SECOND ANNUAL REPORT

Covering Period· January 1998- December 1998

Submitted to the Office Agriculture & Food Security
U S Agency for International Development

CHARACTERIZATION AND MANAGEMENT OF TOMATO AND PEPPER GEMINIVIRUSES

Principal Investigator Dr Henryk (Hanokh) Czosnek
Grantee Institution The Hebrew University of Jerusalem, Israel

Collaborator Dr Wayne A McLaughlin
The University of the West Indies, Kingston, Jamaica
Collaborator Dr Douglas P Maxwell
The University of Madison, Madison, Wisconsin, USA

Project Number C16-020
Grant Number TA-MOU-96-C16-020

Grant Project Officer David W Mulinex

Project Duration January 1st 1997 to December 31, 1999

1. Scientific summary

The project started March 1997, when funds were made available to partners During the first year of reseaich, protocols to characterize in molecular terms the whitefly-borne geminiviruses infecting tomato, pepper and other vegetables in Jamaica have been established at the University of Wisconsin and implemented at the University of West Indies Several whitefly-transmitted geminiviruses affecting tomato and pepper in Jamaica have been cloned and sequenced Tomato genotypes commercially available have been tested in infected fields in controlled experiments in Jamaica New tomato lines suitable for Jamaica have been developed Some of them show tolerance to tomato yellow leaf curl virus while others are immune

During this second year of research, we have continued to characterize geminiviral disease affecting tomato and as well as other vegetables in Jamaica, at the molecular and epidemiological levels The diagnosis of these diseases has been extended by introducing new sets of primers for use in PCR-based tests The immune tomato lines have been tested in large scale field conditions in Israel and yields have been evaluated These and other lines, experimental as well as commercial, are presently evaluated in Jamaica in field conditions Virus-derived strategies have been implemented to engineer tomato plants for resistance against TYLCV and other whitefly-transmitted geminiviruses in Jamaica

Objective 1. Molecular and biological characterisation of geminiviruses of tomato and pepper

Molecular characterization of tomato dwarf leaf curl virus from Jamaica (ToDLCV-Ja)

Tomato plants with severe dwarfing and leaf curling symptoms were analyzed by the polymerase chain reaction (PCR), using universal primers for bipartite geminiviruses DNA extracted from these plants was submitted to Southern blot hybridization with a DNA-A probe specific of whitefly-transmitted bipartite geminiviruses Results showed that these plants were infected by at least two geminiviruses 1 A tomato yellow leaf curl virus resembling the monopartite strain from the Middle East (TYLCV) and 2 The bipartite geminivirus from Jamaica The bipartite tomato geminivirus from Jamaica has been given the

name tomato dwarf leaf curl geminivirus (ToDLCV) because of the severe dwarf and leaf curling symptoms associated with infected plants

Oligonucleotide overlapping primers designed to amplify the entire genome (2 6-kb) of ToDLCV from Jamaica was used with PCR to obtain the entire genome of ToDLCV DNA-A using *vent* DNA polymerase This full-length clone will be tested for its ability to replicate in inoculated explants and in protoplasts, and then sequenced

Molecular characterization of tomato yellow leaf curl virus from Jamaica (TYLCV-Ja)

A full-length replicating TYLCV DNA-A clone from Jamaica was amplified by PCR from DNA extracted from infected tomato A 2 8 kbp PCR fragment was obtained and was cloned The sequence of the clones showed a close relationship with TYLCV from the Middle east, indicating a recent import of this virus in the Caribbean Islands

Objective 2 Establishment of the diagnostic laboratory

Characterization and cloning of cabbage leaf curl geminivirus from Jamaica (CLCV-Ja)

The sap from infected cabbage tissue with leaf curling and slight mosaic symptoms from Jamaica was used to inoculate *Nicotiana benthamiana* test plants Replicative form of CLCV was extracted from symptomatic plants A full-length DNA-A fragment was cloned in a unique *EcoRI* site of the plasmid Bluescript and the full-length DNA-B fragment was cloned in a unique *PstI* site of the same plasmid

