Infectivity of *Tomato yellow leaf curl virus* isolated from imported tomato fruit in Estonia

Kadri JUST[1,2], Muhammad Naeem SATTAR[2,3], Usman ARIF[2,4], Anne LUIK[1],
Anders KVARNHEDEN[1,2]

[1]Institute of Agricultural and Environmental Sciences, Estonian University of Life Sciences
Kreutzwaldi 1, 51014 Tartu, Estonia
E-mail: kadri.just@student.emu.ee

[2]Uppsala BioCenter, Swedish University of Agricultural Sciences and Linnean Center of Plant Biology
P.O. Box 7080, SE-750 07 Uppsala, Sweden

[3]King Faisal University
P.O. Box 420, Al-Ahsaa 31982, Saudi Arabia

[4]School of Biotechnology, Royal Institute of Technology (KTH)
SE-106 91, Stockholm, Sweden

Abstract

There is a risk that *Tomato yellow leaf curl virus* (TYLCV) and its vector, whiteflies of the *Bemisia tabaci* species complex, will become established in greenhouses in temperate regions of the world, including northern Europe. In this study, TYLCV isolated from imported tomato fruit in Estonia (originating from Spain) was shown to be able to infect plants of tomato and *Nicotiana benthamiana* using *Agrobacterium*-mediated inoculation with an infectious clone as well as using biolistic delivery of products from rolling circle amplification (RCA). A 1.8-mer genomic construct of TYLCV was engineered and efficiently agroinfiltrated into plants of tomato and *N. benthamiana*, and induced symptoms characteristic of natural infection. With *Agrobacterium*-mediated inoculation, the infection efficiency was 100% for both tomato and *N. benthamiana*, whereas biolistic inoculation using RCA products resulted in efficiencies of 57% and 36%, respectively. Particle bombardment with monomeric linear genome failed to produce any infection in tomato or *N. benthamiana*. The genome of TYLCV amplified from tomato fruit was infectious confirming that tomato fruit may serve as a source of virus inoculum. This is the first report of agroinfiltration and particle bombardment assay using TYLCV DNA derived from infected tomato fruit tissue.

Key words: agroinfiltration, begomovirus, infectious clone, *Solanum lycopersicum*, tomato fruit.

Introduction

Tomato (*Solanum lycopersicum* L.) is the most important vegetable crop globally and constitutes 72% of the value of fresh vegetables produced worldwide (Hanssen et al., 2010). The production is affected by many pests and infection of tomato by different viruses, such as *Pepino mosaic virus* (PepMV; genus *Potexvirus*, family *Alphaflexiviridae*) and begomoviruses (genus *Begomovirus*, family *Geminiviridae*) may result in significant yield losses. Among the tomato-infecting begomoviruses, *Tomato yellow leaf curl virus* (TYLCV) has the broadest geographical distribution. From its origin in the Middle East, it has spread throughout the Mediterranean Basin and recently also to Asia, Africa, Australia as well as to North America and South America (Lefeuvre et al., 2010; EFSA PLH, 2014). Symptoms induced by TYLCV include upward curling and yellowing of leaves, stunting of the plant, flower abortion and infection can cause up to 100% yield loss. TYLCV has a genome of circular single-stranded (ss)DNA and it is transmitted in a persistent manner by whiteflies of the *Bemisia tabaci* species complex (Navas-Castillo et al., 2011). The rapid global spread of TYLCV is the result of international movement of virus-infected plant material and of plants (including non-hosts of TYLCV) harbouring viruliferous whiteflies (EFSA PLH, 2014). Recently, TYLCV has also been reported to be seed transmitted with high seed transmission rates from infected tomato plants (Kil et al., 2016).

In southern Europe, *B. tabaci* and TYLCV have become established along the Mediterranean coastal regions, while the outdoor conditions in other parts of Europe are unfavourable for its spread and establishment.

