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Short Communication

A Comparative Analysis of the Suppressor Activity of *Tobacco mosaic virus* Proteins in the Tomato Plant

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Abstract

Plant viral suppressors are proteins which have the capability of inhibiting induced host antiviral RNA interference. RNA silencing is part of the cellular machinery that controls the translation of transcripts, and plays a major role in the defense against virus infections. *Tobacco mosaic virus* (TMV)-encoded proteins (Replicase, Movement and Coat proteins) were expressed in pCAMBIA1302 vector, and the suppressor strength was checked on the tomato plant. The suppression activity was detected by assaying for GFP fluorescence at 3dpi, 5dpi, 7dpi and 10dpi. Phenotypic observations were corroborated with semi-quantitative RT-PCR. The results indicated that TMV suppressors of the gene silencing are the coat protein.

Keywords: Tomato; TMV; Co-agroinfiltration; RNA Silencing Suppressor, GFP Silencing Assay

1. Introduction

Most eukaryotic organisms are equipped with an RNA silencing system that is being the most important and significant antiviral defense mechanisms (Hamilton *et al.*, 2002; Matzke and Birchler, 2005).

For plant-viral infections, viruses need to replicate their own genomes inside the cell. However, once the virus infects the plant, the host posttranscriptional gene silencing (PTGS) machinery is activated as a first line of plant defense response (Marathe *et al.*, 2000; Waterhouse *et al*., 2001). RNA silencing suppressors (RSS) are proteins encoded by many plant viruses having the ability to suppress the silencing based virus defense strategy and play an important role in virulence (Brigneti *et al.*, 1998; Baulcombe, 2002; Das and Sanan-Mishra, 2014). The RSS are interacting with the RNA silencing machinery components resulting in a breakdown of the host antiviral defense response (Das and Sanan-Mishra, 2014). However, inactivation of the RSS activity will lead to the recovery of plants from the viral infection (Silhavy *et al.*, 2002; Ziebell *et al.*, 2007).

Various RSS have been reported from diverse viruses and a lot of work is being done to understand their mechanism of action. Among the tobamoviruses, the RSS were shown to bind double-stranded small RNAs, inhibit 2′-O-methylation of small RNAs, and prevent the formation of RNA-induced silencing complexes (Akbergenov *et al.*, 2006; Vogler *et al.*, 2007).

Tobacco mosaic virus (TMV; genus Tobamovirus; family Virgaviridae) is a positive-sense single strand RNA virus (Meshi *et al.*, 1992). The TMV genome encodes three proteins: the replicase components proteins (RP; 130 kDa and 180 kDa) required for transcription and replication, the movement protein (MP; 30 kDa) required for cell-to-cell virus movement, and the coat protein (CP; 17.5-kDa) required for virus coat formation (Dorokhov *et al.*, 1994). The sequence of 130 kDa includes the methyltransferase and RNA helicase motifs, while the sequence of 180 kDa includes the methyltransferase, RNA helicase and RNA-dependent RNA polymerase motif. So the 180 kDa sequence can read-through protein of the 130 kDa open reading frame. TMV infects tomato plants systemically causing heavy losses in the tomato yield worldwide (Sikora *et al.*, 1998).

In addition to its role in the virulence and activation of pathogen-related genes, it was demonstrated that CPs may play an important role in the suppression of the RNA silencing, viral replication, attachment to the site host, cell-to-cell movement, and symptom development (Callaway *et al.*, 2001; Culver, 2002).

For the RSS assays, there are two common methods widely used, the first one is called the Agro-infiltration method which depends on the reversal transient suppression assay and in which model plants stably silenced for a reporter gene like GFP are used. Restoration of the reporter gene expression indicates that the tested construct encodes an RSS. The second method is a modification method called co-Agroinfiltration, in which two *Agrobacterium* strains are used (Johansen and Carrington, 2001; Takeda *et al.*, 2002). This work is aimed at assessing and identifying the suppressor activity of the

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three TMV-encoded proteins in tomato plants using co-agroinfiltrating method.

