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# *Tomato yellow leaf curl virus* confronts host degradation by sheltering in small/midsized protein aggregates

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ABSTRACT

*Tomato yellow leaf curl virus* (TYLCV) is a begomovirus transmitted by the whitefly *Bemisia tabaci* to tomato and other crops. TYLCV proteins are endangered by the host defenses. We have analyzed the capacity of the tomato plant and of the whitefly insect vector to degrade the six proteins encoded by the TYLCV genome. Tomato and whitefly demonstrated the highest proteolytic activity in the fractions containing soluble proteins, less—in large protein aggregates; a significant decrease of TYLCV proteolysis was detected in the intermediate-sized aggregates. All the six TYLCV proteins were differently targeted by the cytoplasmic and nuclear degradation machineries (proteases, ubiquitin 26S proteasome, autophagy). TYLCV could confront host degradation by sheltering in small/midsized aggregates, where viral proteins are less exposed to proteolysis. Indeed, TYLCV proteins were localized in aggregates of various sizes in both host organisms. This is the first study comparing degradation machinery in plant and insect hosts targeting all TYLCV proteins.

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## 1. Introduction

To ensure a successful infection, viruses neutralize the host defense mechanisms aimed at preventing virus replication and spread. Proteolysis is one of the most potent protection mechanisms host organisms use to destroy invading viruses. Indeed, there are various proteolytic enzymes/systems than are potentially able to convert viruses into harmless entities by degrading their proteins. Viruses encounter proteolysis by forming aggregates and by integrating their proteins into inclusion bodies (Kleczkowski and van Kammen, 1961).

*Tomato yellow leaf curl viruses* (TYLCVs) are a complex of begomoviruses, TYLCV from Israel being the member type (Díaz-Pendón et al., 2010). TYLCVs possess ssDNA genome of ~2800 nt encapsidated in a geminate particle. TYLCVs are transmitted by the whitefly *Bemisia tabaci* (Czosnek, 2007). The virion-sense strand comprises two genes, V1 and V2, while the complementary-sense strand comprises four genes, C1–C4 (Navot et al., 1991). The role of the proteins encoded by TYLCV genome has been summarized elsewhere (Díaz-Pendón et al., 2010). V1 encodes the structural coat protein (CP), which is essential for cell-to-cell movement, systemic infection and transmission by the whitefly vector. V2 encodes a multi-functional protein involved in virus movement, in the suppression of post-transcriptional gene silencing (PTGS) and in the suppression of methylation-mediated transcriptional gene silencing (TGS). C1 encodes a replication-associated protein (Rep) which initiates viral replication. C2 is the transcriptional activator protein (TrAP), which interferes with transcriptional gene silencing and PTGS. C3 encodes the replication enhancer protein (REn) involved in viral replication. C4 counteracts PTGS. The TYLCV whitefly vector *B. tabaci* acquires the virus while feeding on the infected plant vascular system. The virus translocates in the insect in a circular and persistent manner (Ghanim et al., 2001), reaches the salivary glands, is deposited in the plant phloem sieve elements during feeding and from which it invades the vascular-associated cells (Medina et al., 2006). It is thought that once in the nucleus, the virion disassembles and the viral DNA is replicated according to a rolling circle mechanism and is transcribed (Hanley-Bowdoin et al., 1999;

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Gafni and Bernard, 2002). After translation in cytoplasm, the CP (as monomers, polymers, particles or aggregates) is transported into the nucleus to be assembled into virions (Kunik et al., 1998). The assembled virions, or nucleoprotein complexes, are transported back to cytoplasm and move to other cells and long-distance *via* the vascular system.

TYLCV infection in tomato plants is accompanied by the accumulation of the CP in aggregates of increasing size as infection progresses (Gorovits et al., 2013a). The appearance of large CP-aggregates is a feature of a successful TYLCV invasion in plants. Using *in vitro* assays, we have shown that TYLCV CP (in soluble and insoluble states) was prone to protease digestion and 26S proteasome degradation; while autophagy-dependent CP degradation has not been analyzed in that study (Gorovits et al., 2014). Highest degradation capacity against CP was detected among tomato leaf soluble proteins and proteins in large aggregates/inclusion bodies. The same fractions contained the highest nuclease activities toward viral DNA. Inhibition of tomato 26S proteasome degradation and particularly autophagy in *in vivo* experiments caused a redistribution of CP aggregates, pointing to the role of these mechanisms in TYLCV-induced aggregation and consequently of TYLCV survival (Gorovits et al., 2014). The TYLCV protein V2 also forms aggregates, degraded by the 26S proteasome machinery. *In vivo* and *in situ* studies demonstrated the dependence of V2 degradation on the integrity of the actin and microtubule cytoskeleton (Moshe et al., 2015a).

In the current study, the six TYLCV proteins over-expressed in *Escherichia coli* were used as substrates for the analysis of proteolytic capacities of the host tomato plant and the whitefly vector. Both organisms use comparable degradation mechanisms toward viral proteins. The main difference between plant and insect ubiquitin 26S proteasome system (UPS) and autophagy in degradating TYLCV proteins is that these mechanisms are conspicuous in plant fractions containing soluble proteins, but not in insect soluble fractions. In both TYLCV hosts, fractions containing small/midsized aggregates had low proteolytic activities. TYLCV proteins were detected specifically in such intermediate-sized aggregates at the beginning of infection in plants and during circulation in the whitefly vector.

