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in Tomato Plants**

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TABLE OF CONTENTS

	Page
ABSTRACT	3
OBJECTIVES	4
I. Background and rationale	4
II. Proposed objectives and research plan	5
RESEARCH ACTIVITIES AND RESULTS	6
I. Introduction	6
II. Scientific studies and achievements	7
1. Paper # 1. <i>Transgenic tomato plants expressing TYLCV capsid protein are resistant to the virus: The role of the nuclear localization signal (NLS) in the resistance.</i>	
2. Paper # 2. <i>Nuclear import of the capsid protein of tomato yellow curl leaf virus (TYLCV) in plant and insect cells.</i>	
3. Paper # 3. <i>The molecular structure of Agrobacterium VirE2-single stranded DNA complexes involved in nuclear import.</i>	
4. Paper # 4. <i>Nucleic acid transport in plant-pathogen interactions.</i>	
5. Paper # 5. <i>Transport of DNA into the nuclei of Xenopus oocytes by a modified VirE2 protein of Agrobacterium.</i>	
6. Paper # 6. <i>The capsid protein of tomato yellow leaf curl virus binds cooperatively to single stranded DNA.</i>	
7. Paper # 7. <i>Nuclear transport of geminiviral proteins.</i>	
8. Paper # 8. <i>Characterization of a tomato karyopherin α that interacts with the Tomato Yellow Leaf Curl Virus (TYLCV) capsid protein.</i>	
EVALUATION OF RESEARCH ACHIEVEMENTS	8
DESCRIPTION OF COOPERATION	8
CONCLUSIONS	9
LIST OF PUBLICATIONS	10

ABSTRACT**NUCLEAR IMPORT OF THE TOMATO YELLOW LEAF CURL VIRUS IN TOMATO PLANTS**

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. In Israel the disease is well known and has an economic significance. In recent years viral symptoms were found in countries of the "New World" and since 1997, in Florida. Surprisingly, little is known about the molecular mechanisms of TYLCV interaction with the host plant cells. This proposal was aimed at expanding our understanding of the molecular mechanisms by which TYLCV enters the host cell nucleus. The main objective was to elucidate the TYLCV protein(s) involved in transport of the viral genomic DNA into the host cell nucleus. This goal was best served by collaboration between our laboratories one of which (V.C.) was already investigating the nuclear import of the T-DNA of *Agrobacterium tumefaciens*, and the other (Y.G.) was studying the effect of TYLCV capsid protein (CP) in transgenic plants, hypothesizing its involvement in the viral nuclear entry.

Three years of our collaborative work have provided significant data that strongly support our original hypothesis of the involvement of TYLCV CP in viral nuclear import. Furthermore, our results have laid a foundation to study fundamental, but as yet practically unresolved, questions about the role of the host cell factors in the nuclear import of geminiviruses within their host plant. As a result, this research may lead to development of new approaches for plant protection based on control of TYLCV import to the host plant cell nucleus.

OBJECTIVES

I. Background and rationale

The tomato yellow curl leaf virus (TYLCV) is a constant threat to tomato growers, causing up to 100% crop loss in the Middle East, North and Central Africa, South East Asia, Taiwan, Europe and Southeastern United States. TYLCV is a geminivirus transmitted by the whitefly *Bemisia tabaci*. Members of the geminivirus group are characterized by a genome of circular single-stranded (ss)DNA encapsidated in geminate (twinned) quasi-isometric particles from which the group derives its name. Geminiviral putative replicative forms are circular double-stranded (ds)DNA molecules organized in chromatin-like structures. Unlike other known whitefly-transmitted geminiviruses which divide their genome between two ssDNA molecules, TYLCV contains only one genomic circular ssDNA molecule of 2,787 nucleotides. The TYLCV genome encodes six open reading frames (ORFs), two on the viral (+) strand (V1 and V2) and four on the complementary (-) strand (C1, C2, C3, and C4). One of these ORFs, V1, codes for the capsid protein (CP) that represents a building block of the viral capsule.

