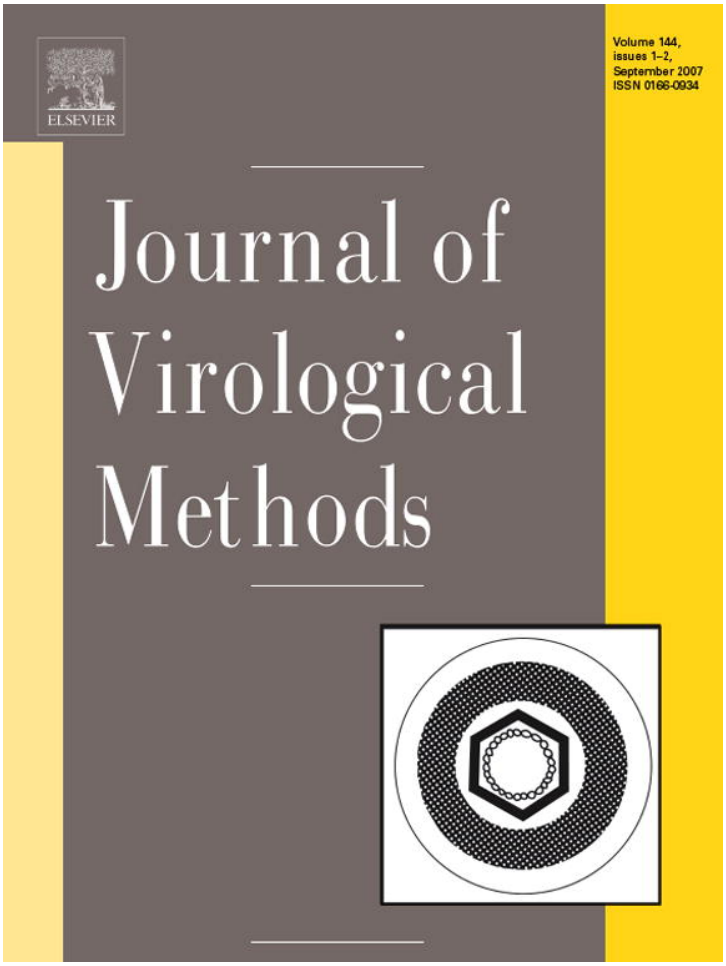

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Journal of Virological Methods
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Biolistic inoculation of plants with *Tomato yellow leaf curl virus* DNA

Moshe Lapidot [a,*], Galit Weil [a], Lidya Cohen [a], Limor Segev [a], Victor Gaba [b]

[a] *Department of Vegetable Research, Institute of Plant Sciences, ARO Volcani Center, P.O. Box 6, Bet Dagan 50250, Israel*
[b] *Department of Plant Pathology and Weed Science, Institute of Plant Protection, ARO Volcani Center, P.O. Box 6, Bet Dagan 50250, Israel*

Received 30 January 2007; received in revised form 25 April 2007; accepted 26 April 2007
Available online 15 June 2007

Abstract

Tomato yellow leaf curl virus (TYLCV) full-length DNA was amplified by PCR and cloned into a bacterial plasmid. The cloned TYLCV DNA was excised from the plasmid, ligated and the resulting monomeric circular double-stranded TYLCV DNA was used to inoculate tomato (*Solanum lycopersicom*) and datura (*Datura stramonium*) plants by particle bombardment. The bombarded plants produced typical disease symptoms, similar to those produced following whitefly-mediated inoculation, albeit 5–7 days later than whitefly-inoculated plants. The success rate of inoculating tomato plants by particle bombardment averaged 37%, whereas with datura plants, it averaged 85%. With whitefly-mediated inoculation of TYLCV, the success rate of inoculation was also higher in datura plants than in tomato plants. Bombardment of datura plants with a linear form of TYLCV DNA also resulted in viral infection, with an inoculation success rate similar to that with the closed-circular TYLCV DNA. Bombarding datura plants with the bacterial plasmid containing the cloned TYLCV DNA did not result in viral infection, but bombardment with a bacterial plasmid containing a cloned dimer of TYLCV DNA yielded an infection rate of 50–100%. This is the first report of TYLCV inoculation of plants using particle bombardment of a cloned monomeric linear or closed-circular form of TYLCV double-stranded DNA.

