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Progressive aggregation of *Tomato yellow leaf curl virus* coat protein in systemically infected tomato plants, susceptible and resistant to the virus

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Tomato yellow leaf curl virus (TYLCV) coat protein (CP) accumulated in tomato leaves during infection. The CP was immuno-detected in the phloem associated cells. At the early stages of infection, punctate signals were detected in the cytoplasm, while in the later stages aggregates of increasing size were localized in cytoplasm and nuclei. Sedimentation of protein extracts through sucrose gradients confirmed that progress of infection was accompanied by the formation of CP aggregates of increasing size. Genomic ssDNA was found in the cytoplasm and in the nucleus, while the dsDNA replicative form was exclusively associated with the nucleus. CP–DNA complexes were detected by immuno-capture PCR in nuclear and cytoplasmic large aggregates. Nuclear aggregates contained infectious particles transmissible to test plants by whiteflies. In contrast to susceptible tomatoes, the formation of large CP aggregates in resistant plants was delayed. By experimentally changing the level of resistance/susceptibility of plants, we showed that maintenance of midsized CP aggregates was associated with resistance, while large aggregates where characteristic of susceptibility. We propose that sequestering of virus CP into midsized aggregates and retarding the formation of large insoluble aggregates containing infectious particles is part of the response of resistant plants to TYLCV.

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1. Introduction

Tomato yellow leaf curl virus (TYLCV) impairs tomato (*Solanum lycopersicum*) cultures worldwide (Czosnek, 2007). TYLCV (genus *Begomovirus*, family *Geminiviridae*) is transmitted by the whitefly *Bemisia tabaci*. The circular ~2.8 kb single-stranded TYLCV DNA (cssDNA) genome replicates in nuclei of infected cells *via* a double-stranded intermediate (dsDNA), by a rolling circle mechanism (Díaz-Pendón et al., 2010). The virus spreads systemically in the host plant (Ber et al., 1990) and is confined to phloem tissues (Wege, 2007). Microscope observation of leaves infected with geminiviruses, including TYLCV, has revealed large aggregates that may include geminate particles (Abouzid et al., 2002; Christie et al., 1986; Russo et al., 1980). The role of these inclusions in the process of geminivirus propagation and in the host immune response is unclear. Similarly to geminiviruses, circoviruses (family *Circoviridae*) are a family of animal viruses with a monopartite ssDNA of ~2 kb and a stem-loop structure involved in the rolling circle mechanism of replication (Todd et al., 2001; Faurez et al., 2009). They too form aggregates in infected cells. For example, the Porcine circovirus 2 (PCV-2) capsid and DNA are abundantly present in cytoplasm of lymphoid tissues, either diffusely distributed or confined to discrete cytoplasmic aggregates and inclusions (Krakowka et al., 2002).

Virus aggregation in animal cells has been extensively studied (reviewed in Netherton and Wileman, 2011; Novoa et al., 2005). Large inclusions are often located in the perinuclear region of the cytoplasm close to the microtubule organizing center (MTOC). They usually concentrate virus-encoded proteins needed for genome replication and particle morphogenesis, and have been named virus factories. Viral inclusions other than virus factories may serve as sequestering units, where virus components are captured by host proteins for storage and subsequent degradation.

In plants, the nature of DNA and RNA virus-induced aggregates has been analyzed with molecular tools only in a few instances. In most cases, their role in the virus cycle and in the plant response to infection remains to be investigated. The AV2 movement protein of the begomovirus *Indian cassava mosaic virus* (ICMV) forms cytoplasmic as well as nuclear inclusion bodies with unknown functions (Rothenstein et al., 2007). In *Cauliflower mosaic virus* (CaMV)-infected turnip cells, viral P2 and P3 proteins, which were first confined in multiple small aggregates, later concentrated in a single large inclusion body that promotes transmission by aphid vectors; these aggregates were shown to be different from virus

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factories (Martinière et al., 2009; Hoh et al., 2010). *Tobacco etch virus* (TEV) CI helicase forms aggregates along the plasma membrane and near the plasmodesmata, while proteases NIa and NIb form nuclear aggregates (Langenberg and Zhang, 1997). *Potato virus X* (PVX) TGBp1 movement protein (MP) is arranged in cytoplasmic and nuclear aggregates (Samuels et al., 2007). *Tobacco mosaic virus* 30K movement protein aggregation was reported to be connected with polyubiquitination and 26S proteosome degradation processes, characteristic of animal viroplasm (Reichel and Beachy, 2000). A *Potato leaf roll virus* (PLRV) MP17 movement protein developed large aggregates in cells treated with the 26S proteosome inhibitor clasto-lactacystin B-lactone (Vogel et al., 2007).

In this report, we tested the hypothesis that progressive coat protein (CP) aggregation is inherent to the progress of TYLCV infection in susceptible tomato plants. The TYLCV CP has a variety of functions. It is the only protein composing the viral capsid: a geminate TYLCV particle is made of 110 CP monomers enveloping one ~2800 nucleotide ssDNA genomic molecule (Czosnek, 2008). In monopartite geminiviruses such as TYLCV, an intact wild type CP is essential for cell-to-cell movement and systemic infection (Wartig et al., 1997), nuclear import (Kunik et al., 1998; Yaakov et al., 2011), particle formation (Noris et al., 1998), and transmission by the whitefly vector (Caciagli et al., 2009), suggesting that the monopartite viruses move within the plant in the form of viral particles. In contrast, some bipartite geminiviruses such as *Tomato golden mosaic virus* (Gardiner et al., 1988), systemically infect plants even when their CPs have been experimentally deleted or truncated; however the amount of viral DNA is usually decreased, symptoms do not develop, and the viruses are not transmissible by the whitefly vector. Although TYLCV CP was shown to bind to cloned viral DNA fragments (Palanichelvam et al., 1998), experiments aimed at showing binding of CP to geminiviral genomic ssDNA (from AbMV) have failed so far (Hehnle et al., 2004). It is not clear how TYLCV and other geminiviruses reach the nuclei of infected cells, whether as particles, or as CP-DNA-host protein complexes (Gafni and Bernard, 2002). It is thought that once in the nucleus, the virion disassembles and the viral DNA is replicated according to a rolling circle mechanism and transcribed (Hanley-Bowdoin et al., 1999). Among other viral mRNAs, CP transcripts cross the nuclear membrane and are translated in the cytoplasm. Then the newly synthesized CP (as monomers, polymers, particles or aggregates) needs to be transported into the nucleus to be assembled into virions (Kunik et al., 1998). The assembled virions, or nucleoprotein complexes involving the CP, move back to the cytoplasm and translocate to other cells and long-distance *via* the vascular system (Gafni, 2002).