Most animal and insect viruses as well as many plant viruses replicate in the nucleus of their host cells. However, relatively little is known about mechanisms by which viral genomic nucleic acid molecules are transported into the cell nucleus. Geminivirus particles are imported into the host plant cell nucleus where DNA replication, transcription, and virus assembly presumably take place. Serological studies using African cassava mosaic virus (ACMV) have demonstrated nuclear import of this virus. Sections of *N. benthamiana* leaves incubation with fluorescent antibody indicated that ACMV particle antigen accumulates in the nuclei of many phloem cells and of some cells in other tissues. Similar results were obtained when the intracellular location of beet curly top virus antigen was studied by scanning electron microscopy. Studies of the bipartite geminivirus squash leaf curl virus (SqLCV) indicated that a protein (BR1) encoded by the DNA B component binds the genomic viral ssDNA and shuttles it into and out of the host cell nucleus. Also, the BR1 protein of another bipartite geminivirus, the bean dwarf mosaic geminivirus (BDMV), has been shown to play a role in the nuclear export of the viral genome. Monopartite geminiviruses, such as TYLCV, lack the DNA B genomic component and do not encode a BR1 protein homologue. Also, as electron microscopy studies of TYLCV-infected tomato tissues showed that the nucleoplasm of the host phloem parenchyma cells is filled with whole virions, it is suggested that viral capsid protein may be involved in nuclear import of the viral particle.

Thus, the mechanisms by which TYLCV genomic ssDNA molecules are imported into the host cell nucleus for replication and transcription and then are exported from the nucleus for cell-to-cell spread were largely unknown. Furthermore, no information existed about the host cell factors involved in these processes.

II. Proposed objectives and research plan

The main goal of the study was to resolve the molecular mechanism by which the TYLCV reaches the nucleus of infected tomato plant cells. For this, the originally proposed research had four specific objectives:

- (1) To determine if the capsid protein (CP) of TYLCV is karyophilic.
- (2) To identify and characterize nuclear localization signal (NLS) sequences of TYLCV CP.
- (3) To develop a detection system for nuclear import of TYLCV virions.
- (4) To determine if NLS mutants of TYLCV CP expressed in transgenic plants interfere with nuclear transport of wild-type CP and entire virion.

For the studies of nuclear import of TYLCV CP we planned on using a reporter gene fused to the open reading frame of the capsid protein and introduced into tomato protoplasts. The reported gene selected for this purpose was GUS enzyme that its activity is easily detected in or out of the nucleus of transformed cells. For the identification of the functional NLS signal(s) of TYLCV CP, we planned to do mutation analysis followed by transient expression studies in protoplasts. Also we planned to study the role of CP nuclear import in transgenic plants, a goal that was replaced later by experiments aimed at learning the ssDNA binding activity of the TYLCV CP. The reason for this change was the following: We believe that it is much better to reveal first the biochemistry and the genetics of the process by which the virus is imported into the nucleus. Only then we will find the "weak" steps in the process which may lead to interference and generation of transgenic plants resistant to the virus.

RESEARCH ACTIVITIES AND RESULTS

I. Introduction

As already mentioned, the main objective of the study was to elucidate the TYLCV protein(s) involved in transport of the viral genomic DNA into the host cell nucleus.

The following findings were obtained during the funding period:

(1) TYLCV CP was shown to carry a functional nuclear localization signal (NLS). This karyophilic activity of CP is a prerequisite for its proposed role in transporting the viral genomic DNA into the host cell nucleus. Our studies characterized the NLS sequence in detail and demonstrated its function both in plant and in insect cells (insects are TYLCV transmission vectors). This work was described in *Acta Hort.* **447**, 387-391 (1997), and in *Plant J.* **13**, 393-399 (1998).

(2) TYLCV CP was demonstrated to possess a single-stranded (ss)DNA binding activity, which, again, is a prerequisite for its role as a nuclear transport protein for the single-stranded viral genome. This binding activity was shown to be absolutely specific for ssDNA yet independent of its nucleotide sequence. This work was described in *J. Gen. Virol.*, **78**, 2829-2833 (1998).

(3) Nuclear import of protein-ssDNA complexes was directly demonstrated using the *Agrobacterium* VirE2 protein associated with fluorescent ssDNA. In addition to its ability to directly transport ssDNA into the cell nucleus, VirE2 was shown to possess plant-specific NLS signals. One amino acid change in the VirE2 NLS converted this sequence into a general-type signal active in animal cells. This work was described in *Plant Cell* **8**, 363-373 (1996).

(4) Molecular structure of a protein-ssDNA complex involved in nuclear import was studied using mass analysis of scanning transmission electron microscopic (STEM) images of the *Agrobacterium* VirE2 protein bound to ssDNA. This complex was shown to have a coiled, telephone cord-like, structure with a diameter exceeding that of a closed nuclear pore but consistent with its open aperture. This work was described in *J. Mol. Biol.* **272**, 718-727 (1997).