Keywords: Begomovirus; Tomato; TYLCV; Particle bombardment

1. Introduction

Today, *Tomato yellow leaf curl virus* (TYLCV) is one of the most devastating viruses of cultivated tomato (*Solanum lycopersicum*) in tropical, subtropical and Mediterranean regions. Although originally found in the eastern Mediterranean (Cohen and Harpaz, 1964), it is now a worldwide problem in tomato cultivation (Moriones and Navas-Castillo, 2000; Polston and Anderson, 1997). The virus is a monopartite begomovirus, transmitted in a persistent circulative manner by the whitefly *Bemisia tabaci* (Gennadius), whose severe population outbreaks are usually associated with high incidence of the disease. Like most begomoviruses, TYLCV cannot be transmitted by mechanical inoculation: it is transmitted only by its whitefly vector. Unlike most begomoviruses, which have a genome composed of two single-stranded DNA (ssDNA) molecules (bipartite) of about 2700 nucleotides (nt) each designated DNA-A and DNA-B, the TYLCV genome is composed of a single circular ssDNA (monopartite) molecule of nearly 2.8 kb, which encodes only six proteins. Similar to other begomoviruses, its DNA-replication cycle relies largely on the use of host cellular DNA-replication proteins. The strategy used by begomoviruses to replicate their ssDNA genome begins with its conversion to double-stranded DNA (dsDNA) intermediates, also known as the replicative form. In the second stage of viral replication, the dsDNA is used as a template to produce ssDNA genomes via a rolling-circle replication mechanism (Gutierrez, 1999; Hanley-Bowdoin et al., 1999).

The whitefly vector of begomoviruses can transmit only virions, i.e. only encapsidated viral DNA: it will not transmit naked DNA. Thus, the use of whiteflies to inoculate plants does not permit manipulation of the viral DNA prior to inoculation. Moreover, it is difficult to apply uniform inoculum pressure when inoculating plants via whiteflies. Thus, due to the need to rear whiteflies in order to develop a controlled inoculation protocol for TYLCV, and due to difficulties derived from vector management, other inoculation procedures are being sought.

Agroinoculation uses *Agrobacterium tumefaciens* to deliver binary vectors containing a tandem repeat (dimer) of a cloned viral DNA into host cells (Grimsley et al., 1986, 1987). As

* Corresponding author. Tel.: +972 3 968 3568; fax: +972 3 960 4180.
E-mail address: lapidotm@volcani.agri.gov.il (M. Lapidot).

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doi:10.1016/j.jviromet.2007.04.011

a result, genome-sized viral DNA is formed, spreads systemically through the plant and induces disease symptoms. Agroinoculation has been used successfully to introduce cloned tandem repeats of TYLCV DNA into leaf disks as well as into whole plants (Czosnek et al., 1993; Kheyr-Pour et al., 1994). However, agroinoculation requires time-consuming subcloning procedures to introduce the viral DNA, which is more than one unit in length, into the binary vector. Moreover, another pathogen – *A. tumefaciens* – is also introduced into the host plant. Moreover, in some cases, agroinoculation of cloned begomovirus DNA does not mimic whitefly transmission, probably due to the difficulties encountered with *A. tumefaciens* itself in the infection of some hosts (Sung and Coutts, 1995). Moreover, it has been shown that the delivery of cloned TYLCV DNA by agroinoculation may overcome the natural resistance of wild tomato species to this virus (Kheyr-Pour et al., 1994).

