Final Scientific Report

Cover Page

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Project Title: Geminivirus-resistant tomato plants: Combining transgenic and conventional strategies for multi-viral resistance

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

Begomovirus, *Tomato yellow leaf curl virus*, *Tomato mottle virus*

Abbreviations commonly used in the report, in alphabetical order:

ToMoV, TYLCV, *Rep* (Replication associated protein),

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Signature
Principal Investigator

Signature
Authorizing Official, Principal Institution

Publication Summary (numbers)

	Joint IS/US authorship	US Authors only	Israeli Authors only	Total
Refereed (published, in press, accepted) BARD support acknowledged				
Submitted, in review, in preparation				
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Postdoctoral Training: List the names and social security/identity numbers of all postdocs who received more than 50% of their funding by the grant.

Cooperation Summary (numbers)

	From US to Israel	From Israel to US	Together, elsewhere	Total
Short Visits & Meetings	1	1	2	4
Longer Visits (Sabbaticals)		1		1

Description of Cooperation:

In essence, the collaboration between the partners of this project can be outlined as follows:

A. The constructs aimed to confer viral resistance were constructed and tested in a protoplasts replication assay by the Hanley-Bowdoin lab (US).

B. Tomato plants were transformed with the above constructs by the Reuveni lab (IS).

C. T0 transgenic tomato plants were screened for presence of the transgene, grown in a greenhouse and seeds of T1 plants collected by the Lapidot lab (IS).

D. The T1 transgenic tomato plants were tested for resistance to TYLCV by the Lapidot lab (IS), the T1 transgenic tomato plants were tested for resistance to ToMoV by the Polston lab (US).

Patent Summary (numbers)

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

Begomoviruses, which constitute one genus of the *Geminiviridae* family, are single-stranded DNA viruses that infect many dicotyledonous crops important to large agricultural industries as well as to subsistence growers. Although all begomoviruses are transmitted by whiteflies (*Bemisia tabaci*), they have proven difficult to manage even with heavy insecticide applications. The begomovirus, *Tomato yellow leaf curl virus* (TYLCV), has been a problem in tomato production in Israel since the 1950s and in the United States since 1997. Approximately 89 begomoviruses have now been reported to infect tomato. Crop losses due to begomoviruses such as TYLCV and *Tomato mottle virus* (ToMoV), are limiting factors in tomato cultivation in Israel, the U.S., and many tomato-growing regions throughout the world. To overcome these limitations, we proposed a two-step strategy that combines transgenic and conventional resistance in order to develop tomato plants that are resistant to multiple begomoviruses. In the first step, we have developed transgenic tomato plants expressing trans-dominant interfering mutants Rep and C3 from TYLCV and ToMoV, and tested whether these plants are resistant to infection by these two viruses. In the second step we have tested whether pyramiding transgenic and conventional resistance is superior to either strategy alone.

The specific objectives of the proposal were:

1. Design and test trans-dominant interfering constructs for TYLCV and ToMoV Rep and C3 in transient replication interference assays.
2. Generate and test transgenic tomato plants expressing mutant Rep and C3 in resistance assays.
3. Generate and test conventional resistant lines that also express mutant Rep and C3.

Two viral replication interfering constructs, expressing the trans-dominant interfering mutants Rep and C3, were designed and constructed during this project. One construct, pNSB1630 was based on TYLCV sequences and the other, pNSB1682, based on ToMoV sequences. The TYLCV transformation construct was tested in a protoplasts replication assay, and was found to inhibit TYLCV replication. The ToMoV transformation construct is yet to be tested in a protoplast assay.

Both transformation vectors, pNSB1630 and pNSB1682, were used to transform four different tomato lines, and generate transgenic plants. The tomato lines used for transformation were: FL7613, MM, TY172, TY199. FL7613 and MM are susceptible to both TYLCV and ToMoV. TY172 and TY199 are breeding lines developed at Volcani Center. TY172 is resistant to TYLCV but susceptible to ToMoV, while TY199 is resistant to both TYLCV and ToMoV.

When transgenic T1 plants expressing the pNSB1630 constructed were screened for TYLCV resistance, it was found that these plants showed very low level of TYLCV resistance, if any. However, some of these lines showed high level of resistance to ToMoV.

Only five transgenic T1 lines expressing the pNSB1682 construct were tested (so far) for resistance to ToMoV. It was found that all five lines express very high level of resistance to ToMoV.