(5) An Arabidopsis gene, designated *AtKAP α* , coding for a cytoplasmic protein that binds specifically to the NLS sequences was isolated and characterized. *AtKAP α* was then shown to mediate nuclear import of the *Agrobacterium* VirD2 protein. This work was described in *Proc. Natl. Acad. Sci. USA* **94**, 10723-10728 (1997) and in *Plant Physiol.* **115**, 314 (PGR97-129) (1997).

(6) A tomato cDNA, designated *LeKAP α 1*, was cloned and shown to share an 86% homology with *AtKAP α* . Its protein product directly interacted with TYLCV CP in the yeast two-hybrid system. With the help of Dr. Zamir (Hebrew University), we also determined that the tomato genome contains at least three members of this gene family which map to chromosomes 6 and 8. This work has been submitted for publication in the *J. of Experimental Biology*.

(7) Amino acid sequence analysis of TYLCV CP was shown to contain a region homologous to the known nuclear export signals (NESs), suggesting that CP may function as a nucleocytoplasmic shuttle for viral genomic ssDNA.

(8) In addition to these publications, Agrobacterium VirE2 protein with a modified NLS that functions in animal cells was patented as a DNA-transporting protein for use in gene therapy (U.S. patent "Protein-Mediated Nuclear Import of DNA" # 08/824,151).

II. Scientific studies and achievements

The primary research objectives were achieved and the following body of the report is mainly presentation of our achievements in the form of scientific papers, which were either published or submitted for publication.

EVALUATION OF RESEARCH ACHIEVEMENTS

The overall objectives of this project were to elucidate the mechanism(s) of nuclear import of a geminivirus exemplified by TYLCV, a monopartite geminivirus. We have suggested in our original hypothesis that the capsid protein of TYLCV is involved in viral nuclear import. The results described in this report strongly support our hypothesis and they have laid a foundation to study fundamental, but as yet practically unresolved, questions about the role of the host cell factors in the nuclear import of geminiviruses within their host plant. Here we describe the characterization of the NLS sequence found in TYLCV CP and its functionality both in plants and in insects. This observation might indirectly support hypothesis suggested by Prof. H. Czosnek from the Hebrew University, that the virus can replicate in the insect as well. We also demonstrated that TYLCV CP possesses a single stranded DNA binding activity. This is a prerequisite for its suggested role as a nuclear transport protein for the single stranded viral genome. Following our isolation of the cytoplasmic receptor AtKAP alpha, a tomato cDNA designated LeKAP alpha was cloned and its protein product directly interacted with TYLCV CP in the yeast two hybrid system. There are probably two or three genes for that protein in the tomato genome. Are any of these three "responding" to viral infection? Or, do viruses that replicate in the nucleus make use of one of these three? The answer for this is not known yet but once we'll have it we can plan controlling the replication of geminiviruses thus establishing new approaches to protect plants from geminiviruses. All this and more can be attributed to the project summarized here.

DESCRIPTION OF COOPERATION

A very close interaction has been established during the course of this study. It has been expressed by frequent communication through electronic mail, frequent visits of both P.I.s in its partner's laboratory and the exchange of genetic material. Constant discussions were held and it led to generation of ideas and experiments throughout the course of this study.

A significant contribution to current research was exchange of genetic material. It included plasmid vectors or constructs that were prepared in one laboratory and utilized by the other.

These everyday contacts led to several joint publications driven by several new discoveries, which were made exclusively because of this increased level of collaboration. This particularly concerns the discovery of the gene for a cytoplasmic receptor that recognizes NLS-bearing proteins: karyopherin alpha.

Both groups freely shared ideas, technologies and materials and the collaboration between the two groups continue. If funded again, it will most probably bring better understanding of the nuclear uptake machinery of nucleic acids-protein complexes.

CONCLUSIONS

The main goal of our study is to learn and understand the biochemistry and genetics of the processes involved in the nuclear import of geminiviruses and other protein-DNA complexes in their host plant cells. In the present study, we have addressed all the objectives listed in the original proposal. Thus we have characterized the capsid protein of TYLCV as a karyophilic protein, identified the nuclear localization signals in this protein, characterized its ssDNA binding activity and cloned and characterized the a cDNA for karyopherin alpha from tomato plants. The latter was made possible by utilizing the karyopherin alpha gene we have cloned earlier from Arabidopsis.

