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BARD

FINAL REPORT

PROJECT NO. I-1110-86

Molecular Approaches for Breeding Tomato Resistant to the Tomato Yellow Leaf Curl Virus

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Date: January 30, 1991

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BARD project No. IS-1110-86

Title:

MOLECULAR APPROACHES FOR BREEDING TOMATO RESISTANT TO THE
TOMATO YELLOW LEAF CURL VIRUS

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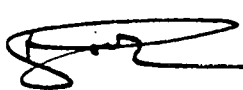
Agricultural Research Organization
Bet Dagan " "

University of Michigan, Ann Arbor

Project's starting date: September 1987

Type of Report: 1st Annual 2nd Annual Final . X

הספריה המרכזית
למדעי החקלאות
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Signature
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Institution's Authorizing Official

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ABSTRACT

This research project finds itself in the framework of our constant fight against the tomato yellow leaf curl disease. We have generated the molecular tools, cloned DNA probes and antibodies, to better understand the disease process and to obtain TYLCV resistant cultivars, through classical breeding and by means of genetic engineering.

TYLCV virions have been isolated. Antibodies have been prepared against the virus coat protein. The TYLCV genome has been cloned and sequenced. It comprises 6 open reading frames. Contrary to all the described whitefly-transmitted geminiviruses, TYLCV has a single genomic component, making it the first geminivirus in its class. This genome has the capacity to induce the whole disease cycle: inoculation, replication, systemic spread, disease symptom and whitefly transmission.

The disease process has been described in molecular terms using cloned TYLCV DNA. This included the determination of tissue susceptibility to infection, symptom development, accumulation of viral DNA and spread of TYLCV in the infected plant.

A quick TYLCV detection test has been designed which allows the detection of infected plants well before disease symptoms are visible. This test is used as a screening device in breeding for resistance and as a tool in epidemiology studies.

A tomato protoplast system has been established which allows the study of TYLCV DNA replication and gene expression at the cell level. The protoplasts support the replication of cloned TYLCV DNA. The cells can be transformed with TYLCV genes: The gene encoding the TYLCV capsid protein was able to be transcribed in transfected protoplasts. This system might be used as a tool to screen for plants which do not support TYLCV expression.

Plasmids which contain each one a different TYLCV gene, in sense and antisense orientations have been constructed. Some of these genes have been cloned into the Ti plasmid of Agrobacterium and used to transform tomato plants. Plants containing the TYLCV coat protein and the C1 gene (implicated in TYLCV replication), in both sense and antisense orientations, have been obtained. These plants are now being tested for TYLCV resistance using both agroinoculation and whitefly-transmission tests. If some plants show some resistance, they will be tested in field conditions during the coming tomato growing season.

OBJECTIVES OF THE ORIGINAL RESEARCH PROPOSAL

Two main objectives were assigned to this project:

1. To study the molecular biology of TYLCV, its replication and gene expression in intact plants and in protoplast cultures. This included:
 - a. The preparation of molecular probes (DNA and antibodies) for the detection of viral genomes and their expression products.
 - b. The study of TYLCV replication cycle in infected plants
 - c. The study of TYLCV replication and gene expression in protoplasts
2. To study viral replication and gene expression in transgenic protoplasts and plants containing TYLCV ORFs in direct and reverse orientations. This included:
 - a. The construction of vectors containing viral DNA sequences, and transfection of tomato cell cultures
 - b. The transformation of tomato leaf discs with Agrobacterium containing recombinant Ti plasmids with TYLCV DNA sequences

All these objectives have been attained.

SCIENTIFIC REPORT

INTRODUCTION

1. The TYLCV disease

The tomato yellow leaf curl virus (TYLCV) causes a severe disease which affects tomato crops (Lycopersicon esculentum Mill.) in Eastern Mediterranean countries and North Africa, leading to reduced yields. Symptoms similar to those of the TYLCV disease have been also described in North and Central Africa, Southeast Asia and Taiwan and Mexico (Makkouk and Laterrot, 1983; Czosnek et al., 1990). The virus is transmitted by the whitefly Bemisia tabaci Genn. (Cohen and Harpaz, 1964) usually in summer and autumn. It cannot be transmitted mechanically (Cohen and Nitzany, 1966).

Disease management is attempted by controlling the whitefly vector, although not very efficiently, by the frequent sprays of insecticides throughout the growing season, or/and by using fine meshed nets. To improve pest management, research efforts have concentrated on understanding the behavior of the virus vector in order to devise efficient control measures (Al-Musa, 1982, Cohen, 1990; Cohen et al. 1988; Gerling, 1990; Mazyad et al., 1979).

2. TYLCV, a whitefly-transmitted geminivirus

TYLCV was included into the geminivirus family on the basis of its epidemiology (Cohen and Harpaz, 1964) and of electron microscopy observations (Russo et al., 1980; Cherif and Russo, 1983). Viruses that belong to this group are characterized by a covalently closed circular single-stranded DNA genome (cssDNA) encapsidated in geminate particles (Goodman, 1977).

So far, the DNA sequence and genome organization of 10 geminiviruses have been published. They are usually classified into 3 subgroups on the basis of their insect vector, host range and genome organization:

The first subgroup includes viruses that are transmitted by the whitefly Bemisia tabaci, infect dicots and possess bipartite genomes consisting of 2 DNA molecules of equimolar amounts, each one of about 2800 nucleotides, denominated DNA 1 and 2 or DNA A and B: African cassava mosaic virus (ACMV; synonym cassava latent virus CLV, Stanley and Gay, 1983; Morris et al., 1990), tomato golden mosaic virus (TGMV, Bisaro et al., 1982; Hamilton et al., 1984), bean golden mosaic virus (BGMV, Howarth et al., 1985; Morinaga et al., 1987), abutilon mosaic virus (AbMV, Frischmuth et al., 1991) and squash leaf curl virus (Lazarowitz and Lazdins, 1991). Systemic spread of the virus and infection depends on the pres-

ence of the 2 genomic molecules in the target plant.

In the second subgroup each virus is transmitted by a different leafhopper species, infects monocots and possess a monopartite genome: wheat dwarf virus (WDV, MacDowell et al., 1985), maize streak virus (MSV, Mullineaux et al., 1984; Howell, 1984; Lazarowitz, 1987), digitaria streak virus (DSV, Donson et al., 1987) and chloris striate mosaic virus (CSMV, Andersen et al., 1988).

The third subgroup included leafhopper-transmitted viruses with a monopartite genome that infect dicots. The only member of this subgroup which was characterized on the molecular level is the beet curly top virus (BCTV, Stanley et al., 1986).

On the basis of this classification, TYLCV was postulated to have a bipartite genome. TYLCV cloning strategy was based on two characteristics of whitefly-transmitted geminiviruses: 1. In plants infected by these viruses, the two viral genomic components are always found. They are equally represented in both the viral genomic ssDNA and its dsDNA replicative form (RF) (Hamilton et al., 1982; Ikegami et al., 1981; Abouzid et al., 1988; Stanley and Townsend, 1985). 2. The genomic components of a whitefly-transmitted geminivirus show little homology to each other, except for a highly conserved common region (CR) of about 200 nucleotides. Within this region resides a conserved inverted repeat that always contains a single HpaII site (reviewed by Stanley, 1985, and by Lazarowitz, 1987). The HpaII site could be used to clone the two putative genomic DNAs of TYLCV in a single cloning event.

3. Molecular analysis of TYLCV infection

Understanding replication and gene expression of TYLCV in infected tissues may help improve methods for breeding resistant cultivars, either by introgressing resistant genes from wild tomato species or by genetic engineering.

1. At the whole plant level

Little is known on the molecular events underlying geminivirus infection. Inoculation, replication and spread of TMV is well documented (see reviews by Atabekov and Dorokhov, 1984, and by Zaitlin and Hull, 1987). The pattern of African cassava mosaic geminivirus accumulation was described using the ELISA technique; it was shown that ACMV content of cassava leaves decreased with leaf age; ACMV was not detected in mature leaves (Fargette et al., 1987).

We have used molecular hybridization with specific TYLCV DNA probes to study the accumulation of viral DNA in relation to symptom expression, to assess the differential susceptibility of the plant leaves to infection, and to follow the spread of the virus from the site of inoculation.

2. At the plant cell (protoplast) level.

Protoplasts have been used to study several types of naturally occurring genetic resistance (reviewed by Sander and Mertes, 1984). Recently, protoplasts have been used to study the molecular basis of genetically engineered cross-protection in transgenic plants expressing virus coat protein (Register and Beachy, 1988). It was found that protoplasts from tobacco expressing constitutively the tobacco mosaic virus coat protein gene did not support TMV RNA replication TMV after transfection.

Protoplasts have also been used to study the replication of geminiviruses and to perform functional analysis of the virus genes. Protoplasts of Nicotiana plumbaginifolia inoculated with cloned ACMV DNA A and DNA B supported the replication of both DNAs when introduced together. When introduced separately, they supported the replication of DNA A but not DNA B (Townsend et al., 1986). They have also been used to perform functional analysis of geminivirus genes (Matzeit et al., 1990, 1991).

Relying on our experience with tobacco protoplasts inoculations (Rozenberg et al., 1988), we have established a system to study the replication of TYLCV DNA in susceptible tomato protoplasts. We have developed a cell suspension line from calli of susceptible tomato, protoplasts of which were transfected with TYLCV DNA (a head-to-tail dimer of the full-length TYLCV genomic DNA cloned into the pTZ18R plasmid) encapsulated into liposomes.

4. The need for a quick TYLCV disease diagnostic test

In order to predict and monitor TYLCV epidemics and to speed up breeding programs for TYLCV resistance, adequate procedures for rapid and specific virus detection are needed. Virus should be detectable in all plant tissues and in insect vectors (whenever relevant) as early as possible in the infection process. In developing a diagnostic test for TYLCV, our goal was to eliminate most preparative steps on the tested material prior to its hybridization with specific probes.

For many years, symptom observation and bioassays (transmission to test plants, either mechanically or using insects or grafting) have been the only means for virus diagnostics (Symons, 1985). New techniques have been introduced recently relying on the preparation of antibodies, usually against the virus coat protein and their immunoabsorption by plant extracts (enzyme-linked immunosorbent assay, ELISA) (Engvall and Perlman, 1971; Van Regenmortel, 1982). There are many viruses which are difficult to purify in sufficient amounts to obtain high quality antisera or which are poorly antigenic, among them TYLCV.

Nucleic acid hybridization is used more and more in diagnostics (Symons, 1985) using clarified plant saps (Owens and

Diener, 1981; Baulcombe et al., 1984).

We have developed a simple, specific, and rapid method for the detection of DNA and RNA viruses in plants and in insect vectors, based on the squashing of the plant or the insect material onto a nylon membrane, followed by hybridization with a virus-specific probe. Some applications are presented.

5. Breeding for TYLCV resistance

Because of its great economic importance, many efforts have been put into breeding tomato for TYLCV resistance over the last fifteen years. The breeding programs are based on introgressing resistant (or tolerant) traits found in some accessions of several wild tomato species (L. cheesmanii, L. chilense, L. hirsutum, L. peruvianum, L. pimpinifolium) into cultivars (L. esculentum) (Hassan et al., 1984; Kasrawi et al., 1988; Pilowsky and Cohen, 1974; Zakay et al., 1991).

Only recently has a commercial variety being released which shows field tolerance (Hazerah, TY20): the plant presents symptoms, has large amounts of virus; good yields can be achieved if sprayed with insecticides during the first month of growth (Pilowsky and Cohen, 1990). True resistant cultivars (no virus, no symptoms) have still to be designed.

In addition to classical breeding TYLCV-resistant cultivars could be also obtained by genetically engineering the tomato genome.

6. Genetic engineering of plants for virus resistance

1. The Agrobacterium Ti plasmid based plant transformation systems

The T-region of the Ti plasmid is integrated into the genome of plant cells after infection (Chilton et al., 1977) and therefore can be used as a vehicle for incorporation of foreign genes into plants (reviewed by Rogers and Klee, 1987).

The structure of the T-DNA is well known. The borders of the T-DNA consist of a 25 bp direct repeat. The T-DNA contains genes involved in the neoplastic growth and in opine production. The precision of the T-DNA integration allows the insertion of foreign DNA between the borders of the T-DNA and ensures its transfer to the plant genome. A second region in the Ti plasmid contains sequences essential for T DNA transfer, the vir region (for review, see Hille et al., 1984).

Three main strategies have been developed to introduce foreign DNA sequences into the T-DNA of the Ti plasmid.

The first is a site-specific mutagenesis technique. A foreign gene is fused with a selectable antibiotic resistance marker and inserted into a subfragment of the T-DNA cloned into a standard E. Coli cloning vehicle. This construct is then introduced into a wide host range plasmid to allow its

introduction into Agrobacterium by recombination with the Ti plasmid. Plant tissue is inoculated with Agrobacterium containing engineered Ti plasmid, isolated after antibiotic selection (Barton and Chilton, 1983).

The second is based on the construction of two small plasmids, one containing the vir region of the Ti plasmid (necessary for tumor induction), the other contains on a wide host-range replicon the T-DNA which was made free of the onc genes and which contains unique restriction sites for cloning of foreign DNA (e.g; pCGN1547, pMON505). An A. tumefaciens strain harboring both plasmids has a tumor-inducing capacity, although neither plasmid is functional alone. With this approach the T-DNA can be easily genetically manipulated using E. Coli as a host (Hoekema et al., 1983,). Transfer of this plasmid into a A. tumefaciens strain harboring the plasmid with the vir region allows introduction of the manipulated DNA into plant cells and the regeneration of mature fertile plants (reviewed by Rogers and Klee, 1988).

The third involves the cloning of the foreign DNA into pBR322 which is introduced by homologous recombination into an intermediate Ti plasmid, in which part of the T-DNA has been replaced with pBR322 sequences. This modified Ti plasmid is then introduced into a large host range Agrobacterium by homologous recombination with the Ti plasmid (Leemans et al., 1981). A non-oncogenic vector (pGV3850) (Zambryski et al., 1983) which contains 1) the T-DNA border regions and all contiguous Ti plasmid sequences outside the T-DNA, 2) the DNA sequences near the right T-DNA border encoding the enzyme nopaline synthase as a marker to identify transformed cells, 3) a deletion in the internal T-DNA genes which prevent tumorigenesis and replacement with pBR322. The cointegrate can be selected and maintained in Agrobacterium by including a kanamycin resistance marker into the pBR322-containing foreign gene. Another useful acceptor Ti plasmid has been constructed (pGV3851). It is similar to pGV3850, except that only the left internal portion of the T-DNA has been deleted and replaced by pBR322. The transformed tissue resulting from in vivo infection of plants with this plasmid can be selected in tissue culture by growth of shoots in the absence of plant hormones. The shoots can then be rooted and normal plants regenerated (Zambryski et al., 1984).

2. Transformation of tomato plants with TYLCV DNA sequences

We have used the two latter methods to introduce TYLCV DNA sequences into tomato plants. Tomato plants were transformed with engineered the Agrobacterium strains using the transformation/regeneration leaf disc system (Horsch et al., 1985; McCormick et al., 1986).

In recent years, genetic engineering of plants for virus resistance has provided promising strategies for control of virus disease. At present, the most promising of these has

been the expression of the coat protein gene in plants transformed with this gene. Other potential methods include the expression of anti-sense viral transcripts in transgenic plants, the application of artificial anti-sense mediated gene regulation to viral systems, and the expression of viral satellite RNAs, RNAs with endoribonuclease activity, antiviral antibody genes, or human interferon genes in plants (recently reviewed by Gadani et al., 1990; by Nelson et al., 1990; and by Beachy et al., 1990).

Using Agrobacterium tumefaciens Ti plasmid-based vectors it should be possible to introduce TYLCV DNA sequences (e.g. gene encoding the coat protein and/or other open reading frames (in the sense or in the reverse polarity) into the genome of cultivars. As a result of the expression of the viral gene(s), replication of the virus DNA might be blocked in the early stages of infection. Such an approach has been successful with several RNA viruses (see reviews by Gadani et al., 1990, Nelson et al., 1990, Beachy et al., 1990);

It has to be stressed that in conventionally bred plants and in genetic engineered plants, the selection of the resistant individuals is not obvious. Progress in breeding programs is slow because of numerous backcrossings and because of a lack of accurate and easy selection means. Similarly, the effectiveness of genetically engineered plants has to be tested for resistance or tolerance in the field.

MATERIALS AND METHODS

1. Materials

NA-Agarose was from Sigma. The pTZ18 cloning vector was from Pharmacia. Restriction and modifying enzymes were obtained from Pharmacia, Biolab and Boeringer. Nylon membranes (Hybond-N) and radionucleotides were obtained from Amersham. Kodak XAR-5 and pre-flashed Fuji films were used for autoradiography.

2. Maintenance of whiteflies and virus cultures.

Virus cultures were maintained in jimsonweed (Datura stramonium L.) and in tomato plants (Lycopersicon esculentum Mill.). Whiteflies were kept on cotton plants (Gossypium hirsutum L.) grown in muslin-covered wooden cages held in an insect-proof temperature room at 30° C. Virus was acquired by the whitefly vector Bemisia tabaci Genn. after an access period of 24 hours on TYLCV-infected plants, unless specified otherwise (Cohen and Nitzany, 1966). Healthy tomato plants were inoculated by access to viruliferous whiteflies for 24 hours, unless specified otherwise. Tomato plants were grown subsequently either in an insect-proof greenhouse or in insect-proof wooden cages. Plants were sprayed biweekly with Senprothrin (Smash).

3. Purification of virus

Jimsonweed plants (Datura stramonium L.) were found to be a better virus source than tomato, although virus was purified from both plants.

Infected tissue was grinded in liquid nitrogen and the preparation was stirred overnight in a buffer containing 0.1 M sodium phosphate pH 7.0, 10 mM sodium sulfite, 0.1 % (v/v) β -mercaptoethanol, 1 % (v/v) Triton X-100. The extract was filtered through muslin and the resulting sap was clarified by the addition of 10 % (v/v) cold chloroform followed by a 10 min centrifugation at 8,000 g. The supernatant was loaded on a 20 % (w/v) sucrose cushion and centrifuged for 3.5 hours at 90,000 g in Sorvall AH 627 rotor. Pellets were resuspended in 0.1 M phosphate buffer pH 7.0 containing 2 mM EDTA, held overnight at 0° C and centrifuged for 5 min at 4,000 g. Virus suspension was layered on top of 10-30 % (v/v) sucrose gradients and centrifuged for 2.5 hours at 90,000 g in Sorvall AH627 rotor. The presence of the virus in the fractions was detected by electron microscopy. Virus yields obtained were in the range of 40-70 μ g per 80 gr of infected tissue.

4. Preparation and characterization of an antibody against TYLCV

Preparation of polyclonal antibodies

Batches of 0.5 mg of purified viral antigen were mixed with incomplete adjuvant (1:1) before being injected. Four intradermal injections were followed by two intramuscular injections at two weeks interval before the first bleeding.

Western blots of TYLCV coat protein and its immunostaining

Crude sap of TYLCV infected tissue was prepared by crushing plant tissue in liquid nitrogen and homogenizing it in the virus extraction buffer. The homogenate was filtered through cheesecloth, mixed with Laemmli's sample buffer (Laemmli, 1970) and boiled for 5 min before being separated on a polyacrylamide gel.

Concentrated crude preparations were prepared by high speed centrifugation (32,000 rpm in Beckman 35 rotor, for 3 hours) of the above mentioned filtrate. The resulting pellets were suspended in 0.1 M phosphate buffer pH 7.0 containing 2.5 mM EDTA. Following a low speed spin, the supernatant was mixed with Laemmli's boiling buffer and run on a polyacrylamide gel.

TYLCV coat protein was separated on 10 % polyacrylamide gel and electroblotted onto nitrocellulose membrane. Immunostaining of the blots was carried out as described (Hibi and Sato, 1985). To reduce unspecific background, antisera was adsorbed with a tissue preparation from uninfected plants (Hibi and sato, 1985).

5. DNA extraction from virions, plants and insects

From virions

Virus was prepared from plants 1-2 wks after the appearance of disease symptoms, by centrifugation of infected leaf extracts on sucrose gradients (Czosnek et al., 1988b). DNA was extracted from virus preparations with phenol-chloroform, following a 2 h incubation at 50°C in 0.4% sodium dodecyl sulfate (SDS) and 50 µg/ml proteinase K (Maniatis et al., 1982).

From plants

DNA was isolated from tomato leaves using the CTAB method as previously described (Taylor and Powell, 1982).

Crude DNA (lysates) was prepared by adapting the CTAB-based DNA extraction procedure. Tomato tissues were frozen in liquid nitrogen and ground to a fine powder. Boiling extraction buffer (containing 2% (w/v) hexadecyltrimethylammonium bromide (CTAB), 100 mM Tris-HCl pH 8.0, 20 mM EDTA, and 1.4 M NaCl) was added (3 ml/gram tissue). After adding 1% (v/v) beta-mercaptoethanol, the mixture was boiled for one min and incubated for 5 min at 55° C. Then an equal volume of chloro-

form-isoamylalcohol (24:1, v/v) was added and the aqueous phase was extracted and collected after a 10 min centrifugation at 6,000.

From whiteflies

Viruliferous female whiteflies were collected and immediately frozen. Whiteflies were grinded in 200 µl of 50 mM Tris-HCl (pH 8.0), containing 0.5% SDS and 100 µg/ml proteinase K. After a 1 h incubation at 50° C, the DNA was extracted with phenol-chloroform (Maniatis et al., 1982).

6. Cloning and sequencing the TYLCV genome

Isolation of TYLCV dsDNA (RF)

DNA from a TYLCV-infected tomato plant was subjected to agarose gel electrophoresis using a 0.1 x 15 cm well. DNA in a 0.6 cm-wide gel lane was blotted and hybridized with radiolabelled virion DNA. The double-stranded forms of the TYLCV genomic DNA were located following autoradiographic detection, excised from the gel and purified by electroelution (Maniatis et al., 1982).

Cloning of TYLCV RF

Supercoiled covalently closed DNA (cccdNA) was digested by HpaII and was cloned into the AccI site of the pTZ18R plasmid. cccDNA was also digested with EcoRI, SacI or PstI, and was ligated with pTZ18R linearized with the same enzymes. Ligation mixtures were used to transform *E. coli* NM522. Clones containing TYLCV DNA were identified by hybridization of colony blots with radiolabelled virion DNA (Czosnek et al., 1988a) and were subjected to restriction endonuclease analysis.

Sequencing

Two independent HpaII clones (pTYH19 and pTYH20), inserted in opposing orientations into the pTZ18R vector were used for sequence analysis. Sequences were created by digestion of pTYH19/20 with EcoRI, SacI, SphI and XbaI followed by self ligation, and further on by subcloning of HaeIII, Sau3AI and RsaI fragments of the initial subclones into pUC18. Clones were subjected to sequencing by the chemical degradation procedure (Maxam and Gilbert, 1980), and by the chain termination method (Sanger et al., 1980). The nucleotide sequence around the single HpaII site used for cloning was verified by partial sequencing of an EcoRI clone (pTYE1). The data were compiled and analyzed using the GCG sequence analysis package (Devereux et al., 1984), and the DNA Strider application for the Macintosh (Marck, 1988). The sequence of cloned TYLCV DNA was compared to sequences of 9 geminiviruses whose sequences were available from GeneBank using the Gap and Bestfit functions of the GCG package.

7. Gel electrophoresis and hybridization with TYLCV-DNA probes

Electrophoresis

Electrophoresis of DNA was performed in 1% agarose gels containing 0.5 µg/ml ethidium bromide, in Tris-Phosphate-EDTA buffer (Maniatis et al., 1982). Samples were blotted onto nitrocellulose membranes after depurination and alkali denaturation (Southern, 1975) .

Molecular hybridization with radiolabelled TYLCV DNA.

Blots were prehybridized for 3 h and hybridized for 16 h (Carmon et al, 1982) with either virion DNA or cloned TYLCV DNA radiolabelled with [32P]dCTP (specific radioactivity of about 108 cpm/µg DNA; about 2x10⁵ cpm/cm² membrane), by nick-translation (Rigby et al., 1977). Blots were washed at 65°C for 30 min (twice) in 0.15 M NaCl - 0.015 M trisodium citrate (1 x SSC) prior to autoradiographic exposure at -80° C, using 2 intensifying screens.

When needed, the amount of TYLCV DNA was estimated by scanning the autoradiograms with a Computing Densitometer (Model 300A, Molecular Dynamics). As standards, known amounts of TYLCV-DNA were submitted to gel electrophoresis, hybridized and visualized as described above.

