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BARD

FINAL REPORT

PROJECT NO. IS-2566-95

**Resistance to Tomato Yellow Leaf Curl Virus by
Combining Expression of a Natural Tolerance
Gene and a Dysfunctional Movement Protein in
a Single Cultivar**

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BARD Final Scientific Report

(Cover Page)

Date of Submission of the report: June 2000

BARD Project Number: IS-2566-95R
Evaluating Panel:

Project Title: Resistance to tomato yellow leaf curl virus by combining expression of a natural tolerance gene and a dysfunctional movement protein in a single cultivar

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Continuation of (Related to) Previous BARD Project:

Yes

No

Number:

Keywords not appearing in the title and in order of importance. Avoid abbreviations. Geminivirus, genetic engineering, marker assisted breeding, plant breeding, whiteflies

Abbreviations used in the report, in alphabetical order:

Budget: IS: \$

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Signature
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	Joint IS/US authorship	US Authors only	Israeli Authors only	Total
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Book chapters			2	2
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Abstracts		2		2
Not refereed (proceedings, reports, etc.)			1	1

**Please list the student by name and provide his social security or identity number.*

Patent Summary (numbers)

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Cooperation Summary (numbers)

	From US to Israel	From Israel to US	Together, elsewhere	Total
Visits/Meetings	2	1	1	4
Sabbaticals				
Postdoctorates				

Cooperation, briefly explain whether synergistic, complementary or supportive.

Synergistic: helped to better understand TYLCV problems in the New and old Worlds _____

Complementary: aspects that could not be dealt in the US or Israel _____

Supportive: visit to learn particular techniques _____

Achievements

Significance of main scientific achievements or innovations

1. The complete sequence of a TYLCV isolate from the Dominican Republic was determined and found to be >97% identical to that of the Israeli isolate of TYLCV, confirming its introduction from the Eastern Mediterranean. Monitoring of TYLCV in whiteflies, using PCR, was found to be a reliable indicator of the incidence of the virus in the DR and was used to demonstrate the effectiveness of a host free period in TYLCV management. Finally, symptomless infections were detected in various weeds suspected an important means by which the virus persists in during the host-free period.
2. Using a mutational analysis of an infectious clone of TYLCV-DR, we have established that mutants in the C4, V1 (precoat) and V2 (coat protein, CP) genes did not effect replication (in protoplasts) but abolished infectivity in tomato. These proteins were then expressed in *E. coli* and used in microinjection experiments to further investigate their role in movement. The CP localized to the nucleus of injected cells but none of the proteins, alone or in combination moved extensively upon injection of mesophyll or epidermal cells. Moreover, none of the proteins, alone or in combination, increased the plasmodesmal size exclusion limit or facilitated cell-to-cell movement of DNA. Thus, on the basis of these experiments, TYLCV does not appear to encode a bone fide cell-to-cell movement protein. All three proteins bound double-stranded and single-stranded DNA in *in vitro* binding assays, consistent with their role in movement of viral DNA. Finally, to identify the intracellular location of these proteins, they were fused with the green fluorescent protein gene (GFP) and electroporated into protoplasts or bombarded into bean or tobacco cells. The CP localized to the nucleus, the V1 protein had a perinuclear distribution and the C4 was localized to the cell periphery. Together, these results are consistent with these proteins coordinating the transport of viral DNA across nuclear and plasmodesmal boundaries and, possibly, in a tissue specific (e.g., phloem-limited) manner. Finally, a series of C4, V1 and CP mutants (ΔN and ΔC mutants) were generated and these, along with the wild-type genes, were placed under control of the 35S promoter and inserted into a binary vector. Transgenic tomato plants were generated with all the genes, with the exception of the wild-type C4 gene that had a deleterious effect on some aspect of the transformation process. T1 families of these lines will be screened for resistance to TYLCV.

3. The green fluorescent protein (GFP) was used to follow TYLCV movement in the tomato plant. A plasmid (pTYLCV:GFP-TYLCV) which contained a head-to-tail TYLCV-Is heterodimer was constructed. While the second copy of the virus was TYLCV-Is wild type, in the first copy the CP ORF has been replaced in frame by the GFP structural gene. When this construct was introduced into tomato by coated-particle bombardment, GFP was expressed at the site of contact and within a few days in the neighboring cells. Within 3 weeks the plants exhibited TYLCV disease symptoms but GFP activity ceased. Analysis of GFP mRNA and protein indicated that the GFP gene was transcribed but that the GFP mRNA was not translated. These findings indicate that a geminiviral promoter could be silenced.

4. TYLCV resistant genes from wild tomato species were used to forward the development of resistant cultivars. The *Ty-1* gene was mapped with increased accuracy and the chromosome segment from the wild tomato species that contains the gene was trimmed down further. In addition resistance gene(s) from other wild tomato species were introgressed into the cultivated tomato. Advanced breeding lines were produced which may serve as the basis for future cultivars.

Potential for application

Immediate

1. The information regarding TYLCV-DR and, in particular, the monitoring of the virus in whiteflies, has been applied in the DR to help with the management of the virus. These tools are presently being used to assist in the evaluation of the host-free period in the DR and to help select the appropriate locations for growing tomatoes in the DR. Management in the DR may serve as model for Florida and Georgia in case TYLCV epidemics worsened.

2. The production of transgenic resistant tomato plants may serve as an additional source of resistance that can be incorporated into existing breeding programs utilizing resistance genes from the wild tomato species.

Medium-term and long-term

1. The MP-expressing transgenic tomato, if proved to be efficient against TYLCV, then this strategy could be applied to other whitefly-transmitted geminiviral diseases.