However, outbreaks of *B. tabaci* and viruses it transmits can nevertheless occur under greenhouse cultivation conditions (Botermans et al., 2009; EFSA PLH, 2013). The present increase in international travel and trade in plant material enables dissemination of the insect vector and associated viruses to greenhouses in northern Europe, including those in regions with protected zone status. For instance, Sweden, Finland, Republic of Ireland and the United Kingdom are officially free of *B. tabaci* (EFSA PLH, 2013), but it is frequently intercepted also in these areas (Cuthbertson et al., 2011; EPPO, 2012). In Lithuania, *B. tabaci* has been detected on ornamental plants in greenhouses (Ivinskis et al., 2009) and in Estonia on imported poinsettia (*Euphorbia pulcherimma*) plants (Estonian Agricultural Board, 2005).

Also traded tomato fruit may play a role in spread of whiteflies and associated viruses (Wege, 2007). *B. tabaci* can be carried on leaves of vine tomatoes as well as on sepals and peduncles, while TYLCV-infected tomato fruit may act as a source for virus transmission by whiteflies (Delatte et al., 2003; EFSA PLH, 2013). Considering the widespread presence of TYLCV in tomato-production areas in southern Europe, it is expected that virus is spread to northern Europe by international trade via infected plant material and insect vector. In tests of tomato fruit imported from the Mediterranean Region to Estonia and Sweden, 28% and 10% of the fruit were infected with TYLCV, respectively (Just et al., 2014). The aim of this project was to test the infectivity of TYLCV DNA found in imported tomato fruit in Estonia. In northern Europe, *B. tabaci* is a quarantine pest and vector transmission tests of TYLCV and other begomoviruses are therefore complicated to carry out. Thus, alternative biotechnological methods such as agroinfiltration and biolistic inoculation were selected. Experiments showed that TYLCV isolated from imported tomato fruit is infectious and able to induce symptoms in tomato plants.

Materials and methods

Construction of an infectious clone for Tomato yellow leaf curl virus (*TYLCV*). Imported tomato fruits were previously used for isolation of TYLCV full-length genomes and isolate TYLCV-[EE-Imp-05-08] (accession No. HF548826) was selected for construction of an infectious clone. During cloning, the 1.0-mer clone was named pGTYEE-1.0 (Just et al., 2014). Experiments were carried out at the Swedish University of Agricultural Sciences and Estonian University of Life Sciences in 2010–2013. The 0.8-mer clone containing the replication origin was released from pGTYEE-1.0 by digestion with *Sac*I and *Nco*I. The resulting fragment was cloned into *Sac*I and *Nco*I digested pLH7000*, which is a modified form of the binary vector pLH7000, and the resulting clone was named pLTY-0.8. The constructs pGTYEE-1.0 and pLTY-0.8 were digested with *Sac*I to release the full-length genome and linearize pLTY-0.8, respectively. The full-length genome was ligated into the linearized pLTY-0.8, resulting in a 1.8-mer construct of the virus genome named pLTY-1.8.

Agrobacterium transformation and inoculation of full-length clone. The partial dimer construct pLTY-1.8 was transformed into cells of *Agrobacterium tumefaciens* strain C58C1 using freeze-thaw transformation (Weigel, Glazebrook, 2006). Polymerase chain reaction (PCR) was run to confirm the presence of begomovirus DNA in agrobacteria as described previously (Leke et al., 2012; Just et al., 2014). Subsequently, 5 ml of culture was used to inoculate 50 ml of Luria-Bertani (LB) containing respective antibiotics, 10 mM 2-(N-morpholino) ethanesulfonic acid (MES) and 150 µM acetosyringone, and the culture was incubated overnight with shaking (28°C, 250 rpm). Bacteria were harvested by centrifugation at 8000 rpm for 15 min and the pellet was resuspended in 20 ml buffer (10 mM $MgCl_2$, 10 mM MES and 150 µM acetosyringone). The optical density of inoculum was 0.6. Leaves of tomato (*Solanum lycopersicum* L.) cv. 'Moneymaker' and *Nicotiana benthamiana* were infiltrated on the lower side with a 2 ml syringe without a needle. For both tomato and *N. benthamiana*, 14 plants were inoculated at two-leaf stage. One negative control plant was inoculated both for tomato and *N. benthamiana* with *A. tumefaciens* harbouring the empty binary vector pLH7000*. Plants were maintained in a climate chamber at 23°C with a photoperiod of 16/8 h light/dark and monitored for symptom development. The plants were tested for viral infection by PCR one month after inoculation.