8. Squash blots

Squash-blotting of tomato tissues and of whiteflies.

Tomato leaves and flowers were squashed onto a dry nylon membrane using a hard object (e.g. glass rod, pen). Stems were either cut longitudinally or sliced serially from the apex to the crown and imprinted on the membrane. Fruits were cut open and imprinted. Frozen whiteflies were sprinkled on the nylon membrane taking care to separate each individual from the others, and squashed with a glass rod. Squash-blots were hybridized with a TYLCV-specific DNA probe as described above.

Squash-blotting and hybridization of tobacco leaves inoculated with RNA virus

Leaves from TMV-infected tobacco plants were squashed onto a nylon membrane pre-soaked with 0.5 % sodium dodecyl sulfate (SDS) and 100 µg/ml proteinase K (to prevent degradation of RNA by plant ribonucleases). The blot was hybridized with a [32P]-dCTP radiolabelled TMV-RNA reverse transcript (Hull, 1985). Leaves from PVY-infected tobacco plants, squashed as described above, were hybridized with a 4 kbp PVY cDNA fragment (5' end of the PVY genome) radiolabelled by nick-translation with [32P]-dCTP. Leaves from non-inoculated tobacco plants were squashed and hybridized with both probes as described above. All squash-blots were hybridized and virus DNA detected as described above.

9. Replication and expression of TYLCV DNA introduced in tomato protoplasts

Establishment of a tomato suspension cell line (831)

Surface-sterilized leaves of a hybrid F1 L. esculentum x L. peruvianum (sensitive to TYLCV) were cut to fine stripes and left on Petri dishes containing MS medium (Linsmaier and Skoog, 1965) in 1% agarose. When enough callus was obtained, it was disintegrated and cells were grown at 25° C in a shaker as a suspension in MS medium supplemented with 2% (w/v) sucrose, 10 mM CaCl₂ and 4.5 µM 2,4 Dichloro-phenoxyacetic acid.

Preparation of protoplasts

Protoplasts are prepared from cell suspension as described (Sela et al., 1984). The enzyme solution TSE-1 containing cellulysin and macerozyme is added (Shahin, 1984). After incubating at 28° C for about 5 hours under light shaking, the digested tissue is filtered through two layers of cheesecloth. Protoplasts collected into test tubes and centrifuged at 1000 x g. The floating protoplasts are collected and washed by additional centrifugations in washing solution TWS (Shahin, 1984). The protoplasts are adjusted in plating medium (Shahin, 1984) to a concentration of 500,000 cells/ml.

Culture of protoplasts

Protoplasts are cultured in a thin layer (2 ml) of TM-2 medium. Protoplasts divide and form colonies in TM-2 medium supplemented with combinations of: 2,4-dichlorophenoxyacetic acid/benzyl-aminopurine (2,4-D/BAP), or 2,4-D/zeatine riboside, or para-chlorophenoxy-acetic acid (p-CPA)/zeatine riboside, or NAA/BAP, or NAA/kinetin, or 2,4-D/kinetin, and p-CPA/BAP, in the range of 0.5-1.0 mg/liter. They are incubated sealed in diffused light (500 lx, 16 hr/day).

DNA isolation from protoplasts

Protoplast (about 4x10⁶ cells) are concentrated by centrifugation (150 x g), suspended in 0.4 % SDS and 100 µg/ml proteinase K and incubated for 2 hours at 50°C. DNA is phenol extracted, concentrated by ethanol precipitation and suspended in sterile water (Rozenberg et al., 1988).

Inoculation of tomato protoplasts with TYLCV nucleic acids encapsulated into liposomes

A. Preparation of liposomes:

Large unilamellar vesicles (LUV) are prepared according to Gad et al. (1986). Liposomes, composed either of phosphatidyl serine: cholesterol PS:CHOL (3:2, molar ratio) or of cardiolipin: phosphatidylcholine:phosphatidylethanolamine CL:PC:PE (3:1:2), are prepared in 0.4 M mannitol, 0.1 M NaCl, 0.1 mM EDTA and 5 mM HEPES pH 7.4 (liposome buffer), with 70 µg TYLCV DNA / 5 µmol Pi. After bath sonication for 30 sec to form emulsion and evaporation of the ether (reverse phase

evaporation) (Szoka and Papahadjopoulos, 1978), the vesicles are extruded through 0.4 μ m followed by 0.2 μ m polycarbonate membranes (Olson et al., 1979). Liposomes are separated from the non-encapsulated DNA by floatation on a ficoll gradient (Fraley et al., 1982). Liposome concentration was measured as lipid phosphorous content (Pi).

B. Incubation of protoplasts with liposomes:

Protoplasts (5×10^5 cells/ml) are resuspended in 550 mM mannitol, 5 mM CaCl_2 , 0.1 mM EDTA, 5 mM HEPES pH 7.4. Fifty μ l of liposomes (250 nmol Pi) containing TYLCV DNA are incubated at room temperature with 2 ml of protoplast suspension, with 15 μ g/ml polylysine. Ten min later, 60 μ g/ml of polyglutamate are added and protoplasts are pelleted for 5 min at 100 x g. Protoplasts are plated at a density of 2×10^5 cells/ml, in MS medium supplemented with 0.5M mannitol, 3% (w/v) sucrose, 2 μ M biotin, 1 μ M folic acid, 30 μ M thiamine, 16 μ M naphthalene acetic acid, 5 μ M benzyl aminopurine and 800 mg/l casein hydrolysate.

Introduction of the TYLCV coat protein gene into the Ti plasmid of Agrobacterium tumefaciens

The HpaII clone of TYLCV (pTYH19) (TYLCV full-length clone into pTZ18R) was cut with the enzymes BclI and BamHI to excise the TYLCV coat protein gene under the control of its own promoter. Following dilution and self-ligation, the resulting plasmid (pTYH19.3, which contains the TYLCV coat protein gene) was cut with EcoRI and PstI and was ligated to pBR322 cut with the same enzymes. As a result the coat protein was cloned into pBR322 (pTY1). The pTY1 plasmid was conjugated with the Ti plasmid pGV3851 (Zambryski et al., 1984) using the triparental method (Rogers and Klee, 1988). The resulting Agrobacterium tumefaciens (pGV3851::pTY1) was used to introduce the TYLCV coat protein into tomato protoplasts.

Co-cultivation of Agrobacterium tumefaciens with tomato protoplasts

Protoplasts were cultivated in the K3 medium (Nagy and Maliga, 1976) at a concentration of 105 cells/ml (5 ml per dish) for 4 days (2 days in the dark followed by 2 days in the light). Bacteria which were grown for 2 days in YEB medium were concentrated a 10 fold and resuspended in K3 medium. Two hundred μ l of bacteria were seeded in each Petri dish and mixed gently. After 2 days in culture, the protoplasts were transferred in K3 medium containing 0.5 g/l clafuran. After one week, the protoplasts were suspended in TM2 medium without zeatin. One month after the beginning of the co-cultivation, microcalli 1-2 mm were visible. The calli were transferred to MSX medium to accelerate growth. Some of the calli were put on Tan's growing medium (1987), but no regeneration from these calli was ever observed.

10. Agroinoculation of tomato plants with a cloned TYLCV DNA dimer

DNA extracted from infected tomato plants was cut with PstI and was subjected to gel electrophoresis. DNA migrating in the position of a 2.8 Kbp marker was eluted from the gel and ligated with a PstI-linearized pTZ18R. The ligation mixture was used to transform E. coli XL1-blue. Colonies containing TYLCV DNA were detected by hybridization with genomic TYLCV DNA. The 2.8 Kbp insert from one clone (pTYP42) was separated from its plasmid vector and subcloned into a PstI-rstricted pCGN1547 binary vector. One clone with a complete head-to-tail dimeric insert was identified (pTY4) and introduced into A. tumefaciens LBA4404 (Hoekema et al., 1983) by transformation, according to the method of An et al. (1988).

A 200 ml culture of LBA4404 containing pTY4 was grown at 30° C for 48 h, concentrated by centrifugation and suspended in 10 ml sterile water. Tomato plants (L. esculentum, cv. Monique) were agroinoculated (Grimsley et al., 1987) by injection of the bacterial culture into the crowns, the stems add into the bases of shoot nodes. Plants were grown in an insect-free growth chamber kept at 25° C with a 16h / day 1800 lux illumination.

11. Transformation of tomato with TYLCV DNA sequences

TYLCV ORFs were cloned into the shooter vector pGV3851 (Zambryski et al., 1984) and into the pCGN1547 binary vector (Calgene). The former plasmid was introduced into Agrobacterium strain C58 and the latter plasmid into Agrobacterium LBA4404.

TYLCV DNA sequences were introduced into tomato using the leaf disc method (McCormick et al., 1985). Tomato discs were incubated in Petri dishes containing MS medium. After 2 days, the explants were incubated with a concentrated solution of bacteria and replated on the same medium for 2 additional days. The discs were then washed with claforan (0.5 g/l) and plated on McCormick's medium without any hormone (pGV3851) or with 100 µg/ml kanamycin (pCGN1547). After 3-6 weeks, shoots begun to appear which were subsequently rooted and plants grown in pots.

RESULTS

1. Isolation of the tomato yellow leaf curl virus and its characterization as a geminivirus

We have previously published a procedure to isolate TYLCV virions (Czosnek et al., 1988b). This procedure which was a modification of methods developed for other geminiviruses did not yield virus in sufficient amounts and clean enough to allow the preparation of antibodies.

A major effort was investigated in finding a purification procedure which will yield a reasonable amount of purified TYLCV virions to be used in the production of antibodies.

The gemini-like particles observed in our preparations showed a characteristic bisegmented structure and had a size (20 x 30 nm) similar to that reported for geminiviruses (Goodman, 1977) (Figure 1, A1). Whiteflies fed on the particles-containing fractions were able to transmit the disease to healthy tomato or Datura plants (Czosnek et al., 1988).

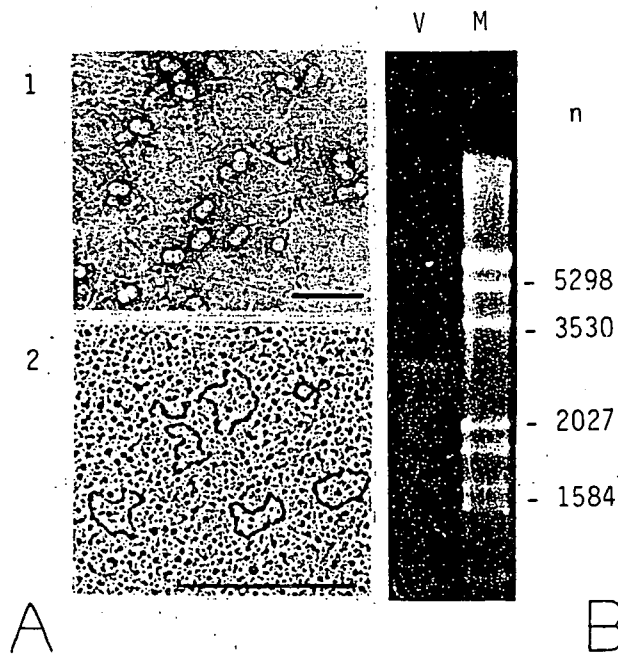


Fig. 1. A: Electron micrographs of (1) TYLCV particles negatively stained with uranyl acetate and of (2) viral DNA spread (KLEINSCHMIDT 1968); bars represent either 100 nm (1) or 500 nm (2). B: Electrophoresis of (V) TYLCV-DNA in a 1.5 % agarose gel stained with 0.5 μ g/ml ethidium bromide and of (M) single-stranded DNA size markers (in nucleotides, n) (EcoRI/Hind III bacteriophage lambda DNA digest made single-stranded after alkali treatment)

The nucleic acid extracted from virus-containing fractions was insensitive to RNAase A, but was digested by both DNAase I and nuclease S1, indicating its single-stranded DNA nature

(Czosnek et al, 1988b). Electron microscopic observation of this DNA (Figure, A2) demonstrated the presence of circular single-strand DNA molecules. Gel electrophoresis of virion DNA, together with ssDNA markers, indicated that the TYLCV genome has about 2800 nucleotides (Figure 1, B).

2. An antiserum against TYLCV

1. Characteristics

The antiserum was tested for its ability to detect the virus antigen in crude extracts by indirect ELISA (Clark and Adams, 1977) and direct ELISA (Koenig, 1981). Indirect ELISA was found unefficient for virus detection in both purified and crude preparations due to high background. However when direct ELISA was used instead, readings from infected tissues were 2-5 times higher than the values obtained for healthy tissue. Best results were obtained when the IgG and the conjugate were at a 1:100 dilution. It was also found that better results were obtained when the tested tissue was grinded in the same buffer used for virus extraction in the purification procedure.

Immunoblot assays are recommended over other immuno detection methods due to their higher sensitivity. These methods are assumed to detect as low as 50 pg of a specific protein.

Using TYLCV antiserum to probe western blots of TYLCV coat protein, we were able to detect the virus coat protein both in purified and crude sap from TYLCV infected plants. Results were more conclusive when the crude sap was concentrated by a high speed ultracentrifugation prior to PAGE separation. This treatment helped to eliminate some of the unspecific background in blots from uninfected material.

2. Serological relationship of TYLCV with other geminiviruses

TYLCV coat protein was separated on 10 % polyacrylamide gel and electroblotted onto membranes. The membranes were immunostained using antisera against the following geminiviruses: TYLCV, ACMV (African cassava mosaic virus, synonym cassava latent virus CLV), SLCV (squash leaf curl virus), and BGMV (bean golden mosaic virus). Antibodies against ACMV, SLCV and BGMV were kindly provided respectively by Dr Harrison BD, Scottish Crop Research Institute, Dundee, UK; Dr. Duffus JE, US Agricultural Research Station, USA; Dr. Hiebert E, University of Florida, Gainesville, USA.

The immunostained blots shown in Fig. indicate a positive reaction with each of the tested antisera which is in agreement with former reports on the close serological relationships in the whitefly-transmitted geminivirus group (Roberts et al., 1984).

3. TYLCV is a whitefly-transmitted geminivirus with a single genomic component

1. Cloning of the TYLCV genome

A single genomic component was cloned from TYLCV-infected plants. Nineteen HpaII clones were obtained; 18 contained a 2.8 Kbp insert which was closely related to each other. One TYLCV isolate was cloned in a PstI site unique for that isolate (Table 1).

Table 1. Forty two TYLCV clones obtained by three methods for viral RF isolation, cloned using four restriction enzymes.

Number of clones	Enzyme	Plant	Origin	Year	Cloning method
19	HpaII	tomato	field	1987	A
2	EcoRI	tomato	field	1988	B
4	EcoRI	tomato	field	1989	C
15	PstI	datura	greenhouse	1989	C
2	SacI	datura+ tomato	greenhouse	1989	C

Clones were subjected to restriction analysis and several were partially sequenced. Representative clones were hybridized with ACMV DNA A but not with B.

2. Sequence and genome organization of TYLCV genome

The sequencing strategy is shown in Figure 2.

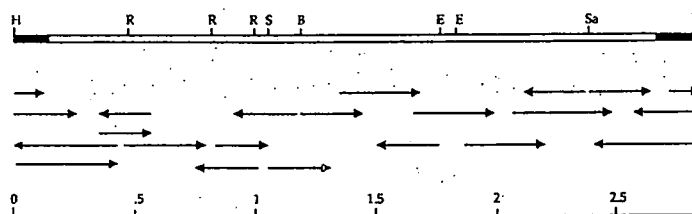


Fig. 2. Sequencing strategy of the TYLCV genome. The cloned TYLCV genome is represented as a thick black line. The inter-genic region is the non-shaded area within the lines. Sites for restriction endonucleases used for subcloning are shown. B: BclI, E: EcoRI, H3: HaeIII, H: HpaII, R: RsaI, Sa: SacI, S3: Sau3AI, S: SphI, X: XbaI. Arrows below the map indicate the direction and extent of sequencing from each subclone. Several clones were sequenced in both directions.

The TYLCV DNA sequence of 2790 nucleotides (27% A, 32% T, 19% C and 22% G) is shown in the virion sense in Figure 3 (EMBL accession number is X15656).

GTGGAATGA	ATCCGTGTC	CTCAAAGCTC	TATGCCAATG	GGCTATCGG	TGCTTACCT	ATACTTGGAC	ACCTAATGSC	TATTTGGTAA	TTCATTAAT	GTTCATFCA	ATTGAAATT
10	20	30	40	50	60	70	80	90	100	110	120
CAAAATTC	AAATCAATC	ATTAAAGCGG	CGATCCGAT	AATATTACCG	CATGGCCCGG	CTTATTCCCT	TTATGTGCTC	CCCACGAGGG	TTACACAGAT	GTATTGTGCA	ACCAATCATA
130	140	150	160	170	180	190	200	210	220	230	240
TTGCATCTC	AAACGTTACA	TAAGTGTGCA	TTTGTCTTAA	TATACITGGT	CCCCAAGTTT	TTTGTCTTGC	AATATGTGGG	ACCCACTTCT	TAATGAATTT	CCGGAATCTG	TTCAAGGATT
250	260	270	280	290	300	310	320	330	340	350	360
TCGTGTATG	TTAGCTATTA	AATATTGTCA	GTCCGTTCAG	GAACCTTAAG	AGCCCAATAC	ATTGGGCCAC	GATTTAATTA	GGGATCTTAT	ATCTGTGTGA	AGGCCCCGGG	ACTATCTCGA
370	380	390	400	410	420	430	440	450	460	470	480
AGCGAACCAG	CGATATAATC	ATTTCCACGC	CCGCTCTGAA	GGTTCGCGCA	AGGCTGAAGT	TCGACAGCCC	ATACAGCAGC	CCCTCTGCTG	TCCCACTTGT	CCAGGGTACA	AACAGGTCAC
490	500	510	520	530	540	550	560	570	580	590	600
GATCTGCGAC	CTACGCGCC	ATGTACCGAA	ACCCCAAGT	ATACAGAATG	TATCGAAGCC	CTGATTTTCC	CCGTGCTATG	GAAGGCCCAT	GTAAAGTCCA	GTCTTATGAG	CAAGGGGATG
610	620	630	640	650	660	670	680	690	700	710	720
ATATTAGCA	TACTGGGATT	GTTCTGTGTC	TTAOTGATCT	TACTCGTCCA	TCTGGAAATTA	CTCAGAGACT	CGGTAAAGAG	TTCTGTCTTA	AATCGATATA	TTTATTAGGT	AAAGTCTGGA
730	740	750	760	770	780	790	800	810	820	830	840
TGGATCATA	TATCAAGAG	CAGATTCACA	CTAATCAGGT	CATGTTCTTC	TTCGTCCGTC	ATACAGGCTC	CTATGGAATC	AGCCCAATGG	ATTTTGCACA	CGTCTTATAT	ATGCTOGATA
850	860	870	880	890	900	910	920	930	940	950	960
ATGAGCCGAG	TACCGCAACC	CTGAAGAAATG	ATTTGCGTGA	TAGGTTTCAA	GIGATCAGGA	AAATTCATGC	TACAGTTATT	GSTGGGCCCT	CTGGATCATA	CCACACGGCA	TTAGTTAAGA
970	980	990	1000	1010	1020	1030	1040	1050	1060	1070	1080
GATTTTATA	AAATTAACAT	CATGTAAGTT	TATTTATATT	CATTCAGGAG	CCAGCAAGT	ACGACAAACA	TACICAAATC	GCCTTGTAT	TGTATATGEC	ATGTAAGCAT	GCCTCTAATC
1090	1100	1110	1120	1130	1140	1150	1160	1170	1180	1190	1200
CATGTATCC	AACTATGAAA	ATAAGCATCT	ATTTCTATCA	TTCAATATCA	AATTAATAAA	ATTTATATTT	TATATCATGA	GTCTCTGTAA	CATTTATGCT	CTTTCAACT	ACATCATACA
1210	1220	1230	1240	1250	1260	1270	1280	1290	1300	1310	1320
ATACATGATC	AACGCTCTCG	ATTACATTGT	TAATGGAAAT	TACAACAAAG	CTATCTAAT	ACTTAAGAAC	TTCTATCTTA	AATACTCTTA	AGAAATGACC	AGTCTGAGCC	TGTATGTCTG
1330	1340	1350	1360	1370	1380	1390	1400	1410	1420	1430	1440
TGCAATTCG	GAATTCAGCA	AAACATTGTT	GAATCCCAT	TACCTTCCTG	ATGTTGTGGT	TGAATCTTAT	CTGAATGAA	ATGATGCTCT	GCTTCATTAG	AAATGGCCCTC	TGGCTGTGTT
1450	1460	1470	1480	1490	1500	1510	1520	1530	1540	1550	1560
CTGTTATCT	GAATAGAGG	GGATTGTGTA	TCTCCAGAT	AAAAACGCCA	TCTCTGCGCT	GAGGAGCAGT	CATGACTTCC	CCCTGCGGCG	AATCCATGAT	TATTGCAATT	GAGCTGGAGG
1570	1580	1590	1600	1610	1620	1630	1640	1650	1660	1670	1680
TAGTATGAGC	AGCCACAGTC	TACGTCTACA	CCCTTACCCC	TTATCTCTTT	CTCTCTGGCT	ATCTCTGTGT	GGACCTTGAT	TGATCTCTCC	GAACAGTGGC	TCGTACAGGG	TGACGAGGCT
1690	1700	1710	1720	1730	1740	1750	1760	1770	1780	1790	1800
TCGATCTTG	AGAGCCCAAT	TTTCAAGGA	TATCTTTTAT	TCTCTGCTTA	GAATCTCTCT	ATATGAGCAG	CTAGGTCTCT	CAITGCGAGG	GAAGATAGTC	GGAATTCCTC	CTTTAATTGG
1810	1820	1830	1840	1850	1860	1870	1880	1890	1900	1910	1920
AATGGGCTTC	CGTACTTTTG	TGTTGCTTTG	CCAGTCCCTC	TGSGCCCCCA	TGAATCTCTT	GAAGTCTTTT	AAATTAATCG	GCTCTACGTC	ATCATATGAG	TTGTAACCTG	CATCATTACT
1930	1940	1950	1960	1970	1980	1990	2000	2010	2020	2030	2040
GTACACCTTT	GACAGACTTA	GCTCTAGATG	TCACATATAA	TAATATATGG	GGCTAGAGA	CCCTGCCCAT	ATGTTTATGC	CTGTCTGTCT	ATCACCTTCA	ATGACAAATC	TTATGGGTCT
2050	2060	2070	2080	2090	2100	2110	2120	2130	2140	2150	2160
CCATGGCCCT	GCAGCGGAAT	ACAAGAGCTT	CTCGGCGACT	CACCTCTTCA	GTTCTATGCG	AATTTGATTA	AAAGAACGAG	AAAGAAATGG	AGAAACATAA	ACTTCTAAAG	GAGGACTAAA
2170	2180	2190	2200	2210	2220	2230	2240	2250	2260	2270	2280
AATCTATCT	AAATTTGAAC	TTAATTTATG	AAATTTGATA	ATATAGTCTT	TTCGGGCTTT	CTCTTTTAAAT	ATATTTAGGG	CCCTGCTATT	ACTCCCTGAA	TTGAGTCTTT	CGGATATGCT
2290	2300	2310	2320	2330	2340	2350	2360	2370	2380	2390	2400
GTCGTTGGCA	GATTCCTGAC	CTCTCTGAGC	TCATCTGCCA	TGATTTTGGG	AACTCTTAAA	ATCAATGAAG	TTCCTGCTTT	TCTCAAGTAA	GCTCTGACA	TCTGTGAGC	TCTTAGCTGC
2410	2420	2430	2440	2450	2460	2470	2480	2490	2500	2510	2520
CTGAATGTT	GATGGGAAT	GTCCTGAGCT	GTTCGGGAT	ACCAAGTCCA	AGAACGTTG	GTTCTTACAT	TGATTTTGGC	CTTCGATTTG	GATAAGCACA	TGAGATGTG	GTTCCTCAT
2530	2540	2550	2560	2570	2580	2590	2600	2610	2620	2630	2640
CTCTGGAGT	TCCTTCAAAA	CTTTGAGCTA	TTTTTATTTT	GTTCGGGTTT	CTAGTTTTTT	TAATTTGGAA	ACTCTCTCTT	CTTTAGAGAG	AGAACATTTG	GGATATGTTA	GGAATTAAT
2650	2660	2670	2680	2690	2700	2710	2720	2730	2740	2750	2760
TTTGGCATAT	ATTTTATAA	AACGAGGCT									
2770	2780	2790									

Fig. 3. Nucleotide sequence of the TYLCV genome. The first nucleotide at the 5' end of the intergenic region was designated at nucleotide 1. Direct and inverted repeats are indicated by arrows beneath and above the sequence. Variable bases are shown above the sequence.