2. The fine-mapping of resistance genes may allow to isolate and study the gene in another 3-5 years. In a similar strategy the *Mi* nematode-resistance gene of tomato has

been cloned after it has been located with sufficient precision following 15 years of selection.

Estimated economic benefits

The processing tomato industry in the DR was essentially wiped out by TYLCV in 1993-94. Partly through the efforts of this project, the industry has rebounded back to pre-TYLCV levels of production. This industry is valued around 10 million US dollars. Perhaps more importantly is the fact that this is an industry that provides considerable income to small farmers in the DR.

The introduction of TYLCV into Florida and recently in Georgia has major implications for the fresh market tomato industry in the US. In Israel, the efforts to breed for TYLCV resistance are considerable. The use of pesticides is one of the highest in the world. More and more cultivated areas are under covers, increasing cost production. The potential impact of TYLCV-resistant varieties could be considerable in the US and Israel, as well as in many TYLCV-stricken countries around the Mediterranean, tropical Africa and Southeast-East Asia. Still the public acceptance of transgenic food has to be thoroughly evaluated.

Details of cooperation

1. TYLCV-DR mutants generated in the US were tested for infectivity in Israel. The mutational analysis was a joint project
2. TYLCV was tagged with the GFP gene during a 6-week-long visit by an Israeli student to UC Davis. This was a joint effort between US and Israeli scientists.
3. The US investigators have visited twice their Israeli partners.

Publications

- Salati, R., Nakhla, M. K., Maxwell, D. P., Carvalho, M. G., Hou, Y.-M., and Gilbertson, R. L. 1997. Complete nucleotide sequence of an infectious clone of tomato yellow leaf curl geminivirus from the Dominican Republic. *Phytopathology* 87:S84 (Abstract).
- Gilbertson, R. L., Ullman, D. E., Salati, R. S., Maxwell, D. P., Grafton-Cardwell, E. E., and Polek, M. 1998. Insect-transmitted viruses threaten agriculture. *California Agriculture* 52: 23-28.

Abstract

Background

The tomato yellow leaf curl disease (TYLCV) has been a major deterrent to tomato production in Israel for the last 20 years. This whitefly-transmitted viral disease has been found in the Caribbean Islands in the early 1990s, probably as an import from the Middle East. In the late 1990s, the virus has spread to the US and is now conspicuous in Florida and Georgia.

Objectives

Because of the urgency facing the TYLCV epidemics, there was a compelling need to mobilize scientists to develop tomato variety resistant to TYLCV. The major goal was to identify the virus movement protein (MP) and to express a defective form of MP in a cultivar that contained the natural *Ty-1* resistance gene. The research included 1. cloning of the TYLCV isolate from the Dominican Republic (DR) which is (or a close variant) also present in the continental USA; 2. defining the role of the MP; 3. mutating the putative MP gene; 4. introducing the modified gene into an advance *Ty-1* line; 5. testing the transgenic plants in the field. The pressing threat to tomato production in the US resulted in an extension of the objectives: more emphasis was placed on characterization of TYLCV in the DR, on determination of the epidemiology of the virus in the DR, and on using new TYLCV resistance sources for tomato breeding.

Achievements and signification

1. The characterization of TYLCV-DR allowed for more effective TYLCV management strategies that are now implemented in the DR.
2. The identification of the TYLCV MPs and, more importantly, insight into their function has provided a model for how these proteins function in TYLCV movement and support the targeting of one or more of these proteins in a dominant lethal strategy to engineer plants for TYLCV resistance.
3. The transgenic plants that are being generated with wild-type and mutated TYLCV MPs will serve to test the hypothesis that interference with one or more of the TYLCV movement proteins will be a strategy for generating TYLCV-resistant plants.
4. The fine mapping of the resistance *Ty-1* gene allowed eliminating deleterious chromosome segments from the wild tomato genitor *L. chilense*. It may in a near future allow the cloning of the first geminivirus resistance gene.
5. Another resistance source from the wild tomato species *L. hirsutum* was introgressed into the domesticated tomato, resulting in the production of resistant breeding lines.

Implications

1. The monitoring of TYLCV in whiteflies has been applied in the DR. These tools are presently being used to assist in the evaluation of the host-free period and to help select the appropriate locations for growing tomatoes in the DR.
2. An overall strategy to obtain resistant against TYLCV has been used. The expression of wild-type or mutated TYLCV MPs in transgenic tomato is another addition to the arsenal used to fight TYLCV, together with marker-assisted breeding and mobilization of additional resistant genes from the wild.

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- Vidavsky, F. and Czosnek, H. 1998 Tomato breeding lines immune and tolerant to tomato yellow leaf curl virus (TYLCV). The second International Congress on Geminiviruses and Whiteflies. Puerto-Rico (Abstract).
- Salati, R., Nahkla, M. K., Maxwell, D. P., and Gilbertson, R. L. 1999. Molecular epidemiology of tomato yellow leaf curl virus in the Dominican Republic: Identification of inoculum sources during a host-free period. *Phytopathology* 89:S68 (Abstract).
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- Salati, R., Nahkla, M., Rojas, M. R., Guzman, P., Jaquez, J., Maxwell, D. P., and Gilbertson, R. L. Epidemiology of tomato yellow leaf curl virus in the Dominican Republic: Characterization of an infectious clone, virus detection in whiteflies, and identification of alternate hosts. *Phytopathology*: submitted for publication.
- Levi, A. Gilbertson, R.L., Lucas, W.J., and Czosnek, H. Silencing of the green fluorescent protein GFP by a geminiviral sequence. In preparation.