Particle bombardment (biolistic inoculation) has also been used to inoculate plants with begomovirus DNA (Garzon-Tiznado et al., 1993; Gilbertson et al., 1991). A high inoculation efficiency was achieved using biolistic inoculation of either unit-length (monomer) or tandem repeats (dimer) of cloned begomovirus DNA, thus eliminating the need for elaborate DNA manipulations and facilitating the genetic analysis of begomoviruses (Bonilla-Ramirez et al., 1997). Although biolistic inoculation of a viral DNA monomer that is still in the cloning plasmid was shown to be possible, higher inoculation rates were achieved following the excision of the viral clone from the plasmid prior to biolistic inoculation (Bonilla-Ramirez et al., 1997). However, biolistic inoculation (as described above) was only demonstrated for bipartite begomoviruses: biolistic inoculation of a partial dimer of DNA cloned from a monopartite begomovirus, *Tomato leaf curl Karnataka virus*, was only demonstrated for the first time in 2002 (Chatchawankanphanich and Maxwell, 2002). The first reported attempt to biolistically inoculate TYLCV was in 2003, using dimeric constructs of TYLCV-[Cu] (TYLCV isolate from Cuba) and *Tomato yellow leaf curl Sardinia virus* (TYLCSV) (Ramos et al., 2003). Neither virus was infectious following biolistic inoculation (for unknown reasons), although the TYLCSV clone was infectious following agroinoculation (Ramos et al., 2003). Recently, Morilla et al. (2005) demonstrated, for the first time, the biolistic inoculation of plants with dimeric forms of DNA cloned from TYLCV-[Alm] (Almeria isolate), TYLCV-Mld (mild strain) and TYLCSV.

In this study, we report the use of particle bombardment to inoculate tomato as well as datura (*Datura stramonium*) plants with a monomeric form of TYLCV dsDNA. The viral DNA was infectious, regardless of whether inoculation was performed with a closed-circular or linear form of the viral dsDNA.

2. Materials and methods

2.1. Cloning of TYLCV DNA

Total nucleic acids were extracted from tomato (*S. lycopersicum*) plants infected with the Israeli strain of TYLCV (Navot et al., 1991) (GenBank accession no. **X15656**). TYLCV full-length DNA was amplified by PCR using the partially overlapping primers TYNCOF (5′-ATA/ATA/**CCA/TGG**/CCG/CGC/AGC/GGA/ATA-3′, nt 2158–2178, forward direction) and TYNCOR (5′-ATA/ATA/**CCA/TGG**/AGA/CCC/ATA/AGT/ATT/GTC-3′, nt 2163–2140, reverse direction). Both primers contain an *Nco*I restriction site (bold) at nt 2158 in the virus sequence, and six non-TYLCV nucleotides were added at the 5′ end (underlined). The amplified TYLCV DNA was cloned into the plasmid pCRII-TOPO (Invitrogen, Carlsbad, CA) using A-T cloning to create pTY-TOPO. The resulting pTY-TOPO clone was verified by restriction analysis and sequencing. The primers were designed such that following amplification, restriction with *Nco*I and ligation would result in a circular full-length TYLCV dsDNA.

2.2. Particle bombardment (biolistic inoculation)

Plants were bombarded with a particle inflow gun (Finer et al., 1992), using a vacuum of 74 mmHg, a gas inflow (He) pressure of 3 bar, with the inflow valve operating for 100 ms. DNA was mixed with Au particles (1.5–3.0 μm, Johnson Matthey Alfa Aesar) prepared as per Gaba and Gal-On (2005). Au particles (4 mg) in 20–40 μl of 50% glycerol were mixed with 5 μg/5 μl DNA and vortexed, then 10 μl fresh 2.5 M $CaCl_2$ was added and vortexed; 4 μl 1 M spermidine (Sigma Chemical Co., St. Louis, MO) was added and vortexed for 3 min, left at room temperature for 1 min, centrifuged up to 5000 rpm and the supernatant removed. The Au/DNA mixture was washed with fresh 70% EtOH and then 96% EtOH. Finally, the pellet was resuspended in 20 μl 96% EtOH. Aliquots of 5 μl/plant were then used for bombardment. In each experiment, 10 plants were bombarded with each construct.

