A tomato kinesin-like protein is associated with *Tobacco mosaic virus* infection

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**ABSTRACT**

*Tobacco mosaic virus* (TMV) causes severe damage and economic losses of tomato in crop yield production and quality worldwide. Tomato plants infected with TMV usually show a mosaic pattern and chlorosis symptoms that result from affected chloroplasts. To elucidate tomato–TMV interactions, the transcriptional profiles of tomato tissues at different time intervals were analysed using a differential display-polymerase chain reaction technique. Compared to mock-inoculated plants, and based on gene expression changes in tomato plants, the representative down- and up-regulated genes were selected for further analysis. The Basic Local Alignment Search Tool at the National Center for Biotechnology Information (NCBI-BLAST) analysis revealed that the three up-regulated gene transcripts at 15 days post-inoculation were coding for chloroplast (MG565980 and MG565979) and kinesin-like proteins (MG565981). Additionally, the down-regulated gene was a chloroplast-related gene (MG565978). Interestingly, the two chloroplast genes, MG565978 and MG565980, shared more than 96% sequence identity, and their transmembrane profiles were nearly identical. On the other hand, many protein kinase C phosphorylation, casein kinase II phosphorylation and N-myristoylation sites were detected within the MG565981 gene. Moreover, the high similarity between the kinesin gene and many *Arabidopsis* defensin genes indicated that it might play an important role in the plant defence system against TMV infection. It should be stressed that studies of the pathways in which chloroplast and kinesin-like protein genes are involved may elucidate the mechanisms of tomato tolerance to viral infection and can lead us to a more comprehensive understanding of tomato–TMV interactions.

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**Introduction**

Plant diseases, especially plant viruses, cause a variety of problems and are responsible for huge losses of crop production and quality throughout the world [1]. Thus, viral infections must be controlled in order to keep up the standard and abundance of food, fibre and feed produced by farmers around the globe [2]. Tomato (*Solanum lycopersicum* L.) is the second most important cultivated and consumed vegetable crop after potato, worldwide [3,4]. Due to their suitability for genome manipulation, tomato plants are used as a model plant for scientific research in order to study the metabolic and fundamental biological processes and analyse plant-pathogen interactions [5].

*Tobacco mosaic virus* (TMV, genus *Tobamovirus*) is one of the most economically relevant viral threats to many economically important plants such as tobacco, tomato, pepper and potato, worldwide [6]. Many plant virologists consider it the most vital and serious plant virus [7]. Tomato plants are systemically infected, resulting in mosaic and chlorosis symptoms, often associated with plant deformations or altered structure and morphology. Moreover, many characteristics and gene expression level changes similar to stress and defence responses have been reported after viral infection [8].

Chloroplasts are plastids, one of the foremost dynamic organelles of plant cells. Chloroplasts perform photosynthesis, synthesise major phytohormones and are crucial for inter-organelle signalling [9]. Chloroplasts, as the main target for viruses, play a crucial role in the defence response and undergo...
enormous structural and functional damage during viral infection [9,10]. There is abundant and increasing evidence that chloroplast-associated genes are the most affected genes during viral infection [11].

Kinesins, a superfamily of microtubule-based motor proteins, are present among all eukaryotic organisms [12–14]. In plants, kinesins directly or indirectly contribute to cell division and growth in diverse tissues [15]. In addition to their roles in mitosis, morphogenesis and signal transduction [16], kinesins affect vesicle transport, organelle distribution, cellulose microfibril order and microtubule organisation [13,15,17,18].

The differential display-polymerase chain reaction (DD-PCR) technique provides a robust methodology for the fast identification of differentially expressed genes through scanning the differentially expressed mRNA in pathogen-infected plants [19–22]. Many studies on virus–host interactions use DD-PCR to isolate and characterise genes related to virus resistance [1,23,24]. Consequently, through using the DD-PCR technique, this study determined whether there were differentially expressed up- and/or down-regulated genes during tomato-TMV interactions, which are essential to better understand the tomato immune defence system against TMV infection. Moreover, further study of the function of these genes and the interactions between them will lead to improved tomato resistance against viral infections.