Appendices

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A. Identification of TYLCV movement protein(s)

1. Cloning and expression of proteins that may be involved in movement of TYLCV in infected tomato plant

Three TYLCV genes coding for the putative movement protein of TYLCV – V1 (encoding the coat protein), V2 and C4 – have been cloned into *E. coli* expression vectors and the proteins have been expressed at high levels (UCD).

2. Production of antisera against the putative TYLCV movement protein

The *E. coli* expressed V1, V2, and C4 fusion proteins were used to produce antisera against each of these proteins. The proteins were expressed in *E. coli* and purified. The proteins were used to immunize rabbits. In western blot experiments, the antisera prepared against each protein recognized the appropriate purified *E. coli* expressed protein. In experiments with extracts prepared from frozen TYLCV-infected plant tissue, a protein of the size expected for the CP (V1) was detected in western blots, whereas not such protein was detected in healthy plant extracts.

3. Role of putative TYLCV movement protein in plants: Cell-to-cell movement of in *Nicotiana benthamiana* and in *Lycopersicon esculentum* following microinjection

All three of the *E. coli* expressed fluorescently labeled proteins appear to have the capacity to move to cell to cell when microinjected into mesophyll cells of *Nicotiana benthamiana*.

Microinjection studies performed in *N. benthamiana* mesophyll cells revealed that the fluorescently-labeled (fluorescein isothiocyanate, FITC) C4 protein has the capacity to move, extensively, cell to cell. In some cases, FITC-labeled CP also was able to move cell to cell, but not as extensively as C4. For example, the CP moved cell to cell in over 50% of the injections (16/30), whereas in the remaining injections it moved to adjacent cells (6/30) or remained in the injected cell. The FITC-V2 failed to move cell to cell or moved only into adjacent cells (Table 1). Co-injection experiments in which viral DNA (as an undigested recombinant plasmid) labeled with the fluorescent dye TOTO-1 was injected with each of the proteins revealed that none of the proteins was able to facilitate the cell to cell movement of viral DNA (Table 1).

Similar experiments to assess the capacity of the TYLCV proteins to move cell to cell were conducted in tomato mesophyll cells. Interestingly, all three proteins, V1, V2, and C4 had the capacity to move cell to cell (Table 1). These results are extremely

significant and suggest that the TYLCV proteins may be specifically host-adapted to tomato cells.

Table 1. TYLCV-DR V1, V2, and C4 proteins and mutants microinjection results in *Nicotiana benthamiana* and *Lycopersicon esculentum*

1. *Nicotiana benthamiana*

<u>Injected agent</u>	<u>Coinjected agent</u>	<u>Results</u>
Lucifer yellow	-	87 (87)
10 kDa F-dextran	-	0 (81)
4 kDa F-dextran	-	0 (11)
FITC-TYLCV V1	-	6 LTAC 16 (30)
FITC-TYLCV V2	-	9 LTAC (14)
FITC-TYLCV C4	-	16 (16)
TYLCV V1	10 kDa F-dextran	1 LTAC (12)
TYLCV V2	10 kDa F-dextran	5 LTAC (15)
TYLCV C4	10 kDa F-dextran	2 LTAC (10)
TYLCV CP	4 kDa F-dextran	5 LTAC (18)
TYLCV V2	4 kDa F-dextran	6 LTAC (13)
TYLCV C4	4 kDa F-dextran	11 (12)
TYLCV V1 Δ N	-	0 (13)
TYLCV V1 Δ C	-	1 LTAC (14)
TYLCV V2 Δ N	-	0 (10)
TYLCV V2 Δ C	-	1 LTAC (10)
TYLCV C4 Δ N	-	0 (11)
TYLCV C4 Δ C	-	3 LTAC (13)
TYLCV V1 DNA-TOTO-1	-	0 (10)
TYLCV V2 DNA-TOTO-1	-	0 (11)
TYLCV C4 DNA-TOTO-1	-	0 (11)
2. <i>Lycopersicon esculentum</i>		
Lucifer yellow	-	23 (23)
10 kDa F-dextran	-	0 (24)
4 kDa F-dextran	-	0 (11)
FITC-TYLCV V1	-	10 (10)
FITC-TYLCV V2	-	9 (11)
FITC-TYLCV C4	-	13 (13)
TYLCV V1	10 kDa F-dextran	0 (11)
TYLCV V2	10 kDa F-dextran	0 (10)
TYLCV C4	10 kDa F-dextran	0 (10)
TYLCV V1	4 kDa F-dextran	3 (11)
TYLCV V2	4 kDa F-dextran	1 LTAC 4 (10)
TYLCV C4	4 kDa F-dextran	3 LTAC 3 (10)

4. Plasmodesmal size exclusion limit induced by putative TYLCV movement protein(s)

Having established that the C4 and, to a lesser extent, the CP (V1), proteins have the capacity to move cell to cell through plasmodesmata, we next explored whether these

proteins possessed the capacity to increase the plasmodesmal molecular size exclusion limit (SEL). Thus, each protein was co-injected into *N. benthamiana* mesophyll cells with FITC-labeled 10 kDa dextran (F-dextran). None of the proteins facilitated the cell-to-cell movement of the 10 kDa F-dextran, although in a few cases some movement of the dextran to adjacent cells was observed. To see if these proteins could modify plasmodesmata to allow for movement of dextrans small than 10 kDa, similar co-injection experiments were performed with 4 kDa F-dextran. Here, the C4 protein facilitated the extensive movement of the 4 kDa F-dextran, which is consistent with its ability to move cell to cell. Such extensive movement of the 4 kDa F-dextran was not observed for the CP or pre-CP, although in some cases the dextran was observed to move into adjacent cells when co-injected with these proteins.