Inoculation of full-length clone by biolistic delivery. Plants of *N. benthamiana* and *S. lycopersicum* cv. 'Moneymaker' were bombarded at two-leaf stage by a particle inflow system gene gun (Clapham et al., 2000) with two types of inoculum: linear genome monomer of the isolate TYLCV-[EE-Imp-05-08] released from the clone pGTYEE-1.0 by restriction with *Sac*I, and rolling circle amplification (RCA) product of religated (circular) genome monomer released from pGTYEE-1.0. Viral DNA (1 µg plant^{-1}) was coated onto gold particles and the pellet was finally re-suspended in 100% ethanol. An aliquot (20 µl plant^{-1}) was deposited on a metal sieve plate and used for bombardment. A partial vacuum of ~7.5 cm Hg was applied by means of a vacuum ejector and the particles were accelerated by releasing a millisecond of pressurized helium (5.8 bar) using a solenoid valve. For each set of inoculations, 14 plants each of tomato and *N. benthamiana* at two-leaf stage were used. As a negative control, one plant for each species was mock-inoculated with gold particles alone. Following inoculation, plants were kept in a growth chamber at 23°C with a photoperiod of 16/8 h light/dark and monitored for symptom development. The plants were tested for viral infection by PCR one month after inoculation. Data was statistically analysed using a Chi-square test for independence with α value 0.05.

Confirmation of infection by PCR. Plant DNA was extracted from a young systemic leaf of inoculated plants by homogenization of tissue in an alkaline solution (0.5 N NaOH) followed by neutralization in 100 mM TrisHCl pH 8.0. A fragment from the *CP* gene of TYLCV (579 bp) was amplified by PCR as described previously (Leke et al., 2012; Just et al., 2014).

Southern blot hybridization. Total DNA was extracted (Weigel, Glazebrook, 2009) from young systemic leaves of plants, which had been inoculated with TYLCV-[EE-Imp-05-08] by either agroinfiltration (three plants each of tomato and *N. benthamiana*) or biolistic delivery (two plants each of tomato and *N. benthamiana*) and that had tested virus positive by PCR as well as from negative control plants. For Southern blot analysis, both undigested DNA and DNA restricted with *Nde*I were included. *Nde*I has a unique restriction site in the genome of TYLCV-[EE-Imp-05-08]. The DNA was separated by electrophoresis in a 1.2% agarose gel in 1% Tris-acetate-EDTA (TAE) buffer. After an initial depurination in 0.25 M HCl for 10 min, the gel was rinsed in deionized H_2O and consecutively soaked in a denaturation solution (0.4 M NaOH) for 30 min. DNA was transferred to Hybond™-XL membranes (GE Healthcare, UK) by capillary force, and the membrane was washed in 2 × saline-sodium citrate (SSC) buffer to remove any gel traces and subsequently dried. DNA was fixed to the membranes using a Stratagene UV crosslinker (Artisan Technology Group, USA) . The membrane was hybridized with a PCR product for the *CP* gene of TYLCV-[EE-Imp-05-08] radioactively labelled with α-^{32}P using Rediprime II DNA Labeling System (GE Healthcare, UK). Hybridization was performed at 65°C for 16 hours. The membrane was washed once with 2 × SSC/1% SDS and once with 2 × SSC/0.1% SDS at 65°C, wrapped in plastic, exposed to white-screens and the signal was detected using a phosphoimager (Bio-Rad, USA).