Materials and methods

Viral infections, sample collection and RNA extraction

The Egyptian TMV isolate used in this study (Acc# MG264131) was continuously maintained in our lab on Nicotiana benthamiana plants through a mechanical inoculation process. At 35 days after planting, two true leaves of Heinz 1706 tomato cultivar (Solanum lycopersicum L.) seedlings were dusted with carborundum. The selected PCR products (up/down-regulated genes) were sequenced directly after being excised and purified from the gel with a PCR clean-up column kit (Qiagen, Germany). Sanger sequencing of selected genes was performed using BigDye® Terminator v3.1 Cycle Sequencing kit and a 3130xl Genetic Analyzer system (Applied Biosystems). The obtained DNA nucleotide sequences were analysed using the Basic Local Alignment Search Tool at the National Center for Biotechnology Information (NCBI-BLAST; https://blast.ncbi.nlm.nih.gov/Blast.cgi) to confirm the identity of the obtained sequences. The phylogenetic tree was analysed and generated by MEGA5 software program using UPGMA (unweighted pair group method with arithmetic mean) statistical analysis method with 2000 bootstrap replicates. The transmembrane domain was performed. The reaction mixture contained 2.5 μL of 5X buffer with MgCl₂, 2.5 μL of 2.5 mmol/L deoxynucleoside triphosphates (dNTPs), 1 μL of oligo(dT) primer (20 pmol/μL), 4 μL of random hexamer primers, 1 μg of purified RNA and 200 U of reverse transcriptase (RT) enzyme (M-MLV; Fermentas). In a thermal cycler (Eppendorf), the reverse transcriptase reaction was performed at 42°C for one hour and deactivated at 72°C for 10 min. After holding at 4°C, the reaction mixture was stored at -20°C until used.

Differential display (DD)-PCR and detection of up/down-regulated genes

The five different arbitrary primers (Table 1) were used to scan the mRNA transcribed genes of infected plants as well as mock-treated plants. For DD-PCR, 1 μL of cDNA was added to 2.5 μL Taq polymerase buffer 10x (Promega, USA) containing a final concentration of 1 mmol/L MgCl₂, 0.2 mmol/L dNTPs, 0.4 mmol/L of primer (Table 1) and 0.2 μL Taq polymerase (5 U/μL) in a final reaction volume of 25 μL. The PCR reaction program started with an initial denaturation at 95°C for two minutes followed by forty cycles. Each cycle was programmed with 95°C for one minute, 30°C for one minute and 72°C for one minute. At the end of the last cycle, a final extension step at 72°C for 10 min was added. The RT-PCR amplification products were electrophoresed in 0.5X TBE (Tris–borate–ethylenediaminetetra-acetic acid buffer) with 1.5% agarose, stained with ethidium bromide and visually analysed by a gel documentation system.

Sequencing analysis and phylogenetic construction

The selected PCR products (up/down-regulated genes) were sequenced directly after being excised and purified from the gel with a PCR clean-up column kit (Qiagen, Germany). Sanger sequencing of selected genes was performed using BigDye® Terminator v3.1 Cycle Sequencing kit and a 3130xl Genetic Analyzer system (Applied Biosystems). The obtained DNA nucleotide sequences were analysed using the Basic Local Alignment Search Tool at the National Center for Biotechnology Information (NCBI-BLAST; https://blast.ncbi.nlm.nih.gov/Blast.cgi) to confirm the identity of the obtained sequences. The phylogenetic tree was analysed and generated by MEGA5 software program using UPGMA (unweighted pair group method with arithmetic mean) statistical analysis method with 2000 bootstrap replicates. The transmembrane domain
profiles were predicted by the TMpred program (https://embnet.vital-it.ch/software/TMPRED.form.html) [26].

**Results and discussion**

Plant viruses are some of the most important problems for food security, where they play significant roles in crop losses all over the world [1]. Among the viruses, the Tobacco mosaic virus (TMV) is one of the most studied. It is used as a model plant virus in fundamental biological research towards understanding of host–virus interactions [27]. TMV infects many plant species, including tomato, causing light or dark green mosaic symptoms and distortions of the leaves [28,29].

In the present study, the TMV-inoculated tomato plants at 15 dpi showed identical mosaic symptoms to those reported previously [29,30]. By using RT-PCR with specific TMV-coat protein (CP) gene primers, a DNA fragment of 700 bp was detected in the infected tissues at 6, 9 and 15 dpi (Figure 1(A)). No virus was detected in mock-inoculated samples.

To better clarify the molecular basis of tomato-TMV interactions with rapid identification of the differentially expressed tomato genes during viral infection, the DD-PCR technique was performed using five arbitrary primers (Table 1). Today, DD-PCR is increasingly effective in isolating and characterising genes differentially expressed amongst cells, tissues or individuals [1,22,24]. Many plants defend themselves against pathogens, such as viruses, bacteria and fungi, through altering their gene expressions [31].

**Differential display PCR (DD-PCR) and up/down regulated genes**

In the present study, the completely differentially expressed genes were carefully investigated through the comparison among the amplified fragments in mock-treated as well as in infected samples at different time points following TMV inoculation. The five primers yielded more than 134 bands with different molecular sizes ranging between 190 and 2500 bp (Figure 1(B–F)). The amplified patterns obtained with the RAPD9 primer were identical mimics, and none of the differentiated bands was detected at any time interval (Figure 1(D)). There were 128 PCR bands held in common between the mock-inoculated and infected samples (monomorphic), whereas six PCR bands were differentially expressed in all the treatments (polymorphic) (Table 1).