To assess whether the capacity of the V1, V2, or C4 proteins to increase the plasmodesmal SEL was different in tomato versus *N. benthamiana*, co-injection studies were performed with the 4 and 10 kDa F-dextrans. Again, none of the proteins facilitated the cell-to-cell movement of the 10 kDa F-dextran, whereas movement of the 4 kDa F-dextran was observed in some cases, particularly for C4 and the pre-CP. These results suggest that, unlike the situation found for protein trafficking, there was little difference in the capacity of these proteins (individually) to increase PD SEL in *N. benthamiana* or tomato.

B. Construction and expression of TYLCV V1, V2, and C4 mutants, and their capacity to move following microinjection in *N. benthamiana*.

1. Generation of mutants of the V1, V2 and C4 genes introducing stop codons

In order to determine the role of the V1, V2 and C4 genes in TYLCV movement, these genes have been mutagenized *in vitro* 1. by introducing stop codons in the coding region of the gene and 2. By changing amino acids to produce putative dysfunctional movement proteins.

(a) Mutants generated at UCD, using the Morph⁺ plasmid DNA mutagenesis procedure:

The upper sequence is the wild-type virus; the lower sequence shows the mutation in bold. Underlined codon indicates a stop.

V1 (from start codon at nucleotide 474):

```
ATG TCG AAG CGA CCA GGC GAT ATA ATC ATT TCC ACG CCC GTC TCG AAG GTT CGC CGA AGG CTG A
ATG TCG AAG CGA CCA GGC GAT ATA ATC ATT TCT AGA TGA CCG TCT CGA AGG TTC GCC GAA GGC TGA
```

V2 (from start codon at nucleotide 314)

ATG TGG GAC CCA CTT CTA AAT GAA TTT CCT GAA TCT GTT CAC GGA TTT CGT TGT TGT ATG TTA GCT
 ATG TGG GAC CCA CTT CTA GAA TGA ATT TCC TGA ATC TGT TCA CGG ATT TCG TTG TTG TAT GTT AGC

C4 (from nucleotide 2596)

C CAC ATC TCC ATG TGC TTA TCC AAT TCG AAG GC
 C CAC ATC TCC ATG TGA TGA TCC AAT TCG AAG GC

C1 overlap in the Mutagenized C4 region (Leu to Met)

CCA CAT CTC CAT GTG CTT ATC CAA TTC GAA GGC
 CCA CAT CTC CAT GTG ATG ATC CAA TTC GAA GGC

(b) Mutants generated at HU using the pALTER plasmid DNA mutagenesis procedure:

V1 (from nucleotide 750):

GTT AGT GAT GTT ACT CGT GGA TCT GGA ATT
 GTT AGT GAT GTT ACT CGT TGA TCT GGA ATT

V2 (from start codon at nucleotide 314)

ATG TCG GAC CCA CTT CTT AAT GAA TTT CCT
 ATG TGG GAC CCA CTT CTA GAA TGA ATT TCC T

C4 (from start codon at nucleotide 2636)

ATG GGG AAC CAC ATC TCC ATG TGC TTA TCC AAT TCG AAG GCA
 ATG GGG AAC CAC ATC TCC ATG TGC TTA TCC AAT TAG AAG GCA

2. Generation of N-terminal and C-terminal mutants of the V1, V2 and C4 genes

N-terminal (Δ N) and C-terminal (Δ C) mutant forms of each protein were designed. In each case, the deletions were designed to eliminate conserved amino acid motifs identified based upon alignments made among TYLCV isolates and other related geminiviruses. In the case of CP, the Δ N mutant had a putative nuclear localization signal (NLS) deleted, whereas in the case of the other mutants deleted motifs have no known function. The sizes and number of deleted amino acids for each protein are indicated in Table 2.

Table 2. The number of amino acids deleted and predicated molecular mass of N-terminal and C-terminal TYLCV deletion mutants.

Protein	Mutant	No. of Amino Acids Deleted (Total)	Predicted Molecular Weight (kDa)*
V1	Δ N	60 (258)	23 (30)
	Δ C	50 (258)	25(30)
V2	Δ N	21 (116)	11(13)
	Δ C	28 (116)	10(13)
C4	Δ N	18 (97)	9 (11)
	Δ C	20 (97)	9 (11)

*does not include the additional 4 kDa added to the N-terminus of the fusion protein

These mutants were then expressed in *E. coli*, purified (using the histidine tag in the N-terminus of the fusion protein), labeled with FITC, renatured and used in

microinjection experiments. As shown in Table 2, the capacity of the mutant proteins to move, cell to cell, were significantly retarded compared to the wild-type proteins. The ΔN mutants of all three proteins remained within the injected cell, whereas the ΔC mutants only moved to adjacent cells at a very low frequency. These results establish that (i) the capacity of the wild-type proteins to move cell to cell represent an actual property of these proteins and (ii) that movement defective mutant proteins, which have the potential to interfere with the viral movement, have been generated.

C. Replication and expression of TYLCV clones in tomato and tobacco

1. Replication in tomato leaf discs after bombardment with cloned TYLCV DNA

Tomato leaf discs (1.3 cm diameter) were bombarded with TYLCV-Isr (full-length head-to-tail dimer contained in binary vector pCGN1547) and with TYLCV-Dom (uncut plasmid). About 1 μ g cloned DNA (as plasmids) was delivered per bombardment event, either with tungsten or with gold. Groups of 3 leaf discs were collected immediately after bombardment to assess viral DNA input. Groups of 3 leaf discs were collected 7 days after bombardment to assess replication of input viral DNA. DNA was prepared from tomato plants infected by viruliferous whiteflies as control. Total DNA was prepared from the different groups of leaf discs using the CTAB method. About 1 μ g of DNA was subjected to gel electrophoresis, blotted and hybridized with a radiolabeled TYLCV-Isr probe.

