tomato plants infected with CMV, *PR-7* was found to have rapid accumulation and significant maximum expression at 10, 11, and 14 dpi, with 4.3-fold, 4.2-fold, and 3.6-fold increases, respectively (Figure 10). On the other hand, in TMV or CMV-infected tomato plants, these two viruses shut down the *PR-7* gene expression at 5, 6, 7, and 8 dpi, and at 9 dpi in TMV only (Figure 10). These results agree with the previous report that stated that the *PR-7* expression level was increased by 11.1-fold at 9 days and reached higher levels of 34.7-fold at 15 days of TMV infection [16]. In another study, the up-regulation of tomato *PR-7* expression in response to pathogen infections was detected [75]. The results of this study showed that the *PR-5* and *PR-7* genes had a similar transcriptional profile in terms of how much they were expressed. It is also possible that these genes are co-regulated in a cis-acting way [72], which shows that they work together.

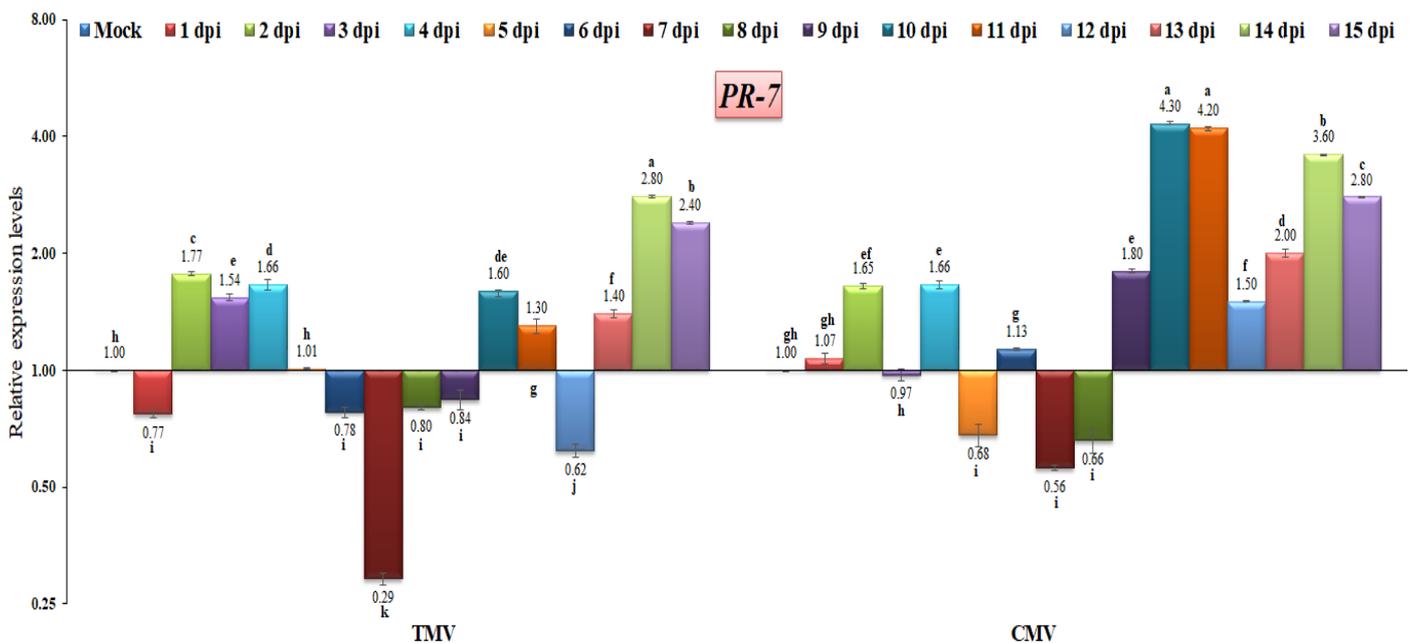


Figure 10. Comparison between the relative transcriptional levels of the tomato *PR-7* gene in TMV- and CMV-infected tissues compared to control at 1 to 15 dpi. The columns show the mean value from five biological replicates, and the bars represent the standard deviation (SD). Means were distinguished using Tukey's HSD test at $p \leq 0.05$ levels, and small letters are used to represent differences. Columns of data showing different letters are significant.

4. Conclusions

Globally, TMV and CMV have a significant impact on the tomato, a model species for the family *Solanaceae*. The comparative expression profiling of two destructive plant viruses infecting tomato plants, TMV and CMV, found a positive correlation between PR gene expression (*PR-1*, *PR-2*, *PR-3*, *PR-4*, *PR-5*, and *PR-7*) during the time course study (1 to 15 dpi). All the tested genes exhibited fluctuations in their expression levels at different time intervals, except *PR-2*, which showed up-regulation for both viruses at all intervals. Based on the expression profiles of other PR genes, we can divide the time course study into three time groups: The first is the virus replication stage or virus incubation period (1–6 dpi), in which almost all genes exhibited up-regulation, indicating a rapid response

of the plant against viral infection. The second group, at the early stage of infection or virus activation (7–10 days), is when most of these genes were down-regulated or exhibited lower expression levels, which may be related to viral suppressor activity. The last group is the late stage of infection or the symptom appearance stage (11–15 days), in which the genes begin to increase again after the virus has succeeded in multiplying and systemically transferring between cells. Finally, the differences between the expression profiles of PR genes for both viruses indicated that the response of the defense system of the same plant might be changed or different from one pathogen to another or from one virus to another. Overall, our results give important information about the biology behind how the tomato, TMV, and CMV interact. Based on the transcriptional data, future research should be able to find the host genes that are needed for a virus to take hold. This could lead to ways to control these and other viruses.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/ijpb14020035/s1>, Figure S1: The cycle threshold (Ct) for all samples with the three housekeeping genes (*EF-1 α* , *18S rRNA*, and *β -actin*).

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