## 2. Materials and methods

### 2.1. Sources of virus, insects and plants

TYLCV was maintained in tomato plants (cv. Daniella) by whitefly-mediated inoculation. *B. tabaci* whiteflies from the Middle East Asia Minor 1 (MEAM1) or B biotype were reared on cotton plants. All plants were grown in a temperature-controlled greenhouse under standard conditions. Tomato plants at their 3–5 true leaf stage were caged with viruliferous whiteflies (about 30 insects per plant at the onset of infection) for the duration of the experiments. Whiteflies were discarded with Imidacloprid before tissue sampling.

### 2.2. Purification of TYLCV proteins expressed in E. coli and preparation of antibodies

The six full-length TYLCV genes were PCR-amplified using specific primers containing two additional restriction sites: *Nde* I at the 5′ end and *Bam* HI at the 3′ end. The amplicons were cloned into the *Nde* I and *Bam* HI sites of plasmid pET-14B (Novagen, USA). The recombinant plasmids were used to transform BL21 *E. coli* cells. Following IPTG induction, the His-tagged TYLCV proteins were purified from the transformed bacteria by Ni-affinity chromatography in the presence of 6 M urea using the HisPur Ni-NTA Resin (Thermo Scientific, USA). *E. coli* protein extracts were mixed with equal volume of equilibration buffer (PBS with 20 mM imidazole), incubated for 1 h at ambient temperature, washed with two resin-bed volumes of wash buffer (PBS with 25 mM imidazole) and eluted with elution buffer (PBS with 250 mM imidazole). The purified proteins were dialyzed for 16 h at 4 °C against 50 mM Tris–HCl pH 8.6, 5 mM EDTA, 500 mM arginine, 500 mM NaCl, 12.5% glycerol, and centrifuged at 12,000 × *g* for 20 min. The TYLCV protein-containing supernatants were stored at −80 °C in 12.5% glycerol and were used to immunize rabbits and prepare an antisera (Hadar Biotech, Rehovot, Israel).

### 2.3. Protein extraction from tomato leaves and from whiteflies, and their separation on sucrose gradients

Cytoplasmic and nuclear proteins were prepared as previously described (Gorovits et al., 2013a). The detection of cytoplasmic HSP70 and nuclear Histone 3 were used as internal markers to assess the purity of the cellular fractions. To extract native total proteins for sucrose gradient analyses, 500 mg of tissue (pooled from three plants or from approximately 7000 viruliferous whiteflies) were homogenized by 10 strokes of pestle A in a Dounce homogenizer in 50 mM Tris–HCl pH 7.5, 80 mM KCl, 10 mM MgCl2, 0.2 mM EDTA, 0.5% Nonidet P40, 1 mM dithiothreitol. Homogenates were incubated on ice for 45 min, vortexed and centrifuged at 1200 × *g* for 10 min at 4 °C. The supernatant (0.5 ml) was loaded on top of 10 ml sucrose gradients 10–50% sucrose gradients. After 20 h centrifugation at 104,000 × *g* at 4 °C (Beckman SW27 rotor), the gradients were fractionated into 10 aliquots (1–10, from top to bottom) as described previously (Bendahmane et al., 1997; Asurmendi et al., 2007; Gorovits et al., 2013, 2014; Moshe et al., 2015a,b).

### 2.4. In vitro proteolysis of TYLCV proteins

Each TYLCV purified viral protein (0.5 μg) was incubated with aliquots (30 μl) of TYLCV infected leaf or viruliferous whiteflies protein extracts, separated by ultracentrifugation on sucrose gradients, for 2 h at 37 °C. The incubation was stopped by addition of standard PAGE loading buffer supplemented with 2% SDS. After boiling for 10 min, samples were subjected to SDS-PAGE electrophoresis and immunoblotted with the TYLCV proteins antibodies. Proteases activity was inhibited by the addition of Complete Protease Inhibitor Mixture (Roche, Mannheim, Germany) in ratio 1:25 to reaction mixture; 26S proteasome activity was inhibited by 50 μM MG132 (Calbiochem, USA), autophagy activity—by 10 μM Wortmannin (Calbiochem, USA). Inhibitors were added together with gradient protein fractions and incubated with purified TYLCV proteins 2 h at 37 °C.

### 2.5. In vivo treatments of detached leaves by Wortmannin and Rapamycin

Detached infected tomato leaves (two-three leaflets per tube, the tips of the petioles soaking in the solutions) were incubated for 48 h at room temperature in micro-tubes containing the autophagy regulators: 10 μM Wortmannin or 10 μM Rapamycin (Calbiochem, USA); DMSO in water served as control. The fresh solutions were replaced every 12 h.

### 2.6. Immunodetection of viral and plant proteins

The origin of the (polyclonal) antibodies used in this study was as follows: TYLCV anti-CP, V2, C1–C4 were raised by the authors, as described in Section 2.2; 20S Proteasome alpha + beta antibodies were purchased from Abcam, UK; ATG8 (AS14 2769), cytoplasmic HSP70 (AS08 371), histone H3 (AS10 710) from Agrisera, Sweden.

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