- 1: input DNA following bombardment of tomato leaf discs with TYLCV-Dom (time 0)
- 2: input DNA following bombardment of tomato leaf discs with TYLCV-Isr (time 0)
- 3: tomato leaf disc 7 days after bombardment with TYLCV-Isr
- 4: tomato leaf disc 7 days after bombardment with TYLCV-Dom
- 5: leaf discs of tomato plants infected by viruliferous whiteflies

Results show that in leaf discs bombarded with cloned TYLCV-Isr and TYLCV-Dom, the input plasmids have given rise to molecules comigrating with the virus genomic ssDNA from infected plants. The TYLCV dsDNA is barely seen in the leaf discs.

2. Bombardment of tomato and *N. benthamiana* plants with cloned TYLCV DNA

Tomato plants (cv. Daniella) at their 3-4 leaf stage and *N. Benthamiana* plants at their 5-6 leaf stage were bombarded individually with cloned TYLCV-Isr and TYLCV-Dom. Conditions of bombardment were as for leaf discs.

Figure 2. 1: tomato plant infected with TYLCV-Isr by viruliferous whiteflies. 2: tomato plant bombarded with TYLCV-Isr DNA. 3: *N. benthamiana* bombarded with TYLCV-Dom. 4: tomato plant bombarded with TYLCV-Isr DNA.

DNA was extracted from young leaves of tomato plants 55 days (sample 2) and 68 days (sample 4) after bombardment. DNA was extracted from young leaves of *N. benthamiana* plants 68 days (sample 3) after bombardment. About 1 µg of DNA was subjected to gel electrophoresis, blotted and hybridized with a radiolabeled TYLCV-Isr probe.

Results presented in Figure 2 show that 2 out of 15 tomato plants contained TYLCV-Isr DNA (samples 2 and 4); these plants presented typical disease symptoms. None of the 15 tomato plants bombarded with TYLCV-Dom were infected (neither viral DNA nor symptoms). One *N. benthamiana* plant out of the 5 plants bombarded with TYLCV-Dom contained viral DNA and showed disease symptoms similar to those of *N. benthamiana* bombarded with TYLCV-Isr (about 50% of *N. benthamiana* plants become infected following bombardment with TYLCV-Isr).

3. Inoculation of tomato plants by whiteflies fed with cloned TYLCV DNA.

Whiteflies were fed for 24 h through membranes with cloned TYLCV-Dom (0.5µg of DNA/ µl of 15% sucrose). Tomato plants at their 4-5 leaf stage were caged for 48 h with the insects using leaf cages (about 20 insects per plants). After 52 days, DNA was extracted from young leaves and 1 µg DNA was subjected to gel electrophoresis, blotted and hybridized with a radiolabeled TYLCV-Isr probe.

Figure 3. 1: tomato plant infected with viruliferous whiteflies.
2: tomato plant inoculated with whiteflies fed with TYLCV-DOM digestion with NcoI.
3: tomato plant inoculated with whiteflies fed with TYLCV-DOM digestion with EcoRV.
4: tomato plant inoculated with whiteflies fed with TYLCV-DOM, as an undigested circular plasmid.

Results presented in Figure 3 show that 2 out of the 3 tomato plants inoculated with TYLCV-DOM digested with NcoI contained viral DNA. One out of the 3 tomato plants inoculated with TYLCV-DOM digested with EcoRV contained viral DNA. Two out of the 3 tomato plants inoculated with TYLCV-DOM undigested contained viral DNA. None of the plants exhibited disease symptoms. In an independent experiment, about 50% of the tomato plants inoculated with whiteflies fed with TYLCV-Isr showed typical disease symptoms and viral DNA forms.

D. Analysis of TYLCV movement using the green fluorescence protein (GFP): replacement of the CP gene by the GFP gene

1. Replacement of the TYLCV CP gene by the GFP gene, under the control of the CP promoter

The TYLCV CP gene was replaced by the GFP gene. In the final plasmid, GFP was under the control of the virus CP promoter. The gene swapping is schematically described in Figure 4. The GFP gene was cloned in the vector pHGFP-S65T (accession number - U43284) and the TYLCV genome was cloned in the vector pBK-RSV. The GFP gene was excised using Pst I and Sal I. For mutagenesis, the full-length TYLCV

genome was excised from its vector using Pst I and was cloned into the pAlter mutagenizing cloning vector (Promega-Altered sites II in vitro mutagenesis system). Two sites (Nsi I and Hind III) were introduced by mutagenesis in the 5' and 3' end of the GFP and the TYLCV coat protein genes. The Nsi I restriction site was located in the N terminal region of the GFP (825 bp) and in the same region of the coat protein gene (476 bp). The Hind III restriction site was located in the C terminal region of the GFP (1546 bp) and in the same region in the coat protein (1240 bp). Two ATGs are found next to the Nsi I site, one nucleotide from each other. We used the T4 DNA Polymerase to remove 4 bases from this site to restore the open reading frame of the GFP gene.