2.3. TYLCV DNA constructs for bombardment

The different TYLCV constructs used for particle bombardment are summarized in Table 1. These clones are:

pTY-TOPO: Plasmid pPCRII-TOPO containing a full-length (monomer) of TYLCV DNA.
Circular TYLCV DNA: pTY-TOPO was restricted with *Nco*I, thus releasing the TYLCV insert. Following gel purification, the TYLCV clone was ligated to create a circular double-stranded clone.
Linear TYLCV DNA: As above, pTY-TOPO was restricted with *Nco*I, but following gel purification, the TYLCV DNA was not ligated and was used as a linear clone.
pTY4-Binary: Received from Dr. H. Czosnek. A tandem direct repeat (dimer) of TYLCV DNA was cloned into the binary vector pCGN1547 as described by Navot et al. (1991).
pTY4-KS: The TYLCV dimer was released from pTY4-Binary by restriction with *Hind*III and *Kpn*I. The TYLCV dimer was then cloned into the plasmid pBluescript KS+.
pTY2-KS: TYLCV DNA monomer was released from pTY4-Binary by restriction with *Pst*I and cloned into the plasmid pBluescript KS+.

Table 1
Different TYLCV constructs used to inoculate plants by particle bombardment

Construct name	Details	Reference
pTY-TOPO	pPCRII-TOPO containing a full-length (monomer) of TYLCV DNA	This work
Circular TYLCV DNA	A circular full-length dsDNA clone of TYLCV obtained following *Nco*I restriction of pTY-TOPO and ligation	This work
Linear TYLCV DNA	A linear full-length dsDNA clone of TYLCV obtained following *Nco*I restriction of pTY-TOPO as above but without ligation	This work
pTY4-Binary	Binary vector pCGN1547 containing a tandem direct repeat (dimer) of TYLCV DNA	Navot et al. (1991)
pTY4-KS	pBluescript KS+ containing a tandem direct repeat (dimer) of TYLCV DNA	This work
pTY2-KS	pBluescript KS+ containing a full-length (monomer) of TYLCV DNA	This work

2.4. TYLCV acquisition and transmission by whiteflies

2.4.1. Acquisition from bombarded plants

To confirm that progeny from infectious cloned viral DNA was transmissible by whiteflies, the bombarded tomato and datura plants were used as inoculum source plants for whitefly acquisition and transmission. Whiteflies were allowed a 48-h acquisition-access period (AAP) on bombarded plants, after which they were provided a 48-h inoculation-access period (IAP) on 10 susceptible Marmande tomato test plants, approximately 25 whiteflies per plant. Following the IAP, whiteflies were removed by treating plants with imidacloprid (Confidor, Bayer, Leverkusen, Germany). The plants were maintained in an insect-proof greenhouse for 4 weeks and monitored for disease symptoms.

2.4.2. Effect of host plant on TYLCV transmission by whiteflies

Whiteflies were given a 48-h AAP, as described above, on the following TYLCV-infected resistant and susceptible source plants: the highly TYLCV-resistant tomato line TY172 (Friedmann et al., 1998; Lapidot et al., 1997), commercial TYLCV-resistant tomato cultivars 8484 (Hazera Genetics Ltd., Brurim, Israel) and Fiona (Sluis & Groot, Enkhuizen, The Netherlands) and tomato line L27 as the susceptible control. Following acquisition, whiteflies were transferred from each TYLCV-infected source plant to 10 young (first-leaf stage) susceptible Marmande tomato or datura test plants, a single whitefly per plant (Lapidot et al., 2001). The test plants were covered with ventilated plastic cups, and the whiteflies were allowed a 24-h IAP. They were then removed by treating plants with imidacloprid, and the plants were kept in an insect-proof greenhouse for 4 weeks and monitored for TYLCV disease development. The experiment was repeated four times.