Results

Symptom appearance and analysis of viral DNA in agroinfiltrated plants. A genomic 1.8-mer construct of TYLCV-[EE-Imp-05-08] successfully infected plants of *S. lycopersicum* and *N. benthamiana* by agroinfiltration and induced symptoms resembling natural infection: leaf curling and distortion, yellowing of leaves and reduction in plant growth (Fig. 1 A, D, F).
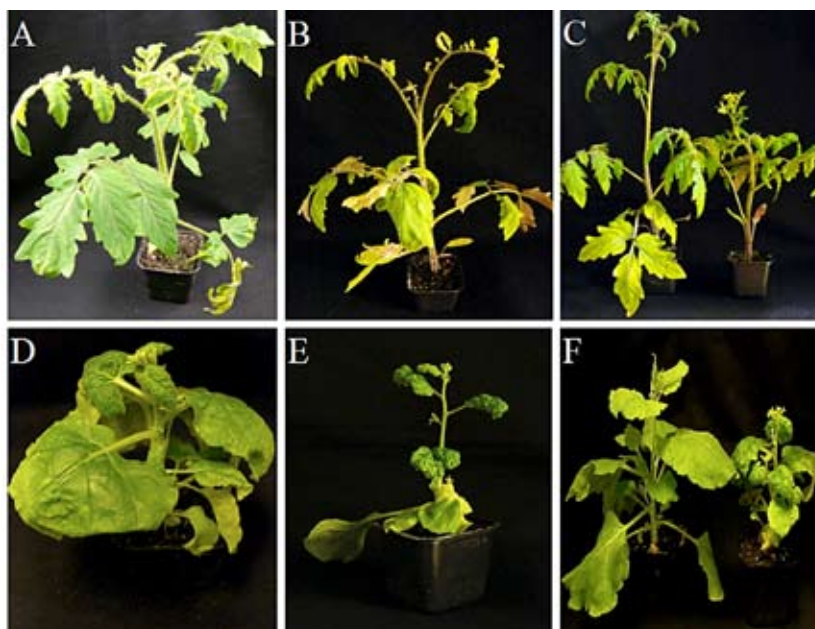
C – mock-inoculated tomato *vs* biolistic delivery of rolling circle amplification (RCA) product, F – mock-inoculated *N. benthamiana vs* agroinfiltration

Figure 1. Representative infection symptoms on plants of tomato (A, B, C) and *Nicotiana benthamiana* (D, E, F) 40 days after inoculation by TYLCV-[EE-Imp-05-08], either inoculated using an infectious clone and *Agrobacterium tumefaciens* (A, D, F) or biolistic delivery of circular genome monomer (B, C, E)

The first symptoms appeared 2–4 weeks after inoculation and one month after inoculation, all 14 plants of both tomato and *N. benthamiana* were positive for TYLCV infection when tested by PCR (Table). Neither viral DNA nor symptoms of infection were observed in plants inoculated with *Agrobacterium* containing empty

Table. Results of agroinfiltration and gene gun bombardment of tomato and *Nicotiana benthamiana* with TYLCV-[EE-Imp-05-08]

Host plant	No. of plants infected / No. of plants inoculated / Inoculation efficiency (%)		
	Agroinfection	Gene gun RCA product	Gene gun linear monomer
Tomato	14 / 14 / 100%	8 / 14 / 57%	0 / 14 / 0%
N. benthamiana	14 / 14 / 100%	5 / 14 / 36%	0 / 14 / 0%

RCA – rolling circle amplification

binary vector. With an inoculation efficiency of 100%, the cloned viral DNA represented a fully biologically active copy of the genome of the TYLCV isolate detected from imported tomato fruit.