The results revealed that TMV induced a variation in gene expression within tomato tissues at all
intervals. These results agree with previous observations [1,24,32,33]. All polymorphic bands were selected, excised from the agarose gel, purified and submitted for sequencing. Some genes may have been missed and others had low-quality sequence values during the sequencing process. Three up-regulated bands and one down-regulated band were successfully sequenced and characterised. Sequence analysis revealed that the up-regulated transcript genes code for a kinesin-like protein (Acc# MG565981) and chloroplast-associated genes (Acc# MG565980 and MG565979). The down-regulated transcripts coded for another chloroplast-associated gene (Acc# MG565978).

Isolation and sequencing of chloroplast-associated genes

The two up-regulated genes coding for chloroplast proteins were induced and detected in the infected tissues at 15 dpi with the A2 primer and at 6 dpi with the A4 primer (Figure 1(B,C)). The first chloroplast-associated gene (Acc# MG565979) was up-regulated and expressed only at 15 dpi with about 450 bp. The second one (Acc# MG565980) had a size about 1100 bp, and was slightly induced in mock-inoculated plants (the band was very faint) compared to that induced at 6 dpi. Intriguingly, the down-regulated gene was found to code for a chloroplast protein. Moreover, it was noticed that the chloroplast genes MG565980 (up-regulated) and MG565978 (down-regulated) are closely related to each other with about 96% similarity, and these results confirm that most likely there are many copies of that gene (Figure 2(A,B)). Based on their DNA sequence alignment, there was some A-T transition reflecting the 4% difference between the two genes.

It is well known that the chloroplast is a common target of plant viruses [34,35]. In addition, it has important roles in plant–virus interactions and is involved in virus replication, movement, symptoms and the systemic defence response [35]. As Manfre et al. [37] report, the production of TMV-like
symptoms at 12 dpi with severe systemic mosaic and chlorosis symptoms at 15 dpi were associated with TMV infection and disturbance of chloroplast components. The chloroplasts and photosynthesis-related genes (CPRGs) that associate with chloroplast membranes or locate in chloroplasts have been reported as the majority of significantly changed proteins in the plants susceptible to virus infection [38–41]. Most of these genes are down-regulated in association with the degree of chlorosis severity [36,42–44]. The up-regulation of the chloroplast-related genes at 6 and 15 dpi suggests that these genes play a major role in the tomato defence system against TMV infection.

Our results agree with those of Bhattacharyya et al. [45] and Reuveni et al. [46], who postulated that the mosaic symptoms were typically related to an altered quantity, shape, variety and structure of chloroplasts. In addition to the reduction of chloroplast ribosomal RNA level [47], the inhibition of the nuclear-encoded CPRGs transcription through feedback signalling upon TMV infection has been reported [48]. Similarly, the coat protein of Cucumber mosaic virus (CP) was most likely to repress the CPRGs transcription via the retrograde signalling from the chloroplast into the nucleus [41]. Additionally, Caplan et al. [10,34] reported that chloroplasts are part of the mechanism of N immune receptor-mediated defence against TMV and are involved in infection by DNA viruses [49].

The down-regulated chloroplast gene (MG565978) was amplified with the two different primers (At1 and At5) at 15 dpi in the mock-inoculated plants but was completely shut down in the infected plants at all dpi. This is evidence that there is a direct relationship between this gene and the TMV viral infection. The gene was inhibited in 15 dpi, which indicates that this kind of inhibition was accompanied by appearance of viral symptoms. The disappearance of the band corresponding to this gene in the infected plant tissues along the examined intervals suggested that the virus had been able to control the cell organelles and began to affect the chloroplasts structurally and functionally once the virus entered the plant cell and started propagation. Additionally, the viral load reached the maximum level for cell control at day 15, with symptoms emerging. Clearly, this gene was found in the cells in different copies with different molecular sizes, two copies were observed in the mock-treated plants (15 dpi) at molecular sizes about 650 (At1) and at 380 bp (At5). Moreover, when the transmembrane domains of the up-regulated (MG565980) and down-regulated (MG565978) genes were predicted using TMpred software, it was observed that the transmembrane domain profile is identical. This confirms that the two genes have the same function but one of them was shut down and the other was not affected by the viral stress (Figure 3). This assumption agrees with the report by Fu et al. [50] that some abnormalities were observed in the leaves of Chinese cabbage infected with Turnip mosaic virus (TuMV), among them membrane vesiculation and breakdown.