2.5. Detection of TYLCV DNA

TYLCV DNA was detected in the inoculated tomato and datura plants by PCR. Total nucleic acids were extracted from the apex of the infected plants according to Dellaporta et al. (1983) and served as templates for PCR amplification. An 856 nt long DNA fragment, corresponding to the 5′ half of the C1 gene of TYLCV, was amplified by means of the primers TYC1F (5′-GGG/CCT/AGA/GAC/CTG/GCC/CAC-3′, nt 2087–2107 forward direction), and TYC1R (5′-CCG/GTA/ATA/TTA/TAC/GGA/TGG/C-3′, nt 171–150 reverse direction), as described previously (Lapidot, 2002).

2.6. Statistical analysis

Statistical analyses were carried out by means of one-way analysis of variance (ANOVA) (SAS Institute, Cary, NC).

3. Results

3.1. Inoculation of a circular monomeric form of TYLCV DNA by particle bombardment

TYLCV (Israeli strain) DNA was amplified by PCR using the partially overlapping primers TYNCOF and TYNCOR. Both primers contained the *Nco*I restriction site at nt 2158 in the viral sequence. The resulting amplified viral DNA was cloned into the plasmid pTOPO, to create pTY-TOPO. The restriction enzyme *Nco*I was used to release a linear full-length TYLCV clone from pTY-TOPO. This clone was ligated to itself, creating a circular full-length dsDNA clone of TYLCV, very much like the viral replicating form.

The circular clone of TYLCV was used to inoculate tomato and datura plants by particle bombardment (Fig. 1, Table 2). The bombarded plants produced typical TYLCV disease symptoms, similar to those produced following whitefly-mediated inoculation, within 2–4 weeks (Fig. 2). However, development of disease symptoms in the bombarded plants occurred 5–7 days later than in those inoculated by whiteflies. The bombarded plants were scored as infected based on symptom development, and by PCR detection of TYLCV DNA in infected plants (Fig. 1). The bombarded plants were also used as TYLCV source plants for whitefly acquisition and transmission. TYLCV acqui-

Table 2
Inoculation of datura and tomato plants with circular TYLCV DNA by particle bombardment

Species[a]	Experiment (% infected plants)						
	I	II	III	IV	V	VI	Average
Tomato	33	40	50	40	40	20	37.2
Datura	80	70	100	80	80	100	85.0
P							<0.001[b]

[a] In each experiment, 10 plants were inoculated per species.
[b] Significance by unpaired *t* test.