Although we haven't finished (yet) the screen of all the transgenic lines, it is already clear that we were able to successfully combine genetic resistance for TYLCV with transgenic resistance to ToMoV.

Achievements

1. Design and test trans-dominant interfering constructs for TYLCV and ToMoV Rep and C3 in transient replication interference assays.

Two viral replication interfering constructs, expressing the trans-dominant interfering mutants Rep and C3, were designed and constructed during this project. One construct was based on TYLCV sequences and the other based on ToMoV sequences.

TYLCV clones: 18 clones were generated during the construction of the TYLCV transformation vector, **pNSB1630**. All mutations were confirmed by DNA sequencing and the final plant transformation cassette was sequenced. A list of primers used for the construction of the different clones in Tables 1 and a list of the different TYLCV clones are in Table 2. The design strategy of selected TYLCV constructs is detailed below.

pNSB1538 – The TYLCV fragment corresponding to C1,2,3 was released from the TYLCV replicon and ligated into the plasmid pUC119. pNSB1538 was the mutagenesis template for the following clones. The C1 coding sequence carries an alanine substitution at residues FQ115. The ATG of the C2 coding sequence was mutated to prevent translation. The C1 coding sequence carries an L145A mutation to prevent interaction with the host RBR protein. The C3 coding sequence carries alanine substitutions at FYK30. C1 FQ115, L145A, C2 ATG and C3 FYK30 mutations were combined in pNSB1538. All the mutations were verified by DNA sequencing.

To make the plant expression cassettes for protoplast experiments, the wild-type or mutant TYLCV C1,2,3 coding regions were released by digestion and cloned into pMON921. The cassettes contain the enhanced 35S promoter and rbcS E9 3' terminator.

To make the plant transformation vectors, the plant expression cassettes corresponding to wild-type or mutant TYLCV C1,2,3 were released by digestion and cloned into pMON721.

pNSB1630 – Plant transformation cassette corresponding to pNSB1553 in pMON721 in the opposite orientation as 35S-nptII-nos. This plasmid was used to transform plants.

TYLCV Protoplast Assays

The Hanley-Bowdoin lab established conditions for TYLCV replication in *Nicotiana tabacum* protoplasts. The protoplasts are mixed with cloned viral DNA and subjected to electroporation. The cells are grown at 25°C for 65 h in indirect light prior to DNA extraction and DNA gel blot analysis. At this time, total DNA was extracted and digested with NcoI and DpnI to linearize nascent viral DNA and to cut residual input plasmid DNA, respectively. The DNA was analyzed on agarose gel blots by hybridization to a ³²P-labeled TYLCV probe. A titration experiment was performed to determine a linear range for the input replicon DNA that would allow detection of interference activity. DNA gel blot analysis showed that 0.75 µg of input DNA was sufficient for

maximal nascent viral DNA accumulation (Figure 1). Based on this result, we performed subsequent interference experiments with 0.5 µg of input TYLCV replicon DNA.

We then asked if the TYLCV expression cassette carrying all four mutations can interfere with TYLCV replication in tobacco cells. Protoplasts were transfected with 0.5 µg of TYLCV replicon DNA in the presence of 40 µg of the empty expression cassette (pMON921) or an expression cassette for TYLCV C1,C2,C3 carrying the C1-FQ115, C1-RBR, C2-ATG and C3-FYK30 mutations (pNSB1552). Negative control transfections contained salmon sperm DNA only. TYLCV accumulation was severely reduced in the presence of the mutant TYLCV C1,C2,C3 cassette but not the empty cassette, establishing that the mutant TYLCV cassette interferes with viral replication (Figure 2).

ToMoV Clones: 19 clones were generated during the construction of the ToMoV transformation vector, **pNSB1682**. The ToMoV C1 trans-dominant negative mutant (mC1; FQ117), the C1 RBR binding mutant (mC1-RBR; L147), the C2 ATG start site mutant (mC2; M1A), and the C3 mutant (mC3; YFK30) were generated using the primers TMVREPTD, TMVREPRB, TMVC2ATG and TMVC3TD, respectively (Table 3). The mutations were made individually or in combinations using the Qiagen Multi Site-Directed Mutagenesis system. All of the mutations were confirmed by DNA sequencing. The ToMoV plasmids are listed in Table 4.

