Final Scientific Report

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Project Title: Molecular Interactions of TYLCV capsid protein during assembly of viral particles

Investigators

Principal Investigator (PI): Yedidya Gafni

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Collaborating Investigators: None

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

Karyopherin alpha, coat protein, homotypic interaction, post transcriptional gene silencing, suppression of gene silencing

Abbreviations commonly used in the report, in alphabetical order:

CP, coat protein, NLS, nuclear localizing sequence, PTGS, post transcriptional gene silencing, siRNA, small interfering RNA, TYLCV, Tomato yellow leaf curl virus

Budget: IS: \$180,000

US: \$150,000

Total: \$330,000

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	3	5	3	11
Submitted, in review, in preparation	1	3	2	6
Invited review papers	--	--	1	
Book chapters	1	--	--	
Books	--	--	--	--
Master theses	--	--	3	3
Ph.D. theses	--	1	2	3
Abstracts	--	--	--	--
Not refereed (proceedings, reports, etc.)	--	--	--	--

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

Cooperation Summary (numbers)

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

Also, we organized a BARD Workshop on ssDNA (TYLCV as one) in Eilat in 2005 which was a great success and was summarized in an article in *Plant Molecular Biology* 61: 357-364. 2006.

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. If funded again, it will most probably bring better understanding of the life cycle of TYLCV and the plant proteins involved. This should eventually lead to developing better approaches to combat the virus and the disease it causes.

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Patent Summary (numbers)

	Israeli inventor only	US inventor only	Joint IS/US inventors	Total
Submitted		1		1
Issued (allowed)				
Licensed		1		1

Molecular Interactions of TYLCV capsid protein during assembly of viral particles

Abstract

Tomato yellow leaf curl geminivirus (TYLCV) is a major pathogen of cultivated tomato, causing up to 100% crop loss in many parts of the world. The present proposal, a continuation of a BARD-funded project, expanded our understanding of the molecular mechanisms by which CP molecules, as well as its pre-coat partner V2, interact with each other (CP), with the viral genome, and with cellular proteins during assembly and movement of the infectious virions. Specifically, two major objectives were proposed:

- I. To study in detail the molecular interactions between CP molecules and between CP and ssDNA leading to assembly of infectious TYLCV virions.**
- II. To study the roles of host cell factors in TYLCV assembly.**

Our research toward these goals has produced the following major achievements:

- Characterization of the CP nuclear shuttling interactor, karyopherin alpha 1, its pattern of expression and the putative involvement of auxin in regulation of its expression. (#1 in our list of publication, Mizrachy, Dabush et al. 2004).
- Identify a single amino acid in the capsid protein's sequence that is critical for normal virus life-cycle. (#2 in our list of publications, Yaakov, Levy et al. in preparation).
- Development of monoclonal antibodies with high specificity to the capsid protein of TYLCV. (#3 in our list of publications, Solmensky, Zrachya et al. in press).
- Generation of Tomato plants resistant to TYLCV by expressing transgene coding for siRNA targeted at the TYLCV CP. (#4 in our list of publications, Zrachya, Kumar et al. in press).
- Characterization of AtVIP1 and its involvement in stable genetic transformation mediated by our ssDNA model pathogen: *Agrobacterium tumefaciens*. (#5 in our list of publications, Li, Krichevsky et al. 2005)

These research findings provided significant insights into (i) the molecular interactions of TYLCV capsid protein with the host cell nuclear shuttling receptor, and (ii) the mechanism by which TYLCV V2 is involved in the silencing of PTGS and contributes to the virus pathogenicity effect. Furthermore, the obtained knowledge helped us to develop specific strategies to attenuate TYLCV infection, for example, by blocking viral entry into and/or exit out of the host cell nucleus via siRNA as we showed in our publication recently (# 4 in our list of publications). Finally, in addition to the study of TYLCV nuclear import and export, our research contributed to our understanding of general mechanisms for nucleocytoplasmic shuttling of proteins and nucleic acids in plant cells. Also integration for stable transformation of ssDNA mediated by our model pathogen *Agrobacterium tumefaciens* led to identification of plant specific proteins involved.