2. Material and Methods

2.1. Primer Design and TMV Genes Amplification

Based on the published sequence of TMV genome (Ac# KF972436), two degenerate primers were designed by NCBI Primer blast software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) to amplify the full length of replicase (RP; 130 kDa) and movement proteins. The primers sequence was checked by the NCBI-BLAST online software. The source of the TMV used in this study was isolated by our laboratory in Egypt and maintained continuously on *N. banthemiana*. One microgram of viral RNA was used to synthesize cDNA using oligo (dT) primer with Superscript reverse transcriptase (Invitrogen, USA) according to the manufacturer's instructions. A volume of 1 µl of cDNA was used for the PCR amplification of the replicase, movement and coat protein genes using TMV-specific primers (Table 1). The PCR amplified products were analyzed on 1.2 % agarose gel.

Table 1. Primer sequences and their relative position in the respective TMV genome

Gene		Primer sequence 5'……..3'	Position	Amplification Size (bp)	Reference
CP	Forward	ATTTAAGTGGASGGAAAAVCACT	5542 to 5562	695	Letscher *et al.*, 2002
	Reverse	CGGTCAGTGCCGAACAAGAA	6236 to 6213		
MV	Forward	GCTCGCAGATTTTGATTTTG	3872 to 3891	1827	This study
	Reverse	ACGAACTGAGATGGAGTAGT	5698 to 5679		
RP	Forward	CAATGATCTAGCAAAGCGTCG	89 to 109	3356	This study
	Reverse	CAGAAATATCACCAGTCTTTGGC	3444 to 3422		

2.2. Plasmid Construct

Each individual gene of the RP, MV and CP genes were purified, and cloned into a pGEMTeasy vector (Promega, USA) to adapt ligation site with the pCAMBIA1302-GFP binary vector sites. The three recombinant plasmids were transformed into *Agrobacterium tumefaciens* strain LBA4404 to start the agroinfiltration study after the ligation and cloning of the three modified genes into pCAMBIA1302 under the CaMV 35S promoter. The transformants were checked by plasmid isolation, restriction enzyme analysis and colony PCR.

2.3. Preparation of Agrobacterium Competent Cells and Transformation Protocol

Agrobacterium tumefaciens strain LBA4404 was grown in 5 mL YEM medium supplemented with 50 mg L^{-1} streptomycin and 25 mg L^{-1} rifampicin at 28 ^{o}C in a shaking incubator at 180 rpm. The primary culture was added to 50 mL YEM medium supplemented with streptomycin and rifampicin, and was incubated at 28 ^{o}C with shaking for 4-5h till an optical density (OD) reaching 0.5-1.0 at 600 nm. The culture was chilled on ice for thirty minutes, and the cells were harvested by centrifugation at 5,000 rpm, at 4 ^{o}C. The pellet was suspended gently in 20 mL cold, sterile 100 mM $CaCl_2$, and was kept on ice for thirty minutes. The *Agrobacterium* suspension was centrifuged at 5000 rpm, and the pellet was re-suspended in 1 mL of chilled, sterile $CaCl_2$ plus 1 ml of 50 % sterile glycerol, and was mixed gently by pipetting. Each 100 µL competent cells were aliquoted into a sterile microfuge tube, and were stored at -80 °C for further use.

For transformation, *Agrobacterium* competent cells were thawed by being kept on ice for ten minutes. A quantity of 1-2 µg plasmid DNA was added to the *Agrobacterium* cell tube, and was mixed well and incubated on ice for thirty minutes. The cells were quickly frozen by immersing the microfuge tube in liquid nitrogen for one minute; heat shocked at 37 ^{o}C in the water bath for five minutes, and were immediately kept on ice again for five minutes. A volume of 1 mL LB medium was added to the cells, and was incubated at 28 ^{o}C for three-five hours with shaking. A 100µl of the suspension was spread onto LB-agar plate containing 50 mg L^{-1} kanamycin, 50 mg L^{-1} streptomycin and 25 mg L^{-1} rifampicin, and was incubated at 28 ^{o}C. Transformed colonies that appeared after two-three days were screened by colony PCR, plasmid isolation, and restriction enzyme digestion analysis.