To make the plant expression cassettes for protoplast experiments, the wild-type or mutant ToMoV C1,2,3 coding regions were released by digestion and cloned into pMON921. The cassettes contain the enhanced 35S promoter and rbcS E9 3' end.

To make the plant transformation vectors, the wild-type or mutant coding regions in the ToMoV C1,2,3 expression cassettes were amplified by PCR, digested and cloned into pBI121. The resulting transformation vectors contained the enhanced 35S promoter and the nos 3' end. The C1,2,3 inserts in the transformation vectors were verified by single-pass sequencing.

We are currently finishing the ToMoV replicon clone and will use it to test the equivalent ToMoV C1,C2,C3 mutant expression cassette in replication interference assays. We will also ask if the TYLCV and ToMoV mutant expression cassettes interfere with the heterologous viral replicon. These studies will be combined with the tomato transformation and resistance studies performed by the Lapidot and Polston groups in a paper describing the efficacy of combining C1 and C3 trans-dominant negative mutants to confer resistance to more than one geminivirus.

2. Generate and test transgenic tomato plants expressing mutant Rep and C3 in resistance assays.

The above TYLCV transformation vector, pNSB1630, and the ToMoV transformation vector, pNSB1682, were used to transform four different tomato lines, and generate transgenic plants.

The tomato lines used for transformation were FL7613, MM, TY172, TY199. FL7613 and MM are susceptible to both TYLCV and ToMoV. TY172 and TY199 are breeding lines developed at Volcani center. TY172 is resistant to TYLCV but susceptible to ToMoV, while TY199 is resistant to both TYLCV and ToMoV. T0 transgenic plants were tested for presence of the transgene by PCR. Plants that were found positive were transplanted to a greenhouse and grown for fruit, and seeds were produced. The resulting T1 transgenic plants were tested for their reaction to inoculation with TYLCV or ToMoV (Table 5).

14-21 days old T1 transgenic plants (from three parental lines, FL7613, MM and TY172) expressing the TYLCV transformation vector, pNSB1630, were inoculated with either TYLCV or ToMoV using whiteflies. Following inoculation the whiteflies were removed, and the plants grown in a greenhouse. Plants were screened 30 days post inoculation. The plants were scored for effect of viral inoculation using a disease severity index (DSI) of 0 to 4 – (0) no visible symptoms; (1) very slight yellowing of leaflet margins on apical leaf; (2) some yellowing and curling of leaves; (3) a wide range of severe leaf yellowing, curling and cupping, yet plants continue to develop; (4) very severe plant stunting and yellowing, and pronounced cupping and curling; plants stop growth. The results are summarized in Table 5.

The transgenic plants showed low level of resistance to TYLCV, if any. The “best” T1 line was 1630-A-08-T0-26 that showed a mediocre level of resistance (mean DSI of 2.5 and a range of 2-4). Surprisingly, some of these lines, despite expressing a TYLCV expression vector, showed a high level of resistance to ToMoV (Table 5). For instance, 1630-B-08-T0-63 (mean DSI of 1.5 and a range of 0-1), 1630-D-08-T0-70 (mean DSI of 0.8, and a range of 0-2), 1630-D-08-T0-73 (mean DSI of 0.7 and a range of 0.5-1), 1630-D-08-T0-74 (mean DSI of 0.6 and a range of 0-1). Plants from the above lines are being grown in the greenhouse and seeds of T2 plants will be collected.

We were able to test only five transgenic lines expressing the ToMoV transformation vector, pNSB1682. All five lines expressed a very high level of resistance to ToMoV (Table 6). Most of the pNSB1682 transgenic plants are still at the T0 stage. Seeds will be collected from these plants and the resulting T1 plants will be tested for resistance to both ToMoV and TYLCV. The same is the case with TY199 transgenic plants expressing pNSB1630, which will be tested in the future. A list of these lines is summarized in Table 7.