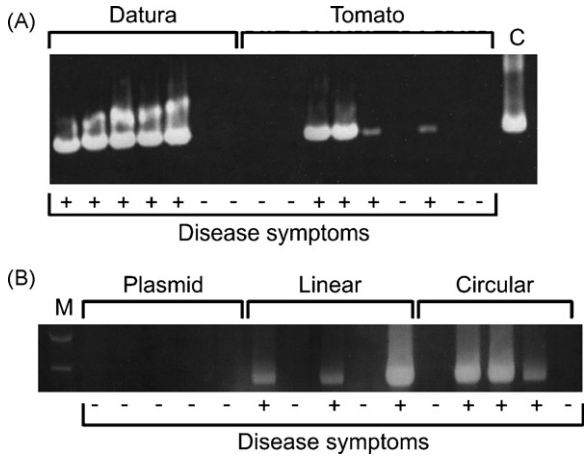

Fig. 1. Inoculation of tomato and datura plants with TYLCV DNA by particle bombardment. Plants were inoculated with TYLCV DNA as described in Section 2. Four weeks after inoculation, total DNA was extracted from the apex of the inoculated plants, followed by PCR amplification of TYLCV DNA using primers TYC1F and TYC1R (Lapidot, 2002 and Section 2). The PCR products were subjected to agarose gel electrophoresis, where the expected 850 nt amplification band can be seen. The inoculated plants were also monitored for disease symptom development: + and − denote plants with and without disease symptoms, respectively. (A) Inoculation of tomato and datura plants with circular TYLCV DNA. DNA was also extracted from control (C) TYLCV-infected tomato plants. (B) Inoculation of datura plants with different forms of TYLCV DNA: plasmid, pTY-TOPO, TY clone in the pTOPO plasmid; linear, linear clone of TYLCV; circular, circular clone of TYLCV; M, molecular weight markers.

sition and transmission from the bombarded plants was identical to acquisition and transmission from any other TYLCV-infected plant; all of the inoculated test plants produced normal disease symptoms (data not shown).

When the efficiency of particle bombardment inoculation of tomato plants was compared to that of datura plants, the infection rate in the latter was double that of the former (Table 2). Moreover, the bombarded datura plants developed TYLCV disease symptoms a day or two prior to the tomato plants (not shown).

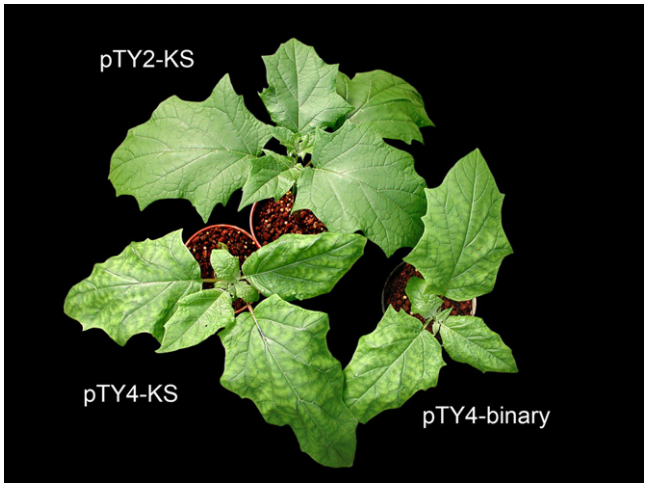

Fig. 2. Inoculation of datura plants with different multimeric forms of TYLCV DNA. Datura plants were inoculated with pTY4-Binary, pTY4-KS and pTY2-KS as described in Section 2. Picture was taken 30 days after inoculation.

Table 3
Inoculation of datura plants with different TYLCV DNA constructs by particle bombardment

Construct[a]	Experiment[b] (% infected plants)				
	I	II	III	IV	Average
Circular	80	100	60	60	75[a]
Linear	60	60	60	40	60[a]
pTY-TOPO	0	0	0	0	0[b]

[a] DNA constructs used for bombardment: circular, circular clone of TYLCV; linear, linear clone of TYLCV; pTY-TOPO, TYLCV clone in pTOPO plasmid.
[b] In each experiment, 10 plants were inoculated per construct. Means with different letters differ significantly at $P < 0.05$ when analyzed by one-way ANOVA.

3.2. Inoculation of a linear form of TYLCV DNA

As documented in Table 2, we were able to inoculate plants by bombarding with a circular viral dsDNA which resembles the viral replicating form. To test whether circular DNA is necessary for inoculation, the plants were inoculated with a linear form of the viral DNA—the viral DNA was excised from the pTY-TOPO plasmid and bombarded, without the ligation step, onto datura plants (Table 3). The linear form was found to be infectious, showing the same efficiency as the circular form (Table 3). Datura plants were also inoculated with the plasmid containing the viral DNA (pTY-TOPO) without excising the viral DNA from the bacterial plasmid: without prior excision, none of the plants became infected (Table 3).