The 5' end of the intergenic region (IR) was designated as nucleotide 1. Minor sequence variations were encountered between the two HpaII clones and the ECoRI clone, as the latter was partially sequenced. The variable bases found in pTYE1 are shown above the complete sequence in Figure 3.

Sequence comparisons on the DNA level between TYLCV and 10 geminiviruses whose sequences were published showed that TYLCV is most closely related to the DNA A component of ACMV with 73% similarity between their aligned sequences.

In order to locate potential genes, the DNA sequence was screened on both strands for open reading frames (ORFs) with coding potential for proteins of m.w. greater than 10 Kd. The ORFs are described in Table 2 and their organization along the genome is depicted in Figure 4.

Table 2. Open reading frames in TYLCV DNA and homologies with their counterparts in four geminiviruses. Percent of conserved homology in amino acid sequence is followed (in brackets) by percent of direct homology. Reading frames were labeled (from 1 to 3) beginning with the first ORF on each strand.

ORF	Reading frame	Start	Stop	Amino acids	Mol. wt.	Homologies			
						CLV	TGMV	BCTV	WDV
V 1	+2	474	1256	260	30300	90.7 (80.2)	82.6 (74.1)	48.7 (23.1)	41.5 (18.2)
V 2	+1	314	664	116	13450	91.2 (72.6) ^a	-	43.1 (17.0)	55.4 (25.7)
C 1	-1	2790	1714	358	40738	81.0 (71.4)	79.7 (69.5)	74.6 (61.0)	54.6 (34.1) ^b
C 2	-2	1805	1400	135	15601	77.8 (63.7)	69.3 (52.0)	51.2 (29.6)	-
C 3	-3	1657	1255	134	15929	86.6 (73.1)	71.0 (56.5)	66.4 (40.6)	-
C 4	-2	2639	2346	97	11091	44.3 (34.0) ^c	58.8 (49.4)	60.0 (49.4)	-

^a 13.1 kDd ORF in CLV

^b Compared to the predicted sequence of the spliced mRNA of WDV (Schalk, H.J. et al.)

^c Compared to the protein translated from the second AUG.

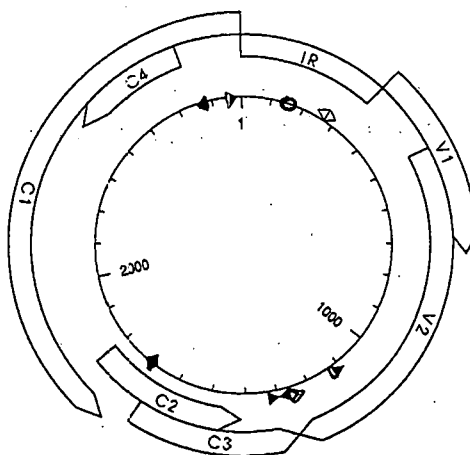


Fig. 4. Potential coding regions in TYLCV DNA. Open reading frames (ORFs) that start with an ATG and have the potential to code for proteins with molecular weight of 10 KDa or more are displayed. The name of each ORF is indicated, corresponding to Table 2. Potential TATA boxes are indicated as open triangles. Polyadenylation signals with the sequence G/AATAAA are indicated as solid triangles. The intergenic region (IR) is also shown.

All the ORFs have an A residue the the 3rd base preceding their 1st ATG. Two ORFs were located on the virion strand. ORF V2, which precedes V1 (the coat protein gene) is highly homologous in amino acid sequence to an unnamed ORF of 13.1 Kd found in the same location in DNA A of ACMV, but absent from the genomes of other whitefly-transmitted geminiviruses (Table 2). On the complementary strand, we found 3 ORFs analogous to the AC1, AC2 and AC3 (designated also AL1, AL2 and AL3) of the whitefly-transmitted geminiviruses (Davies and Stanley, 1989), and a 4th ORF (C4) which is present in the same region in the genomes of all dicot-infecting geminiviruses.

Potential promoters conforming to the consensus sequence TATAT/AA (Breathnach and Chambon, 1981) were located in positions similar to that of promoters found in ACMV and TGMV (Figure 4). The positions of putative polyadenylation signals with the consensus sequence G/AATAAA (Messing et al., 1983) are shown in Figure 4. Direct and inverted repeats, which were shown to be involved in enhancement of transcription and binding of regulatory proteins (Wasylyk et al., 1983; Birchmeier et al., 1982) were found in several positions are shown in Figure 5.

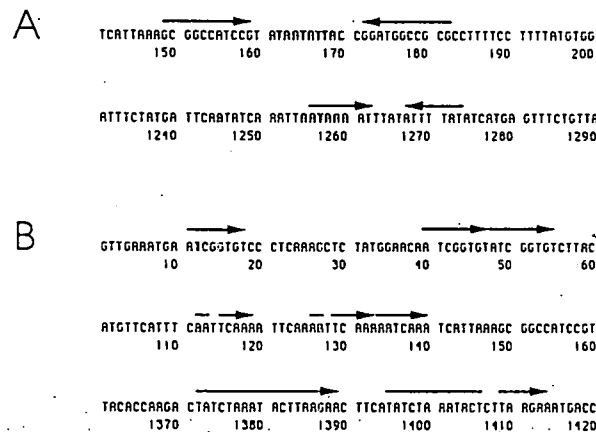


Fig. 5. A: Conserved inverted repeats in the TYLCV genome: the stem and loop structure (nucleotides 148-191) found in the IR of all geminiviruses, the AT rich region in the convergence point of V2 and C3 ORFs (nucleotides 1254 to 1271). B: Direct repeats in the TYLCV genome; imperfect repeats are indicated by a break in the arrow above the sequence.

3. Comparison with other geminiviruses

Table 2 shows homologies between TYLCV ORFs and that of several other geminiviruses. From all the geminiviruses whose sequence has been published, ACMV is the closest to TYLCV. Figure 6 shows a dot matrix comparison (Mullineaux et al., 1985) between TYLCV DNA sequence and that of ACMV, BCTV and WDV. It can be seen from that Figure 6 and from Table 2 that TYLCV has a high level of homology with ACMV.

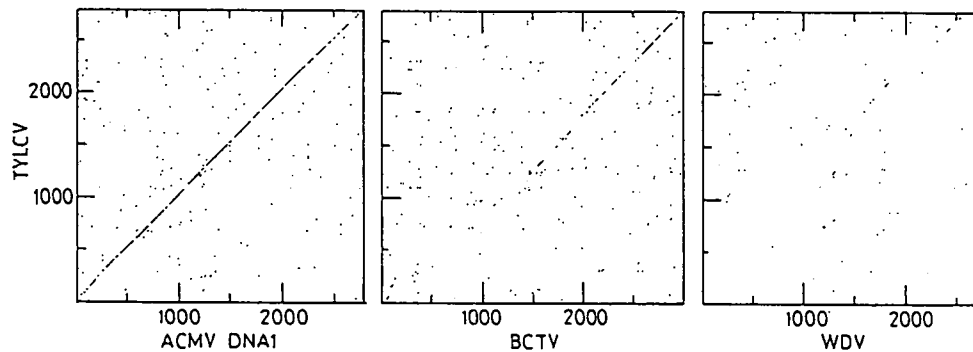


Fig. 6. Dot matrix comparison (Mullineaux et al., 1985) between the genomes of TYLCV, ACMV DNA A, BCTV and WDV.

Homology with BCTV, a leafhopper-transmitted geminivirus that affects dicots, is mainly in the region of the C1 ORF (implicated in virus DNA replication). Little homology is found with WDV, a leafhopper-transmitted virus that affects monocots.

The amino acid sequence of the TYLCV coat protein is given in Figure 7. It is compared with that of ACMV, TGMV and BGMV. TYLCV coat protein is most homologous to that of ACMV.

TYLCV	1	MSKRPGDIIISTPVSKVRRRLNFDSPYSSRAAVPIVQGTNKRKRSWTYRPMYRKPRIYRMYRSPDVPKCGECPCKVQSYEQRDDIKHT	87
ACMV	1G.....RN..TA.T.HV..RK.A.VN.....TM.....I.....F.....V..L	87
TGMV	1	.P..DAPWRLMAGT...S.SA...P.GSL...DA.VN.....SL.G...K.....H..SLV	77
BGMV	1	MAGT...S.SG.YS...PSGGMGSKS...ANA.VN.....K.....K.....H..S.V	71
TYLCV	88	GIVRCVSDVTRGSGITHRVGKRFCKVSIYFLGKVVMDENIKKQNHNTNQVMFFLVRDRRPYGNSPMDFGQVFNMFNDNEPSTATVKNDL	174
ACMV	88	..CKVI.....P.L.....I...I..L.T.....N.I.Y.L.....A.Q...I.....I....	174
TGMV	78	.K.M.I.....N.....V.I...I.....LK...S...W.....T.....	163
BGMV	72	.K.M.I...I...N.....V.I...I.....MLK...S.I.W.....T.....F	157
TYLCV	175	RDRFQVMRKFHATVIGGSPGMKECALVKRFFKINSHVTLFIFIQEAARYENHTENALLLYMACTHASNPVYATMKIRIYFYDSISN	260
ACMV	175L.....V...Y.....YRL.H...YNH...G.....L.....G...258	
TGMV	164IHR...K.T.QYASN...R..W.V.NN.V YNH...G.....L.....T...247	
BGMV	158	...Y...HR.N.K.S.QYASND...R..W.V.N..V YNH...G.....L...V...T...241	

Fig. 7. Comparison between the coat protein genes of TYLCV and ACMV, TGMV and BGMV. Sequences were aligned using the Bestfit program of the UWCGG software package. Gaps were inserted in order to maximize homology. Only differences in amino acids are shown, unchanged residues are marked by a dot. Stop codons are denoted with an asterisc.

Similarly to all geminiviruses analyzed so-far, TYLCV possesses in its intergenic region a sequence of about 30 nucleotides which may form a hair-pin structure (nucleotides 148-191). This sequence is highly homologous to that of the whitefly-transmitted geminiviruses, but different from that of the leafhopper-transmitted geminiviruses (Figure 8).

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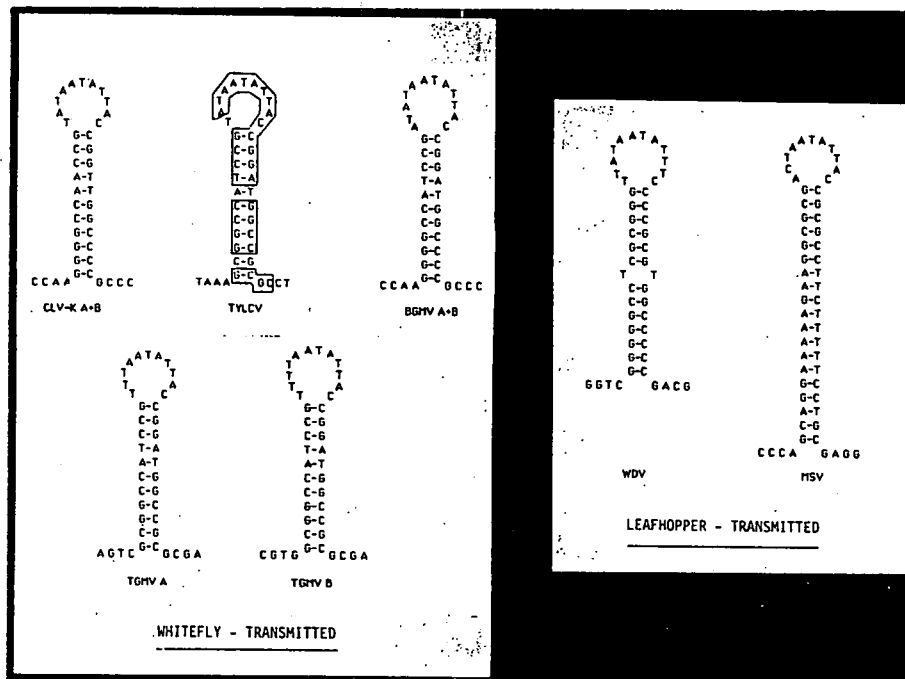


Fig. 8. Comparison between potential stem-loop structure present in the IR of whitefly- and leafhopper-transmitted geminiviruses. Consensus sequences between TYLCV and ACMV (CLV), BGMV and TGMV are boxed.

4. Infectivity of the TYLCV genome

A complete head-to-tail dimer of the cloned TYLCV genome was inserted in the *A. tumefaciens* vector pCGN1547 and used to agroinoculate 10 healthy tomato plants. Viral DNA was detected in all the plants 6 days post inoculation by squash blotting. Symptoms which appeared about 15 days post inoculation were indistinguishable of those associated with natural whitefly-mediated infection (Figure 9).

The disease was passed from the agroinoculated plant to test plants using whiteflies. TYLCV DNA was detected after 1 week and disease symptoms appeared in 15 out of the 16 plants 3 weeks postinoculation. TYLCV genomic DNA and its replicative form was present in roots and leaves of these plants.

5. No TYLCV second genomic DNA could be found in infected plants and in viruliferous whiteflies

The search for the putative TYLCV B genomic component was based on two assumptions:

1. The sequences of genomes A and B of geminiviruses with a bipartite genome share almost no homology outside the common region (CR), and their restriction maps are different.
2. The common region could be used as a probe to detect both putative components.

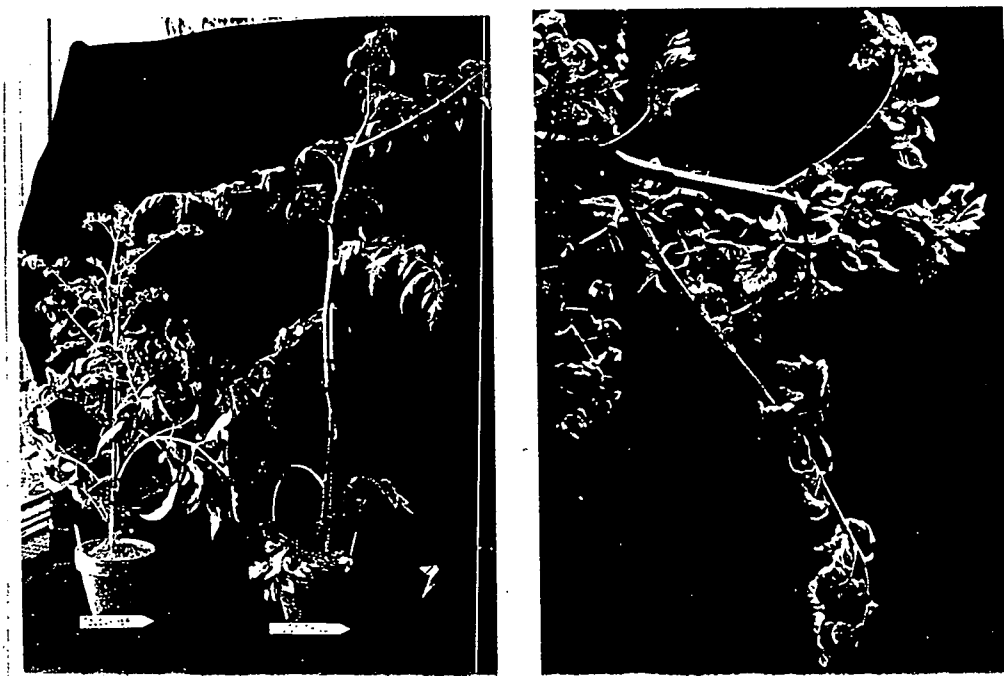


Fig. 9. Disease caused by agroinoculation of a complete head-to-tail TYLCV DNA dimer, cloned into the pCGN1547 binary vector and introduced into *Agrobacterium* LBA4404. Left pannel: agroinoculated tomato plant 30 days after inoculation (left) and noninoculated plant of the same age (right). Right pannel: details showing typical TYLCV disease symptoms.

a. Among TYLCV RF in infected plants

Assuming TYLCV has 2 genomic components, cleavage of the viral dsDNA by restriction enzymes would produce fragments whose length would total 5.6 Kbp. If recognition sites for a given enzyme exist in only one of the components, fragments that add up to 2.8 Kbp and uncut dsDNA will be seen. A probe containing the CR would detect at least one gramtent from each of the components. A probe specific for DNA A would not detect the uncut dsDNA species.

If TYLCV has a single genomic component, its restriction would produce fragments that add up to 2.8 Kbp and agree with the rstriction map derived from the sequence of the cloned component.

Total DNA extracted from infected plants was digested with 16 different restriction enzymes. The dsDNA was identified by hybridization of Southern blots with virus plus- and minus-strand-specific DNA probes. Enzymes for which no site exist in the sequenced cloned TYLCV genome did not cut TYLCV dsDNA. Enzymes for which there are recognition sites in the cloned molecule of TYLCV, either linearized the viral dsDNA or transformed it fragments whose length added up to 2.8 Kbp. Hybridization with the intergenic region (IR) probe did not reveal any bands in addition to those deduced from the restriction map of the cloned TYLCV DNA or already detected

by the DNA A-specific probes. Results of an analysis with several restriction enzymes are shown in Figure 10.

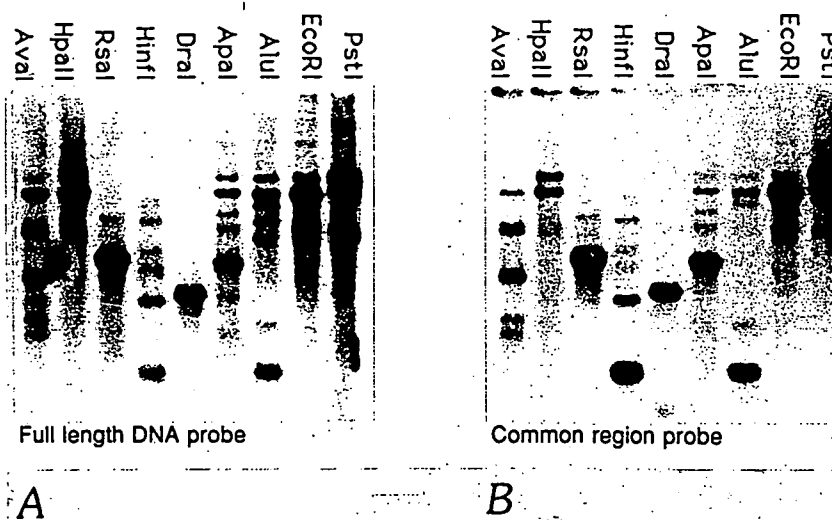


Fig. 10. Restriction and hybridization analysis of TYLCV dsDNA. Total nucleic acids extracted from a tomato plant infected in the field (with typical disease symptoms) was incubated with 9 restriction enzymes. Following gel electrophoresis and blotting, samples were hybridized with the full length TYLCV clone (A) and with a AluI/AluI fragment containing the IR.

b. Among TYLCV genomic ssDNA in plants

The same approach used in our search for component B in TYLCV dsDNA was followed here, using the restriction enzyme AatII. Sequence variation exists at the recognition site for this enzyme in the TYLCV genome. Although there are no sites for AatII in the HpaII clones that were sequenced, on other clones (e.g. pTYE1), a unique AatII site exists. Restriction mapping and partial sequencing of several clones indicated that it is located in the IR (Figure 3, nucleotides 218-223). When total DNA from an infected plant was incubated with AatII, the dsDNA was completely linearized to a 2.8 Kbp band while from the ssDNA yielded 2 fragments (Figure 11, A lane 3). When infected plant DNA was incubated with BclI, the dsDNA was linearized as expected while the ssDNA remained unaffected (Figure 11, A lane 4). The same ssDNA fragments and linearized dsDNA were detected when blots were hybridized either with the IR probe (Figure 11, B lane 2) or with DNA A specific probes (Figure 11, B lanes 1, 3). By hybridization of the same blots with two DNA A-specific probes. We delimited the second AatII restriction site in the TYLCV ssDNA to a region between nucleotide 1902 to nucleotide 2165. The fact that the DNA A-specific probes detect the same ss and dsDNA fragments as the IR probe did, excludes the possibility that any of the fragments comes from the putative B component.

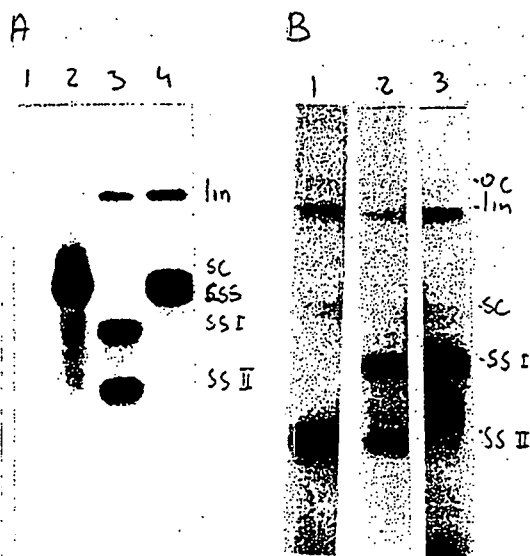


Fig. 11. Restriction and hybridization of TYLCV ssDNA. A: Effect of treatment with AatII on TYLCV ssDNA. Total nucleic acids from an uninfected plant (lane 1) and from infected plants (lanes 2-4), incubated with AatII (lane 3) and BclI (lane 4), were subjected to electrophoresis and blots were probed with a full length clone of TYLCV. The positions of the linear (lin), supercoiled (sc), circular single stranded (css) and cleaved ssDNA forms (ssI and ssII) are indicated. B: Mapping of the AatII site to the IR. Total nucleic acid extract from an infected plant was incubated with AatII. Blots were hybridized with a IR probe (lane 2) and with DNA A-specific probes (lanes 2 and 3). Positions of the open circular (oc), linear (lin), supercoiled (sc), circular single-stranded (css) and cleaved ssDNA forms (ssI and ssII) are indicated.

c. In viruliferous whiteflies

To discriminate between DNA A and the putative DNA B present in whiteflies, we aimed to cut the ssDNA of the A component in several fragments without affecting the B component. Upon hybridization with the CR probe, an uncut DNA species (representing the putative DNA B molecule) was expected to be seen, in addition to the fragments resulting from cleavage of DNA A.

TYLCV ssDNA extracted from viruliferous whiteflies was rendered locally double-stranded. Based on the sequence of the cloned TYLCV genome, three 18-mer oligonucleotides complementary to different regions in the ssDNA genome were synthesized. Each oligomer contained a single recognition site for one of the following restriction sites: ApaI (from nucleotide 456 to 473), XbaI (2057-2074) and SacI (2501-2518) (Figure 12, A). The 3 oligomers were annealed with TYLCV ssDNA in a DNA extract from 200 viruliferous whiteflies, as the mixture was heated to 75° C and slowly cooled to 45° C. The partially dsDNA was incubated for 2 h with a mixture of XbaI, SacI and ApaI, submitted to gel electrophoresis and Southern blotted. Blots were hybridized to a DNA A specific probe (pTYH20.23). Three bands were detected

(Fig 12, B lane 2), as expected from the sequence of the cloned TYLCV DNA A-like genome. When the IR probe was used, only the middle size band (corresponding to the ApaI-SacI fragment of 745 nucleotides) was seen (Figure 12, B lane 3). Unrestricted ssDNA was not detected by either probes.