Details of cooperation

The achievements of this project are the result of full collaboration between the four partners. Each partner was in charge of a specific stage of the project, which once completed was passed to

the next partner for completion of the next stage of the project. The details of the collaboration between the partners of this project are as follows:

1. A ToMoV DNA-A construct was developed by the Polston lab (US), and a TYLCV construct was developed by the Lapidot lab (IS). Both constructs were sent to the Hanley-Bowdoin lab (US).
2. The TYLCV and ToMoV replication-inhibition constructs were designed, constructed and tested in a protoplasts replication assay by the Hanley-Bowdoin lab (US).
3. Tomato plants were transformed with the above constructs by the Reuveni lab (IS).
4. T0 transgenic tomato plants were screened for presence of the transgene, grown in a greenhouse and seeds of T1 plants collected by the Lapidot lab (IS).
5. The T1 transgenic tomato plants were tested for resistance to ToMoV by the Polston lab (US), and to TYLCV by the Lapidot lab (IS).

Appendix

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Table 1. List and sequence of primers used for construction of TYLCV clones.

Primer	Sequence	Usage
BGLII for	CAAGCTGACTCTAGCAGATCTcAACatgcctcggtta	cloning into pUC119
BGLIIrev	taaacgaggcatGTTgAGATCTGCTAGAGTCAGCTTG	cloning into pUC119
C1for	CTTCATTGATTTTGGAGTTGCCGCAATCGATGGCAGATC	C1 mutagenesis
C1rev	GATCTGCCATCGATTGCGGCAACTCCAAAATCAATGAAG	C1 mutagenesis
RBRfor	CAGTAAATCCGAGGCCGCCAATATATTTAAAAGAGAAG	C1 mutagenesis
RBRrev	CTTCTCTTTTAATATATTGGCGGCCTCGGATTTACTG	C1 mutagenesis
C2for	GAAAAATTGGGCTCTCAAGAACGCAACCTTCGTCACC	C2 mutagenesis
C2rev	GGTGACGAAGGTTGCGTTCTTGAGAGCCCAATTTTC	C2 mutagenesis
C3for	GATAACAATCCCCTCGCTGCCGCGATAACAGAACACAG	C3 mutagenesis
C3rev	CTGTGTTCTGTTATCGCGGCAGCGAGGGGATTGTTTATC	C3 mutagenesis

Table 2. List of the different TYLCV clones.

Plasmid	Vector	Insert	Usage
pNSB1538	pUC119	Wild-type TYLCV C1-C2-C3	
pNSB1541	pUC119	C1-FQ115 mutant	
pNSB1544	pUC119	C2-ATG mutant	
pNSB1547	pUC119	C1-RBR mutant (L145A)	
pNSB1550	pUC119	C3 FYK30 mutant	
pNSB1552	pUC119	C1-FQ115,L145; C2-ATG; C3 FYK30	
pNSB1538	pMON921	Wild-type TYLCV C1-C2-C3	For expression in protoplasts
pNSB1542	pMON921	C1-FQ115	For expression in protoplasts
pNSB1545	pMON921	C2-ATG	For expression in protoplasts
pNSB1548	pMON921	L145	For expression in protoplasts
pNSB1551	pMON921	C3 FYK30	For expression in protoplasts
pNSB1553	pMON921	C1-FQ115,L145; C2-ATG; C3 FYK30	For expression in protoplasts
pNSB1630	pMON721	C1-FQ115,L145; C2-ATG; C3 FYK30	For expression in tomato plants
pNSB1631	pBI121	C1-FQ115,L145; C2-ATG; C3 FYK30 (opposite orientation)	For expression in tomato plants

Table 3. List and sequence of primers used for construction of ToMoV clones.

Primer	Sequence	Usage
T3SEQ	AATTAACCCTCACTAAAGGG	sequencing
T7TERMINATOR	TATGCTAGTTATTGCTCAG	sequencing
TOMOVAL1F	CGGAATTCAAAAATGCCCCACCAAAG	cloning into pUC119
TOMOVAL3R	CGCGGATCCTTAATAAAATTTGAATTTTATTG	cloning into pUC119
TOMOVAL1F2	CGCGGATCCATGCCCCACCAAAGAA	cloning into pBI121
TOMOVAL3R2	TGCAGGATATCTTAATAAAATTTGAATTTTATTG	cloning into pBI121
TMVC2ATG	CTGGACTATCAAGAACGCGATCTTCATCACC	C2 M1A mutation
TMVREPTD	CAATCGAATGGGGAGATGCTGCTATCGACGGCAGATCTG	Rep F116A-Q117A mutation
TMVREPRB	GTCGGTTCAATCTGCCGCTGCGGTTCTAAGGGAAG	Rep L147A mutation
TMVC3TD	GCTAGAAAATCCCCTTGCTGCTGCTATACACAGAGTAGAGG	C3 Y28A-F29A-K30A mutation

Table 4. List of the different ToMoV clones.