Nucleotide sequence analysis using the DNAStar program showed that DNA-B of the Jamaican cabbage virus was >96% identical to cabbage leaf curl virus from Florida (CLCV-Fl) This was the first report of CLCV outside of Florida To date approximately 92% or 2400 of the 2600 nucleotides of a replicating full length DNA-A clone of the cabbage-infecting geminivirus from Jamaica was also sequenced Nucleotide sequence analysis of DNA-A of the Jamaican cabbage virus showed that 1600 nucleotides which includes the entire *av1*, *ac2*, and *ac3* genes and the c-terminal part of the *ac1* gene was 90% identical to CLCV-Fl The remaining 800 nucleotides was <70% similar to CLCV-Fl and showed greatest nucleotide similarity (86%) to *Sida* golden mosaic geminivirus from Jamaica (SiGMV-Ja) Only partial nucleotide sequence of the common region for the DNA-A of the

Jamaican cabbage virus was obtained However nucleotide sequence of this partial common region sequence to homologues region of DNA-B showed <70% similarity This suggests that the common regions of DNA-A and DNA-B of the Jamaican cabbage virus are distinct and that two distinct geminiviruses exists in our cabbage sample The common regions of DNA-A and DNA-B of the same bipartite geminivirus must be >90% identical to be considered the same virus However nucleotide identity of >90% for the entire *av1*, *ac2*, and *ac3* genes and the c-terminal part of the *ac1* gene suggest that DNA-A component of the cabbage virus from Jamaica may be a recombinant virus between CLCV-Fl and SiGMV-JM Other full-length clones are being isolated and sequenced to determine mixed infection of 2 or more cabbage viruses or if the cabbage virus from Jamaica is a recombinant virus Recombination among geminiviruses is a new phenomenon and has been shown to be a major evolutionary tool among geminiviruses

Host range, symptomatology, and transmission characteristics of the TYLCV strain from Jamaica in pepper

Whitefly transmission studies were set up to establish that Jamaican hot pepper (*Capsicum chinense*) is a new host of TYLCV-Ja Two-week-old seedlings were whitefly inoculated with TYLCV-Ja, in whitefly-proof cages Each plant were exposed to 100 viruliferous whiteflies (*B tabaci*) in a whitefly-proof cage for 7 days, then sprayed with an insecticide Pepper plants developed mild symptoms of leaf curling and mottling TYLCV in leaf samples were confirmed by PCR using primers specific for the Middle eastern strain of the virus

Objective 3: Reaction of geminivirus tolerant tomato germplasm to TYLCV-Ja and ToDLCV-Ja

Breeding and evaluation of tomato lines resistant to TYLCV

We have started a breeding program aimed at obtaining tomato lines immune to a range of geminiviruses as wide as possible, including those present in Jamaica The wild tomato species *L hirsutum* served as the source for immunity TYLCV-immune *L hirsutum* plants were crossed with the domesticated tomato *L esculentum* and a series of selfing was

performed At each generation, individuals were selected for immunity (no symptoms and undetectable viral DNA), following massive and repeated inoculations with viruliferous whiteflies A stable BC1F4 line (denominated 902) which does not segregate for immunity was obtained The line has good horticultural characteristics and bears 80-120 g red fruits The immune line needs not to be protected by insecticides and/or nets F1 hybrids have been produced by crossing the immune line with superior tomato varieties and more are developed

Behavior of immune tomato lines in field tests in Israel

The immune homozygous parents and their F1 hybrids were tested in open field The behavior of the lines was compared with that of susceptible varieties as well as with commercial varieties claimed to be tolerant to TYLCV To ensure that all plants were properly inoculated, seedlings were caged for 4 days with viruliferous whiteflies, about 20 insects per plant The seedlings were then planted in a field where the population of whiteflies was very high (beginning of July) Squashes of leaves from each plant were sampled every 2 weeks and hybridized with a probe specific for TYLCV Fruits were harvested 14 weeks after planting (starting end of October) and every 4 weeks thereafter The results of the yields obtained are summarized in the following table Numbers show the cumulative yield (in kilograms) obtained with 55 plants

Date	Immune line	F1	Commercial	Susceptible
Oct 29	46	0	0	0
Nov 24	149	51	8	0
Dec 18	209	142	36	0