Achievements

The main significant achievements of the present project are:

1. **Characterization of the CP nuclear shuttling interactor, karyopherin alpha 1, its pattern of expression and the putative involvement of auxin in regulation of its expression.**

Geminiviruses in general and TYLCV in particular are considered as nuclear replicating viruses and the role of the coat protein was suggested by us to serve as a shuttle protein for the viral genome importing it into the plant cell nucleus. As assembly of the virus capsule is believed to take place inside the nucleus too, the involvement of karyopherin alpha is crucial for both processes. During the second year, much attention was given to karyopherin α and its specific expression in the plant. The karyopherin α 1 (*LeKAP α 1*) gene of tomato (*Lycopersicon esculentum*) encodes a receptor involved in nuclear import. To analyze the expression pattern of this gene, a genomic clone containing its upstream region was isolated and sequenced. To study the promoter functionality, a 2170 bp fragment (LM1), was fused to GUS and introduced into petunia cells by particle bombardment. For further characterization of the promoter, one inverse and three deletion constructs were studied in cell suspension. To follow its expression in tobacco leaves, transgenic plants expressing GUS under the control of the LM1 promoter were made. Expression of LM1-GUS (Figure 1) was largely restricted to actively growing leaf regions, suggesting possible involvement of active cell division and plant growth regulators in *LeKAP α 1* expression.

Figure 1. Histochemical localization of GUS activity during leaf-primordium morphogenesis. *LeKAPal::GUS* gene expression in transformed tobacco plants, showing. (a) shows strong GUS expression in the tip (arrow) of a young leaf primordium. (b) basipetal progress of GUS activity along the margins. (c) incision control showing GUS-free cut surfaces along the parallel incisions (arrows) made in the lamina, demonstrating that the *GUS* expression is restricted only to the margins. (d) leaf-fragment control showing GUS expression only along the margins (arrows). (e) *GUS* expression at a late developmental stage showing *GUS* expression (arrows) at the base of the leaf, and also in the petiole (e and f). (g) *GUS* expression along the margins (arrowhead) of a young leaf primordium and inside the blade (arrow) of an older leaf. (h) incision control showing *GUS* activity inside the blade (arrow) of an older leaf, while the exposed surfaces along the parallel cuts in the lamina remained GUS free. Scale bars = 1 cm.

2. Identify a single amino acid in the capsid protein's sequence that is critical for normal virus life-cycle.

The coat protein (CP) of *Tomato yellow leaf curl virus* (TYLCV), encoded by the *vc1* gene, is the only known component of the viral capsid. Point mutation that was introduced into the conserved NLS region of the CP changing Arginine number 19 to Leucine, had a significant effect on its homotypic interaction as well as on its interaction with karyopherin alpha and GroEL. These were observed first by exploiting the yeast two-hybrid assay and confirmed *in planta* by measuring fluorescence resonance energy transfer (FRET) between YFP- and CFP-tagged proteins (Figure 2). In addition, when the mutation was introduced into the TYLCV genome, it affected its ability to replicate, spread, cause symptoms and be acquired by the whitefly insect vector.

Figure 2. FRET in tobacco protoplasts showing interaction between TYLCV CP and Karyopherin α 1. A&C before photobleaching, B&D after photobleaching.

TABLE 1. Summary of FRET assays done in tobacco protoplasts.

Interacting proteins	FRET efficiency %	Standard deviation
CP-CP	12.5%	7.5
CP-Karyopherin α	10.5%	3.3
CP mut3-CP mut3	4.4%	2.7
CFP control	0.2%	6
CFP-YFP	-0.106%	4.11

