Final Scientific Report

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BARD Project Number: US-3917-06C

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Project Title: Suppression of RNA silencing by TYLCV-IS during viral infection.

Investigators

Principal Investigator (PI): Vitaly Citovsky

Co-Principal Investigator (Co-PI): Yedidya Gafni

Collaborating Investigators:

Institutions

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Keywords *not* appearing in the title and in order of importance. Avoid abbreviations.

Suppression of RNA silencing; Protein-protein interaction; intracellular localization; cysteine protease; protein degradation

Abbreviations commonly used in the report, in alphabetical order:

BiFC, bimolecular fluorescent complementation; PTGS, post transcriptional gene silencing; SGS3, suppressor of gene silencing 3; TYLCV, tomato yellow leaf curl virus;

Budget: IS: \$165,000

US: \$135,000

Total: \$300,000

Signature
Principal Investigator

Signature
Authorizing Official, Principal Institution

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Publication Summary (numbers)

	Joint IS/US authorship	US Authors only	Israeli Authors only	Total
Refereed (published, in press, accepted) BARD support acknowledged	4	7	2	13
Submitted, in review, in preparation	1	4	2	7
Invited review papers	2	0	1	3
Book chapters	0	2	0	2
Books	0	1	0	1
Master theses	0	0	3	3
Ph.D. theses	0	0	2	2
Abstracts	0	1	1	2
Not refereed (proceedings, reports, etc.)	1	2	2	5

Postdoctoral Training: List the names and social security/identity numbers of all postdocs who received more than 50% of their funding by the grant.

Benoît Lacroix, Stony Brook, SS# (Last four digits) 5551

Lakhmir Singh, Volcani Center, Pass. # F5952306

Cooperation Summary (numbers)

	From US to Israel	From Israel to US	Together, elsewhere	Total
Short Visits & Meetings	1	2	0	3
Longer Visits (Sabbaticals)	0	0	0	0

Description Cooperation:

Both PIs closely collaborated by frequent communication through electronic mail and frequent visits to each other's laboratories. Constant discussions were held, generating ideas and experiments throughout the project. A significant contribution to current research was exchange of genetic material, including constructs prepared in one laboratory and utilized by the other. These everyday contacts led to several joint publications driven by new discoveries, which were made exclusively due of this increased level of collaboration. Both groups freely shared ideas, technologies and materials and the collaboration between the two groups continues. This collaboration will continue with the new grant received, and it will most probably bring better

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understanding of the life cycle of TYLCV and give us better ways to combat it and the disease it causes.

Patent Summary (numbers)

	Israeli inventor only	US inventor only	Joint IS/US inventors	Total
Submitted	0	0	0	0
Issued (allowed)	0	0	0	0
Licensed	0	0	0	0

SUPPRESSION OF RNA SILENCING BY TYLCV-IS DURING VIRAL INFECTION

Abstract

The Israeli isolate of *Tomato yellow leaf curl geminivirus* (TYLCV-Is) is a major tomato pathogen, causing extensive (up to 100%) crop losses in Israel and in the south-eastern U.S. (e.g., Georgia, Florida). Surprisingly, however, little is known about the molecular mechanisms of TYLCV-Is interactions with tomato cells. In the current BARD project, we have identified a TYLCV-Is protein, V2, which acts as a suppressor of RNA silencing, and showed that V2 interacts with the tomato (*L. esculentum*) member of the SGS3 (LeSGS3) protein family known to be involved in RNA silencing. This proposal will use our data as a foundation to study one of the most intriguing, yet poorly understood, aspects of TYLCV-Is interactions with its host plants – possible involvement of the host innate immune system, i.e., RNA silencing, in plant defense against TYLCV-Is and the molecular pathway(s) by which TYLCV-Is may counter this defense. Our project sought two objectives:

I. Study of the roles of RNA silencing and its suppression by V2 in TYLCV-Is infection of tomato plants.

II. Study of the mechanism by which V2 suppresses RNA silencing.

Our research towards these goals has produced the following main achievements:

- Identification and characterization of TYLCV V2 protein as a suppressor of RNA silencing. (#1 in the list of publications).
- Characterization of the V2 protein as a cytoplasmic protein interacting with the plant protein SlSGS3 and localized mainly in specific, not yet identified, bodies. (#2 in the list of publications).
- Development of new tools to study subcellular localization of interacting proteins (#3 in the list of publications).
- Characterization of TYLCV V2 as a F-BOX protein and its possible role in target protein(s) degradation.
- Characterization of TYLCV V2 interaction with a tomato cysteine protease that acts as an anti-viral agent.