It has to be noted that a very strong heat wave affected the field immediately after planting and lasted for 2 months, affecting fruit set It has also to be noted that, apart for two insecticide spray one and two weeks after planting, the field was not treated against whiteflies Finally, at the time this report is written, the immune line and the F1 hybrid are still producing fruits

The immune lines and their hybrid have also been tested successfully in India (Bangalore), Taiwan, Egypt and Guatemala

Testing tomato lines resistant/tolerant to TYLCV from Israel in Jamaica

Since TYLCV in Jamaica is similar to the strain present in Israel, we wish to find out whether the lines developed in Israel could be of use in Jamaica In addition, the behavior of these lines in field infested with the bipartite tomato geminivirus from Jamaica is of great interest to find out whether resistance can be achieved for a broad range of Old and New World whitefly-transmitted geminiviruses

A field test is conducted in Jamaica to evaluate the immune tomato lines and their hybrids in climatic and insect pressure of Jamaica Seeds of tomato lines bread in Israel have been sent to Jamaica together with commercial varieties Whitefly mediated inoculation of tomato plants with TYLCV-Ja were undertaken Tomato seedlings from 6 lines were established and whitefly inoculated with TYLCV The plants are currently being monitored for symptom development and accumulation of viral DNA

Objective 4. Virus derived strategies for conferring virus resistance

Construction of potential trans-dominant lethal mutants in the *rep* of TYLCV-Is

A full-length infectious clone of a TYLCV-Is isolate from Egypt (Ismailia governorate) (TYLCV-Is EG1) was cloned (denominated pTYEG14) This clone was used as a wild type template for mutant preparation Mutants were designed to provide a *rep* product that might interfere in *trans* with the replication of wild-type TYLCV These mutants were K^{104} to R, and D^{259} to R The former is a conserved lysine within the putative nicking motif III The latter mutant is conserved residue within the NTP binding site of *rep*

Construction of mutants in pTYEG14

Following a Kunkle-based mutagenesis procedure using pTYEG14, colonies were generated containing mutant sequences from mutagenic primers *rep*v2000, and *rep*v2467 Primer *rep*v2000 matches part of the putative NTP-binding motif and contains the codon change D^{259} to R Primer *rep*v2467 matches part of the putative nicking motif and contains the codon change K^{104} to R

Colonies were produced for each site directed mutagenesis reaction Putative mutant containing colonies were streaked for single colony isolation on 2x YT Amp + Tet

+IPTG/X-GAL media and plasmid preparations from these colonies were used for restriction digestion, and sequencing using Sequenase version 2 0 in a double stranded protocol Colonies were named for the plasmid they contained with p and the single letter AA abbreviation followed by the AA position and the mutant AA with a designated colony number (i e pTYD^{259}R # 5) Plasmid pTYD^{259}R # 5 was determined to contain the codon change D^{259} to R Plasmid pTYK^{104}R # 1 was determined to contain the codon change K^{104} to R. These colonies were stored as glycerol stocks at -80°C The following figure describes the mutants obtained and the changes in amino acid codons for the NTP-binding motif and DNA-nicking motif of *rep* ORF of TYLCV-Is EGI1

NTP-binding site mutation

Wild type TYLCV-Is	AA	D V D **D** I V N Y
Wild type TYLCV-Is	nt[a]	GG GTC TAC GTC **ATC** AAT GAC GTT GTA CC
Wild type TYLCV-Ja	AA	D V D **D** I V N Y
Wild type TYLCV-Ja	nt[a]	GG GTC TAC GTC **ATC** AAT GAC GTT GTA CC
Wild type pTYEG14	AA	D V D **D** I V N Y
Wild type pTYEG14	nt[a]	GG GTC TAC GTC **ATC** AAT GAC GTT ATA CC
D^{259} to R	AA	D V D **R** I V N Y
Primer *rep*v2000	nt[a]	GG GTC TAC GTC **TCT** AAT GAC GTT GTA CC

Nicking motif III mutation

Wild type TYLCV-Is	AA	N G D **K** E V Y
Wild type TYLCV-Is	nt[a]	GTT TCC GTC **TTT** CTC CAC GTA GG
Wild type TYLCV-Ja	AA	S P D **K** E V Y
Wild type TYLCV-Ja	nt[a]	TCT CCC GTC **TTT** CTC CAC GTA GG
Wild type pTYEG14	AA	N G D **K** E V Y
Wild type pTYEG14	nt[a]	GTT TCC GTC **TTT** CTC CAC GTA GG
K^{104} to R	AA	N G D **R** E V Y
Primer *rep*v2467	nt[a]	GTT TCC GTC **TCG** CTC CAC GTA GG