### The up-regulation of kinesin-like protein

Although kinesin-like protein gene (Acc# MG565981) with a molecular weight about 750 bp was detected in the mock-inoculated plants in low intensity, this band was noticed with high intensity in the infected plants at 15 dpi post inoculation (Figure 1(E)). NCBI-BLAST and phylogenetic analysis revealed that it is closely related to the kinesin-like protein gene (Acc# XM_004233996) isolated from tomato Heinz 1706 cultivar in the UK with 98% similarity (Figure 4). The bioinformatics analysis detected three motifs in the kinesin-like protein sequence (Figure 5). These motifs are the Protein kinase C phosphorylation site (three
Figure 3. Diagram showing the predicted transmembrane domains of the deduced amino-acid sequences of the isolated genes.

Figure 4. Phylogenetic analysis showing the genetic relationship between the nucleotide sequence of the tomato (S. lycopersicum) kinesin-like protein gene (MG565981) and other kinesin-like protein genes available in GenBank. The phylogeny was tested with 2000 bootstrap replicates based on the UPGMA statistical method.
aa), the Casein kinase II phosphorylation site (four aa) and the N-myristoylation site (six aa) (Figure 5).

Bioinformatics tools have been widely used to analyse and identify motifs from DNA and protein sequences [51]. Keating and Striker [52] reported that the cellular protein phosphorylation process has major effects on both viral infection and replication. Moreover, the addition of phosphate group by kinases can regulate the stability, activity and interactions of a viral protein with other cellular and viral proteins [53]. Interestingly, the kinesin gene showed about 60% similarity with one of the up-regulated chloroplast genes (MG56979) but their transmembrane profiles are different (Figure 3), which confirmed that the two genes were structurally similar but functionally different as previously reported [54]. Evidently, viruses transport their macromolecules like RNA and proteins from cell to cell through the cell membrane [55,56].

Moreover, kinesin genes are maternal DNA and are one of the superfamily of microtubule motor proteins found in all eukaryotic organisms; they are important for chloroplast movement and anchoring to the plasma membrane [57]. Accordingly, the high expression level of the kinesin gene in infected plants could be considered a marker for plant defence against viral infection, and this assumption was in agreement with the previous results [58] that the kinesin-like proteins play a fundamental role in the oriented deposition of cellulose microfibrils and the strength of the cell wall. In addition, both of the mitochondrial and chloroplast genes are associated in guiding the cell during pathogen attacks. This suggestion agrees with Kong and Hanley-Bowdoin [57], who reported that kinesin-like protein interacts with the gemivirus AL1 protein in a yeast 2-hybrid assay. Moreover, Kellmann et al. [59] showed that interconnection between, movement

Figure 5. DNA nucleotide sequence of tomato kinesin-like protein gene. The deduced amino acid sequence of the protein is shown below the DNA sequence. Potential Protein kinase C phosphorylation site (three aa), Casein kinase II Phosphorylation site (four aa) and N-myristoylation site (six aa) are shadowed and underlined. Black colour indicates interaction between two sites.
protein (MP) of *Tomato spotted wilt tospovirus* (TSWV) (NSm) and the kinesin-like protein permits intracellular movement of TSWV nucleocapsids through utilising the cytoskeleton or vesicle sorting systems. Consequently, plants, as well as viruses transport their macromolecules like RNA and proteins from cell to cell through plasmodesmata [55,56], and such transport activities could also require kinesins [14].

To study whether the kinesin gene was one of the defence genes in the plant or if it belonged to this large family, the obtained DNA sequence of kinesin was compared with 18 defence genes in the GenBank database (Figure 6). It was observed that 19 genes were divided into two lineages. The first lineage was split into two main clusters including 11 genes, with kinesin among them. The second cluster was grouped into two groups and kinesin was isolated in one group, with high similarity to the other four genes (2nd group). The four defence genes are isolated from *Arabidopsis*. The results showed that this gene might have a special defensive function against viral infections. We assumed that the similarities between the kinesin gene and the other defence genes indicate that the kinesin gene may play an important role in the plant defence against viral infections.

**Conclusions**

Based on the present studies and in line with previous reports, we concluded that chloroplast and kinesin-like protein genes are targets during TMV infection, and the virus is able to modulate some of them either in its movement or during propagation. There are different copies of the chloroplast genes that were affected by the viral infection; some of these genes may shut down, whereas others are up-regulated. The gene selectivity depends on the epitopes existing either on the expressed proteins on the chloroplast membrane or on the viral coat protein. Furthermore, studies of the pathways in which chloroplast and kinesin-like protein genes are involved may elucidate the mechanisms of tomato tolerance to the viral infection and can lead us to a more comprehensive understanding of tomato–TMV interactions.

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