2.4. Co-agroinfiltration and GFP Detection

Seeds of Heinz 1706 tomato (*Solanum lycopersicum* L.) cultivar were grown in a greenhouse under controlled conditions. At forty-five days, *A. tumefaciens* harboring the appropriate plasmid construct was infiltrated according to Karjee *et al.*, (2008) method with some modification. Briefly, *A. tumefaciens* was inoculated to LB medium with appropriate antibiotics, and was grown until OD 600 reached one. Cells were harvested by centrifugation at 4 ^{o}C with 5000 rpm and were re-suspended in YEM to adjust a final OD600 ≈ 0.8-1.2. With the help of a finger on the dorsal side of the tomato leaf, pressure infiltration (by a syringe without a needle on the leaf ventral side) was carefully applied. At 3, 5, 7, and 10 days, post-inoculation (dpi) the infiltrated leaves were collected and GFP fluorescent was visualized under fluorescent UV.

2.5. GFP Reporter Transcript

For semi-quantitative RT-PCR, 1 µg of total RNA isolated from the infiltrated leaf tissues at different

intervals was used to synthesize cDNA. After the treatment of cDNA with DNaseI for thirty minutes, 1 µL was used as a template for PCR amplification. The PCR program with initial denaturation at 95°C for three minutes followed by thirty-five cycles at 94˚C for thirty seconds, 58˚C for thirty seconds, 72˚C for thirty seconds, and final extension at 72˚C for five minutes. The tomato actin gene forward 5'-ATGCCATTCTCCGTCTTGACTTG-3', reverse 5'-GAGTTGTATGTAGTCTCGTGGATT-3' primer (Naqvi *et al.,* 2010) was applied as a reference gene to evaluate the cDNA content. RT-PCR products were analyzed by electrophoresis in 1.2 % agarose gel, and the band intensities were quantified using Alpha Imager Imaging System.

3. Results and Discussion

To identify the potential RNA silencing suppressors (RSS) of TMV, the three open reading frames of RP, MV and CP were amplified using RT-PCR with specific primers. An individual amplicon with a molecular size of about 695 bp for CP, 1827 bp for MV, and 3356 bp for RP genes (Figure 1) was cloned into the pCAMBIA1302 binary vector under CaMV 35S promoter, and was then transformed into in the *A. tumefaciens* strain LBA4404.

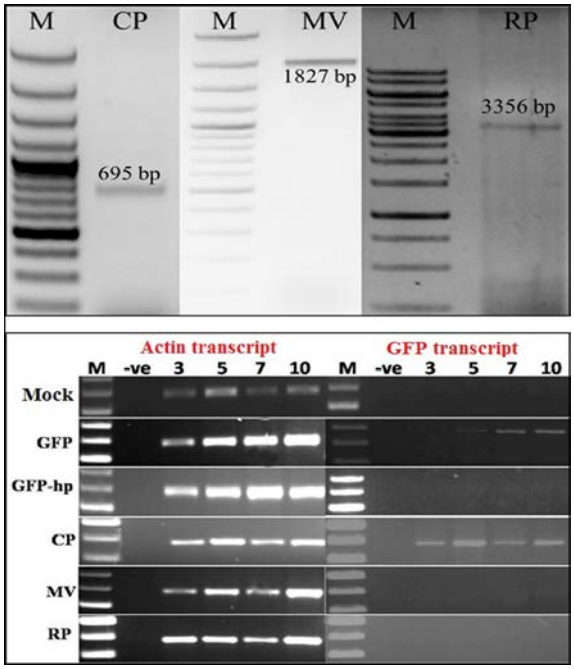

Figure 1. Agarose gel electrophoresis (1.2%) in TAE buffer stained with ethidium bromide showing (Upper): RT-PCR amplification of TMV-coat protein (CP), TMV-movement protein (MV), and TMV-replicase protein (RP) of TMV-infected tomato; (Down): analysis of GFP transcripts in total RNA obtained from tomato leaves infiltrated six treatments that referred by mock (*Agrobacterium* empty), GFP (pCAMBIA1302), GFP-hp (pCAMBIA1302 plus GFP-hairpin), CP (GFP-hp+CP), MV (GFP-hp+MV) and RP (GFP-hp+RP).