Plasmid	Vector	Mutation contained	Usage
pNSB1632	pUC119	Wild-type Rep-C2-C3	
pNSB1659	pUC119	C2-ATG	
pNSB1651	pUC119	C2-ATG/Rep-RBR	
pNSB1652	pUC119	C2-ATG/Rep-TD	
pNSB1660	pUC119	C2-ATG/C3-TD	
pNSB1669	pUC119	C2-ATG/Rep-RBR/Rep-TD/C3-TD	
(pNSB1661)	pUC119	C2-ATG/Rep-RBR/Rep-TD	
pNSB1671	pMON921	Wild-type Rep-C2-C3	For expression in protoplasts
pNSB1672	pMON921	C2-ATG	For expression in protoplasts
pNSB1673	pMON921	C2-ATG/Rep-RBR	For expression in protoplasts
pNSB1674	pMON921	C2-ATG/Rep-TD	For expression in protoplasts
pNSB1675	pMON921	C2-ATG/C3-TD	For expression in protoplasts
pNSB1676	pMON921	C2-ATG/Rep-RBR/Rep-TD/C3-TD	For expression in protoplasts
pNSB1677	pBI121	Wild-type Rep-C2-C3	For expression in tomato plants
pNSB1678	pBI121	C2-ATG	For expression in tomato plants
pNSB1679	pBI121	C2-ATG/Rep-RBR	For expression in tomato plants
pNSB1680	pBI121	C2-ATG/Rep-TD	For expression in tomato plants
pNSB1681	pBI121	C2-ATG/C3-TD	For expression in tomato plants
pNSB1682	pBI121	C2-ATG/Rep-RBR/Rep-TD/C3-TD	For expression in tomato plants

Table 5. Screening of transgenic tomato lines, expressing the TYLCV transformation vector pNSB1630, for resistance to TYLCV and ToMoV.

Parental line	Remarks*	Number	TYLCV screen		ToMoV screen	
			Mean DSI	DSI Range	Mean DSI	DSI range
FL7613	Control, S		4	4	3.5	2.8-4.0
TY172	Control, R				3.8	3.5-4.0
TY172	Resistant	1630-B-08-T0-4				
FL7613	Susceptible	1630-A-08-T0-5	4	4	3.7	3.5-4.0
TY172	Resistant	1630-B-08-T0-6				
TY172	Resistant	1630-B-08-T0-8				
FL7613	Susceptible	1630-A-08-T0-10	4	4	2.5	0.0-4.0
TY172	Resistant	1630-B-08-T0-11				
TY172	Resistant	1630-B-08-T0-12				
FL7613	Susceptible	1630-A-08-T0-14	4	4	2.8	2.0-4.0
Fl 7613	Susceptible	1630-A-08-T0-15				
TY 172	Resistant	1630-B-08-T0-16				
TY 172	Resistant	1630-B-08-T0-17				
Fl 7613	Susceptible	1630-A-08-T0-19	3.8	3.5-4.0	3.5	3.5-4.0
Fl 7613	Susceptible	1630-A-08-T0-20	3.7	3.5-4.0	3.6	3.5-4.0
TY 172	Resistant	1630-B-08-T0-21				
Fl 7613	Susceptible	1630-A-08-T0-22				
Fl7613	Susceptible	1630-A-08-T0-26	2.5	2.0-4.0		
Fl7613	Susceptible	1630-A-08-T0-27	4	4	2.6	2.0-3.5
Fl7613	Susceptible	1630-A-08-T0-33	3.7	3.5-4.0		
Fl7613	Susceptible	1630-A-08-T0-35	4	4		
TY 172	Resistant	1630-B-08-T0-40				
TY 172	Resistant	1630-B-08-T0-41			2.3	0.0-4.0
Fl 7613	Susceptible	1630-A-08-T0-45	4	4	3.2	0.0-4.0
TY 172	Resistant	1630-B-08-T0-46				
Fl 7613	Susceptible	1630-A-08-T0-50	4	4	2.1	0.0-3.0
TY 172	Resistant	1630-B-08-T0-51				
TY 172	Resistant	1630-B-08-T0-54			1.8	0.0-4.0
MM	Susceptible	1630-D-08-T0-58	3	2.5-4.0	3.1	2.5-3.5
MM	Susceptible	1630-D-08-T0-59	3.5	3.3-4.0	2.1	0.0-3.5
MM	Susceptible	1630-D-08-T0-60	3.5	3.3-4.0	2.6	0.0-3.5
Fl 7613	Susceptible	1630-A-08-T0-62	4	4	3.7	3.0-4.0
TY172	Resistant	1630-B-08-T0-63			0.1	0.0-1.0
TY172	Resistant	1630-B-08-T0-64			1.5	0.0-3.5
TY172	Resistant	1630-B-08-T0-65			1.8	0.0-3.5
Fl 7613	Susceptible	1630-A-08-T0-66	4	4	2.3	1.5-4.0
Fl 7613	Susceptible	1630-A-08-T0-67	4	4	2.9	1.5-4.0
MM	Susceptible	1630-D-08-T0-70			0.8	0.0-2.0
MM	Susceptible	1630-D-08-T0-71			1.1	0.5-2.0
MM	Susceptible	1630-D-08-T0-72			1.0	0.5-2.0