Using *in situ* immuno-detection and cell fractionation, we have shown that TYLCV CP aggregation increased with the progress of systemic infection from small to large aggregates, which are located first in the cytoplasm then in the nuclei of phloem-associated cells. By comparing two inbred tomato lines resulting from the same breeding program using *Solanum habrochaites* as the source of resistance, one resistant to TYLCV (R), the other susceptible (S) (Vidavsky and Czosnek, 1998) we have shown that TYLCV CP aggregation is slower in R than in S plants. Moreover the aggregation process can be modified by experimentally changing the plant phenotype from resistant to susceptible, and from susceptible to resistant.

2. Materials and methods

2.1. Sources of virus, insects and plants and infection of plants with TYLCV

TYLCV (Navot et al., 1991) was maintained in tomato plants by whitefly-mediated inoculation (*Bemisia tabaci*, MEAM1 species) as described (Zeidan and Czosnek, 1991). The TYLCV resistant (R) and susceptible (S) inbred tomato lines (respectively lines 902 and 906-4) result from the same breeding program aimed at introgressing resistance from wild tomato species into the domesticated tomato, which started with the cross *S. habrochaites* LA1777/LA786 (source of resistance) × *S. lycopersicum* (see pedigree of lines in Vidavsky and Czosnek, 1998). S and R lines have horticultural qualities equivalent to those of commercial cultivars. Resistance is a dominant trait (Vidavski et al., 2008). Upon TYLCV inoculation, S plants remain stunted, accumulate large amounts of virus, and do not yield fruits. By comparison, R plants grow as if not infected, accumulate lower amounts of virus than S plants and produce fruits. R and S plants can be distinguished by a single nucleotide polymorphism (SNP) present in an hsp70 gene (Eybishtz et al., 2009); this SNP is not linked to resistance. GroEL-expressing *Nicotiana benthamiana* plants and *LeHT1*-silenced R tomatoes were generated as described by Edelbaum et al. (2009) and Eybishtz et al. (2010), respectively. *N. benthamiana* plants have been transformed with a whitefly GroEL gene, expressed under an *Arabidopsis* phloem-specific promoter. These plants were homozygous for the GroEL gene from the T4 generation. Here we used inbred progenies of these homozygous plants. R plants were not transformed. They have been inoculated with *Tobacco rattle virus* (TRV) containing a silencing LeHt1 DNA fragment. All plants were grown in a greenhouse according to the regulations of the Israel Plant Protection Authorities. Plants at their 3–5 true leaf (1 cm length or more) stage were caged with viruliferous whiteflies (about 50 insects per plant at the onset of infection) for the duration of the experiments. Two new true leaves appeared approximately every two weeks. Whiteflies were discarded before tissue sampling.

2.2. Preparation of an antibody directed against TYLCV CP expressed in Escherichia coli

A PCR-amplified fragment of the CP gene (nucleotides 1–783) was cloned into the *Nde*I and *Bam*HI sites of pET-14B (Novagen). The recombinant plasmid was used to transform *E. coli* cells, strain BL21 (DE3). Following IPTG induction, the His-tagged TYLCV CP was purified from recombinant bacteria by Ni-affinity chromatography in the presence of 6 M urea using His-Bind Resin (Novagen) as described (Gottfried et al., 2000). The purified CP was 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 supernatant was used to immunize rabbits and prepare an antiserum (Hadar Biotech, Rehovot, Israel).

2.3. Visualization in situ of TYLCV CP in tomato leaves

For histological analyses, two infected tomato apical true leaves were cut into 0.5 cm squares and processed as described (Paciorek et al., 2006; Sauer et al., 2006). Briefly, after fixation in 4% paraformaldehyde in MTSB (50 mM PIPES, 5 mM EGTA, 5 mM $MgSO_4$, pH 7) leaf samples were embedded in wax (PEG 400 distearate and 1-hexadecanol – both from Sigma – mixed at a ratio of 9:1). Fifteen micrometer-thick wax-embedded tissues were sectioned with a microtome (HM340E, Waldorf, Germany), rehydrated and blocked for 1 h at 25 °C in 2% BSA/MTSB prior to incubation for 18 h at 4 °C with anti-TYLCV-CP primary antibody diluted 1:100 in 2% BSA/MTSB. After washing with MTSB the samples were incubated for 1.5 h at 25 °C with a Cy3-conjugated anti-rabbit secondary antibody (Jackson Immunoresearch, USA) diluted 1:200. The samples were inspected using a stereoscopic fluorescent zoom microscope (SMZ1500, Nikon, Japan) and fluorescent microscope (Eclipse 80i, Nikon, Japan); CP was detected as red fluorescent signals. Plant nuclei were stained with DAPI (Thermo Scientific DAPI,

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