Analysis of viral DNA in plants inoculated by biolistic delivery. Tomato and *N. benthamiana* plants inoculated with RCA product of TYLCV-[EE-Imp-05-08] started to display typical symptoms of TYLCV infection 2–3 weeks after inoculation (Fig. 1 B, C, E). The mock-inoculated plants used as negative control did not show any symptoms. Out of 14 *N. benthamiana* plants and 14 tomato plants inoculated with the RCA product, 5 and 8 plants were symptomatic, respectively (Table). Infection was confirmed by PCR one month after inoculation. The inoculation efficiency of RCA product by particle bombardment was 57% and 36% for tomato and *N. benthamiana*, respectively. The null hypothesis for a Chi-squared test for independence stated that there is no statistical relationship between species and infectivity. Since the calculated Chi-square value (1.29) was lower than the critical value (3.84) and the probability value (0.26) was higher than the alpha value (0.05), the null hypothesis could not be rejected. After particle bombardment with the linear genome monomer of TYLCV-[EE-Imp-05-08] released from the clone pGTYEE-1, none of the 14 plants of tomato or 14 plants of *N. benthamiana* became infected.
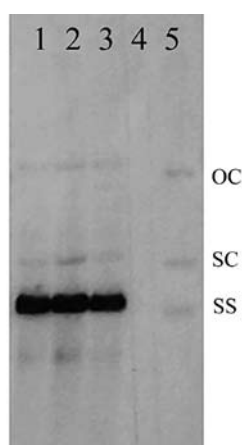
Notes. The plants were sampled at 45 days after inoculation. Each lane represents an individual plant. The positions of open circle (oc) and supercoiled (sc) forms of double-stranded viral DNA are indicated as well as viral single-stranded DNA (ss).

Figure 2. Southern blot analysis of total DNA extracted from tomato plants inoculated with TYLCV-[EE-Imp-05-08] using agroinfiltration (lanes 1–3) and biolistics (lane 5) or agroinfiltrated with empty binary vector (lane 4)

Confirmation of infectivity by Southern blot hybridization. Accumulation of genomic ssDNA of TYLCV and its replicative forms (open circular and supercoiled dsDNA) in systemic leaves of agroinfiltrated and bombarded plants was verified by Southern blot analysis of plants sampled at 45 days after inoculation both for tomato (Fig. 2) and *N. benthamiana* (not shown).

As expected, restriction digest with *Nde*I, which cuts once in the genome of TYLCV-[EE-Imp-05-08], resulted in a linearization of the dsDNA forms (results not shown). No probe hybridization occurred for negative controls.

Discussion

Infectivity of an Estonian TYLCV isolate from imported tomato fruit was demonstrated using agroinfiltration and biolistic inoculation. Agroinoculation has been an important technique for viral inoculation studies since the mid-1980s (Vaghchhipawala, Mysore, 2008), and it has successfully been used to inoculate tomato plants with cloned tandem repeats of TYLCV DNA derived from leaf tissues (Lapidot et al., 2007). Agroinoculation with the infectious 1.8-mer construct of TYLCV-[EE-Imp-05-08] amplified from tomato fruit resulted in an infectivity of 100% and the development of TYLCV symptoms both on tomato and *N. benthamiana* plants. In addition, plants of tomato and *N. benthamiana* were bombarded with RCA product and infectivity of 57% and 36% was obtained, respectively. The first successful biolistic inoculation of TYLCV was done by Morilla et al. (2005) using dimeric forms of DNA of TYLCV derived from pepper leaf tissue. Similar inoculation efficiencies were obtained as in the current study: 50% and 75% on tomato and *N. benthamiana*, respectively. Lapidot et al. (2007) bombarded tomato with circular full-length DNA of TYLCV with an inoculation efficiency of 37%. They also demonstrated infection after bombardment of *Datura stramonium* plants with a linear monomer of the TYLCV genome. However, none of the inoculated plants of tomato or *N. benthamiana* in the current study became infected after bombardment with a linear TYLCV genome monomer.