MM	Susceptible	1630-D-08-T0-73			0.7	0.5-1.0
MM	Susceptible	1630-D-08-T0-74			0.6	0.0-1.0

* Resistant and susceptible denotes the reaction of the parental lines to inoculation with TYLCV. Controls are the same genotypes as the transformed plants, prior to transformation.

Table 6. Screening of transgenic tomato lines, expressing the ToMoV transformation vector pNSB1682, for resistance to ToMoV.

Parental line	Remarks*	Number	TYLCV screen		ToMoV screen	
			Mean DSI	DSI Range	Mean DSI	DSI Range
FL7613	Control, S				3.0	2.5-3.5
TY172	Control, R				2.9	2.5-3.3
TY172	Resistant	1682-B-09-100			1.0	0.0-3.0
TY172	Resistant	1682-B-09-101			0.3	0.0-2.0
TY172	Resistant	1682-B-09-102			0.3	0.0-3.0
TY172	Resistant	1682-B-09-103			0.1	0.0-1.0
TY172	Resistant	1682-B-09-104			0.9	0.0-4.0

* Resistant and susceptible denotes the reaction of the parental lines to inoculation with TYLCV. Controls are the same genotypes as the transformed plants, prior to transformation.

Table 7. A list of transgenic TY199 plants expressing the TYLCV transformation construct pNSB1630.

Parental line	Number	Parental line	Number
TY199	1630-C-08-T0-7	TY199	1630-C-08-T0-38
TY199	1630-C-08-T0-9	TY199	1630-C-08-T0-39
TY199	1630-C-08-T0-13	TY 199	1630-C-08-T0-42
TY199	1630-C-08-T0-24	TY 199	1630-C-08-T0-43
TY199	1630-C-08-T0-25	TY 199	1630-C-08-T0-44
TY199	1630-C-08-T0-29	TY 199	1630-C-08-T0-48
TY199	1630-C-08-T0-31	TY 199	1630-C-08-T0-49
TY199	1630-C-08-T0-32	TY 199	1630-C-08-T0-52
TY199	1630-C-08-T0-34	TY 199	1630-C-08-T0-53
TY199	1630-C-08-T0-35	TY 199	1630-C-08-T0-55
TY199	1630-C-08-T0-36	TY 199	1630-C-08-T0-56
TY199	1630-C-08-T0-37	TY 199	1630-C-08-T0-57

Figure 1. A titration assay to determined a linear range for the input replicon DNA that would allow detection of interference and complementation activities. Tobacco protoplasts were transfected with the indicated amount of TYLCV replicon DNA and analyzed for the accumulation of nascent double stranded (ds) viral DNA on DNA gel blots.

Figure 2. Replication interference assay in protoplasts for TYLCV. Tobacco protoplasts were transfected with 0.5 μ g of the TYLCV replicon DNA and 40 μ g of the indicated plant expression cassette. Total DNA was isolated at 65 h post transfection and analyzed on DNA gel blots. The mutant TYLCV expression cassette is depicted below the blot. The blue arrow is the enhanced 35S CaMV promoter, the red arrow is the TYLCV C1,C2,C3 ORFs, and the green arrow is *rbcS* E9 3' end.