These research findings provided significant insights into (I) the suppression of RNA silencing executed by the TYLCV V2 protein and (II) characterization some parts of the mechanism(s) involved in this suppression. The obtained knowledge will help to develop specific strategies to attenuate TYLCV infection, for example, by blocking the activity of the viral suppressor of gene silencing thus enabling the host cell silencing machinery combat the virus.

Achievements

The main significant achievements of the present project are:

1. Identification and characterization of TYLCV V2 protein as a suppressor of RNA silencing.

To identify the potential RNA silencing suppressor of TYLCV-Is, we tested all six proteins encoded by the genome of this virus for their ability to prevent silencing of a *GFP* reporter gene in plant tissues. Strong expression of GFP was observed two days post infiltration (dpi) was almost completely silenced at 7 dpi as assessed by observation of GFP fluorescence (Fig. 1B, paper #1 in the appendixes). Only one TYLCV-Is protein, V2 (see Fig. 1B, paper #1 in the appendixes), suppressed silencing, substantially elevating the levels of GFP expression. These data suggest that TYLCV-Is V2 functions as a suppressor of gene silencing and this was indeed published as a novel finding (Paper #1 in the appendixes).

2. Characterization TYLCV-Is V2 as a cytoplasmic protein interacting with SISGS3.

Sub-cellular localization of TYLCV-Is V2 was examined using autofluorescent tags GFP and its yellow spectral variant, YFP. To control for the position of the tag relative to TYLCV-Is V2, YFP was fused to the N-terminus of V2 and GFP was fused to the C-terminus of V2 and expressed in tobacco protoplasts and *Nicotiana benthamiana* leaf tissues. Fig. 3 (paper #2 in the appendixes) shows that, in protoplasts, both YFP-V2 and V2-GFP were distributed in the cell cytoplasm, associated with cytoplasmic strands and accumulating in inclusion bodies or aggregates which were observed mainly at the cell periphery. Similar sub-cellular localization patterns were observed following expression in leaf epidermis (Fig. 3, paper #2 in the appendixes).

When V2 was used for screening a tomato cDNA library to find proteins with which it can interact, a protein involved in gene silencing machinery was found to strongly interact with V2. This protein, SISGS3 was firstly characterized in *Arabidopsis* and we were the first to characterize and study in tomato (Paper # 2 in the appendixes).

Collectively, our observations identify V2 as a viral factor likely responsible for counteracting the anti-viral RNA silencing defense of the host plants against the TYLCV-Is infection. This response occurs in the plant cell cytoplasm in yet an unknown mechanism(s). (Paper #2 in the appendixes).

3. Development of new tools to study subcellular localization of interacting proteins.

In addition to this work we spend time improving the research tools required for the ongoing work in our labs. Specifically, we designed two extensive vector systems to follow protein-protein interactions and nuclear import in plant cells. For the interaction assay, we used bimolecular fluorescent complementation (BiFC) (Fig. 1, paper #3 in the appendixes).

To facilitate the application of BiFC to plant research, we designed a series of vectors (Fig. 3, paper #3 in the appendixes) for easy construction of N- and C- terminal fusions of the target protein to the YFP fragments. These vectors carry constitutive expression cassettes with an expanded multi-cloning site. In addition, these vectors facilitate the assembly of BiFC expression cassette into *Agrobacterium* multi-gene expression binary plasmids for co-expression of interacting partners and additional autofluorescent proteins as internal transformation controls and markers of subcellular compartments.

For the nuclear import assay, we developed a system of vectors which allows screening for cDNAs coding for nuclear proteins in a simple genetic assay in yeast cells, the verification of nuclear accumulation *in planta* (Fig. 5, paper #3 in the appendixes) following one-step transfer and autofluorescent tagging of the identified clones into multiple cloning site-compatible and reading frame-compatible plant expression vector, followed by a binary multigene expression vector.