3. Development of monoclonal antibodies with high specificity to the capsid protein of TYLCV

Although TYLCV is recognized as a serious threat to tomato crops in many countries, causing losses of up to 100% of the fruit's harvest, no monoclonal antibodies (mAbs) have ever been produced against this pathogen. Current immunodiagnostic methods for TYLCV detection employ only either polyclonal sera or heterologous mAbs. However, mAbs are more specific and reproducible than polyclonal sera and likewise, it is more accurate to use mAbs raised against the virus of interest than those raised against other related viruses as the immunogen. The coat protein (CP) of TYLCV is the only known building block of the viral capsid. Therefore, in this study we used CP as an antigen for mAb production. We employed a strategy in which the CP was truncated at its C-terminus to avoid intra- and inter-protein interactions that could impair epitope exposure. For the same reason, we used a denaturated antigen to expose linear epitopes during the immunization. This strategy yielded three mAbs: they were characterized both biochemically and immunologically, and their epitopes were mapped. We show their potential application as diagnostic tools (Fig. 3), and analyze the possibility of using them as single-chain variable fragment (scFv) plantibodies for neutralization of viral assembly and/or movement within the plant cells, or for studying the interactions of the virus with itself, the host plant and its vector.

Figure 3. Western blot for the detection for TYLCV in tomato plants. Infected and non-infected plant extracts were electrophoresed. The proteins were transferred to a nitrocellulose membrane that was blocked with skim milk, and subjected to overnight incubation with B8 and ECL detection. Lanes 1 and 2—non-infected plants, lanes 3 and 4—TYLCV-infected plants. Each lane represents an individual plant. A strong band at approximately 30 kDa (corresponding to CP) and a weak one at approximately 55 kDa (possibly corresponding to aggregating CP) were revealed in the infected plant, while those signals did not appear with the healthy plant sample.

4. Generation of Tomato plants resistant to TYLCV by expressing transgene coding for siRNA targeted at the TYLCV CP.

The coat protein (CP) of *Tomato yellow leaf curl virus* (TYLCV), encoded by the *vc1* gene, is the only known component of the viral capsid. In addition, based on our former BARD-supported research, we now know that the CP plays a role in the virus transport into the host cell nucleus where viral genes are replicated and transcribed. In this study, we analyzed the effect of small interfering double-stranded RNAs (siRNAs), derived from an intron-hairpin RNA (ihpRNA) construct and targeting the *vc1* gene product, on CP accumulation. Transient assays involving agroinfiltration of the CP-silencing construct followed by infiltration of a fused GFP-CP (green fluorescent protein-coat protein) gene showed down-regulation of GFP expression in *Nicotiana benthamiana*. Some of the transgenic tomato plants (cv. Micro-Tom), expressing the siRNA targeted against the TYLCV CP gene, did not show disease symptoms 7 weeks post-inoculation with the virus, while non-transgenic control plants were infected within 2 weeks post

inoculation (Fig. 4). The present study demonstrates, for the first time, that siRNA targeted against the CP of TYLCV can confer resistance to the virus in transgenic tomato plants, thereby enabling flowering and fruit production.

Figure 4. Transgenic and non-transgenic Micro-Tom tomato plant response to TYLCV infection. Two-week-old plants were inoculated with viruliferous whiteflies. Typical symptoms appeared on wild type plants 3 weeks post-inoculation, shown from above. Transgenic plants expressing the “silencing construct” showed no symptoms even five weeks post infection.

5. Characterization of AtVIP1 and its involvement in stable genetic transformation mediated by our ssDNA model pathogen: *Agrobacterium tumefaciens*.

The advanced study of *Agrobacterium tumefaciens* is of high importance to understand the intracellular biology of TYLCV. Both are ssDNA pathogens of the plant cell, moving their DNA into the plant cell nucleus. Recently, we discovered a plant protein named AtVIP1 that was shown to interact with the T-DNA complex. We functionally dissected AtVIP1 using reverse genetics and directly demonstrated its involvement in stable genetic transformation by *Agrobacterium*. Specifically, we isolated an *Arabidopsis* mutant, *vip1-1*, homozygous for a single T-DNA insertion in the *AtVIP1* gene (also, see below for additional *vip1* mutants).

Figure 5. N-terminal portion of AtVIP1 produced in *vip1-1* plants. **(A)** RT-PCR detection of the 5'-terminal half of the *AtVIP1* transcripts in various tissues of wild-type and *vip1-1* plants. The specific RT-PCR product is 280 bp-long (arrowhead on right). **(B)** RT-PCR detection of the full-length *AtVIP1* transcripts in various tissues of wild-type and *vip1-1* plants. The specific RT-PCR product is 780 bp-long (arrowhead on right). Lane 1, total wild-type seedlings; lane 2, total *vip1-1* seedlings; lane 3, wild-type roots; lane 4, *vip1-1* roots; lane 5, wild-type leaves; lane 6, *vip1-1* leaves.