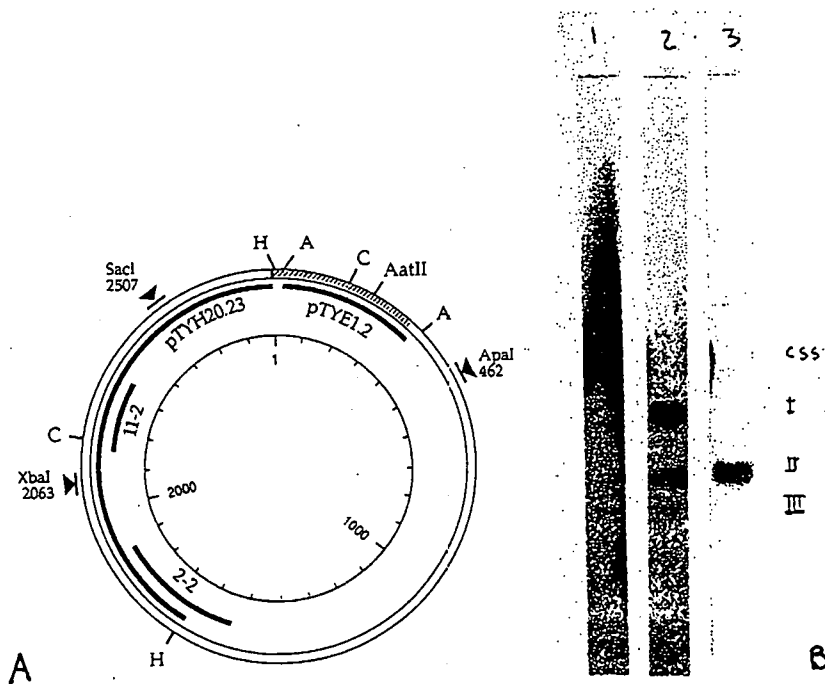


Fig. 12. A: Probes and complementary nucleotides used for restriction and analysis of TYLCV DNA. The genome is displayed as a double circle with the IR shaded in black. Probes used for hybridization analysis are depicted inside the circle as thick black lines. The location of the complementary oligonucleotides used for analysis of ssDNA in whiteflies are shown as short black lines with a black triangle pointing to the restriction site within each region. Sites of restriction endonucleases used in restriction analysis and subcloning of the probes are indicated: A: AluI, C: CfoI, H3: HaeIII, H1: HinfI, S3: Sau3AI.

B: Restriction and hybridization analysis of TYLCV DNA present in whiteflies. Total nucleic acids from 200 insects were hybridized in solution with 3 ss 18-mer oligonucleotides, complementary to 3 regions in the viral DNA, each containing a restriction for either ApaI, XbaI or SacI. The partially dsDNA was incubated with a mixture of the 3 enzymes (lanes 2 and 3) or left untreated (lane 1). After electrophoresis, the blot was probed with a DNA A-specific probe (lanes 1 and 2), autoradiographed, stripped from the probe and rehybridized with a IR probe (lane 3). The positions of the circular single-strand (css) and cleaved ssDNA froms (I-III) are indicated.

4. TYLCV DNA forms in the virion, in infected plants and in the insect vector

1. TYLCV DNA in the viral capsid

DNA was purified from the geminate particles isolated as

described above. In the electron microscope, the DNA preparations contained mainly circular molecules with a size of about 850 nm (Figure 1, A). When electrophoresed in an agarose gel in the presence of single-stranded DNA size markers, the viral DNA migrated as a discrete band with a mobility corresponding to a 2800 nucleotide-long molecule (Figure 1, B). This agreed with the length of the TYLCV genome (2790 nucleotides) as found after cloning and sequencing (Navot et al., 1991).

2. Virus-related DNA species in infected plants

Genomic TYLCV-DNA was used as a probe to detect viral DNA sequences in plants and in insects. Although TYLCV-DNA is single-stranded, it could be radiolabelled by nick-translation, either because of local double-stranded structures (similar to those described for other geminiviruses, reviewed by Lazarowitz, 1987) or because of contaminating plant DNA serving as primer.

Virus genomic single-stranded DNA and virus-related double-stranded DNA species could be detected in TYLCV-infected plants. DNA extracted from individual healthy and infected tomato plants was subjected to electrophoresis in two different agarose gels (A and B, Figure 13).

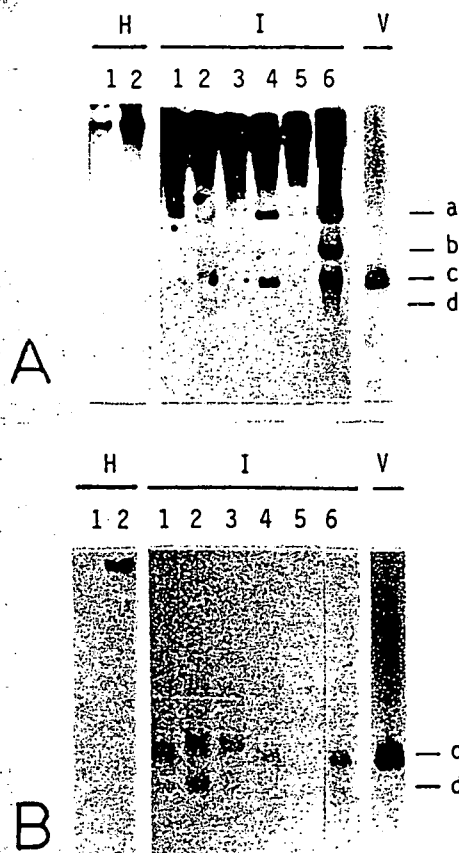


Fig. 13. Identification of single-stranded and double-stranded TYLCV-related DNA species in infected tomato plants by differential hybridization.

Total DNA extracted from two healthy plants (H), from six TYLCV-infected plants (I) and from virus particles (V) was subjected to electrophoresis in two different gels. The samples in gel A were denatured with alkali prior to blotting; the samples in gel B were blotted without prior denaturation. Blots A and B were hybridized with nick-translated TYLCV-DNA and processed for autoradiography. Each lane contains DNA prepared from an individual plant. Note that DNA species *a* and *b* hybridize only after alkali denaturation (double-stranded DNA) while DNA species *c* and *d* hybridize whether denatured or not (single-stranded DNA).

In the gels, the fast migrating viral DNA separated from the slow migrating high molecular weight plant DNA. After electrophoresis, the DNA samples in gel A were alkali denatured, blotted and hybridized with radiolabelled TYLCV-DNA (Figure 13, A); the DNA samples in gel B were similarly processed, but without the alkali DNA denaturing step (Figure 13, B). This differential hybridization allowed to discriminate between single-stranded and double-stranded viral DNA.

The autoradiographic analysis of samples in gel A indicated that the viral probe could detect at least four defined DNA species (designated a to d) which appeared only in DNA preparations from TYLCV-infected plants (Figure 13, A). The signal obtained with high molecular weight DNA, from both healthy and infected plants, was due to contamination of the probe with fragments of plant genomic DNA.

Since band c comigrated with TYLCV genomic DNA (whether the gel was treated with alkali or not, Figure 13, A and B), this band contained the single-stranded viral genome. The single-stranded DNA in band d had a molecular weight lower (about half-size) than that of TYLCV DNA in band c (Figure 13, A and B); it could be an incomplete form of the TYLCV genome, similar to the defective viral genomes described in plants infected by ACMV or TGMV (Stanley and Townsend, 1985, Hamilton et al., 1983, respectively). The DNA in band d is probably encapsidated since we found half-size DNA molecules among the DNA molecules isolated from TYLCV particles (Czosnek et al., 1988b). It has to be noted that this TYLCV DNA species was detected by hybridization in about 10% of the infected plants only. In those plants where it was detectable, its amount was similar to that of full-length TYLCV genomic DNA (Figure 13, B, plant 2, bands c and d).

Bands a and b appeared only if the electrophoresed DNA was treated with alkali prior to blotting and hybridization (Figure 13, A), and therefore contained double-stranded DNA. Band a and b represent putative intermediate double-stranded DNA replicative forms (RF) of the viral genome (band c). Similar viral double-stranded DNA molecules have been described in plants infected by ACMV (Stanley and Townsend, 1985) and by TGMV (Hamilton et al., 1983); by analogy, we postulate that a and b contain the RF in its relaxed and supercoiled forms, respectively.

The double-stranded virus-related DNA forms were isolated from TYLCV-infected tomato plant DNA. Following gel electrophoresis of the infected plant DNA, hybridization with the viral probe and autoradiographic identification (Figure 14, lane 6) the various viral DNA species were purified by electroelution (Figure 14, lanes 3-5).

We have used the supercoiled RF of the viral DNA (band b) in order to clone the TYLCV genome.

3. TYLCV DNA sequences in viruliferous whiteflies

DNA was extracted from viruliferous whiteflies, following

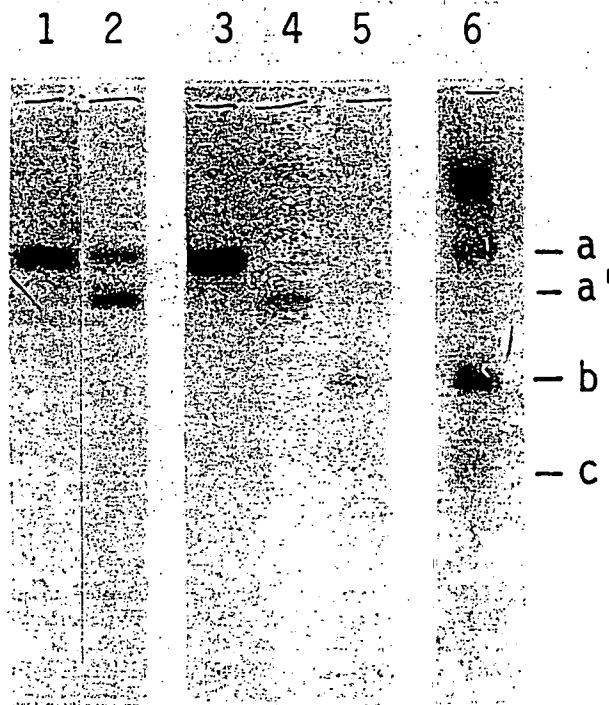
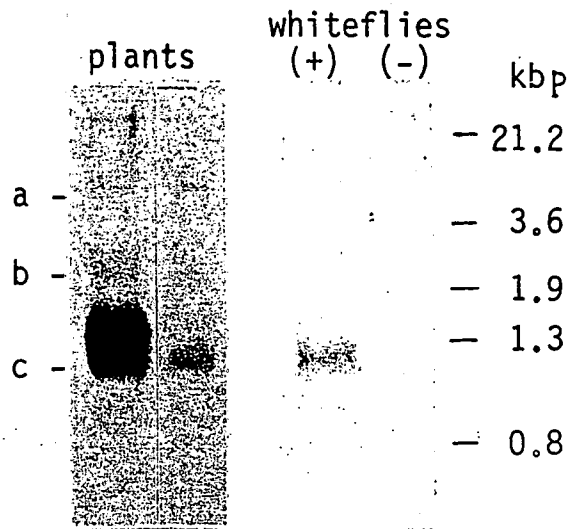


Fig. 14. Isolation of double-stranded replicative form of the TYLCV genome.

DNA from a TYLCV-infected tomato plant was subjected to gel electrophoresis, blotted and hybridized with the TYLCV DNA probe (lane 6). After autoradiographic detection, the double-stranded forms (band *a*: relaxed, band *b*: supercoiled) of the TYLCV genomic DNA (band *c*) were excised from the gel and purified by electroelution (lanes 3 and 5, respectively). Band *a'* (lane 4) is a linearized form of the circular TYLCV-DNA which can be generated by incubating DNA *a* (lane 1) with EcoRI for one hour (lane 2).

Fig. 15. Autoradiographic detection of TYLCV-related DNA sequences in the whitefly vector. DNA extracted from viruliferous (+) and control (-) insects (right panel), was subjected to gel electrophoresis together with DNA from infected tomato plants (left panel). The samples were blotted and hybridized with TYLCV-DNA. Size markers on the right (in kilobase-pairs, kbp) are from a ECoRI/Hind III digest of bacteriophage lambda DNA. Note that only TYLCV genomic DNA could be detected in the insects.



a 18 h acquisition period on a TYLCV-infected plant. It was subjected to gel electrophoresis and blotted. DNA prepared from whiteflies maintained on healthy plants was processed in the same way. Upon hybridization with the TYLCV-DNA probe, a unique DNA band comigrating with the virus genomic DNA (band *c*) could be detected, only in DNA preparations from viruliferous whiteflies (Figure 15). The TYLCV dsDNA species (*a* and *b*) which were conspicuous in infected plants were not detected in the whitefly. Therefore, it is likely that the virus is not replicating in its insect vector.

TYLCV DNA can be detected in DNA preparations isolated from a single viruliferous whitefly.

5. Molecular analysis of tomato infection by TYLCV: susceptibility to infection, symptom development and accumulation of viral DNA

1. Correlation of symptom development with the occurrence of viral DNA

After an acquisition access period of 48 hours on TYLCV-infected jimsonweed, female whiteflies (*B. tabaci*) were transferred to tomato plants (*L. esculentum* cv. M82). Plants at the 6-leaf stage were exposed for a period of 48 hours to 100 whiteflies each.

Fifty eight tomato plants were monitored for the appearance of disease symptoms 1, 4, 8, 11, 15, 18, 22, 25, 29 and 33 days after having been caged with whiteflies. At each time from 1 to 25 days post-inoculation, a different group of seven plants was assayed for TYLCV-DNA (except at day 18 post-inoculation, 9 plants). Plants which were assayed at day 1, 4, 8, 11 and 15 were assayed again either at day 22, 25, 29 or 33 to ensure that inoculation had occurred. Assays for TYLCV-DNA were done on crude DNA extracts (lysates) from pooled samples of the third and fourth leaves (from the apex) of each plant (a leaf is counted if it is longer than 1.5 cm). In several plants, assays also were done on apex, all leaves, stem, cotyledons and roots.

Expression of the disease symptoms was correlated with the appearance of viral DNA was monitored in the 58 plants (Table 3).

Table 3. Frequency distribution of symptom development and appearance of detectable TYLCV-DNA with time in TYLCV-infected tomato

Days after inoculation	Plants with symptoms (%)		Plants with viral DNA (%)	
1	0/58 ^a	(0)	0/7 ^a	(0)
4	0/58	(0)	0/7	(0)
8	0/58	(0)	5/7	(71)
11	0/58	(0)	11/12	(92)
15	22/58	(38)	21/22	(95)
18	45/58	(77)	30/32	(94)
22	50/58	(86)	40/41	(97)
25	56/58	(96)	50/51	(98)
29	57/58	(98)	51/52	(98)
33	57/58	(98)	57/58	(98)

Tomato plants at the 6-leaf stage were inoculated in the greenhouse with viruliferous whiteflies. All plants were checked periodically for the appearance of typical disease symptoms, while some plants were assayed for TYLCV-DNA. Results are expressed as percent of plants with disease symptoms and with TYLCV-DNA

^a Plants analyzed

Characteristic symptoms appeared 15 days after inoculation in 22 of 58 plants (38%). Twenty nine days after inoculation, 57 plants had symptoms (98%). One plant remained symptomless and viral DNA could not be detected in its tissues.

To ensure that for each plant the assay is performed on tissues from a similar developmental stage, TYLCV-DNA was assayed in samples of pooled third and fourth leaf (from the shoot apex). Data obtained from all plants were grouped according to the day the disease symptoms first appeared (Figure 16).

2. TYLCV DNA accumulation in tomato leaves upon infection

Figure 16 A shows the time-course of TYLCV-DNA accumulation in plants where symptoms were first observed 15 days after inoculation. The probe was able to detect TYLCV genomic circular single stranded DNA (CSS), as well as the circular double-stranded TYLCV-DNA replicative form in its supercoiled (covalently closed circular, CCC) and relaxed (open circular, OC) forms. Also were detected higher molecular weight TYLCV-related DNA which might be CCC polymers.

Significant amounts of TYLCV genomic DNA (about 10 pg/ μ g plant DNA) could be detected 8 days after inoculation; no viral DNA was detectable 4 days after inoculation. Eleven days after inoculation, the concentration of viral genomic DNA in the infected leaves had markedly increased although it varied from plant to plant (0.5 to 3.3 ng/ μ g plant DNA). By the time that symptoms were visible the concentration of virus DNA in the infected tissues seemed to have reached a maximum (3.8 to 4.4 ng/ μ g plant DNA). The concentration of TYLCV-DNA replicative form was much lower than that of the viral genomic DNA; the replicative form could not be detected earlier than 15 days after inoculation.

A similar pattern was found in plants where symptoms appeared 18 days after inoculation (Figure 16 B). Detectable amounts of viral DNA (0.2 to 1 pg/ μ g plant DNA) could be found in some of the plants 8 days after inoculation, 10 days before disease symptoms were recorded. Eleven days post-inoculation, the amount of TYLCV-DNA has increased by a factor of 1000 (about 2.9 ng/ μ g plant DNA). Fifteen days post-inoculation, the concentration of TYLCV-DNA has reached that present the day disease symptoms appeared (3.5 to 4.1 ng/ μ g plant DNA).

When symptoms were delayed, the appearance of viral DNA was also delayed. For example (Figure 16 C), when symptoms appeared 25 days after inoculation, TYLCV-DNA could not be detected earlier than 18 days post-inoculation (2 pg/ μ g plant DNA). At the day symptoms appeared, the concentration of TYLCV-DNA was similar to that found in plants with earlier symptoms (about 5 ng/ μ g plant DNA).

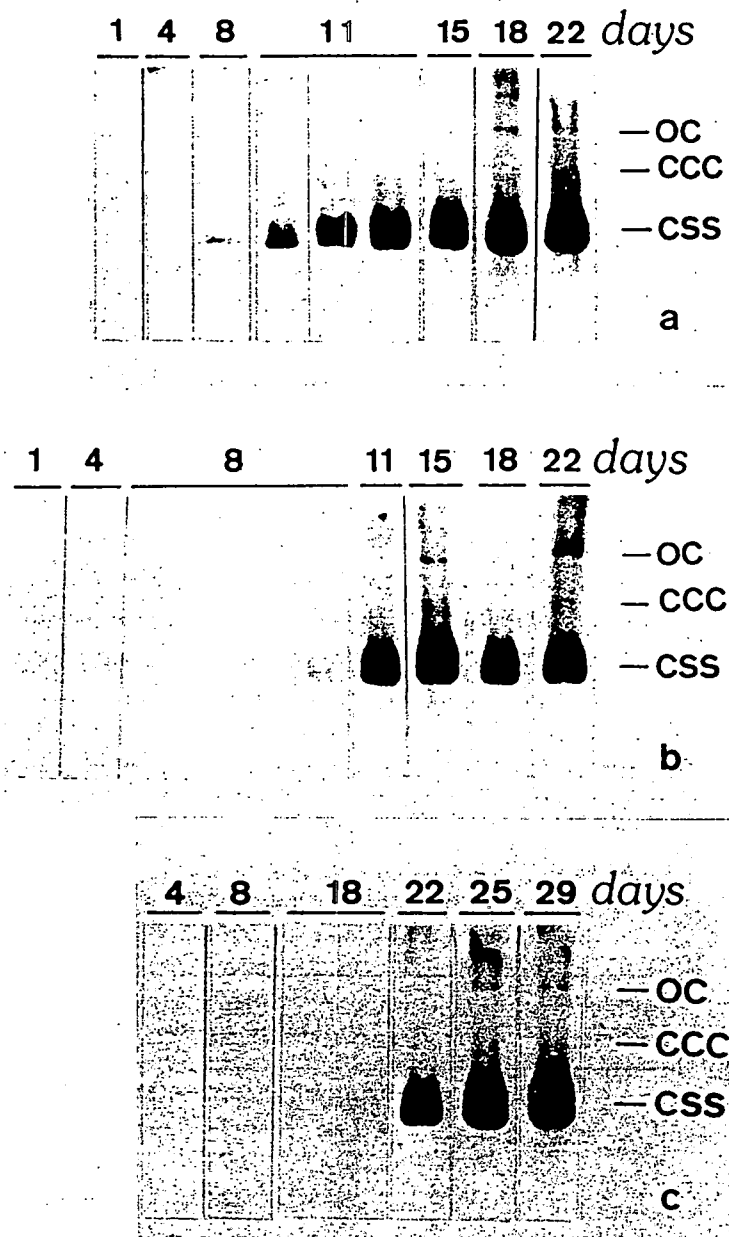


Fig. 16. - Time-course of TYLCV-DNA accumulation and symptom development. Autoradiograms showing the detection of TYLCV-DNA in plants that developed symptoms 15 days (a), 18 days (b), and 25 days (c) post-inoculation. Each lane represents an individual plant. Numbers above the lanes are the day plants were sampled following inoculation. CSS TYLCV genomic circular single-stranded DNA; CCC circular double-stranded DNA replicative form of the viral genome in its supercoiled (covalently closed circular) form; OC replicative form in its relaxed (open circular) form; the uppermost band probably represents a CCC polymer

Table 4 summarizes the data correlating symptom recording and appearance of detectable TYLCV-DNA. TYLCV-DNA could not be detected earlier than 10-11 days before the appearance of disease symptoms. At this time, 4 out of the 7 plants analyzed carried detectable amounts of viral DNA; in these 4 plants symptoms appeared 18 days post inoculation or later.

Table 4. Frequency distribution of TYLCV-DNA detection with time of symptom appearance

Days before appearance of symptoms	Plants with viral DNA (%)	
0	19/19*	(100)
3-4	10/10	(100)
7	3/4	(75)
10-11	4/7	(57)
14	0/5	(0)
17-18	0/6	(0)

* Plants analyzed

Seven days before appearance of symptoms, 3 out of the 4 plants analyzed had detectable TYLCV-DNA; this group included plants showing symptoms 15, 18 and 25 days post inoculation. Three to four days before symptoms appeared, all 10 plants analyzed contained large amounts of viral DNA.

3. Distribution of TYLCV-DNA in different plant tissues

The relative concentration of TYLCV-DNA in various tissues of infected plants was assessed (Figure 17).

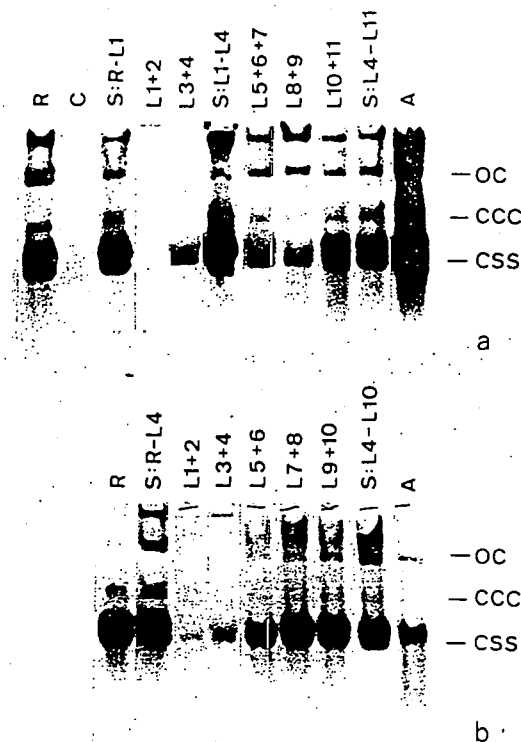


Fig. 17. Autoradiograms showing the distribution of TYLCV-DNA in tomato tissues. a Plant sampled 15 days after inoculation when initial symptoms appeared. b Plant sampled 25 days after inoculation (one week following the appearance of the symptoms). L Leaves, numbered from cotyledons (C) to shoot apex (A). R Roots. S: R-L Stem section from root to leaf. S: L-L Stem section between indicated leaves

The distribution of TYLCV-DNA in a plant on the day symptoms were first noticed (15 days after inoculation) is shown in Figure 17 A. It can be seen that viral DNA was not evenly distributed throughout the plant tissues, although the infection took place in cages where insects were free to inoculate the entire plant, except the roots. The highest concentrations of viral DNA were found in the shoot apex (9 ng/ μ g plant DNA), the roots (5.8 ng/ μ g plant DNA), the young leaves (4.4 ng/ μ g DNA of leaves 10 and 11), and in the stems (4.5 to 5.5 ng/ μ g DNA in the 3 stem sections analyzed). Less viral DNA was found in the older leaves (10 pg/ μ g DNA of leaves 1 and 2); very little viral DNA could be detected in the cotyledons (about 3 pg/ μ g plant DNA). The stems supporting the older leaves and cotyledons had high concentrations of viral DNA (4.4 ng/ μ g plant DNA). Similar results were found in all the plants tested the day symptoms appeared.

The relative concentration of viral DNA in the tested tissues remained almost unchanged during the development of the disease. Twenty five days after inoculation, 10 days after the appearance of the first disease symptoms, the concentrations of viral DNA in the older leaves were still lower than that in the younger tissues (0.1 ng/ μ g DNA of leaves 1 and 2, and 0.7 ng/ μ g DNA of leaves 3 and 4, compared to 5.4 ng/ μ g DNA of leaves 7 and 8, and 5 ng/ μ g DNA of leaves 9 and 10) (Figure 17 B).

4. Developmental stage - dependant susceptibility to infection, and spread of virus in infected plant

The efficiency of infection and spread of viral DNA relative to the site of inoculation was investigated (Figure 18).

Twenty eight tomato plants at the 8-leaf stage were divided into four groups and exposed to whiteflies using leaf cages (20 insects per cage) for a period of 48 h. After exposure, all plants were sprayed with the insecticide Senprothrin (Smash) and kept in an insectproof greenhouse.