4. Characterizing V2 as a F-Box protein and study its role in SGS3 degradation.

We examined different models for the mechanism(s) by which TYLCV-Is V2 affects the host SGS3 to suppress the RNA silencing defense of the plant. One which was found to be used in other cases of suppression of gene silencing is degradation of SGS3 through the ubiquitin proteasome system led by V2.

Regulation of protein stability through the ubiquitin proteasome system is an important mechanism underlying many cellular processes, including virus-host plant interactions. During this process, the target protein is tagged by covalent attachment of ubiquitin and then degraded by a multicatalytic protease complex, the 26S proteasome. The substrate specificity of the ubiquitin conjugation pathway is determined mainly by the E3 ligase, SCF, which is composed of four major proteins: Cul1, Skp1, Roc1, and an F-box protein. F-box protein functions by recognizing the specific substrate protein to be targeted for degradation and associating, via its F-box motif, with the Skp1

component [or its homologs, such as the *Arabidopsis* Skp1-like (ASK) proteins] of the SCF complex. Our amino acid analysis of V2 revealed an F-box-like motif at the N-terminus of the protein (see below). Thus, we tested whether V2 can interact with ASK1.

MWDPLLNEFPESVHGFRCLAIKYLQSVEETYEP
NTLGHDLIRDLISVVRARDYVEATRRYNHFHARL
EGSPKAELRQPIQQPCCCPHCPRHKQATIMDVQA
HVPKAQNIQNVSK

Amino acid sequence of TYLCV-Is. V2 (GenBank accession number CAA33687) with its 50-residue-long F-box-like motif indicated by yellow colored background.

Initially, we examined this interaction in the yeast two-hybrid system, which is often used to discover interactions of different F-box proteins with SKP1 proteins and their plant homologs.

As seen in the figure below, V2 does indeed interact with the *Arabidopsis* ASK1 (line 4) and yeast Skp1 (not shown) and that this interaction was specific, not occurring with diverse non-specific preys (lanes 1-3). **These observations support the idea that V2 may function by targeting its interacting substrates, such as SGS3, for proteasomal degradation via the SCF pathway.** Presently, we are confirming the V2-ASK1 interaction *in vivo*, using bimolecular fluorescent complementation (BiFC) vector systems developed in our labs (see paper #3 in the appendixes).

V2-ASK1 interaction in the yeast-two-hybrid assay. Cell growth on leucine-deficient medium represents selective conditions for protein-protein interactions. Lane 1, V2+Kap α 1; Lane 2, V2+ TYLCV-Is CP; Lane 3, V2+lamin C; Lane 4, V2+ASK1.

5. V2 interactts with a cysteine protease that acts as anti-viral agent

To better understand the molecular pathway by which V2 suppresses RNA silencing, we used the yeast two-hybrid system to screen our tomato two-hybrid cDNA library with V2 as bait and search for more interactors in addition to SGS3. We identified a full-length cDNA clone that encoded a protein product which interacted with V2 in the two-hybrid assay as detected by cell growth in the absence of histidine (not shown). Amino acid sequence analysis of this V2 interactor identified it as a tomato cystein protease that belongs to the papaine like group of cysteine proteases and was first cloned in senescenced leaves by Dr. A. Lers from Volcani (GenBank accession number: AJ003137).

Schematic diagram of tomato Cysteine protease CYP1. The full length cDNA of CYP1 is 1398 bp coding for a 466 a.a. long protein that contains a domain of 113 a.a. named granulin at its C-terminus. The enzymatic active site resides in the center between a.a. 137 and 353.

The interaction of V2 with tomato CYP1 and its derivatives (400 and 800, see the above schematic diagram) was then demonstrated directly *in planta* by fluorescence resonance energy transfer (FRET). YFP-CYP1, CYP1-400 and Cyp1-800 were transiently coexpressed with V2-CFP in tobacco protoplasts, and FRET between these proteins was detected by acceptor photobleaching microscopy. The following figure shows colocalization and FRET between YFP-Cyp1 and its fragments and V2-CFP. These data demonstrate that TYLCV-Is V2 interacts with Cyp1 within plant cells.