In further studies we intend to continue the study of nuclear import of viral genome as well as focusing on mechanisms by which TYLCV genomic ssDNA molecules are exported from the nucleus. This process is crucial for viral cell-to-cell spread and it is largely unknown. We would like to study both the viral and the plant factors involved and develop new strategies to control these pathways by genetically modify tomato plants with better resistance against geminiviruses.

LIST OF PUBLICATIONS

• Publications describing TYLCV CP functions directly related to the project

1. Gafni, Y., Kunik T., Czosnek, H., & Citovsky, V. (1997). Transgenic tomato plants expressing TYLCV capsid protein are resistant to the virus: the role of the nuclear localization signal (NLS) in the resistance. *Acta Hort.* **447**, 387-391.
2. Kunik, T., Palanichelvam, K., Czosnek, H., Citovsky, V., & Gafni, Y. (1998) Nuclear import of the capsid protein of tomato yellow leaf curl virus (TYLCV) in plant and insect cells. *Plant J.* **13**, 393-399.
3. Gafni, Y. & Kunik, T. (1998) Nuclear transport of geminiviral proteins. *Trends Plant Path.* **1**, 65-70.
4. Palanichelvam, K., Czosnek, H., Citovsky, V., & Gafni, Y. (1998) The capsid protein of tomato yellow leaf curl virus binds cooperatively to single-stranded DNA. *J. Gen. Virol.*, **78**, 2829-2833.
5. Gafni, Y. (1998) Nucleocytoplasmic trafficking of geminiviral proteins. *Current Science* **in press**.
6. Kunik, T., Mizrachy, L., Citovsky, V. and Gafni, Y. Characterization of a tomato karyopherin α that interacts with the Tomato Yellow Leaf Curl Virus (TYLCV) capsid protein. *J. Experimental Botany* submitted.

• Publications describing utilization of Agrobacterium-plant interaction system to develop experimental tools for use in the original project

1. Guralnick, B., Thomsen, G. & Citovsky, V. (1996) Transport of DNA into the nuclei of *Xenopus* oocytes by a modified VirE2 protein of *Agrobacterium*. *Plant Cell* **8**, 363-373.
2. Citovsky, V., Guralnick, B., Simon, M., & Wall, J., (1997) The molecular structure of *Agrobacterium* VirE2-single stranded DNA complexes involved in nuclear import. *J. Mol. Biol.* **272**, 718-727.
3. Ballas, N. & Citovsky, V. (1997) AtKAP α gene from *Arabidopsis* (Accession No. U69533) encodes a protein that mediates nuclear import of *Agrobacterium* VirD2 protein (PGR97-129). *Plant Physiol.* **115**, 314.

4. Ballas, N. & Citovsky, V. (1997) Nuclear localization signal binding protein from Arabidopsis mediates nuclear import of Agrobacterium VirD2 protein. *Proc. Natl. Acad. Sci. USA* **94**, 10723-10728.

• **Publications that acknowledge BARD support but are tangential to the original project, describing another viral transport process, i.e. cell-to-cell movement of plant viruses**

1. Lartey, R., Ghoshroy, S., Hoe, J., & Citovsky, V. (1997) Movement and subcellular localization of a tobamovirus in Arabidopsis. *Plant J.* **12**, 537-545.
2. Ghoshroy, S., Feldman, K., & Citovsky, V. (1998) Inhibition of plant viral systemic movement by non-toxic concentrations of cadmium. *Plant J.* **13**, 591-602.
3. Lartey, R., Ghoshroy, S., & Citovsky, V. (1998) Identification of an *Arabidopsis thaliana* mutation (*vsm1*) that restricts systemic movement of tobamoviruses. *Molec. Plant-Microbe Interact.*, in press.
4. Sheng, J., Lartey, R., Ghoshroy, S., & Citovsky, V. (1998) An Arabidopsis mutant with virus-inducible phenotype. *Virology*, in press.
5. Citovsky, V., Ghoshroy, S., Tsui, F., & Klessig, D.F. (1998) Non-toxic concentrations of cadmium inhibit tobamoviral systemic movement by a salicylic acid-independent mechanism. *Plant J.*, in press.
6. Ghoshroy, S. & Citovsky, V. (1998) Preservation of plant cell ultrastructure during immunolocalization of virus particles. *J. Virol. Methods*, in press.
7. Sheng, J.S., Waigmann, L., Ghoshroy, S., Hind, J., Zambryski, P., & Citovsky, V. (1998) A 38-kDa plant cell wall receptor for the tobacco mosaic virus cell-to-cell movement protein (MP) has protein kinase activity that regulates MP function. submitted .