Based on the transient assay for the suppression of GFP silencing (Johansen and Carrington, 2001), the RSS activity of each gene was estimated. The construct of pCAMBIA1302 carrying the green fluorescent protein was used as GFP expressing positive control. Mixture with an equal volume of the two constructs of pCAMBIA1302 and GFP-hairpin (GFP-hp) co-infiltrated together were used as GFP silencing control. For RP, MV and CP, GFP-hp construct was mixed and co-agroinfiltrated with pCAMBIA1302-RP, pCAMBIA1302-MV and pCAMBIA1302-CP. The infiltrated zones were analyzed for the suppression of GFP silencing after 3, 5, 7 and 10 dpi (Figure 2). The results of this study revealed that the leaves infiltrated by pCAMBIA1302 alone exhibited a GFP fluorescence in the infiltrated zones at 5dpi, and the fluorescence increased by 7dpi and 10dpi (Figure 2). These initial observations indicated that the transient infiltration of pCAMBIA1302 in tomato leaves resulted in GFP expression, and this increased with time till 10dpi. This result is in agreement with earlier observations by Kokkirala *et al.,* (2010). However, no color for the GFP expression was detected in leaves infiltrated with mock (*Agrobacterium* free), or co-infiltrated with GFP-hp at all time (Figure 2). The GFP-hp results in the production of siRNA against GFP and these silence its expression through the PTGS (Simmer *et al.,* 2010). The regions co-infiltrated with GPF-hp+CP showed enhanced expression starting at 3dpi, and increased with time reaching a maximum at 10dpi (Figure 2). This showed that CP could suppress the hp-induced silencing resulting in greater accumulation of GFP. In addition, the GPF-hp+RP and GPF-hp+MV did not show any green color for the GFP expression (Figure 2). This data suggested that the RP and MV proteins lacked the RSS activity, or it was too weak to be visually detected.

By comparing the three proteins together, it was observed that the CP only had RSS activity, so it can be designated as the stronger suppressor protein of the TMV genome. In addition, the CP itself in the absence of other TMV-encoded proteins was able to suppress host silencing machine. These results are similar with the previous data that reported the CPs of several plant viruses that have been identified as suppressors of gene silencing (Roth *et al.,* 2004; Soosaar *et al.,* 2005; Ren *et al.,* 2005). These results do not agree with Conti *et al.,* (2017) who demonstrated that the replicase protein carried the TMV suppression of silencing activity. Although those authors noted that during the TMV infections, RNA decay pathways were induced by the action of movement and coat protein.

Semi-quantitative RT-PCR was applied to quantify the degree of suppressor activity based on the transcriptional of the GFP reporter. Molecular analysis has confirmed the results of GFP silencing assay. It was observed that there was no GFP transcript accumulation detected in the infiltrated regions with mock, GFP-hp and TMV-RP (Figure 1). On the other hand, GFP transcript was detected in both GFP and GFP-hp-CP infiltrated leaves (Figure 1). Qu *et al*., (2003); Thomas *et al*., (2003) and Meng *et al*., (2006) reported that the CP of both *Turnip crinkle virus* and *Chlorotic ringspot virus* are the strong suppressors of RNA silencing.