Our DNA sequencing experiments demonstrated that the mutagenic T-DNA was inserted within the second half of the *AtVIP1* ORF, i.e., after amino acid position 164 (not shown). Thus, it is possible that the first half of the *AtVIP1* ORF still produces a truncated transcript. To test this idea, we used RT-PCR to detect *AtVIP1*-specific transcripts in the wild-type and *vip1-1* plants. Fig. 5A shows that both types of plants accumulated RT-PCR fragments corresponding to the sequences within the 5'-terminal half of the *AtVIP1* ORF in various tissues, such as total seedlings (lanes 1, 2), roots (lanes 3, 4), and leaves (lanes 5, 6). These 280-bp RT-PCR products were derived from the *AtVIP1* mRNA rather than from the genomic sequence because the latter would have generated larger, 380-bp PCR fragments due to the presence of an intron. As expected, no full-length *AtVIP1* transcripts were detected in the *vip1-1* plants whereas the wild-type plants accumulated RT-PCR products corresponding to the complete, 780 bp-long *AtVIP1* mRNA (Fig. 5B). In control experiments, actin-specific transcripts generated similar amounts of PCR products in all samples, indicating equal efficiencies of the RT-PCR reactions (not shown).

That *vip1-1* was not a null allele, allowed us to examine whether different parts of the AtVIP1 protein (i.e., its N- and C-terminal portions) may play different and distinct roles in the process of *Agrobacterium*-mediated genetic transformation. First, we produced a recombinant cDNA corresponding to the residual *AtVIP1* sequence in the *vip1-1* mutant. The protein product encoded by this cDNA, designated AtVIP1-N164, was assayed for two known biological activities of AtVIP1, import into the plant cell nucleus and interaction with *Agrobacterium* VirE2.

To assay for nuclear import, AtVIP1-N164 was tagged with GFP [because AtVIP1-N164 is a small protein, ~18 kDa, we fused it to a GFP dimer to yield a ~71 kDa fusion product that exceeds the 40-60 kDa size exclusion limit of the nuclear pore]. Fig. 6A shows that

(GFP)₂-AtVIP1-N164, transiently expressed in tobacco leaves following biolistic delivery of its encoding DNA, accumulated almost exclusively in the cell nucleus. AtVIP1-N164, as well as the full-length AtVIP1, also interacted with VirE2 in a Y2H assay (Fig. 6B, lanes 1, 2, respectively); this interaction was specific because it did not occur with lamin C (lane 3), a known non-specific activator in the Y2H system (Bartel, Chien et al. 1993). In control, non-selective conditions, all combinations of the tested proteins resulted in cell growth (Fig. 6C).

Figure 6. Biological activities of AtVIP1-N164. **(A)** Nuclear import of (GFP)₂-AtVIP1-N164 in tobacco cells. **(B)** AtVIP1-N164 interaction with VirE2 in Y2H assay. Cells grown without histidine. **(C)** AtVIP1-N164 interaction with VirE2 in Y2H assay. Cells grown in the presence of histidine. Lane 1, AtVIP1-N164 + VirE2; lane 2, AtVIP1 + VirE2; lane 3, AtVIP1-N164 + lamin C. **(D)** HeLa cells expressing GFP-VirE2. **(E)** HeLa cells coexpressing GFP-VirE2 and unlabeled AtVIP1. **(F)** HeLa cells coexpressing GFP-VirE2 and unlabeled AtVIP1-N164. All images are single confocal sections.