Leaves were numbered from the oldest leaf (leaf 1) towards the youngest (leaf 8). With the first group of plants, leaf 1 was exposed, with the second group leaf 4, with the third group leaf 7, and with the fourth group the shoot apex was exposed. Assays for TYLCV-DNA was done on all leaves, shoot apices, and roots, 7, 9, and 11 days after exposure to whiteflies (2 plants from each group were analyzed each sampling day). One plant from each group was maintained to monitor symptom development.

The most efficient inoculation was through the shoot apex (Figure 18 A). Nine days after inoculation, large amounts of viral DNA had accumulated at the site of infection (now leaf 9) and some TYLCV-DNA has moved to the roots. Eleven days after inoculation, the concentration of TYLCV-DNA at the site of inoculation and in the roots had increased, and TYLCV-DNA was also found in the new shoot apex, in the neighboring upper leaf (leaf 10) and in the flowers (Figure 18 A).

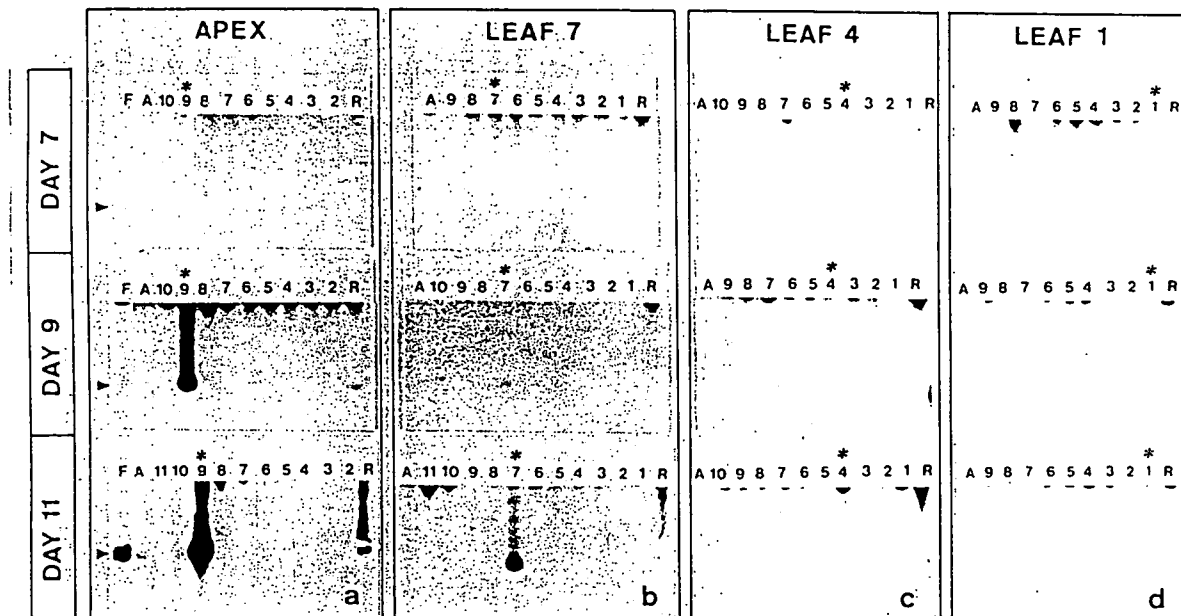


Fig. 18. Spread of TYLCV-DNA in tomato plants inoculated through defined tissues. Twenty eight plants at the 8 leaf-stage were divided into four groups. a Group inoculated on the shoot apex, b on a young leaf (#7), c on a middle-aged leaf (#4), and d on the oldest leaf (#1). In each group, presence of TYLCV-DNA was determined 7, 9, and 11 days after inoculation. Each panel represents an individual plant and each lane represents a sample from either a leaf [numbered from the oldest (1) to the youngest (10)], from the roots (R), from the shoot apex (A), or from the flowers (F). * Inoculation site. ► Location of the TYLCV genomic DNA in the gel

When plants were inoculated through the youngest leaves (leaf 7), the rate of TYLCV-DNA accumulation at the site of inoculation was slower than when the inoculation took place through the shoot apex (Figure 18, A and B). Eleven days after inoculation of leaf 7, the concentration of TYLCV-DNA was still lower than that found 9 days after inoculation of the shoot apex (Figure 18, A and B). Transport of virus DNA to the roots was also delayed; detectable amounts of TYLCV-DNA were found in the roots only 11 days post-inoculation. At that time, TYLCV-DNA was still undetectable in the new shoot apex or in leaves other than those inoculated.

Inoculation of middle-aged leaves (leaf 4) was much less efficient than inoculation of younger tissues. Only 11 days after inoculation was TYLCV-DNA barely detectable at the site of inoculation (Figure 18 C).

Inoculation of old leaves (leaf 1) was inefficient. TYLCV-DNA remained undetectable even 11 days after inoculation (Figure 18 D).

Disease symptoms appeared 16 days post-inoculation in the plants inoculated on the shoot apex and the young leaves. The plants inoculated on middle-aged leaves had delayed symptoms while the plants inoculated on the older leaves never developed symptoms.

5. Time course of TYLCV multiplication in jimsonweed plants

Jimsonweed plants (*Datura stramonium* L.) were inoculated by TYLCV using viruliferous whiteflies. Plants were divided into 9 groups with 5 plants each. Each group was sampled once at a given time point at 3 days intervals by removing 6 discs from the upper developed leaf. Samples were taken also from uninfected plants at each time point. Samples were kept at -20°C before being processed.

Samples from each time point (5 replica of 3 leaf discs in each) were crushed in virus extraction buffer and tested by double antibody sandwich ELISA. The rest of the samples (5 replicas of 3 leaf discs) were crushed in liquid nitrogen and spotted on a nylon membrane. Hybridization was carried out using TYLCV viral DNA as a probe. Quantitative evaluation of the spotted DNA was done by liquid counting.

There is a continuous increase of the nucleic acid content in the plant growing tip during the first 30 days after inoculation. However the virus coat protein was undetectable beyond 14 days after infection. The results are in agreement with our failure to purify virions from plants which were infected for a longer period of time.

6. Expression of the coat protein gene during TYLCV disease development

Tomato plants at their 4-leaf stage were infected by viruliferous whiteflies. DNA and RNA was extracted from leaves 4, 9, 16, 22 and 29 days post infection.

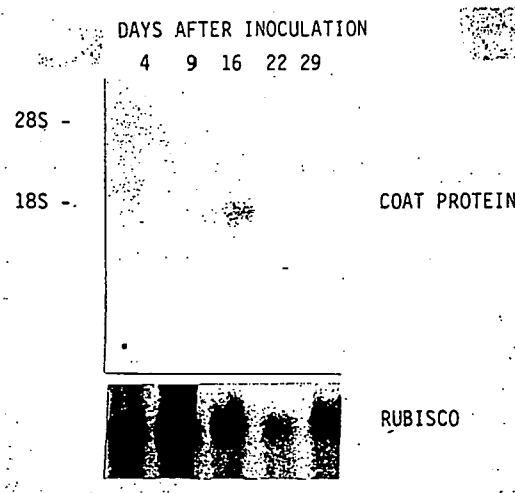


Fig. 19. transcription of the TYLCV coat protein gene in infected tomato plants. RNA was isolated from tomato plants 4, 9, 16, 22, and 29 days after whitefly-mediated inoculation. After Northern blotting (Maniatis et al., 1982), the blots were hybridized with a coat protein-specific TYLCV DNA clone (pTYH19.3). After removal of the probe, the blot was hybridized with a probe for the pea RbCs gene. The place of the tomato ribosomal RNA 28S and 18S migration in the gel is indicated.

The DNA was submitted to gel electrophoresis, blotted and hybridized with radiolabelled TYLCV DNA. The RNA was submitted to gel electrophoresis, blotted and hybridized with a fragment of the cloned TYLCV genome encoding the virus coat protein. From Figure 19, it can be seen that the TYLCV coat protein gene is transcribed strongly only between week 2 and 3. following infection. The level of transcription of the small subunit of the ribulose biphosphate carboxylase gene (RbcS) remains stable for at least 4 weeks post infection. During this time, the amount of TYLCV DNA increases regularly.

6. A simple and rapid method for the detection of TYLCV in plants and in whiteflies

1. The squash-blot procedure

Leaves from healthy and from TYLCV-infected tomato plants are squashed onto a nylon membrane (Hybond-N, Amersham) using a hard object (pen, glass cylinder, etc.). Squashes are permanently fixed onto the membrane by a 3-min irradiation on a ultraviolet table transilluminator.

TYLCV DNA can be detected directly in squashes of the tested tissues, without any pretreatment of the samples. Tomato leaves squashed onto a dry nylon membrane were hybridized with the TYLCV cloned probe. A strong signal was obtained with TYLCV-infected plant tissues, only (Figure 20).

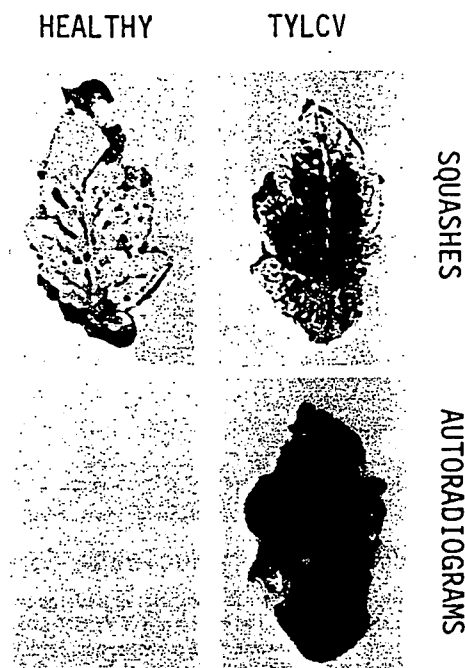


Fig. 20 Autoradiograph of tomato yellow leaf curl virus (TYLCV)-infected tomato leaf squash. Top row: photograph of leaf squash blot from TYLCV-infected and healthy tomato plants. Bottom row: corresponding autoradiograms, following hybridization with the cloned TYLCV-DNA probe.

Squash-blotted material is very stable and can be kept at ambient temperature (free in the air) for several months without losing its hybridization capacity. The blots sustain boiling (for removing probes), alkali treatment (to make double-stranded DNA available to hybridization), and repeated freezing and thawing, without losing their hybridization capacity.

2. TYLCV-DNA probe hybridizes mainly with viral genomic single-stranded DNA present in infected plant squashes

Blots containing squashes of TYLCV-infected leaves were squashed were incubated with nucleases prior to hybridization. Both DNAase I and nuclease S1 completely prevented hybridization, while treatment with DNAase-free RNAase A slightly decreased the hybridization signal. These results indicated that single-stranded TYLCV genomic DNA is the major viral DNA class hybridizing with the TYLCV-DNA probe (Navot et al., 1989).

3. Quantitation of detectable virus in TYLCV infected leaves

Infected leaf tissues were diluted with healthy tissues in defined proportions (infected tissues constituted 100, 50, 20, 10, 5, 2 and 1% of the mixture, by weight). Twenty mg of the mixtures were squashed on a 0.3 cm² dot matrix (sufficient to contain the entire sample). Known amounts of cloned TYLCV DNA were used as standard). The membranes were hybridized simultaneously with the TYLCV probe (Figure 21).

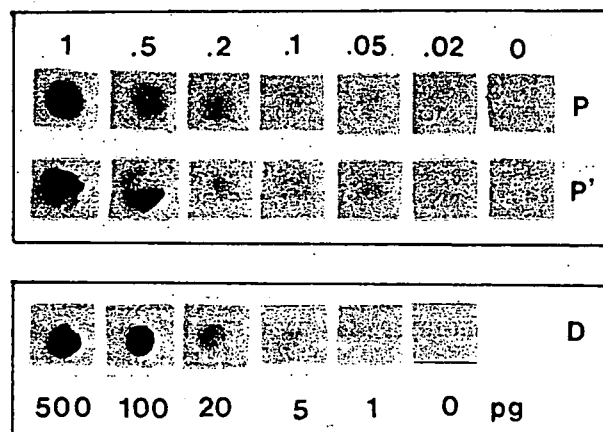


Fig. 21. Quantitation of viral DNA in tomato yellow leaf curl virus (TYLCV)-infected leaf tissues. A leaf from a TYLCV-infected plant and one from an uninfected plant were shredded with a scalpel. The tissues were mixed together in defined portions (w/w), and 20 mg of tissue mix was squashed onto a membrane using a 0.6-cm-diameter dot matrix, sufficient to contain the entire sample. Leaves from two infected plants were used (P and P'). The percentages of infected tissues in the leaf mixes were: 100 (1), 50 (.5), 20 (.2), 10 (.1), 5 (.05), 2 (.02), and 0% (0). Known amounts (in picograms [pg]) of alkali-denatured cloned TYLCV-DNA were spotted (0.4-cm-diameter spot) on the membrane and used as standard (D). One-picogram cloned viral DNA corresponds to 0.3 million copies of the viral genome. The membranes were hybridized with the radiolabelled TYLCV probe and processed for autoradiography.

TYLCV DNA amounts detectable in plant tissues was estimated by comparing visually the hybridization signals. Signals obtained with squashes from two infected plants P and P' were comparable to those obtained with 100 and 20 pg cloned viral DNA (1 pg DNA = 300,000 viral genomes). Hybridization of leaf mixture containing 20% of infected tissue corresponded to 15 million viral genome copies; hybridization of leaf mixtures containing 5% of infected tissues corresponded to 3.5 million viral genome copies. Although the determination of TYLCV amounts is very approximative, it can be estimated that the number of virus genome copies in a leaf from an infected plant is in the range of one million per mg tissue.

The method allows detection of virus amounts corresponding to a few percent of that present in a plant with typical disease symptoms.

4. Detection of TYLCV DNA in infected tomato tissues

The presence of viral nucleic acid was visualized in squashes of roots, stems, leaves, flowers and fruits and a differential distribution of the virus in these tissues was observed. For example, hybridization of cross or longitudinal stem sections indicate that viral nucleic acids in these tissues are found mainly in the vascular system (Fig.22).

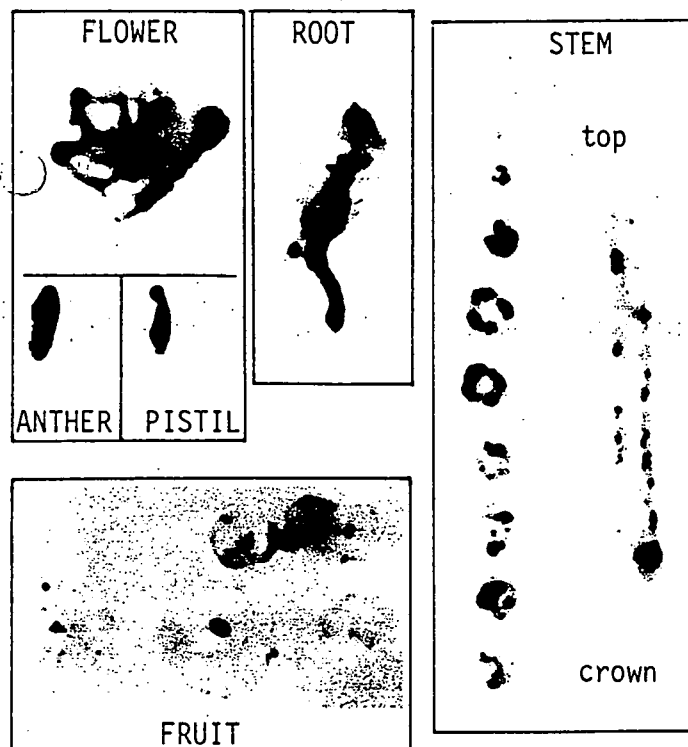


Fig.22. Autoradiograph of tomato yellow leaf curl virus (TYLCV) DNA sequences in squashes of different tissues from a TYLCV-infected plant.

5. Specificity of hybridization with the TYLCV probe

Leaves from tomato plants infected with either cucumber mosaic virus, PVY, or with TMV did not hybridize with the TYLCV probe. Squash-blot from *Abutilon sellovianum* leaves infected with the abutilon mosaic virus (AbMV), a geminivirus (Abouzid and Jeske, 1984), did not react with the TYLCV probe (Navot et al., 1989).

6. Detection of TYLCV DNA in squash-blot of viruliferous whiteflies

Whiteflies kept on a TYLCV-infected or a healthy *Datura* plant for 18 h were individually squashed onto the nylon membrane and hybridized with the viral probe (Figure 23). Viral sequences were detected by autoradiography in each of the whiteflies which fed on the infected plant only. The amount of virus carried by the insects was estimated by spotting known quantities of cloned TYLCV-DNA on the same membrane (from 100 to 1 pg DNA, equivalent to 30 to 0.3 million copies of the viral genome). Although the feeding period of the insects on the infected plant was the same for all the whiteflies, the intensity of the hybridization signals showed considerable variability from one individual to the other. By visual comparison of intensities of hybridization with standards, we estimated that the insects carried between one to 100 million copies of the viral genome.

Viruliferous whiteflies



100 50 10 5 1 pg DNA

Fig. 23. Detection and quantification of tomato yellow leaf curl virus (TYLCV)-DNA sequences in viruliferous whiteflies. Female whiteflies kept on a TYLCV-infected *Datura* plant for an 18-hr virus acquisition period were collected, frozen at -20°C , individually squashed onto a membrane, and hybridized with the viral probe. Quantification was done by spotting known amounts of alkali-denatured TYLCV DNA (100–1 picograms [pg]) onto the same blot, in a range equivalent to 30 to 0.3 million copies of the viral genome. Each spot is the autoradiographic signal given by an individual insect; nonviruliferous whiteflies do not hybridize at all.

7. Detection of TMV- and PVY-RNA in squashes of infected tobacco leaves

The squash-blot method can be used, with minor modifications, to detect RNA viruses. Leaves from uninfected tobacco and from TMV- and PVY-infected tobacco plants were squash-blotted onto a nylon membrane previously saturated with SDS and proteinase K and dried (to denude the viral RNA and protect it from nucleases). TMV was detected after hybridization of squashes with a radiolabelled TMV cDNA. PVY was detected after hybridization with a radiolabelled PVY cDNA clone. Strong and specific signals were obtained with the tissues infected by these two RNA viruses while no signal was obtained with the uninfected tissues (Figure 24).

For RNA detection, pretreatment of the membrane with SDS and proteinase K is obligatory (8 M guanidine-HCl is also adequate). Samples squashed on an untreated membrane hybridize poorly.

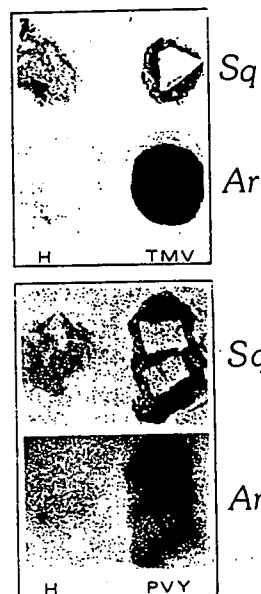


Fig. 24. Detection of tobacco mosaic virus (TMV) and potato virus Y (PVY) RNA sequences in squash blots of TMV- and PVY-infected tobacco leaves. Tissues were squashed onto a nylon membrane presoaked in 0.5% sodium dodecyl sulfate, 100 μ g/ml proteinase K and hybridized with the virus-specific probes. Top panel, first row: photograph (Sq) of leaves from healthy uninoculated (H) and TMV-infected (TMV) tobacco plants; second row: autoradiogram (Ar) following hybridization of squashes with a TMV-RNA reverse transcript. Bottom panel, first row: photograph (Sq) of leaf squashes from healthy uninoculated (H) and PVY-infected (PVY) tobacco plants; second row: autoradiogram (Ar) following hybridization of squashes with a PVY cDNA clone.

8. Use of squash-blots to screen for infected plants in the field; applications in breeding programs

Squashes can be easily done in the field and assayed for the presence of TYLCV DNA sequences in the laboratory. As an example, we have sampled a plot of 170 tomato plants. The tip of cross-sectioned stems was applied once (just to wet the membrane) on a 0.5 x 0.5 cm square, so that all 170 samples could fit on a 12 x 3.5 cm membrane. This was done within one hour. The membrane was then hybridized with the radiolabelled viral probe and TYLCV-infected plants could be detected by autoradiography (Figure 25). Although disease symptoms were not apparent in these plants at the time of sampling, they developed within two weeks.

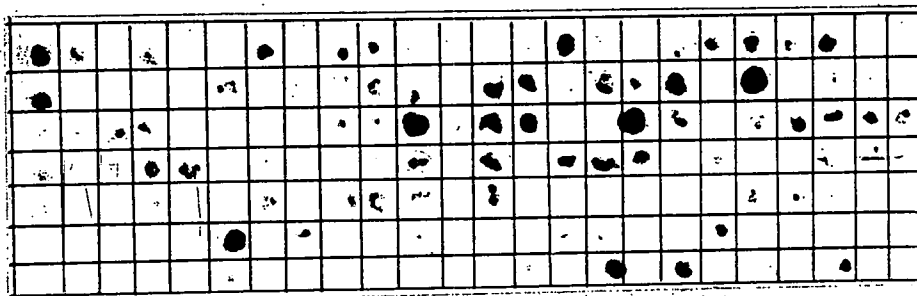


Fig. 25. Field analysis of TYLCV infection. Stems from 170 plants were cross-sectioned and applied once onto a 0.5x0.5 cm grid drawn on a nylon membrane. Samples were hybridized with a TYLCV DNA probe and infected plants detected by autoradiography.

Squash-blots were used to screen large number of plants generated during a breeding program for TYLCV resistance. All the cultivated tomato species (*L. esculentum*) are susceptible to the yellow leaf curl disease. Efforts are in being made to introgress disease resistance traits found in some wild tomato species into tomato cultivars. In three growing seasons, the wild tomato species, *L. chilense* (LA 1969) has not developed the typical viral disease symptoms when grown in fields where *L. esculentum* (cv. M82) was totally infected. No virus was detected in leaf squashes from this wild tomato species, whereas virus was detected in squashes from the cultivated tomato. We are using the squash blot procedure to rapidly screen for TYLCV resistant plant during introgression of resistant genes from the wild species to the tomato cultivar, allowing selection at an early stage with the advantage of accuracy, space and economy of time and manpower (Zakay et al., 1991).

9. Diagnosis of TYLCV epidemics

Squash-blot tests are very stable; they can be mailed from one country to another for sampling and analysis. On November 1987, we sent a membrane containing leaf squashes of healthy control and TYLCV-inoculated tomato from Israel to Turkey. Two months later, leaves from tomato plants grown in glasshouses in the Mersin region were squashed on this membrane. The samples included plants with yellow leaf curl symptoms as well as plants without any symptom. The membrane was returned to us and hybridized with the TYLCV probe on February 1988 (Fig. 10).

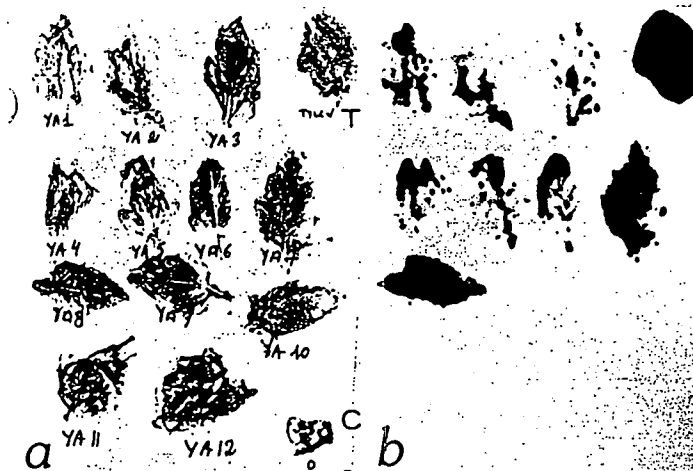


Fig. 26. Diagnosis of tomato yellow leaf curl virus (TYLCV)-infected plants from Turkey. A membrane already containing squashes from control (C) and TYLCV-infected (T) tomato plants applied in Israel was used as a surface on which tomato leaves were squashed in Turkey. The samples, from glasshouse cultures in the Mersin region, included symptomless plants as well as plants showing symptoms similar to those induced by TYLCV (YA1-YA12). The membrane was mailed back to Israel for autoradiographic analysis with the TYLCV-specific probe. A, Photograph of leaf squashes. B, Autoradiogram following hybridization with the TYLCV probe. All samples hybridizing with the TYLCV probe were from plants exhibiting various degrees of yellow leaf curling.