Figure 4. TYLCV CP replacement by GFP
The GFP and TYLCV genes were cloned into the pAlter mutagenizing cloning vector. Nsi I and Hind III sites were introduced by mutagenesis in the 5' and 3' end of the GFP CP genes. Two ATGs are found next to the Nsi I site, one nucleotide from each other. The T4 DNA Polymerase was used to remove 4 bases from this site to restore the GFP open reading frame

The activity of the GFP cloned into TYLCV was assayed. The TYLCV-GFP cloned DNA was introduced into tobacco protoplasts by electroporation. Figure 5 shows the expression of GFP in the transfected protoplasts. Bean (a TYLCV host) radicles were also bombarded with the TYLCV-GFP cloned DNA. GFP activity was detected 5 days after bombardment.

Figure 5. Expression of s-GFP under the control of the TYLCV capsid protein gene promoter. The plasmid containing the s-GFP was introduced into tobacco protoplasts by electroporation (upper panel) and into bean radicles by bombardment (lower panel).

2. Replacement of the TYLCV CP gene by the GFP gene in an heterodimer pTYLCV:GFP-TYLCV where in the first copy the CP ORF has been replaced by GFP and the second copy is TYLCV wild type

To study the movement of TYLCV in infected tomato plants we have constructed a plasmid (pTYLCV:GFP-TYLCV) which contained a head-to-tail TYLCV-Is heterodimer cloned in the *Pst*I site of plasmid pBK-RSV (at TYLCV nt 1665) (Figure 6). While the second copy of the virus was TYLCV-Is wild type, in the first copy the CP ORF has been replaced in frame by the green fluorescent protein structural gene (GFP-S65T, U43284). Linear forms of the full-length TYLCV and TYLCV:GFP could be released after digesting the plasmid with *Pst*I.

Figure 6: Genetic map of the heterodimer TYLCV:GFP-TYLCV cloned in the *Pst*I site of plasmid pBK-RSV. In the first copy, the CP has been replaced in frame with GFP; the second copy is TYLCV wild type. To release the full-length TYLCV and TYLCV:GFP moieties, the plasmid is digested with *Pst*I. The location of the primers used to amplify TYLCV CP and GFP are indicated.

When tomato leaves were bombarded with particles coated with the heterodimeric plasmid, GFP-related fluorescence could be seen at the site of bombardement (Figure 7). No fluorescence was observed in untreated leaves (A). Within 3-5 days, fluorescence has invaded the neighboring cells (B) and was sometimes also found in the vascular system of the bombarded leaf (C).

Figure 7. GFP-related fluorescence in tomato leaves bombarded with particles coated with the heterodimer pTYLCV:GFP-TYLCV. Note the fluorescence in cells of panel B and in the vascular system in panel C. No fluorescence is seen in untreated leaves (panel A).

Three weeks after bombardment, the treated plants presented TYLCV disease symptoms, but GFP-related fluorescence was conspicuously absent. The fate of TYLCV and of GFP was investigated. Tissues from infected plants were analyzed by Southern blot hybridization for the presence of TYLCV and GFP DNA (Figure 8).

Figure 8. A tomato plant, 4 weeks after inoculation was tested for the presence of GFP and TYLCV DNA, using Southern blot hybridization with specific radiolabeled DNA probes. The following tissues were tested, 1: roots; 2: stem; 3: mature leaf; 4: young leaf; 5: shoot apex. H: leaf from an healthy tomato; INF: leaf from an infected tomato 6. Note that both viral DNA and GFP DNA have translocated from the site of inoculation, although no GFP-related fluorescence was observed.

The results shown in Figure 8 indicated that the viral DNA as well as the GFP DNA has moved from the inoculated leaf to other tissues of the plant. GFP and TYLCV DNAs were found in the roots, stem and in young leaves.

The reason for the absence of GFP expression, as a fluorescent protein, was investigated. It was possible that the GFP gene was either not transcribed, or that the GFP mRNA was not transcribed. The presence of GFP mRNA was assessed by RT-PCT using the 2 GFP-specific primers shown in Figure 6. The results presented in Figure 9 clearly show that GFP mRNA molecules are found in the leaves, stem and roots of the treated plant,

indicating that the absence of an active fluorescent protein was not due to an inhibition of transcription of the GFP gene.

Figure 9. A tomato plant, 4 weeks after inoculation was tested for the presence of GFP mRNA, using RT-PCR. The reaction products were Southern blot analyzed using a radiolabeled cloned GFP DNA probe. The following tissues were tested, 1: mature leaf; 2: young leaf; 3: roots; 4: shoot apex; 5: stem. H: leaf from a healthy tomato; INF: leaf from an infected tomato. The thick arrow points to the amplified 460 bp amplified fragment; the thin arrow points to the PCR primers. Note that the GFP mRNA is present in the treated.

The presence of GFP in the treated tomato plants was assessed with a commercially available antibody raised against GFP. Protein extracts were western blot analyzed and the GFP was immunolocalized. Figure 10 shows that GFP was not detectable in the treated plant extracts, indicating that the protein was not transcribed from the GFP mRNA.

Figure 10. A tomato plant, 4 weeks after inoculation was tested for the presence of GFP using a specific antibody. Protein extracts from treated leaves (1 and 2) were western blot analyzed using a GFP antibody. INF: TYLCV infected plant. G: purified GFP protein (arrow).

The results presented in this section indicate that GFP expression was silenced most likely according to a post-translational model. The observation of fluorescence-associated GFP following bombardment of tomato leaves with the TYLCV-GFP-containing heterodimer indicated that some active protein was expressed in the first days after inoculation. With time, it seems that the translation of the GFP gene ceases, while TYLCV is fully expressed. GFP mRNA is found in tomato organs even 4

weeks after inoculation indicating that the GFP gene is actively transcribed or that the GFP mRNA is particularly stable. We may have here a case of suppression of gene expression by a viral sequence.