3.3. Inoculating plants with a dimeric form of TYLCV DNA

It is possible to agroinoculate plants with a tandem repeat of TYLCV (and other begomoviruses) DNA cloned into a binary vector (Czosnek et al., 1993). To determine whether plants can be inoculated with the same *A. tumefaciens* construct without the bacteria, they were bombarded with pTY4-Binary, a binary vector containing a tandem repeat of TYLCV DNA, and a high infection rate was achieved (Table 4). To determine whether the binary vector is necessary for infection, the viral tandem repeat DNA was excised from pTY4-Binary and cloned into a bacterial plasmid (pBluescript KS), creating pTY4-KS. pTY4-KS was found to be highly infectious, with the same efficiency as pTY4-Binary, following bombardment of datura plants (Table 4). Hence, particle bombardment can replace the

Table 4
Inoculation of datura plants with TYLCV dimer and monomer

Construct[a]	Experiment[b] (% infected plants)				
	I	II	III	IV	Average
pTY4-Binary	100	50	67	83	75[a]
pTY4-KS	100	67	60	67	73[a]
pTY2-KS	0	0	0	0	0[b]

[a] DNA constructs used for bombardment: pTY4-binary, TYLCV dimer in binary vector; pTY2-KS, TYLCV dimer in plasmid pBluescript; pTY2-KS, TYLCV monomer in plasmid pBluescript.
[b] In each experiment, 20 plants were inoculated per construct. Means with different letters differ significantly at $P < 0.05$ when analyzed by one-way ANOVA.

Table 5
Effect of source cultivar and host species on TYLCV transmission by whiteflies

Source plant	Host plant	TYLCV transmission[a]
L27	Tomato	11.7%
	Datura	83.2%
	P	<0.01
8484	Tomato	48.1%
	Datura	88.1%
	P	<0.01
Fiona	Tomato	17.5%
	Datura	66.2%
	P	<0.05
TY172	Tomato	25%
	Datura	56.2%
	P	<0.02

[a] Significance by unpaired *t* test.

use of a binary vector and *A. tumefaciens*. To test whether a TYLCV DNA monomer in a bacterial plasmid can be infectious, the viral DNA was excised from pTY4-Binary and a monomeric form was cloned into pBluescript, creating pTY2-KS. Like pTY-TOPO, bombardment with pTY2-KS failed to induce viral disease (Table 4).

3.4. Effect of host plant on TYLCV transmission with whiteflies

Our results showed that datura plants are more easily inoculated than tomato with TYLCV via particle bombardment (Table 2). Is this because datura plants are more susceptible to particle bombardment than tomato, or because they are more susceptible to TYLCV? To answer this question, we compared transmission efficiencies of TYLCV to datura and tomato using a single whitefly per plant. Four different TYLCV-infected source plants were used, a susceptible tomato line (L27) and three different resistant tomato plants expressing different levels of resistance (8484, Fiona, TY172) (Lapidot et al., 2001). Regardless of the source plant from which the virus was acquired, transmission to datura was always more efficient than to tomato (Table 5): following feeding on L27 plants, TYLCV transmission to datura plants was sevenfold higher; when feeding was performed on 8484 plants, TYLCV transmission to datura was nearly double; a fourfold higher TYLCV transmission efficiency to datura was observed following feeding on Fiona, and twofold higher efficiency was seen following feeding on TY172 plants (Table 5).

4. Discussion

In this study, we demonstrate the ability to inoculate plants with cloned TYLCV DNA via particle bombardment. Inoculation efficiency was about 40% with tomato plants, and reached 85% with datura plants. We compared inoculation of tomato and datura plants using the whitefly vector to test whether the higher inoculation rate of the latter was due to its higher receptiveness to particle bombardment. In this case as well, higher inoculation rates were achieved with datura plants. We also tested a number of different TYLCV source plants – a susceptible plant and three types of resistant plants exhibiting different degrees of viral resistance – but regardless of the inoculum source plant, inoculation of datura plants resulted in higher infection rates than inoculation of tomato plants. This suggests that datura plants are more susceptible to TYLCV infection than tomato plants.