The results showed that out of the 12 samples, 8 were infected with TYLCV. The samples positive for TYLCV were from plants with and without symptoms. All negatives were from symptomless plants. Similarly, we have used the squash-blot method to diagnose the TYLCV disease in Italy, Senegal, Mali, Cape Verde, Taiwan and Thailand (Czosnek et al., 1990).

7. Transformation of tomato protoplasts with TYLCV DNA

1. Replication of TYLCV DNA in tomato protoplasts.

Protoplasts were prepared from a cell suspension originating from a callus of susceptible tomato (line 831). A

head-to-tail full-length TYLCV genomic clone dimer in the pTZ18R plasmid (pTYH20.7) was encapsulated into liposomes, either as a closed circle plasmid or freed from its vector, and was delivered to the cells. The protoplasts were cultured and samples were taken at the times indicated, DNA extracted, electrophoresed, blotted, hybridized with TYLCV DNA and auto-radiographed.

Figure 27 shows that the TYLCV dimer plasmid pTYH20.7 gave rise to molecules which have mobilities similar to those of the TYLCV RFs (supercoiled and relaxed) and to the TYLCV genomic DNA present in TYLCV infected plants. The TYLCV genomic ssDNA is best seen when the dimere is freed from its vector (Figure 27, B).

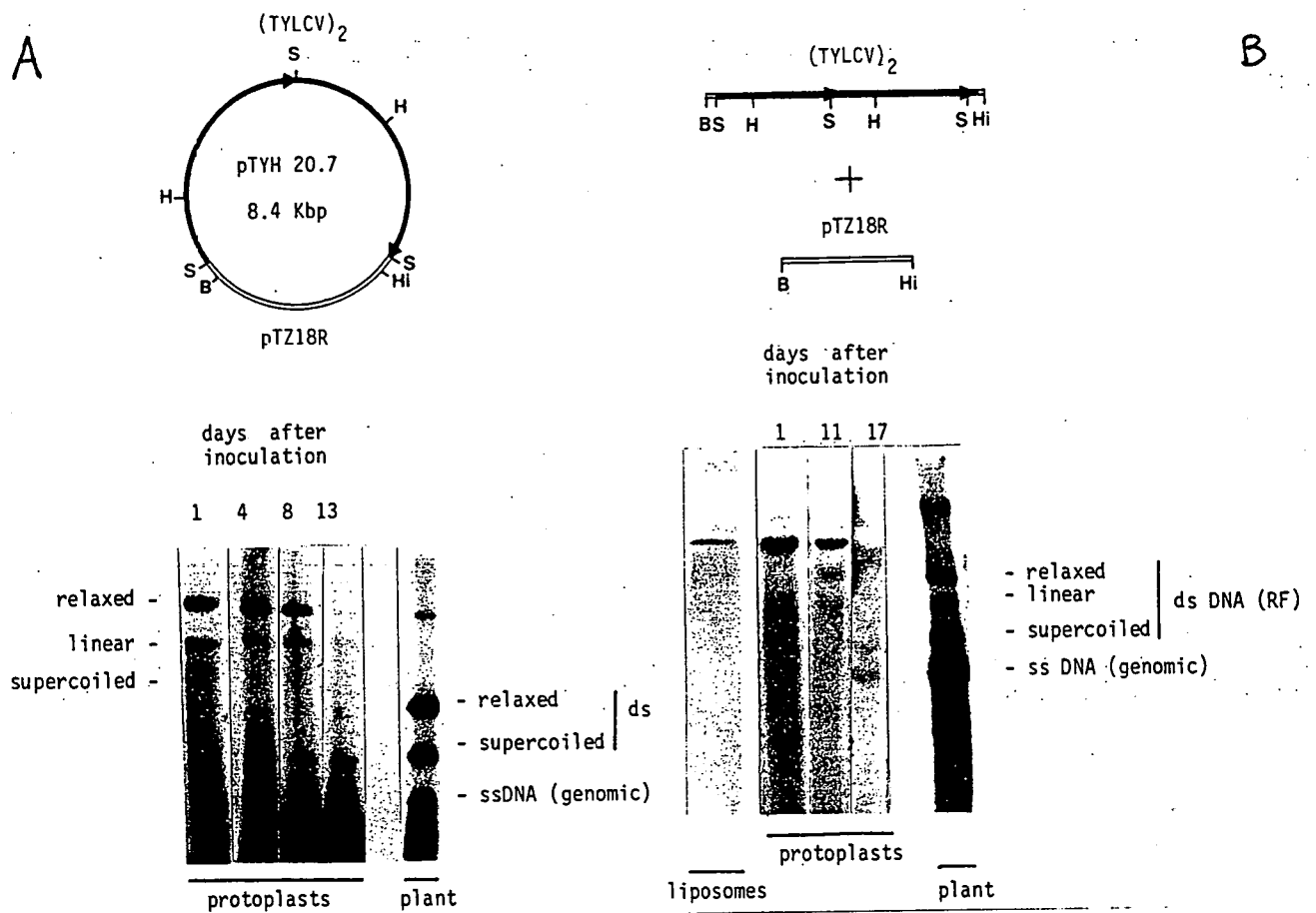


Fig. 27. Replication of TYLCV DNA in tomato protoplasts. A complete head-to-tail TYLCV DNA dimer, either in its vector (A) or freed from its vector (B) was introduced into protoplasts using liposomes. Following inoculation, samples were taken at time intervals as indicated, DNA extracted, subjected to electrophoresis and hybridized with a full-length TYLCV DNA clone. The position of the different TYLCV DNA forms is indicated.

It is likely that a genomic length TYLCV DNA molecule has been excised from the plasmid, probably by homologous recombination, and is replicating in the transfected cells. Therefore the TYLCV DNA dimer has the capacity to replicate in tomato cells from susceptible plants.

A cell line was prepared lately from *L. chilense* which is resistant to TYLCV infection (no virus, no symptoms). Preliminary results indicate that protoplasts from this tomato do not support replication of cloned TYLCV DNA.

2. Transformation of tomato protoplasts with the TYLCV gene encoding the virus capsid proteins and its expression.

The gene encoding the TYLCV coat protein was introduced into the Ti plasmid pGV43851 of *A. tumefaciens* with its own promoter as described in Materials and Methods Section (Figure 28).

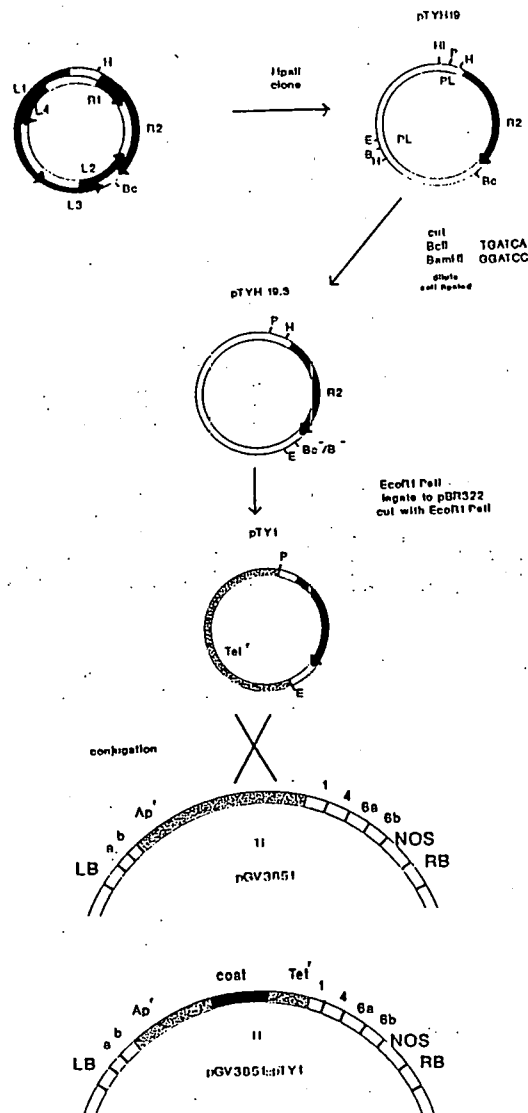


Fig. 28. Introduction of the TYLCV coat protein gene into the *Agrobacterium* Ti plasmid vector pGV3851. See Methods Section for details.

Tomato protoplasts from the tomato suspension cell line 831 were co-cultivated with the *A. tumefaciens*-containing TYLCV coat protein (pGV3851::pTY1). Selection of transformed cells was made by growing them without cytokinins in the culture medium. Calli were formed after one month in culture (Figure 29).

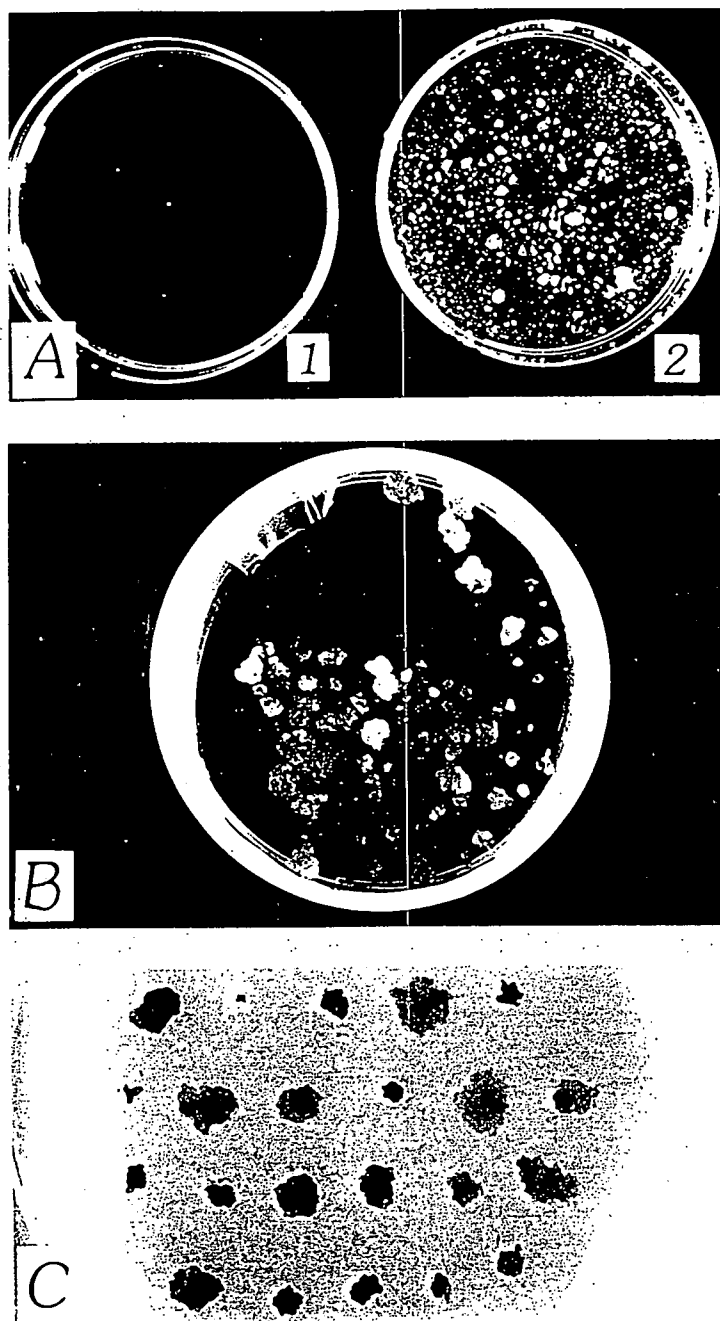


Fig. 29. Calli obtained after co-cultivation of *Agrobacterium* containing the TYLCV coat protein gene (pGV3851::pTY1). A: calli obtained after protoplasts have been cultivated with bacteria for one month (plate 1) and without any bacteria (plate 2). B: Calli in MSX media for acceleration of growth. C: Calli in Tan's greening medium.

Five calli were analyzed; 3 of them contained TYLCV DNA. Two calli expressed the coat protein RNA as determined by Northern analysis. This RNA migrated in a gel with a velocity similar to a molecule present in infected plants (Figure 30).

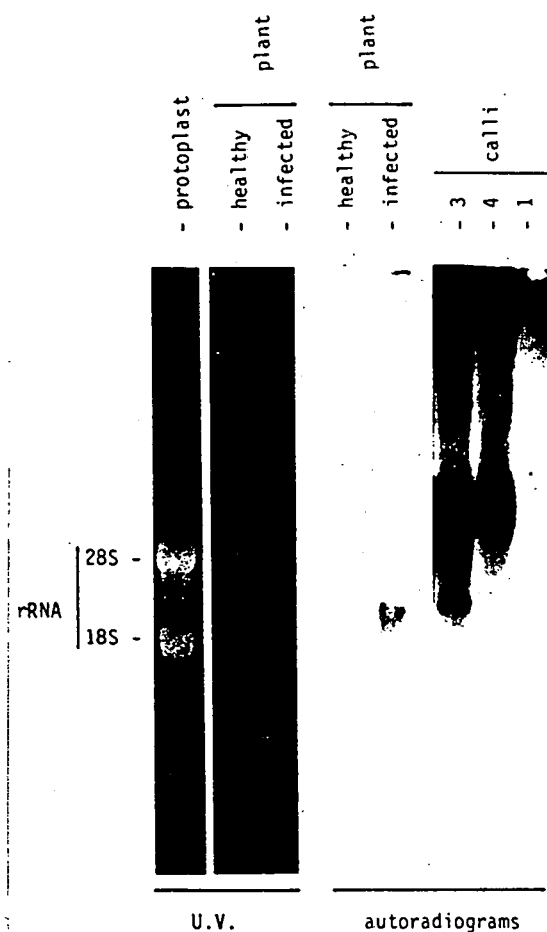


Fig. 30. Transcription of TYLCV coat protein gene in tomato calli following co-cultivation of protoplasts and *Agrobacterium* pGV3851::pTY1. RNA extracted from noninoculated protoplasts, uninfected and TYLCV infected plants, and calli resulting from cocultivation was subjected to electrophoresis, Northern blotted and hybridized with a coat protein-specific DNA probe (PTYH19.3).

Regeneration of whole plants from the TYLCV coat protein positive calli was not possible, despite our efforts in changing culture conditions.

8. Constructions of plasmids containing TYLCV DNA sequences with the potential to induce TYLCV resistance once introduced into tomato plants

The TYLCV genome (Figure 4) was dissected in order to subclone the different open reading frames, with their own

promoter, or with the CaMV 35S promoter, single and double.

ORFs were cloned in the sense orientation as well as in the reverse orientation, as referred to the promoter (anti-sense), either in pBR322 or in pTZ18R. Figure 31 shows the different constructs in the sense and in the antisense orientations. Table 5 gives the nomenclature of the plasmids obtained as well as the TYLCV DNA sequences they contain.

Some of the TYLCV ORFs were introduced either into the pGV3851 Ti plasmid or into the pCGN1547 binary vector.

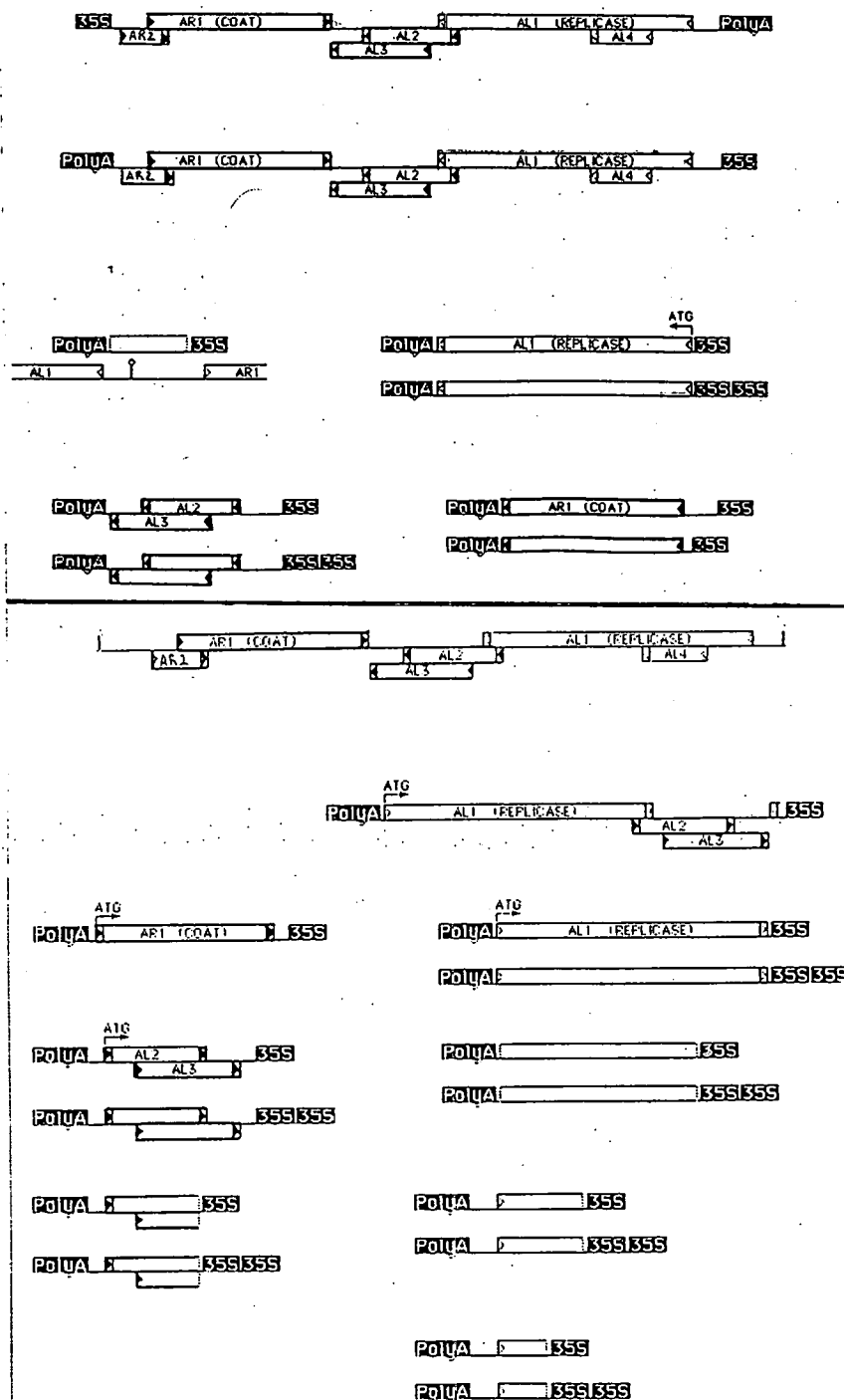


Fig. 31. Constructs containing TYLCV DNA sequences in the sense and antisense orientations, with the potential to be used to transform tomato plants.

Table 5. Plasmids generated during this study.

pLY plasmids (pTY1 pTY2...)			
pLY...	Origin	Date	Description
1	pTYH19.3		ARI + own promotor in pBR322
2	pTYH20.7		TYLCV dimer in pPCV720 (BamHI/HindIII)
3	pTYP33	16.1.89	PstI dimer of pTYP33 in pCGN1547 (PstI)
4	pTYP42	16.1.89	PstI dimer of pTYP42 in 1547 (PstI)
5	pTYP33	16.1.89	PstI monomer of pTYP33 in 1547 (PstI)
6	pTYH19.6	15.1.89	viral frag of pTYH19.6 in pSG Bgl 3525 (BamHI/HindIII)
7	pTYP42	16.1.89	PstI monomer of pTYP42 in 1547 (PstI)
8	pTYH19.3	Pichersky	(syn=UC(4)pSG) ARI with its own promotor in 3525 - Sense
9	pTYH19.3		(syn=pSG) ARI without its own promotor in 3525 - Sense
10	pTYH19.3		(syn=CSAnti) AluI-SphI frag. of ARI in 3525 - AntiSense
11	pTYH19.11	16.2.89	pTYH19.11 viral frag in 3535 (BamHI/PstI)
12	pTYH19.9	16.2.89	pTYH19.9 viral frag in 3535 (BamHI/PstI)
13	pTYH19	16.2.89	pTYH19 viral frag in 3525 (BamHI/HindIII)
14	pTYH20	16.2.89	pTYH20 viral frag in 3525 (BamHI/HindIII)
15	pTYH19S	16.2.89	pTYH19S viral frag in 3525 (BamHI/HindIII)
16	pTY6	17.2.89	pTY6 BglII frag in 1547 (BamHI) orientation A
17	pTY6	17.2.89	pTY6 BglII frag in 1547 (BamHI) orientation B
18	CS Anti	17.2.89	CS Anti BglII frag in 1547 (BamHI) orientation A
19	CS Anti	17.2.89	CS Anti BglII frag in 1547 (BamHI) orientation B
20	pTYH19.8	1.3.89	pTYH19.8 frag in 3535 (BamHI/PstI) Anti Sense for 5' of AL1
21	pTY1		cointegrate of pTY1 in pGV3850
22	pTY11	1.3.89	pTY11 BglII frag in pCGN1547 (BamHI) orient A
23	pTY11	1.3.89	pTY11 BglII frag in pCGN1547 (BamHI) orient B
24	pTY12	1.3.89	pTY12 BglII frag in pCGN1547 (BamHI) orient A
25	pTY12	1.3.89	pTY12 BglII frag in pCGN1547 (BamHI) orient B
26	pTY13	1.3.89	pTY13 BglII frag in pCGN1547 (BamHI) orient B
27	pTY14	1.3.89	pTY14 BglII frag in pCGN1547 (BamHI) orient A
28	pTY15	1.3.89	pTY15 BglII frag in pCGN1547 (BamHI) orient B
29	pTY19.13	10.3.89	pTY19.13 B/P frag in 3525 (B/P) AntiSense
30	pTYH19.11	15.3.89	pTYH19.11 B/P frag in 3525 (B/P) AntiSense
31	pTYH19.8	15.3.89	pTYH19.8 B/P frag in 3525 (B/P) AntiSense
32	pTYH20.9	15.3.89	pTYH20.9 B/P frag in 3525 (B/P) AntiSense
33	pTYH20.9	15.3.89	pTYH20.9 B/P frag in 3535 (B/P) AntiSense
34	pTYH20.11	15.3.89	pTYH20.11 B/P frag in 3525 (B/P) Sense
35	pTYH20.11	15.3.89	pTYH20.11 B/P frag in 3535 (B/P) Sense
36	pTYH20.12	15.3.89	pTYH20.12 B/P frag in 3525 (B/P)
37	pTYH20.12	15.3.89	pTYH20.12 B/P frag in 3535 (B/P)
38	pTYE1.1	15.3.89	pTYE1.1 B/P frag in 3525 (B/P)
39	pTYH19.9	15.3.89	pTYH19.9 B/P frag in 3525 (B/P)
40	pTY20	25.4.89	pTY20 BglII frag in pCGN1547 (BamHI) orient B
41	pTY29	25.4.89	pTY29 BglII frag in pCGN1547 (BamHI) orient A
42	pTY29	25.4.89	pTY29 BglII frag in pCGN1547 (BamHI) orient B
43	pTY29	25.4.89	pTY29 BglII frag DIMER in pCGN1547 (BamHI) orient B
44	pTY30	25.4.89	pTY30 BglII frag in pCGN1547 (BamHI) orient B
45	pTY31	25.4.89	pTY31 BglII frag in pCGN1547 (BamHI) orient A
46	pTY31	25.4.89	pTY31 BglII frag in pCGN1547 (BamHI) orient B
47	pTY33	25.4.89	pTY33 BglII frag in pCGN1547 (BamHI) orient A
48	pTY33	25.4.89	pTY33 BglII frag DIMER in pCGN1547 (BamHI) orient A
49	pTY39	25.4.89	pTY39 BglII frag in pCGN1547 (BamHI) orient B
50	pTY39	25.4.89	pTY39 BglII frag DIMER in pCGN1547 (BamHI) orient B

51	UC(4)	25.4.89	UC(4) BglII frag in pCGN1547 (BamHI) orient B
52	pSG	25.4.89	pGS BglII frag in pCGN1547 (BamHI) orient B
53	PTY32	5.5.89	PTY32 " " " A
54	PTY38	"	PTY38 " " " "
55	PTY34	9.5.94	PTY34 " " " A
56	PTY35	"	PTY35 " " " "
57	PTY36	"	PTY36 " " " "
58	PTY37	"	PTY37 " " " "