E. Transformation of tomato plants with wild type and mutated putative TYLCV movement protein

1. Transgenic plants expressing the C1 and C4 wild type and mutants

The C1 (Rep) and C4 (putative movement proteins), wild type and mutants, have been cloned in the binary vector (pBI-109) in the *Sma*I-BamHI sites. The recombinant plasmid has been introduced into *Agrobacterium tumefaciens* LBA4404. Cotyledons from seedlings grown in sterile agar medium from seeds of lines Ty-50 and Ty-52 have been co-cultivated with the *Agrobacterium*. Plantlets that had regenerated in selective medium are presently grown in pots. The analysis for transgenesis and for resistance/susceptibility to TYLCV will be tested in the near future.

2. Generation and localization of chimeric TYLCV/GFP proteins

To obtain additional insight into the function of the TYLCV CP, pre-CP and C4 proteins, translational fusions were made for each protein with the GFP gene (mGFP5) (Figure 11). GFP fusions were also made with the deletion mutants.

Figure 11. Strategy used to construct TYLCV/GFP chimeric V1, V2, and C4 proteins

A: wild-type

B: Deletion Mutants:

The following mutants were made:

N-terminus deletion mutants: V1 Δ N60 (N-terminus 60 amino acids deletion)

V2 Δ N21 (N-terminus 21 amino acids deletion)

C4 Δ N18 (N-terminus 18 amino acids deletion)

C-terminus deletion mutants: V1 Δ C50 (C-terminus 50 amino acids deletion)

V2 Δ C28 (C-terminus 28 amino acids deletion)

C4 Δ C20 (C-terminus 20 amino acids deletion)

Two methods were used to allow for localization of the chimeric proteins in live cells: (i) radicles of germinating bean seeds were bombarded using particle acceleration and (ii) the constructs were introduced into tobacco protoplasts by electroporation. In both systems, fluorescence associated with the V1/GFP chimera was observed in the nuclei of cells, consistent with the intranuclear location of virions and with a recent report indicating that TYLCV CP localizes to the cell nucleus. This result also raises the important question of whether the TYLCV CP serves as the nuclear export factor for TYLCV (analogous to the BV1 protein for bipartite geminiviruses). In both systems, the fluorescence associated with the C4/GFP chimera was observed at the cell periphery (i.e. near the cell wall). This finding is consistent with microinjection studies showing that the C4 protein moves through plasmodesmata. To date, no clear site of intracellular localization has been identified for the V2/GFP chimera. Thus, in general, results obtained with these TYLCV chimeras are consistent with those obtained with microinjection studies for these proteins. Experiments are ongoing to improve methods of localization of chimeric proteins in cells and to determine the localization of chimeras made with the V1, V2, and C4 mutants.

These constructs have been introduced into *Agrobacterium* and used to transform tomato plants. The transgenic plants are under evaluation.

F. Utilization of resistance found in additional wild tomato genotypes in breeding programs for TYLCV resistance

Besides studying the resistant/tolerant line Ty-52 that contains the *L. chilense* *Ty-1* resistance gene, we have screened additional wild tomato species for genes conferring resistance to TYLCV. We have found that accessions of *L. hirsutum* contained individuals immune to TYLCV whitefly-mediated inoculation.

Two TYLCV-resistant plants from accessions LA 1777 and LA 386 of the wild tomato species *Lycopersicon hirsutum* have been crossed. The resulting resistant F1 plants were crossed with the domesticated tomato *L. esculentum* and a series of selfing was performed.

At each generation, individuals were selected for resistance (no symptoms and undetectable viral DNA) and tolerance (no symptoms but with detectable viral DNA), following controlled massive and repeated inoculations with viruliferous whiteflies. A stable BC1F4 line (denominated 902) which does not segregate for resistance was obtained. This line does not support virus accumulation even upon extensive whitefly-mediated inoculation of young seedlings and does not need protection with nets and/or insecticides. Another stable BC1F4 line (denominated 908) was tolerant to the virus. Both lines have good horticultural characteristics and bear 80-120 g red fruits. Analysis of segregation of susceptibility, tolerance and resistance during the BC1F1 to BC1F4 crosses indicated that tolerance is controlled by a dominant major gene, and resistance by 2 to 3 additive recessive genes. The resistant and tolerant lines do not need to be protected by insecticides and/or nets.

G. Fine mapping of Ty-1, the TYLCV tolerance gene originating from *L. chilense* and *L. peruvianum*.

The TYLCV tolerance gene was mapped to an interval of approximately 15 cM on chromosome 6. Fine mapping of this gene is necessary both for its utilization in marker assisted selection and for future attempts to clone the gene by positional technology.

As a first stage in the fine mapping effort, two wild species sources were used for the fine mapping experiment. The first is a *L. chilense* source (TY52) and derivative lines from it that have smaller introgressed segments in the targeted region. The lines presented in Figure 1 were field inoculated in 1997 1998 and 1999 and were found to be tolerant/resistant to TYLCV. DNA from these lines was digested with four different restriction enzymes together with a control line that contains the *L. pennellii* introgression in the targeted region (IL6-1). Blots were produced and probed with DNA markers that cover the relevant region on chromosome 6. The results yielded a better definition of the introgressed region in the various resistant lines and information regarding the polymorphic enzymes/probes combinations. Based on the results presented in Figure 1 we conclude that the 'Southern' most marker in the tolerant lines of the *L. chilense* origin is TG232 while in the short arm tolerant lines do not carry the wild species allele of TG297. We crossed the Line 140-1 to a susceptible *L. esculentum* line and conducted RFLP analysis of 800 plants in order to select for recombinants between TG590 and TG232. In

1999 five recombinant families were inoculated and results indicate that the resistance gene is located between the two markers.