As mentioned above, one of the functional hallmarks of the AtVIP1-VirE2 interaction *in vivo* is that it promotes nuclear import of VirE2 in plant and non-plant cells. Because only plant genomes encode AtVIP1, VirE2 nuclear import is plant-specific. In yeast and mammalian cells, it occurs only in the presence of transiently-expressed plant AtVIP1. AtVIP1-N164 retained this ability of the full-length AtVIP1 to facilitate nuclear uptake of GFP-tagged VirE2 in mammalian cells. GFP-VirE2 expressed in HeLa cells was unable to enter the cell nucleus and remained exclusively cytoplasmic (Fig. 6D). Coexpression of GFP-VirE2 with either AtVIP1 or AtVIP1-N164, however, resulted in a significant proportion of the fluorescent signal redirected to the cell nucleus (Fig. 6E, F, respectively). [Note that the data in Fig. 6D-E represent confocal optical sections with the plane of focus through the cell nucleus, which detect intranuclear accumulation rather than perinuclear binding.] Quantification of the fluorescent signal on a per cell basis demonstrated that AtVIP1-N164 redirected 40-60% of GFP-VirE2 to the cell nucleus, an efficiency comparable to that reported for the full-length AtVIP1. Potentially, transient expression of GFP-VirE2 and AtVIP1 or AtVIP1-N164 from separate plasmids may not have generated proteins concentrations necessary for the complete nuclear import of VirE2.

Figure 7. Effects of VIP1-N164 on transient and stable genetic transformation of *Arabidopsis* by *Agrobacterium*. **(A)** Infected root segments from the wild-type *Arabidopsis* plants. **(B)** Infected root segments from the *vip1-1* plants. **(C)** Tumor formation in *Agrobacterium*-infected root segments of the wild-type plants. **(D)** Reduced tumor formation in *Agrobacterium*-infected *vip1-1* plants.

That AtVIP1-N164 retained the ability of the full-length protein to bind VirE2 and promote its nuclear import allowed us to examine the potential role of AtVIP1 at later stages of the transformation process. To this end, we assayed transient and stable genetic transformation of *vip1-1* plants. For transient transformation, *Arabidopsis* root segments were inoculated with an *Agrobacterium* strain carrying in its T-DNA a GUS reporter, and GUS activity was detected histochemically 36 hours after inoculation. [Note that standard *Agrobacterium* transformation assays utilize leaf disks in tobacco and root segments in *Arabidopsis*.] Because the *GUS* gene contained an intron which prevents its expression in bacteria, our measurements represented the GUS activity directed by the T-DNA after its transfer to plant cells. Both wild-type and *vip1-1* plants exhibited comparable degrees of transient T-DNA expression as estimated from the size and number of the stained areas of root segments (Fig. 7A, B) and from quantitation of GUS activity by a fluorimetric assay (not shown).

Stable genetic transformation was assayed by the formation of tumors following inoculation with wild-type, oncogenic *Agrobacterium*. Fig. 7C shows that *Agrobacterium* elicited numerous tumors on root segments derived from the wild-type *Arabidopsis* plants. In contrast, the *vip1-1* mutant plants exhibited a dramatic decrease in their susceptibility to *Agrobacterium* tumorigenicity, developing relatively few tumors following inoculation with *Agrobacterium* (Fig. 7D). These data provide the first indication for AtVIP1 activity “beyond nuclear import” and suggest that the C-terminal portion of AtVIP1 carries protein determinants likely required for its function within the cell nucleus, i.e., during T-DNA intranuclear transport and integration.

LIST OF PUBLICATIONS

- 1. Mizrachy, L., D. Dabush, et al. (2004). Cloning and characterization of the tomato karyopherin ALPHA 1 gene promoter. 46: 515-522.**
- 2. Yaakov, N. Y. Levy, et al. Single mutation in the NLS domain of Tomato yellow leaf curl virus (TYLCV) coat protein (CP) identifies an amino acid critical for normal virus life-cycle. In preparation.**
- 3. Solmensky, L., A. Zrachya, et al. (2007). Preparation and characterization of monoclonal antibodies against Tomato yellow leaf curl virus (TYLCV). Arch. Virol. In Press.**
- 4. Zrachya, A., P.P. Kumar, et al. (2006). Production of siRNA targeted against TYLCV coat protein transcripts leads to silencing of its expression and resistance to the virus. Transgenic Res. In Press.**
- 5. Li, J. A. Krichevsky, et al. (2005) Uncoupling of the functions of the *Arabidopsis* VIP1 protein in transient and stable plant genetic transformation by *Agrobacterium*. Proc. Natl. Acad. Sci. 102: 5733-5738.**