PTYH19 derived plasmids

pTYH	Origin	Date	Description
19.1	PTYH19		(syn - 19S) SphI cut and self ligated pTYH19
19.2	"		(syn - 19E) EcoRII cut and self ligated pTYH19
19.3	"		BclII/BamHI cut and self ligated pTYH19
19.5	"		XbaI cut and self ligated pTYH19
19.6	PTYH19.5		SphI cut and self ligated pTYH19.5
19.8	"	5.2.89	Sau3A 531bp frag of pTYH19 in pTZ18 (BamHI)
19.9	"	5.2.89	TaqI/BamHI 370bp frag of pTYH19 in pTZ18 (BamHI/AccI)
19.11	PTYH19.5	7.2.89	TaqI/BamHI 400bp frag of pTYH19.5 in pTZ18 (BamHI/AccI)
19.12	PTYH19.1	24.2.89	1147bp HinfI frag containing AL1 in pTZ18U (HincII) Sense
19.13	PTYH19.1	24.2.89	1147bp HinfI frag containing AL1 in pTZ18U (") AntiSense
19.14	19.1	Pichowsky	(syn-19S45) SacI cut and self ligated pTYH19.1
19.15	"	"	(syn-10-1) ~600bp Sau3A frag. of pTYH19.1 in pUC18 Bam
19.16	"	"	" 2-2 ~360bp HaeIII " HincII
19.17	"	"	" 8-2 ~200bp Sau3A " Bam

PTYH20 derived plasmids

pTYH	Origin	Date	Description
20.1	PTYH20		1.33 x TYLCV genome = pTYH20 + HincII/BamHI frag of pTYE
20.3	PTYH20.1		TYLCV SacI dimer in pTZ18 (SacI) has no viral HincII sites
20.4	PTYH20		1.33 x TYLCV genome = pTYH20 + HincII/BamHI frag of pTYE
20.5	PTYH20.4		TYLCV SphI monomer in pTZ18 (SphI) has the HincII sites
20.6	PTYH20.4		TYLCV SphI dimer in pTZ18 (SphI) has the HincII sites Dr. A
20.7	PTYH20.4		TYLCV SphI dimer in pTZ18 (SphI) has the HincII sites Dr. B
20.8	PTYH20	24.2.89	HinfI fragment containing AL1 in pTZ18U (HincII) AntiSense
20.9	"	"	DraI fragment of AL1 in pTZ18U (HincII) AntiSense
20.10	"	"	DraI fragment containing AL2/3 in pTZ18U (HincII) AntiSense
20.11	"	"	DraI fragment containing AL2/3 in pTZ18U (HincII) Sense
20.12	"	3.3.89	HinfI fragment containing AL1 in pTZ18U (HincII) Sense
20.13	"	"	(syn-20E) EcoRI cut and self ligated pTYH20
20.14	"	"	(syn-20S) SphI " "
20.15	20.13	Pichowsky	(" 11-2) ~150bp HaeIII frag. of pTYH20.13 in pUC18 HincII
20.16	"	"	(" 11-4) ~500bp " " "
20.17	"	"	SacI cut and self ligated pTYH20.13
20.18	20.14	"	(syn-6-5) ~150bp Sau3A frag. of pTYH20.14 in pUC18 BamHI
20.19	"	"	(" 7-8) ~450bp " " "
20.20	"	"	(" 9) ~900bp AsaI subclone of pTYH20.14
20.21	"	"	(" 14) ~450bp " " "
20.22	"	"	(" 15) ~750bp " " "

9. Transformation of tomato leaf discs with *A. tumefaciens* containing recombinant Ti plasmids with TYLCV DNA sequences

The TYLCV coat protein gene with its own promoter cloned into pBR322 was introduced into the shooter Ti plasmid pGV3851 in Agrobacterium C58, using the triparental method. The TYLCV coat protein was also cloned in pBR322 in the antisense polarity relative to its own promoter and was introduced into C58 as above.

The C1 ORF (or AL1) which encodes a protein necessary for TYLCV replication was cloned (in sense and antisense orientations) in the pCGN1547 binary vector (which provides resistance to agromycin) and introduced into competent Agrobacterium LBA4404 cells by the liquid nitrogen method.

Tomato plants were transformed using the leaf disc method. Plants transformed with the pGV3851 vector were selected on a medium without hormones; transformed shoots were confirmed by Southern blot analysis. Plants transformed with pCGN1547 were selected on gentamycin and transformation confirmed by Southern analysis.

Transformed plants regenerated and mature plants were obtained. These plants are presently tested for TYLCV resistance using the whitefly-mediated inoculation system.

DISCUSSION

1. Purification of TYLCV

The difficulties in obtaining high yields of TYLCV are a serious limit in the production of a high quality antiserum. Beside the relatively low titer of our antiserum, it also reacted unspecifically with some of the plant components. These limits can be overcome by using our antiserum for immunostaining of western blots derived from concentrated crude extracts from tested plants.

TYLCV was shown to be serologically related to other whitefly-transmitted geminiviruses.

2. TYLCV, a whitefly-transmitted geminivirus with a single genomic component

On the basis of the genome-structure of whitefly-transmitted geminiviruses, it was assumed that a bipartite genome will be also found for TYLCV. However, all the clones obtained from the viral RF were from a DNA A-like component (Navot et al., 1991). We demonstrated that the single component of the TYLCV genome has the capacity to cause systemic infection in tomato through agroinoculation. However, in using this method to produce an insect-transmitted viral disease one might miss a second genomic component or a viral function, essential for the plant-to-plant transmission of the virus. To verify the capacity of the cloned genome to complete the infection cycle, we used whiteflies to transfer the disease from the agroinoculated plants to test plants. Typical disease appeared in all but one of the 16 plants. Viral nucleic acid were detected in all the tissues of the infected plants. Therefore the unique genomic molecule of TYLCV carries all the information needed for its transmission by insects, replication and systemic spread. In other whitefly-transmitted geminiviruses, the presence of the B component is necessary for virus movement through the plant (Rogers et al., 1986; Sunter et al., 1987; Etessami et al., 1988). A Thai isolate of TYLCV from which 2 genomic components were cloned is an exception to that rule. When dimeric copies of DNA A alone were agroinoculated into tobacco and tomato plants, this component was capable both of symptom production and of systemic movement in the infected tissue (Rochester et al., 1990). A TYLCV isolate from Sardinia, Italy, has a genomic organization and behaves like the TYLCV from Israel (Gronenborn et al., in preparation). It is possible that these 3 viruses represent a time in the evolution of whitefly-transmitted geminiviruses, prior to the specialization of a second genomic component, before DNA A became reliant on it for production of systemic infection. The absence of genome B in the Israeli and Italian TYLCV isolate supports that DNA B has no function in the interaction of a whitefly-transmitted geminivirus with its vector (Etessami et al., 1988).

3. Analysis of TYLCV genome

Sequence analysis of the cloned TYLCV genome revealed the presence of 6 open reading frames encoding proteins larger than 10 KDa: 2 on the virion strand and 4 on the complementary strand. The second ORF on the virion strand in addition to the ORF for the coat protein (designated V2), was analogous to a 13.1 KDa ORF found in ACMV, but not in other whitefly-transmitted geminiviruses (e.g. AbMV, BGMV, TGMV). A comparable ORF (designated V1 or R2) is present in the genomes of all the leafhopper-transmitted geminiviruses though the homology it shares with V2 of TYLCV is limited. Deletion of V1 from the genome of WDV had no effect on its ability to replicate in protoplasts of cereals (Laufs et al., 1990). Mutational analysis of this ORF in MSV showed that it encodes an essential function, most likely associated with systemic spread of the virus (Boulton et al., 1989; Lazarowitz et al., 1989). In contrast, its complete deletion in ACMV had no effect on the ability of the mutated DNA A to cause systemic infection, following its introduction, together with DNA B, into *N. benthamiana* (Etessami et al., 1989; Kinkenberg et al., 1989). It might be that the 13.1 KDa ORF in ACMV has the same function as the V1 of MSV but is used by the virus to facilitate its spread only in some of its plant hosts. The loss of this gene in other whitefly-transmitted geminiviruses might have occurred during their evolution, as they, or their progenitor virus, acquired new hosts for whom no such function was needed. Mutational analysis of V2 in TYLCV is expected to assist in determining the function of this ORF.

Substantial differences in nucleotide sequence exist among the 3 isolates of TYLCV. The 75% identity between the DNA sequences in the Israeli and Sardinian isolates is comparable to that found between TYLCV and ACMV. This variance, considered together with the presence of a B component in the Thai isolate raises the question of their taxonomy. Should they be called strains of TYLCV or designated as different virus species? The inadequacy of symptom analogy for determining relatedness was already discussed (Howarth and Vandemark, 1989). Classification on basis of functional aspects of the viral genome might be more meaningful.

4. TYLCV in the viral capsid, the infected plant and the whitefly vector

TYLCV DNA was used as a probe to identify and analyze virus-related DNAs in the viral capsid, in infected tomato plants and in the virus vector, the whitefly. In addition to the single-stranded viral genomic DNA, double-stranded virus-related DNA molecules were detected in infected plants. Not all of the virus-related DNA forms are present simultaneously in the infected plant. The double-stranded molecules, which are probably the replicative form of the viral genome, have been purified from an infected tomato plant and used to clone

the TYLCV genome. In the viruliferous whitefly, only the single-stranded unit-size viral genome was detected (Czosnek et al., 1989).

5. Molecular analysis of TYLCV infection

Virus accumulation and spread during the development of the tomato yellow leaf curl disease have not been previously characterized. We have investigated correlations between development of disease symptoms and accumulation of TYLCV-DNA, and between susceptibility to infection and stage of growth of leaf (Ber et al., 1990).

TYLCV-DNA can be detected in lysates from infected plant tissues by hybridization with a TYLCV DNA probe (Czosnek et al., 1988a). The use of lysates provided an accurate estimation of virus concentration in these tissues, since it minimized nucleic acid losses which are inevitable in the process of DNA purification.

Using these methods we followed the time-course of TYLCV-DNA accumulation in tissues of the same physiological age, following inoculation by whitefly.

While disease symptoms became visible 15 days post-inoculation, TYLCV-DNA was detectable in these tissues as early as eight days after inoculation, or seven days before disease symptoms were observed. In plants with symptoms appearing 18 days after inoculation, TYLCV-DNA could be detected 10 days earlier. Similar results were obtained when the appearance of symptoms was correlated with the presence of TYLCV-DNA in plants from an experimental field (L. esculentum cv. M82). In more than 60% of the plants, TYLCV-DNA could be detected at least two weeks before the appearance of symptoms. The lag between appearance of detectable amounts of viral DNA and that of disease symptoms could be used for early detection of TYLCV infection in the field and greenhouse.

The analysis of the distribution of the viral DNA in infected plants has shown that large amounts of TYLCV-DNA accumulated in the shoot apex. The high concentration of TYLCV-DNA found in the roots seems to be due mainly to viral DNA transport and not to replication in the root tip cells (the root tissues analyzed included the root tips which in the 8-leaf stage tomato constitute about 1/1000 of the root weight). High concentrations of viral DNA were also found in young leaves, but not in older leaves and cotyledons. The stem also contained large amounts of TYLCV, probably in the vascular tissue.

Double-stranded DNA-viruses and RNA-viruses have different accumulation patterns. The concentration of cauliflower mosaic virus (CaMV) DNA and protein products in the developing leaves of turnip have been measured by spot hybridization and Western blot analysis. No correlation was found between CaMV multiplication and host DNA synthesis; viral DNA increased in concentration with leaf age (Maule et al.,

1989). Similar observations were made upon inoculation of Nicotiana glutinosa plants with tobacco mosaic virus (TMV): virus concentration was higher in middle-aged and older leaves than in young ones (reviewed by Matthews, 1981).

To better understand the process of TYLCV infection, we have inoculated plants at the 8-leaf stage at specific sites using leaf cages. The spread of the virus in the plant tissues was followed thereafter using molecular hybridization of lysates with the virus-specific probe. We found that youngest tissues are the best targets for virus inoculation. In these tissues, the viral DNA replicated quickly at the site of inoculation and was rapidly transported, first to the roots, then to the shoot apex, neighboring leaves and flowers. If inoculation was done on middle-aged leaves, viral DNA replication and transport followed the same pattern but was slower than when inoculation was on younger tissues. In the oldest tissues, inoculation was very inefficient.

These findings indicate that replication and transport of TYLCV-DNA is dependent on the developmental stage of the inoculated tissues. It seems that there is a gradient of susceptibility to TYLCV infection in the tomato plant, upper leaves being more susceptible than lower leaves. In cassava, it was suggested that the low virus DNA content of older leaves was due to the fact that whiteflies feed preferentially on young leaves, their number decreasing progressively with leaf age (Fargette et al., 1987). In our experiments where equal numbers of whiteflies were used to inoculate young and old tomato leaves, the low virus DNA content of old leaves was not correlated with the number of feeding insects, but probability resulted from a decrease in susceptibility accompanying leaf aging. In tomato, it is possible that phloem cells in older leaves are less accessible to the whitefly than in younger tissues, or that virus replication is somehow inhibited in these tissues.

Long distance movement of viruses in whole plants has been studied mainly with TMV (reviewed by Atabekov and Dorokhov, 1984, and Zaitlin and Hull, 1987). In the classical experiments of Samuel (1934), TMV, upon inoculation into a terminal leaflet of a middle-aged tomato plant, moved first to the roots, then to the young leaves, and finally to the leaflets close to the infected one. Some time later, the middle-aged and older leaves became infected. Although the pattern of TMV and TYLCV accumulation in the plant leaves appears to be different (Matthews, 1981), the time-course of TMV movement upon inoculation resembles that of TYLCV.

It is interesting to note the similarity between the transport of TYLCV in the tomato plant and the movement of assimilates (Ho and Hewitt, 1987). The younger leaves export assimilates mainly to the roots, similar to the transport of TYLCV from the site of inoculation. The flowers which are strong sinks for assimilates also accumulate large quantities of the virus. Since assimilates and TYLCV both move through the phloem vessels, the association between them is not sur-

prising.

The form in which TYLCV moves in the plant is not clear. Virus-like particles have been observed in the nuclei of phloem parenchymal cells from TYLCV-infected tomato (Cherif and Russo, 1983; Russo et al., 1980). On the other hand, it has been demonstrated for TGMV, another whitefly-transmitted geminivirus, that the coat protein is not required for virus replication and systemic spread of virus DNA (Brough et al., 1988). It is therefore possible that TYLCV is transported through tomato tissues as unencapsidated DNA molecules.

The presence of virions in the infected tissue is limited for a distinct period of time during the early stages after infection. However the viral nucleic acid is detectable at any time after infection. Therefore, the use of a DNA probe for diagnostics is preferable to the use of antibodies.

6. Replication of cloned TYLCV DNA in tomato protoplasts

Clone TYLCV DNA can replicate once introduced into protoplasts from tomato plants susceptible to TYLCV. This system will allow to study TYLCV replication and expression at the cell level. It will also help to dissect the functions of the 6 TYLCV ORFs.

In addition, the in vitro TYLCV inoculation system may be developed into a tool to screen and select TYLCV-resistant plants in the course of a breeding program. In such a system cloned TYLCV DNA could be used for direct inoculation of protoplasts from the plant population to be selected. It is likely, as indicated by preliminary experiments, that cells from TYLCV resistant plants (L. chilense) would not support TYLCV replication.

This approach should eliminate the need to grow plants to maturity, to inoculate them via the insect vector and to perform extensive field tests already in the first stages of the breeding program.

7. Rapid diagnosis of TYLCV

The squash-blot procedure which we have described provides a specific, rapid and simple means of using molecular hybridization techniques to detect infected plant tissues (Navot et al., 1989).

The diagnosis of TYLCV-infected tissues by hybridization was performed directly on tissues squashed onto a membrane. No pretreatment of the samples (e.g. preparation of saps, lysates, nucleic acids) was necessary. Nylon membranes are the support of choice for squashed samples; nitrocellulose membranes are too fragile. Incubation of squash-blot with either SDS and proteinase K (to denude viral nucleic acids) prior to hybridization did not significantly increase hybridization signals. Therefore the hybridizable viral DNA is either not encapsidated in the plant cells or is released from the capsid during squashing as a result of a mechanical

or a proteolytic effect.

With our method, we estimated that in a leaf with typical symptoms, TYLCV DNA is present in the range of one million genome copies per mg infected tissue. Dilution experiments showed that with this method it is possible to detect as few as 50,000 genome copies per mg tissue (0.15 pg virus DNA) (less if autoradiographic exposure is extended to several days). Sensitivity of the method can be favourably compared with that of spot and Southern blot hybridizations. To reach levels of detection similar to that provided by squash-blot, spotted DNA samples should contain at least 1 µg plant DNA. Such DNA amounts are obtained after processing 200-500 mg leaf tissue. Therefore, because samples do not have to be processed prior to detection, the squash-blot method minimizes losses of hybridizable viral DNA.

As a probe we employed full-length clones or cloned ORFs. For routine TYLCV diagnostics, these probes are adequate since there are no other geminiviruses infecting tomato in our region. In the case where a plant may be infected by two geminiviruses, virus-specific probes can be prepared from the 200 nucleotide intergenic region which seems to be unique to each one of the geminiviruses.

The squash-blot method can be applied to the detection of other geminiviruses. The method can be slightly modified to enable diagnosis of double-stranded DNA and RNA viruses. For the detection of double-stranded DNA viruses, it may be necessary to preincubate the squash-blot in alkali prior to hybridization with a specific probe. As we have demonstrated for the diagnosis of TMV- and PVY-infected tissues, a prerequisite for the detection of RNA viruses is the preincubation of the membrane with RNAase inhibitors such as SDS and protease K.

Squash-blotting provides a tool for rapid large-scale diagnostics of TYLCV and other viruses as discussed above. A small piece of tissue is sufficient for analysis, and an entire field can be sampled on the spot in a short time by untrained personnel.

TYLCV can be detected in squashes of whiteflies. Single-stranded TYLCV genomic DNA is the only viral DNA form found in viruliferous whiteflies. Because the virus circulates in the insect's body, it is assumed that the viral genome is kept intact only if encapsidated. Therefore, it is likely that the viral genome is denuded (mechanically or enzymatically) during squashing. TYLCV concentration in whiteflies can be estimated using TYLCV DNA standards. The comparison between the amounts of virus detectable in the viruliferous whiteflies (in the range of one million per microgram tissue) with those found in the infected leaf (in the range of one million per milligram tissue) indicated that the insect concentrates the virus in its body in several orders of magnitude.

Squash-blotting samples are surprisingly stable. Squash-blotting kept for six months at ambient temperatures, did not

show any reduction in their hybridization capacity. We have shown that blots could be mailed from one country to another for sampling and analysis. Therefore the method can be used for large-scale epidemiological studies conducted either by monitoring the spread of viruliferous insect vectors or by the identification of virus-infected plants (Czosnek et al., 1990). Squash-blotting can also be used in quality control and in the monitoring of plant resistance to viral diseases in the course of breeding programs (Zakay et al., 1990).

8. Transgenic tomato plants containing TYLCV DNA sequences

We have generated a battery of plasmids which contain the TYLCV ORFs, in the sense and antisense orientations, relative to their own promoter or to the CaMV 35S promoter. The TYLCV DNA sequences were introduced into plants via Agrobacterium vectors, and transgenic plants have been obtained which contain two TYLCV ORFs: the coat protein gene and the C1 ORF which is thought to be implicated in virus replication.

Up to now, engineering plants with the virus coat protein (in both sense and antisense orientations) was the favorite of the plant geneticists; this approach has been successful with several RNA viruses (see reviews by Beachy, 1990; and by Nelson et al., 1990). It is likely that it was also attempted with geminiviruses, but nothing has been published yet. By trying to transform and express TYLCV genes, the coat protein and the replication-related protein, we may increase our chances to obtain transgenic plants resistant to TYLCV infection. Such plants are presently tested.

LITERATURE CITED

- Abuzid AM, Frischmuth T and Jeske H (1988) A putative replicative form of the Abutilon mosaic virus (gemini group) in a chromatin-like structure. *Mol. Gen. Genet.* 212:252-258.
- Abouzid AM and Jeske H (1984) The purification and characterization of gemini particles from Abutilon mosaic virus infected Malvacea. *J. Phytopathology* 115:344-353.
- Al-Musa A, (1982) Incidence, economic importance, and control of tomato yellow leaf curl in Jordan. *Plant Dis* 66:561-563.
- An G, Ebert PR, Mitra A and Ha SB (1988) Binary vectors. In: *Plant Molecular Biology Manual*. Gelvin SB, Schilperoort RA and Verma DPS eds. Section A3, pp. 1-19. Kluwer Academic Publishers, Dordrecht. The Netherlands.
- Andersen MT, Richardson KA, Harbison S and Morris BAM (1988) Nucleotide sequence of the geminivirus chloris striate mosaic virus. *Virology* 164:443-449.
- Atabekov JG, Dorokhov YL (1984) Plant virus-specific transport function and resistance of plants to viruses. *Advances in Virus Research* 29:313-364.
- Baulcombe DC, Flavell RB, Boulton RE and Jellis GJ (1984) The use of cloned hybridization probes to detect viral infection in a potato breeding programme. In: *Annual Proceedings of the Phytochemical Society of Europe*, Lea PJ and Stewart GR eds., Clarendon Press, Oxford, 23:183-195. .
- Beachy RN, Loesch-Fries S and Tumer NE (1990) Coat protein-mediated resistance against virus infection. *Annu. Rev. Phytopathol.* 28:451-474.
- Ber R, Navot N, Zamir D, Antignus Y, Cohen S and Czosnek H (1990) Infection of tomato by the tomato yellow leaf curl virus: susceptibility to infection, symptom development, and accumulation of viral DNA. *Arch. Virol.* 112:169-180.
- Birchmeier C, Grosschedl R and Biernstiel ML (1982) Generation of authentic 3' termini of an H2A mRNA in vivo is dependant on a short inverted DNA repeat and on spacer sequences. *Cell* 28: 739-745.
- Bisaro DM, Hamilton WDO, Coutts RHA and Buck KW (1982) Molecular cloning of the two DNA components of tomato golden mosaic virus. *Nucleic Acids Res.* 10:4913-4922.
- Boulton MI and Markham PG (1986) The use of squash-blotting to detect plant pathogens in insect vectors. In: *Developments and Applications in Virus Testing*, Jones AC and Torrance L, eds., The Lavenham Press Ltd., Lavenham, Sudbury, Great Britain, pp. 55-69.
- Boulton MI, Steinkellner H, Donson J, Markham PG, King DI and Davies JW (1989) Mutational analysis of the virion-sense genes of maize streak virus. *J. gen. Virol.* 70:2309-2323.
- Breatnach R and Chambon P (1981) Organization and expression of eucaryotic split genes coding for proteins. *Annu. Rev. Biochem.* 50: 349-383.
- Brough CL, Hayes RJ, Morgan AJ, Coutts RHA, Buck KW (1988) Effects of mutagenesis on the ability of cloned tomato

golden mosaic virus DNA to infect Nicotiana benthamiana plants. J Gen Virol 69:503-514.

Carmon Y, Czosnek H, Nudel U, Shani M and Yaffe D (1982) DNAase I sensitivity of genes expressed during myogenesis. Nucleic Acids Res. 10:3085-3098.

Cherif C and Russo M (1983) Cytological evidence of the association of a geminivirus with the tomato yellow leaf curl disease in Tunisia. Phytopath Z 108:221-225.

Chilton M-D, Drummond MH, Merlo DJ, Sciaky D, Montoya AL, Gordon MP and Nester EW (1977) Stable incorporation of plasmid DNA into higher plants: the molecular basis of crown gall tumorigenesis. Cell 11:263-271.

Clarck MF and Adams AN (1977) Characteristics of microplate method of enzyme-linked immunosorbant assay for the detection of plant viruses. J. Gen. Virol. 34:475-483.

Cohen S (1990) Epidemiology of whitefly-transmitted viruses. In: Gerling D (ed.) (1990) Whiteflies: their bionomics, pest status and management. Intercept Ltd, Andover, Hans, UK. pp. 211-225.

Cohen S and Harpaz I (1964) Periodic, rather than continual acquisition of a new tomato virus by its vector, the tobacco whitefly (Bemisia tabaci Gennadus). Ent Exp Appl 7:155-166.

Cohen S, Kern J, Harpaz I and Ben-Joseph R (1988) Epidemiological studies of the tomato yellow leaf curl virus (TYLCV) in the Jordan Valley, Israel. Phytoparasitica 16:259-270.

Cohen S and Nitzany FE (1966) Transmission and host range of the tomato yellow leaf curl virus. Phytopathology 78:508-512.