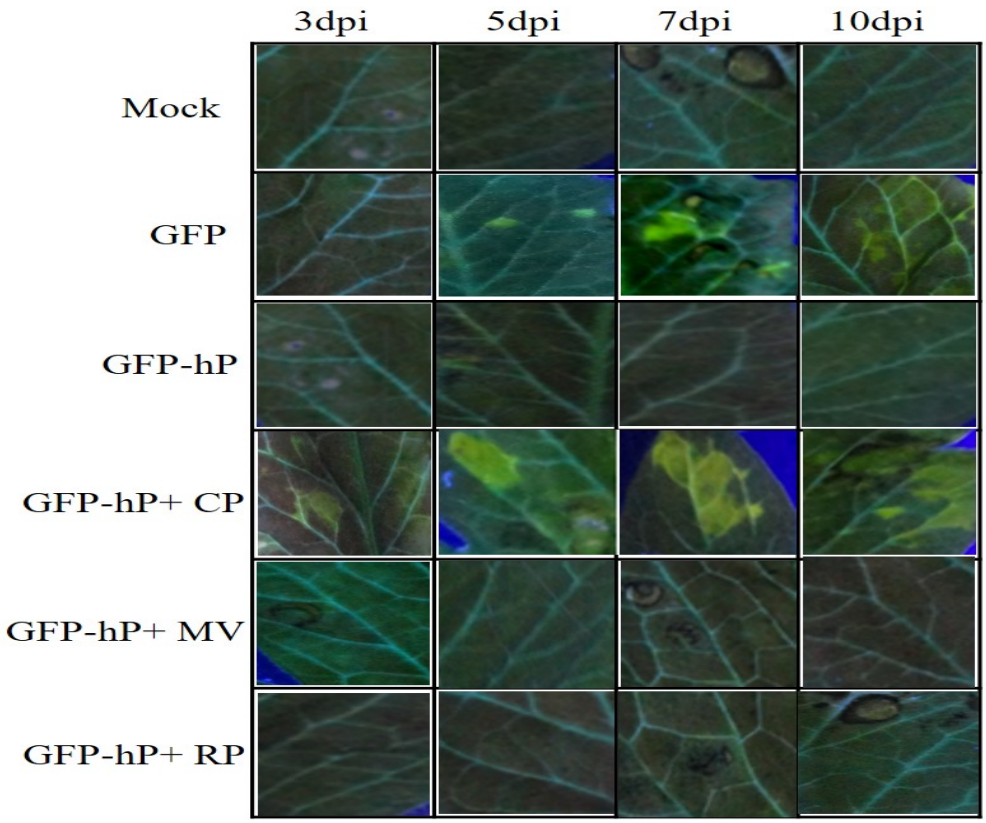

Figure 2. GFP fluorescent in tomato leaves under UV. Tomato leaves were co-agroinfiltrated with six treatments that referred by mock (*Agrobacterium* empty), GFP (pCAMBIA1302), GFP-hp (pCAMBIA1302 plus GFP-hairpin), GFP-hp+CP, GFP-hp+MV and GFP-hp+RP.

At 3dpi, the GFP transcript band was detected only with GFP-hp-CP infiltration, which indicated the suppression activity of CP that starts early, and could suppress the hp-induced silencing of the GFP construct (Figure 1). The band intensity was highly increased after 5dpi and 10dpi (Figure 1). While the GFP transcript with GFP construct was detected as a faint band at 5dpi, but it increased at 10dpi (Figure 1). This may be due to the initiation of host-induced RNAi response against the infiltrated GFP constructs. The results are in harmony with those results obtained by Das and Sanan-Mishra, (2014) and not compatible with the results obtained by Csorba *et al.*, (2007). At 7 dpi till 10 dpi, the GFP transcription with MV protein was detected as very low bands (Figure 1). Luna *et al.*, (2017) reported that the *Beet curly top virus* V2 protein is involved in the virulence determination and viral spread acted as a potent PTGS suppressor.

From the obtained results we can concluded that the 17.5 kDa coat protein of TMV is a potent silencing-suppressor protein, such results will give attention to detect a suppressor-host virus relationship, and can help to establish a strategy for gene expression aiming to enhance the plant viral immune system.

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