Current inoculation experiments show that infectious TYLCV is present in imported fruit in northern Europe and that it poses a potential virus source for transmission if the insect vector is introduced. In 2007, infection of TYLCV was recorded in 19 tomato greenhouse production sites in the Netherlands. The outbreak was probably the result of a single introduction of the virus, and introduced vector accounted for local spread (Botermans et al., 2009). Another example of an infectious virus detected in imported tomato fruit is PepMV in Lithuania (Staniulis et al., 2012). Using mechanical transmission, it was shown that PepMV from imported tomato fruit could be transmitted to tomato and several other host plants. In surveys of Lithuania, PepMV was also detected in tomato samples from commercial greenhouses (Staniulis et al., 2012; Šneideris et al., 2013). PepMV has rapidly spread throughout Europe and dissemination by infected fruit is a possible way (Hanssen et al., 2010). Thus, fruit and vegetables represent a virus dissemination pathway that is currently largely unregulated. Although many inoculation assays on TYLCV have been performed using DNA derived from leaf tissues, this is the first report of agroinfiltration and particle bombardment assay using TYLCV DNA

derived from infected tomato fruit. To conclude, tomato fruit can act as a pathway of entry for TYLCV, which has to be considered.

Conclusions

1. DNA of *Tomato yellow leaf curl virus* (TYLCV) amplified from imported tomato fruit was infective, confirming that tomato fruit may serve as a source of virus inoculum.

2. Agroinfiltration and gene gun bombardment with rolling circle amplification (RCA) product of circular genome monomer resulted in symptoms resembling those of natural infection.

3. Presence of genomic ssDNA of TYLCV and its replicative open circular and supercoiled dsDNA forms in inoculated plants was confirmed by Southern blot hybridization.

Acknowledgements

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Pomidorų lapų geltonosios garbanės viruso, izoliuoto iš į Estiją įvežtų pomidorų vaisių, infektyvumas

K. Just[1,2], M. N. Sattar[2,3], U. Arif[2,4], A. Luik[1], A. Kvarnheden[1,2]

[1]Estijos gyvybės mokslų universiteto Žemės ūkio ir aplinkos mokslų institutas
[2]Švedijos žemės ūkio universiteto ir Linėjaus augalų biologijos centro Upsalos biocentras
[3]Saudo Arabijos Karaliaus Faisal universitetas
[4]Švedijos Karališkojo technologijos instituto Biotechnologijos mokykla

Santrauka

Yra pavojus, kad pomidorų mozaikos geltonasis (garbanojimosi) virusas (TYLCV) ir jo pernešėjas baltosios *Bemisia tabaci* rūšies komplekso muselės paplis šiltnamiuose pasaulio vidutinio klimato regionuose, taip pat ir Europos šiaurinėje dalyje. Tyrimo metu buvo nustatyta, kad TYLCV, izoliuotas iš į Estiją įvežtų (ispaniškos kilmės) pomidorų vaisių, galėjo infekuoti pomidorų ir *Nicotiana benthamiana* augalus juos inokuliuojant *Agrobacterium* klonu su viruso genomu ir bolistiniu metodu įvedant viruso genomo riedančio rato amplifikacijos produktus. Parengtas konstruktas su TYLCV genomu buvo efektyviai transformuotas į pomidoro bei *N. benthamiana* augalus ir sukėlė natūraliai infekcijai būdingus simptomus. Inokuliuojant su *Agrobacterium*, infekcijos efektyvumas pomidorams ir *N. benthamiana* buvo 100 %, o biolistinio inokuliavimo naudojant riedančio rato amplifikacijos produktus efektyvumas buvo atitinkamai 57 ir 36 %. Bombarduojant dalelėmis, padengtomis linijiniu viruso genomu nepavyko sukelti infekcijos nei pomidoruose, nei *N. benthamiana*. Iš pomidorų vaisių amplifikuotas TYLCV genomas gebėjo užkrėsti augalus, ir tai patvirtino prielaidą, kad pomidorų vaisiai gali būti viruso užkrato šaltinis. Šiame straipsnyje pirmą kartą pranešama apie agroinfiltravimo ir bombardavimo dalelėmis tyrimą naudojant TYLCV DNR, išskirtą iš užkrėstų pomidorų audinių.

Reikšminiai žodžiai: agroinfiltracija, begomovirusas, infekcinis klonas, pomidorų vaisiai, *Solanum lycopersicum*.