Czosnek H, Ber R, Navot N, Zamir D, Antignus Y and Cohen S (1988a) Detection of tomato yellow leaf curl virus in lysates of plants and insects by hybridization with a viral DNA probe. Plant Dis 72:949-951.

Czosnek H, Ber R, Antignus Y, Cohen S and Zamir D (1988b) Isolation of tomato yellow leaf curl virus, a geminivirus. Phytopathology 78:508-512.

Czosnek H, Ber R, Navot N, Antignus Y, Cohen S and Zamir D (1989) Tomato yellow leaf curl virus DNA forms in the viral capsid, in infected plants and in the insect vector. J Phytopathology. 125:47-54.

Czosnek H and Navot N (1988) Virus detection in squash-blots of plants and insects: application in diagnostics, epidemiology and breeding. In: Biotechnology in Agriculture, Mizrahi A ed., Alan A Liss Inc, NY, pp. 83-96.

Czosnek H, Navot N and Laterrot H (1990) Geographical distribution of tomato yellow leaf curl virus. A first survey using a specific DNA probe. Phytopath. Medit. 29:1-6

Davies JW (1987) Geminivirus genomes. Microbiological Sciences 4:18-23.

Davies JW and Stanley J (1989) Geminivirus genes and vectors. Trends in Genetics 5:77-81.

Devereux J, Haerberli P and Smithies O (1984) A compre-

hensive set of sequence analysis for the VAX. Nucleic Acids Res. 12: 387-395.

Donson J, Accotto GP, Boulton MI, Mullineaux PM and Davies JW (1987) The nucleotide sequence of a geminivirus from Digitaria sanguinalis. Virology 161:160-169.

Engvall E and Perlman P (1971) Enzyme-linked immunoabsorbent assay (ELISA) - quantitative assay of immunoglobulin G. Immunochemistry 8:871-890.

Etessami P, Callis R, Ellwood S and Stanley J (1988) Delimitation of essential genes of cassava latent virus DNA 2. Nucleic Acids res. 16:7043-7060.

Etessami P, Watts J and Stanley J (1989) Size reversion of African cassava mosaic virus coat protein gene mutants during infection of Nicotiana benthamiana. J. Gen. Virol. 70:277-289.

Fargette D, Thouvenel J-C and Fauquet C (1987) Virus content of leaves of cassava infected by African cassava mosaic virus. Ann Appl Biol. 110:65-73.

Feinberg AP and Vogelstein B (1984) A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity, (Addendum). Anal. Biochem. 137:266-269.

Frischmuth T, Zimmat G and Jeske H (1990) The nucleotide sequence of the Abutilon mosaic virus reveals prokaryotic as well as eukaryotic features. Virology 178:461-468.

Gad AE, Elyashiv G and Rosenberg N (1986) The induction of large unilamellar vesicle fusion by cationic polypeptides: the effect of mannitol, size, charge density and hydrophobicity of cationic polypeptides. Biochim. Biophys. Acta 860:314-324.

Ganadi F, Mansky LM, Medici R, Miller WA and Hill JH (1990) Genetic engineering of plants for virus resistance. Arch. Virol. 115:1-21.

Gerling D (ed.) (1990) Whiteflies: their bionomics, pest status and management. Intercept Ltd, Andover, Hans, UK.

Goodman RM (1977) Single-stranded DNA genome in a whitefly-transmitted plant virus. Virology 83:171-179.

Grimsley N, Hohn B, Hohn T and Walden R (1986) "Agroinfection," an alternative route for viral infection of plants by using the Ti plasmid. Proc. Natl. Acad. Sci. USA 83:3282-3286.

Hain R, Stabel P, Czernilowsky AP, Steinbiss HH, Herrera-Estrella L and Schell J (1985) Uptake, integration, expression and genetic transmission of a selectable chimaeric gene by plant protoplasts. Mol. Gen. Genet. 199:161-168.

Hamilton WDO, Bisaro DM and Buck KW (1982). Identification of novel DNA forms in tomato golden mosaic virus infected tissue. Evidence for a two component genome. Nucleic Acids Res. 10:4901-4912.

Hamilton WDO, Bisaro DM, Coutts RHA and Buck KW (1983) Demonstration of the bipartite nature of the genome of a single-stranded DNA plant virus by infection with the cloned DNA components. Nucleic Acids Res. 11, 7387-7396.

Hamilton WDO, Stein VE, Coutts RHA and Buck KW (1984) Complete nucleotide sequence of the infectious cloned DNA components of tomato golden mosaic virus: potential coding regions and regulatory sequences. EMBO J. 3: 2197-2205.

Hassan AA, Mazayad HM, Moustafa SE, Nassar SH, Nakhla MK, and Sims WL (1984) Inheritance of resistance to tomato yellow leaf curl virus derived from Lycopersicon cheesmanii and Lycopersicon hirsutum HortSci. 19:574-575.

Hibi T and Saito Y (1985) A dot immunobinding assay for the detection of tobacco mosaic virus in infected tissues. J. Gen. Virol. 66:1191-1194.

Hille J, Hoekema A, Hooykas P and Schilperoort R (1984) Gene organization of the Ti-plasmid. In: Gene Involved in Microbe-Plant Microbe Interactions. Dennis ES, Hohn B, Hohn T, King PJ, Schell JS and Verma DPS eds., Springer Verlag, New York Wien. pp. 287-309.

Ho LC and Hewitt JD (1987) Fruit development. In: Ather-ton JG, Rudich J (eds) The Tomato Crop, a Scientific Basis for Improvement. Chapman and Hall, New York, USA, pp 201-239.

Hoekema A, Hirsh PR, Hooykaas PJJ and Schilperoort RA (1983) A binary plant vector strategy based on separation of the vir and T region of the Agrobacterium tumefaciens Ti-plasmid. Nature (London) 303:179-180.

Horsh RB, Fry JE, Hoffman NL, Wallroth M, Eichholtz D, Rogers SG and Fraley RT (1985) A simple and general method for transferring cloned genes into plants. Science 227:1229-1231.

Howarth AJ, Caton J, Bossert M and Goodman RM (1985) Nucleotide sequence of bean golden mosaic virus and a model for gene regulation in geminiviruses. Proc. Natl. Acad. Sci. USA 82, 3572-3576.

Howarth AJ and Vandemark GJ (1989) Phylogeny of geminiviruses. J. gen. Virol. 70:2717-2727

Howell SH (1984) Physical structure and genetic organisation of the genome of maize streak virus (Kenyan isolate) Nucleic Acids Res. 12, 7359-7375.

Hull R (1985) Purification, biophysical and biochemical characterization of viruses with a special reference to plant viruses. In: Virology, a Practical Approach, Mahy B.W.J. ed. IRL Press, Oxford, pp. 1-24.

Ikegami M, Haber S and Goodman (1981) Isolation and characterization of virus-specific double-stranded DNA from tissues infected by bean golden mosaic virus. Proc. Natl. Acad. Sci. USA 78:4102-4106.

Kasrawi MA, Suwwan MA and Mansour A (1988) Sources of resistance to tomato-yellow-leaf-curl-virus (TYLCV) in Lycopersicon species. Euphytica 37:61-64.

Kleinschmidt AK (1968) Monolayer techniques in electron microscopy of nucleic acid molecules. In: Methods in Enzymology. Vol 12B, Grossman L and K Moldave (Eds.) Academic Press, New York, pp. 361-376.

Klinkenberg FA, Ellwood S and Stanley J (1989) Fate of African mosaic virus coat protein deletion mutants in

agroinoculation. J. Gen. Virol. 70:1837-1844.

Koenig R (1981) Indirect ELISA for the broad specificity detection of plants viruses. J. Gen. Virol. 55:53-56.

Laemmli UK (1970) Cleavage of structural proteins during the assembly of head bacteriophage T4. Nature (London) 227:680-685.

Laufs J, Wirtz U, Kammann M, Matzeit V, Schaefer S, Schell J, Czernilowsky AP, Baker B and Gronenborn B (1990) Wheat dwarf virus Ac/Ds vectors: expression and excision of transposable elements introduced into various cereals by a viral replicon. Proc. Natl. Acad. Sci. USA 86:7752-7756.

Lazarowitz SG (1987) The molecular characterization of geminiviruses. Plant Mol. Biol. Rep. 4:177-192.

Lazarowitz SG (1988) Infectivity and complete nucleotide sequence of the genome of a South African isolate of maize streak virus. Nucleic Acids Res. 16:229-249.

Lazarowitz S and Lazdins IB (1991) Infectivity and complete nucleotide sequence of the cloned genomic components of a bipartite squash leaf curl geminivirus with a broad host range phenotype. Virology 180:58-69.

Lazarowitz SG, Pinder JA, Damsteegt VD and Rogers SG (1989) Maize streak virus genes essential for systemic spread and symptom development. EMBO J 8:1023-1032.

Linsmaier EM and Skoog F (1965) Organic growth factor requirements of tobacco tissue cultures. Physiol. Plant 100-127.

MacDowell SW, Macdonald H, Hamilton WDO, Coutts RHA and Buck KW (1985) The nucleotide sequence of cloned wheat dwarf virus DNA. EMBO J. 4, 2173-2180.

Makkouk KM and Laterrot H (1983) Epidemiology and control of tomato yellow leaf curl virus. In: Plant Virus Epidemiology, Plumb RT and Thresh JM, eds. Blackwell, Oxford, pp. 315-321.

Maniatis T, Fritsch EF and Sambrook J (1982) Molecular Cloning, a Laboratory Manual. Cold Spring Harbor Laboratories, Cold Spring Harbor, USA.

Marck C (1988) "DNA Strider": a "C" program for the fast analysis of DNA and protein sequences on the Apple Macintosh family of computers. Nucleic Acids Res. 16:1829-1836.

Matthews REF (1981) Plant Virology. Academic Press, New York.

Matzeit V, Laufs J, Kammann M, Schaefer S, Schalk H-J, Schmidt B, Wirtz U, Baker B and Gronenborn B (1990) The molecular Biology of wheat dwarf virus (WDV) and its potential application in plant genetic engineering. In: Horticultural Biotechnology, Bennett A ed., Alan R Liss Inc, NY, pp. 71-82.

Matzeit V, Schaefer S, Kammann M, Schalk H-J, Schell J and Gronenborn B (1991) Wheat dwarf virus vectors replicate and express foreign genes in cells of monocotyledonous plants. Plant Cell. In press.

Maule AJ, Harker CL and Wilson IG (1989) The pattern of accumulation of cauliflower mosaic virus-specific products in

infected turnips. Virology 169:436-446.

Maxam AM and Gilbert W (1980) Sequencing end-labelled DNA with base-specific chemical cleavages. Methods in Enzymology 65:499-560.

Mazyad HM, Omar F, Al-Taher K and Salha M (1979) Observations on the epidemiology of tomato yellow leaf curl disease on tomato plants. Plant Dis Reprtr 63:695-698.

McCormick SM, Niedermeyer J, Fry J, Barnason A, Horsch R and Fraley R (1986) Leaf-disc transformation of cultivated tomato (*L. esculentum*) using *Agrobacterium tumefaciens*. Plant Cell Reports 5:81-84.

Meshi T and Okada Y (1987) Systemic movement of viruses. In: Plant-Microbe Interactions, Molecular and Genetic Perspectives. Kosuge T Nester EW (eds) Macmillan, New York, USA. pp 285-304.

Messing J, Geraghty D, Heidecker D, Hu NT, Kridl J and Rubinstein I (1983) In: Genetic Engineering of Plants, Kasuge T, Meredith CP and Hollander A, eds. Plenum press, New York, pp. 211-227.

Morinaga T, Ikegami M, Shimotohno K and Miura K (1987) Total nucleotide sequences of the infectious cloned DNAs of bean golden mosaic virus DNA. Microbiology and Immunology 31:147-154.

Morris M, Coates L, Lowe S, Richardson K and Eddy P (1990) Nucleotide sequence of the infectious cloned DNA components of African cassava mosaic virus (nigerian strain). Nucleic Acids res. 18:197-198.

Mullineaux PM, Donson J, Morris-Krsinich BAM, Boulton MI and Davies JW (1984) The nucleotide sequence of maize streak virus DNA. EMBO J. 3:3063-3068.

Mullineaux PM, Donson J, Stanley J, Boulton MI, Morris-Krsinich BAM, Markham PG and Davies JW (1985) Computer analysis identifies sequence homologies between potential gene products of maize streak virus and those of cassava latent virus and tomato golden mosaic virus. Plant Mol. Biol. 5:125-131.

Navot N, Ber R and Czosnek H (1989) Rapid detection of tomato yellow leaf curl virus in squashes of plants and insect vectors. Phytopathology 79:562-568.

Nagy JI and Laliga P (1976) Callus induction and plant regeneration from mesophyll protoplasts. Z. Pflanzenphysiol. 78:453-455.

Nelson RS, Powell PA and Beachy RN (1990) Coat protein-mediated protection against virus infection. In: Genetic Engineering of Crop Plants. Lycett GW and Grierson D eds., Butterworths, London UK, pp. 13-24.

Owens RA and Diener TO (1981) Sensitive and rapid diagnosis of potato spindle tuber viroid disease by nucleic acid hybridization. Science:213:670-672.

Pilowsky M and Cohen S (1974) Inheritance of resistance to tomato yellow leaf curl virus in tomatoes. Phytopathology 64:632-635.

Pilowsky M and Cohen S (1990) Tolerance to tomato yellow

leaf curl virus derived from Lycopersicon peruvianum. Plant Dis. 74:248-250.

Register JC and Beachy RN (1988) Resistance to TMV in transgenic plants results from interference with an early event in infection. Virology 166:524-532.

Rigby PWJ, Dieckman M, Rhodes C and Berg P (1977) Labeling deoxyribonucleic acid to high specific activity in vitro by nick translation with DNA polymerase I. J. Mol. Biol., 113:237-251.

Roberts IM, Robinson DJ and Harrison BD (1984) Serological relationships and genome homologies among geminiviruses. J. Gen. Virol. 65:1723-1730.

Rochester DE, Kositratana W and Beachy RN (1990) Systemic movement and symptom production following agroinoculation with a single DNA of tomato yellow leaf curl geminivirus (Thailand). Virology 178:520-526.

Rogers SG, Bisaro DM, Horsch RB, Fraley RT, Hoffman NL, Brand L, Elmer JS and Lloyd AM (1986) Tomato golden mosaic virus A component DNA replicates autonomously in transgenic plants. Cell 45:593-600.

Rogers SG and Klee H (1987) Pathways to plant genetic manipulation employing Agrobacterium. In: Plant DNA Infectious Agents, Hohn T and Schell JS eds., Springer-Verlag, New York Wien, pp.179-203.

Rozenberg N, Gad AE, Altman A, Navot N and Czosnek H (1988) Liposome-mediated introduction of the chloramphenicol acetyl transferase (CAT) gene and its expression in tobacco protoplasts. Plant Mol. Biol. 10:185-191.

Russo M, Cohen S and Martelli GP (1980) Virus-like particles in tomato plants affected by the yellow leaf curl disease. J Gen Virol 49:209-213.

Sambrook J, Fritsch EF and Maniatis T (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

Samuel G (1934) The movement of tobacco mosaic virus within the plant. Ann Appl Biol 21:90-111.

Sander E and Mertes G (1984) Use of protoplasts and separated cells in plant virus research. Adv. Virus Res. 29:215-262.

Sanger F, Nicklen S and Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74:5463-5467.

Sela I, Reichman M and Weissbach A (1984) Comparison of dot molecular hybridization and enzyme-linked immunosorbent assay for detecting tobacco mosaic virus in plant tissues and protoplasts. Phytopathology 74:385-389.

Shahin EA (1984) Isolation and culture of protoplasts:tomato protoplasts. In: Cell Culture and Somatic Genetics of Plants, Vasil EK ed., Academic Press, 1:370-380.

Southern EM (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98:503-517.

Stanley J (1983) Infectivity of the cloned geminivirus

genome requires sequences from both DNAs. *Nature* 305:643-645.

Stanley J (1985) The molecular biology of geminiviruses. *Advances in Virus Res.* 30:139-177.

Stanley J and Gay MR (1983) Nucleotide sequence of cassava latent virus DNA. *Nature* 301, 260-262.

Stanley J, Markham PG, Callis RJ and Pinner MS (1986) The nucleotide sequence of an infectious clone of the geminivirus beet curly top virus. *EMBO J.* 5, 1761-1767.

Stanley J and Townsend R (1985) Characterization of DNA forms associated with cassava latent virus infection. *Nucleic Acids Res.* 13:2189-2206.

Sunter G, Gardiner WE, Rushing AE, Rogers SG and Bisaro DM (1987) Independent encapsidation of tomato golden mosaic virus A component DNA in transgenic plants. *Plant Mol. Biol. Rep.* 8:477-484.

Symons RH (1985) Diagnostic approaches for the rapid and specific detection of plant viruses and viroids. Pages 93-124. In: *Plant-Microbe Interactions, Molecular and Genetic Perspectives Vol 1*, Kosuge T. and Nester W.E. eds. MacMillan, London.

Szoka FC and Papahadjopoulos D (1978) Preparation of liposomes with large internal aqueous space and high capture by reverse-phase evaporation. *Proc. Natl. Acad. Sci. USA* 75:4194-4198.

Olson F, Hunt CA, Szoka FC, Vail WJ and Papahadjopoulos D (1979) *Biochim. Biophys. Acta* 557:9-23.

Tan MLMC, Rietveld EM, Van Marrewijk GAM and Kool AJ (1987) Regeneration of leaf mesophyll protoplasts of tomato cultivars (L. esculentum): factors important for efficient protoplast culture and plant regeneration. *Plant Cell Res.* 6:172-175.

Taylor B and Powell A (1982) Isolation of plant RNA and DNA. Bethesda Research Laboratories (BRL) Focus 4:4-6.

Thomas JE, Massalski PR and Harrison BD (1986). Production of monoclonal antibodies to african cassava mosaic virus and differences in their reactivities with other whitefly-transmitted geminiviruses. *J. Gen. Virol.* 67:2739-2748.

Townsend R, Watts J and Stanley J (1986) Synthesis of viral DNA forms in Nicotiana plumbaginifolia protoplasts incubated with cassava latent virus (CLV); evidence for the independent replication of one component of the CLV genome *Nucleic Acids Res.* 14:1253-1265.

Van Regenmortel (1982) (ed.) *Serology and Immunochemistry of Plant Viruses*. Academic Press, New York.

Wasylyk B, Wasylyk C, Augereau P and Chambon P (1983) The SV40 73 bp repeat preferentially potentiates transcription starting from proximal natural or substitute promoter elements. *Cell* 32:503-514.

Zakay Y, Navot N, Zeidan M, Kedar N, Rabinowitch H, Czosnek H and Zamir D (1990) Screening of Lycopersicon accessions for resistance to tomato yellow leaf curl virus: presence of viral DNA and symptom development. *Plant Disease*. In press.

Zaitlin M and Hull R (1987) Plant virus-host interactions. *Ann Rev Plant Physiol* 38:291-315.

Zambryski P, Joos H, Genetello C, Leemans J, Van Montagu

M and Schell J (1983) Ti plasmid vector for the introduction of DNA into plant cells without alteration of their normal regeneration capacity. EMBO J 2:2143-2150.

Zambryski P, Herrera-Estrella L, De Block M, Van Montagu and Schell J (1984) The use of the Ti plasmid of Agrobacterium to study the transfer and expression of foreign DNA in plant cells: New vectors and methods. In: Setlow JK and Hollander A eds., Genetic Engineering, Principles and Methods, plenum Press, New York.

DESCRIPTION OF COOPERATION

This research project is a collaborative effort between researchers from three institutes in two countries: Dr. E. Pichersky at The University of Michigan (UM), Ann Arbor, USA, Drs. Y. Antignus and S. Cohen at the Agricultural Research Organization (ARO), Bet-Dagan, Israel and Drs. D. Zamir and H. Czosnek at the Faculty of Agriculture (FAR), Rehovot, Israel.

Much of the achievements were the result of a close collaboration between the three groups as itemized below:

The isolation and characterization of TYLCV was done at ARO and FAR

The cloning of the TYLCV genome was done at FAR. Sequencing was done at UM when Nir Navot, a PhD student at FAR spent one month in the USA.

The preparation of an antibody to TYLCV and its characterization was done at ARO.

The molecular analysis of TYLCV infection was done at FAR and ARO.

Quick diagnostic tests were developed at FAR.

The demonstration of the uniqueness of the TYLCV genome was done at FAR and UM.

The tomato protoplast inoculation system and TYLCV replication and expression was done at FAR.

Plasmids containing TYLCV genes and their transfer to the Ti plasmid of Agrobacterium was done at UM and FAR.

Transgenic plants containing TYLCV DNA were generated at ARO and FAR.

As mentioned above, Nir Navot, a PhD student at FAR spent one month in the laboratory of Dr. E. Pichersky at UM, in the summer of 1987. Dr. E. Pichersky came twice to Israel, in 1988 and 1989.

EVALUATION OF RESEARCH ACHIEVEMENTS

This research project finds itself in the framework of our constant fight against the tomato yellow leaf curl disease. Breeding resistant cultivars encounters many difficulties. Many of these difficulties are the result of our poor understanding of the disease process.

To understand this process at the molecular level, we have generated cloned TYLCV DNA probes as well as antibodies against TYLCV. The DNA probes have been used to describe the events which accompany the development of the TYLCV infection. In particular, the dependance of infection on the developmental stage of the target tissue has been defined as well as the pathway of virus spread in the plant.

Breeding necessitates accurate diagnostic and selection means. We have developed a quick and easy diagnostic test which allows to detect infected plants well before disease symptoms are visible. We have also developed a system where tomato protoplasts can be inoculated with cloned TYLCV DNA. The replication and expression of the TYLCV DNA in the cells can serve as a test to screen for resistant plants. Protoplasts from resistant plants would not support TYLCV DNA replication while protoplasts from sensitive plants would do so.

On the basis of the genome-structure of whitefly-transmitted geminiviruses, it was assumed that a bipartite genome would be also found for TYLCV. However, we demonstrated that TYLCV has a single genomic component, making it the first geminivirus in its class. The single component of the TYLCV genome has the capacity to induce the whole disease cycle: inoculation, replication, systemic spread, disease symptom and whitefly transmission.

Cloning and sequencing the TYLCV genome has allowed us to dissect the genome and to construct plasmids which contain each one a different TYLCV gene, in sense and antisense orientations. Some of these genes have been cloned into the Ti plasmid of Agrobacterium and used to transform tomato plants. Plants containing the TYLCV coat protein and the C1 gene (implicated in TYLCV replication), in both sense and antisense orientations, have been obtained. These plants are now being tested for TYLCV resistance using both agroinoculation and whitefly-transmission tests. If some plants show some resistance, they will be tested in field conditions during the coming tomato growing season. Obtaining TYLCV resistant cultivars through genetic engineering would be a tremendous achievement.

LIST OF PUBLICATIONS

Czosnek H, Ber R, Navot N, Zamir D, Antignus Y and Cohen S (1988) Detection of tomato yellow leaf curl virus in lysates of plants and insects by hybridization with a viral DNA probe. Plant Dis. 72:949-951.

Czosnek H and Navot N (1988) Virus detection in squash-blots of plants and insects: applications in diagnostics, epidemiology and breeding. In 'Biotechnology in Agriculture', Mizrahi A ed., Alan R Liss Inc., pp.83-96.

Czosnek H, Ber R, Navot R, Antignus Y, Cohen S and Zamir D (1989) Characterization of tomato yellow leaf curl virus DNA forms in the viral capsid, in infected plants and in the insect vector. J. Phytopathology 125:47-54.

Navot N, Ber R and Czosnek H (1989) Rapid detection of tomato yellow leaf curl virus in squashes of plants and insect vectors. Phytopathology. 79: 562-568.

Czosnek H, Navot N, Ovadia S, Reinhartz A, Herzberg M and Zilberstein (1989) A quick diagnostic test for tomato yellow leaf curl virus. In: Integrated management Practices for Tomato and Pepper Production in the Tropics. Green KS ed. pp. 260-265. Tainan, Taiwan.

Ber R, Navot N, Zamir D, Antignus Y, Cohen S and Czosnek H (1990) Infection of tomato by the tomato yellow leaf curl virus: susceptibility to infection, symptom development and accumulation of viral DNA. Arch. Virology, 112:169-180.

Navot N, Pichersky E, Zamir D and Czosnek H (1990) Tomato yellow leaf curl virus, a whitefly-transmitted geminivirus with a single genome. Submitted to Virology.