Inoculating plants (tomato and datura) with cloned TYLCV DNA by particle bombardment resulted in the development of normal disease symptoms in the infected plants, although symptom appearance was sometimes delayed by a few days compared to whitefly transmission. Nevertheless, the bombarded plants could serve as an inoculum source for acquisition and transmission by whiteflies just like any other source plant. The slight delay observed in symptom development might be due to the amount of viral DNA inoculated. It is quite possible that the amount of viral DNA that reaches the nucleus of the host cell via particle bombardment is lower than that following inoculation with whiteflies, causing a small delay in disease symptom development. Another possibility is that the lack of capsid protein following particle bombardment could cause an initial delay in shuttling viral DNA to and from the nucleus, since the capsid protein of TYLCV has been shown to be a nuclear shuttle protein (Gafni, 2003; Kunik et al., 1998).

The Finer-type of particle bombardment apparatus (as used here) is commonly used for plant transformation, with the advantage that much lower gas pressures are necessary than the common commercially available Bio-Rad PDS-1000, and consumable costs are reduced. In preliminary reports it seems that tissue penetration depth with the Finer-type apparatus may be greater than with the Bio-Rad PDS-1000 particle gun (Gaba et al., unpublished data). However, the type of particle bombardment apparatus used does not necessarily dictate inoculation success rate. In two previous reports the Bio-Rad PDS-1000 particle gun was used to attempt biolistic inoculation of TYLCV. The results however were different—while Morilla et al. (2005) were able to inoculate plants with a dimeric form of TYLCV-[Alm] DNA, Ramos et al. (2003) were unsuccessful in their attempts to inoculate TYLCV-[Cu], despite using the same apparatus.

Bombardment with viral DNA was infectious, regardless of whether closed-circular or linear forms were used. There was no statistical difference in infection efficiency following inoculation with these two forms. The viral clone was constructed in such a way that the restriction enzyme *Nco*I would release a linear full-length TYLCV clone from pTY-TOPO. The linear full-length clone could then be ligated to itself, creating a closed-circular full-length dsDNA clone (monomer) of TYLC, very much like the infectious viral replicating form. However, the linear form proved to be just as infectious. This is somewhat surprising, since the *Nco*I restriction site is within the C1 open reading frame, which is essential for TYLCV replication (Hanley-Bowdoin et al., 1999). One explanation may be that the linear form can spontaneously close on itself, thus creating a closed-circular form. Nonetheless, to the best of our knowledge, this is the first demonstration of the infectivity of TYLCV DNA monomers following particle bombardment.

The monomeric form of the viral DNA was not infectious when pTY-TOPO was bombarded prior to excision of the viral clone from the cloning plasmid. This is in contrast to biolistic inoculation of bipartite begomoviruses (Bonilla-Ramirez et al., 1997). However, when a dimer of TYLCV DNA in a binary vector (pTY4-Binary) was bombarded, it was highly infectious. The binary vector was not essential, since the TYLCV DNA dimer was still infectious after it was moved to pBluescript, a non-binary plasmid (pTY4-KS). Since pTY4 contains a different TYLCV clone than the one used up to this point, we used it to prepare pTY2-KS—a TYLCV DNA monomer, in pBluescript. However, this was noninfectious, similar to pTY-TOPO. Thus, to inoculate TYLCV DNA by particle bombardment, one needs to either use a plasmid containing a TYLCV DNA dimer, or excise the viral clone from the plasmid and use it in its closed-circular or linear form.

Acknowledgements

The authors wish to thank Prof. Hanokh Czosnek for kindly providing pTY4-Binary. This work was supported in part by USAID-MERC grant no. M21-037 to M.L. Contribution from the Agricultural Research Organization, the Volcani Center, Bet Dagan, Israel, Number 104/2007.

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