[a] viral sense sequence, amino acids derived from complementary sense strand

PCR amplification of *rep* mutants and cloning

The entire *rep* was cloned by PCR from the full-length mutant containing plasmids using the TA cloning kit from Invitrogen The *rep* ORF's were amplified using primers *rep*v1707 and CRc4 and the PCR fragment cloned Resulting colonies were streaked for single colony isolation and screened for the presence of the desired mutations by sequencing as above Plasmids p*rep*D^{259}R # 2, p*rep*K^{104}R # 2 were determined to have the appropriate mutations

Introduction of the *rep* mutants into *Agrobacterium* for transformation of tomato plants

A full-length copy of the mutated viral clones were excised with the restriction enzyme *Sph*I and purified In parallel the *Agrobacterium* binary vector pBin19 was cut and linearized after incubation with the restriction enzyme *Sph*I The viral clones were ligated with the pBIN19 vector in a 3,1 ratio in order to favor the production of head-to-tail TYLCV dimers The dimers were introduced into *Agrobacterium* LBA4404 by direct transformation These recombinant Agrobacterium strains are being used to transform tomato plants, using the leaf disc and cotyledon co-cultivation technique

2 Scientific issues

The results obtained during the second year of the research program do not justify any change to the proposal

3 Managerial issues

The project is developing according to schedule

4 Special concerns

None

5 Collaborative activities

1 Meeting of partners

The three partners and their students met during the International Workshop on *Bemisia* and Geminiviruses that was held in San Juan, Puerto Rico, June 7-12, 1998 During this meeting, we have discussed results and planned experiments and collaborations for the following research program

2 Presentation of results at scientific meeting

The partners have presented their results as posters and as oral reports

1 Brown S, McLaughlin W, Cabarello R and Brown J Characterization, distribution, and host plants of whiteflies in Jamaica Abstract L-19
2 Roye ME, McLaughlin WA, Nakhla MK and Maxwell DP Molecular characterization of geminiviruses from Jamaica Abstract P-64
3 Milo J, Arancibia E and Czosnek H Roots from TYLCV-tolerant genotypes do not inhibit virus movement to susceptible tissues in tolerant/susceptible grafts Abstract P-50
4 Czosnek H, Ghanim M, Rubinstein G, Morin S, Atzmon H, van Hoss H and Czosnek H Whitefly-tomato yellow leaf curl virus interactions, or are geminiviruses insect pathogens? Abstract L-30
5 Hanson SF and maxwell DP Inhibition of geminivirus replication by trans-dominant *rep* gene mutants Abstract L-57

3 Visit of Dr Zeidan from The Hebrew University of Jerusalem to The University of the West Indies

Following the meeting, Dr Muhammad Zeidan from the Hebrew University has been visiting for 3 weeks Dr Wayne McLaughlin at the University of West Indies During this period, Dr Zeidan has given a series of lectures on geminivirus-related resistance of vegetables Dr Zeidan has also collaborated in the establishment of a whitefly colony and of implementing protocols for whitefly-mediated inoculation of tomato plants

4 Visit of Dr Roye from the University of the West Indies to The University of Wisconsin

Dr Marcia Roye has visited the laboratory of Prof Maxwell for the last two months Dr Roye has cloned TYLCV and another whitefly-transmitted geminivirus affecting tomato in Jamaica

5 Visit of Prof Maxwell to The Hebrew University of Jerusalem

Prof Maxwell has visited the laboratory of Prof Czosnek during a 8 day period, May 1997 During this visit the two partners have reviewed the results of the collaboration and planed experiments for the next research period

6 Scheduled visits

Dr McLaughlin is scheduled to come to Israel during 1999 In addition a Ph D student of Dr McLaughlin, Miss Sherline Brown is scheduled to work in the laboratory of Dr H Czosnek for a one-month extendable period During her stay she will learn techniques related to tomato plant transformation