The second source for the TYLCV mapping originated from *L. peruvianum* and confers complete immunity to the disease. Our first assumption was that both species carry resistance or tolerant alleles at the same locus. Therefore we assumed that this line provided a better definition of the Ty-1 locus since the Southern most marker was TG231; The tolerant line 141-2 does carry the marker TG297. It is worth noting that in the region around Ty-1 additional resistance genes were mapped (Figure 1). It turned out that our assumption was wrong. The line 141-2 indeed carried a *L. peruvianum* introgression in chromosome 6, however upon analysis of an F2 population resulting from a cross of 141-2 and a susceptible line, the introgression segregated independently of the resistance. This was revealed by RFLP analysis of 100 F2 plants followed by field inoculations. This result suggested that there is an additional locus responsible for the resistance that maps to a different location in the genome as Ty-1. Efforts were initiated to map this locus through the probing of resistant and susceptible families (resulting from the F2 population) with random genomic markers.

An additional direction that is being pursued is in the search of additional resistance sources in the *Lycopersicon* genus. Throughout the project, lines involving crosses with *L. pennellii*, *L. peruvianum*, *L. hirsutum* and *Solanum lycopersicoides* were field-tested and in no case a new locus was identified.

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WHITEFLIES: VECTORS, AND VICTIMS (?), OF GEMINIVIRUSES

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ABSTRACT

Most of viruses infecting plants rely on insects to move from one host to another. Some remain associated with the mouth parts and can be inoculated within seconds or minutes (non-circulative transmission). Others are transmitted only several hours after acquisition (circulative transmission). During this latent period, virions translocate into the insect alimentary tract, cross the gut into the primitive blood stream and reach the salivary glands. Contrary to many circulative mammalian viruses, circulative plant viruses by en large do not replicate in their arthropod vector and apparently do not adversely affect their insect host. Nonetheless several plant viruses have evolved, or conserved, mechanisms allowing replication (circulative propagative) in both plant and animal hosts. Three of the five families of the phytoarbovirus taxonomic group, the rhabdoviruses, reoviruses and bunyaviruses, contain members which do replicate in both their host plants and in their aphid or leafhopper vector. In addition, the tomato spotted wilt tospovirus replicates in thrips. Most of these viruses depend on their arthropod host for survival and do not adversely affects them. However a number of phytoarboviruses are deleterious and impair the insect longevity and fecundity. Virus replication and deleterious effects on the insect host have been associated with transovarial transmission to insect progeny. In most cases it is not known whether the virus genes necessary for replication in plants and in insects are the same, and whether identical strategies are implemented. Yet in reoviruses the gene products required for replication in the insect and in the plant are different.

It is not fully understood how viruses became pathogens of both plants and insects. Recombination between a virus infecting plants and another infecting animals may be the driving force enabling plant viruses to extend their host range to insects. Indeed evidence has been recently presented suggesting that a plant virus has acquired a vertebrate host by recombining with a vertebrate-infecting virus. Plant viruses such as luteoviruses and geminiviruses seem to use recombination as a way to broaden their host range and possibly to diversity their functions. In particular, geminiviruses may constitute a family of plant viruses who are in the process of acquiring, or loosing, abilities to interact actively with their insect vector, the whitefly *Bemisia tabaci*, to a point reminiscent of an host-pathogen relationship. This assumption is particularly pertinent when examining the relationship between *B. tabaci* and an isolate of the tomato yellow leaf curl geminivirus from Israel (TYLCV-Is)). Some of our last decade investigations will be presented and discussed in this review in the light of the geminivirus insect-pathogen hypothesis.

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TOMATO YELLOW LEAF CURL VIRUS, A SEXUALLY TRANSMITTED DISEASE OF WHITEFLIES

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Abstract

Tomato yellow leaf curl virus from Israel (TYLCV-Is) is a geminivirus (family: *Geminiviridae*, genus: *Begomovirus*) transmitted by the whitefly *Bemisia tabaci*, biotype B. This virus has many features reminiscent of an insect pathogen. TYLCV-Is is acquired and transmitted within 5-10 min. The latent period is 8-10 h. The virus that circulates in the insect is found in the midgut 1 h after the beginning of acquisition feeding, in the haemolymph after 1.5 h and in the salivary glands after 7h. Circulative transmission of TYLCV-Is is mediated by a GroEL homologue synthesized by the whitefly endosymbiotic bacteria and released into the haemolymph. *B. tabaci* GroEL and TYLCV-Is coat protein interact in the yeast two-hybrid system, indicating that a similar interaction may occur in the haemolymph of the whitefly. Disturbing this interaction in the haemolymph leads to the degradation of the virus and to a dramatic decrease in virus transmission. Following a 48 h acquisition access period, most of the virus remains associated with *B. tabaci*. While the viral DNA is detectable during the entire life span of the insect, the coat protein vanishes after 2 weeks. Although infectivity decreases with time, insects are able to inoculate tomato plants for their entire life. The life-long association of TYLCV-Is with *B. tabaci* is associated with a marked decrease in longevity and in fertility. Besides these remarkable effects on the biology of the insect, TYLCV-Is can be transmitted among whiteflies in a sex-dependant manner, in the absence of any other source of virus. The virus can be transmitted from viruliferous males to females and from viruliferous females to males, but not among insects of the same sex. The recipient insects were able to efficiently inoculate tomato test plants. Insect to insect virus transmission was instrumental in increasing the number of whiteflies capable of infecting tomato test plants in a whitefly population. Based on the features of TYLCV-Is, we may consider this virus as sexually transmitted insect virus.