FRET assay: Protein-protein interaction was monitored by FRET microscopy of living *N. tabacum* protoplasts co-expressing V2-CFP and either YFP-Full length Cysteine protease (Cyp1), or its YFP-fragments (400 and 800, see Fig. 3). Representative acceptor photobleaching images show CFP (donor) and YFP (acceptor) channels before and after bleaching. After bleaching, CFP fluorescence increases and YFP fluorescence decreases in the bleached areas. All images are projections of several confocal sections.

Cooperation

A significant cooperation was implemented throughout the entire project period. The US and the Israeli PIs were in constant contact by e-mail and phone and several visits to each other labs were arranged. The good cooperation between the two PIs also led to organization of a BARD Workshop on the subject of their research ("on ssDNA pathogens", 2005, Eilat, Israel) and another BARD Workshop is scheduled for March 2010 ("RNA silencing and its suppression", Eilat, Israel).

Beside these, contacts and discussions, the collaborating labs exchange genetic material, i.e., expression constructs, this greatly facilitated experiments by both groups. Close cooperation between the PIs and the significant findings of this project were instrumental in generating another successful BARD-funded research by the same groups as well as establishment of an applied research program headed by Prof. Gafni aimed at preventing geminiviral infection *in planta* and funded by the Chief Scientist of the Ministry of Agriculture of Israel.

List of publications:

1. Zrachya A, Glick E, Levy Y, Arazi T, Citovsky V, Gafni Y. (2007) Suppressor of RNA silencing encoded by Tomato yellow leaf curl virus-Israel. *Virology* **358**: 159 – 165.
2. Glick, E., Zrachya, A., Levy, Y., Mett, A., Gidoni, D., Belausov, E., Citovsky, V. and Gafni, Y. (2008) Interaction with host SGS3 is required for suppression of RNA silencing by tomato yellow leaf curl virus V2 protein. *Proc. Natl.Acad. Sci. USA* **105**: 157- 161.
3. Citovsky, V., Gafni, Y. and Tzfira, T. (2008) Localizing protein-protein interactions by bimolecular fluorescence complementation in planta. *Methods* **45**: 196-206.
4. Zaltsman A, Yi BY, Krichevsky A, Gafni Y, Citovsky V. (2007) Yeast-Plant Coupled Vector System for Identification of Nuclear Proteins. *Plant Physiol.***145**: 1264 – 1271.
5. Zrachya A, Kumar PP, Ramakrishnan U, Levy Y, Loyter A, Arazi T, Lapidot M, Gafni Y. (2007) Production of siRNA targeted against TYLCV coat protein transcripts leads to silencing of its expression and resistance to the virus. *Transgenic Res.* **16**: 385 – 398.
6. Citovsky V, Lee LY, Vyas S, Glick E, Chen MH, Vainstein A, Gafni Y, Gelvin SB, Tzfira T. (2006) Subcellular localization of interacting proteins by bimolecular fluorescence complementation in planta. *J. Mol. Biol.* **362**: 1120 – 1131.
7. Kumar P P, Usha R, Zrachya A, Levy Y, Spanov H, Gafni Y. (2006) Protein-protein interactions and nuclear trafficking of coat protein and betaC1 protein associated with Bhendi yellow vein mosaic disease. *Virus Res.* **122**: 127 – 136.
8. Kass G, Arad G, Rosenbluh J, Gafni Y, Graessmann A, Rojas MR, Gilbertson RL, Loyter A. (2006) Permeabilized mammalian cells as an experimental system for nuclear import of geminiviral karyophilic proteins and of synthetic peptides derived from their nuclear localization signal regions. *J. Gen. Virol.* **87**: 2709 – 2720.
9. Tzfira T, Citovsky V. (2009) Systems biology of plant-pathogen interactions. *Semin Cell Dev Biol.* **20**: 1015-1016.
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11. Krichevsky, A., Zaltsman, A., Kozlovsky, S.V., Tian, G.W. and Citovsky, V. (2009) Regulation of root elongation by histone acetylation in Arabidopsis. *J. Mol. Biol.* **385**: 45-50.
12. Lacroix B, Loyter A, Citovsky V. (2008) Association of the Agrobacterium T-DNA-protein complex with plant nucleosomes. . *Proc. Natl.Acad. Sci. USA* **105**: 15429- 15434.