

# Transgenic tobacco cells: a permissive system for the assessment of resistance strategies against Tomato yellow leaf curl virus

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RESEARCH

## ABSTRACT

Tomato yellow leaf curl virus (TYLCV) is a major threat to tomato production in the tropics and sub-tropics around the world. The application of genetic engineering and pathogen derived resistance mechanisms to obtain tomatoes that are resistant to this pathogen is considered a promising alternative to the current protective practice against the virus. However, the development of transgenic tomato plants that are resistant to the virus is a resource-consuming and time-consuming procedure, often with unpredictable efficiency, which hinders the evaluation of genetic designs. For this reason an assessment of the strategies against TYLCV replication preceding transgenic tomato development would ensure the protective potential of the candidate transgene. Attempting to circumvent this issue, the present study demonstrated the feasibility of using tobacco cell lines to study the consequences of *c1* antisense expression on TYLCV replication. As a result, the transgenic tobacco cell lines were able to produce siRNA that is complementary to the *c1* sequence and inhibited TYLCV multiplication, forecasting what would happen in transgenic plants harboring this antiviral strategy.

Keywords: begomovirus, TYLCV, tobacco, post-transcriptional gene silencing, pathogen derived resistance, tomato

*Biotecnología Aplicada* 2009;26:127-132

## RESUMEN

Tomato yellow leaf curl virus (TYLCV) constituye el mayor peligro para la producción de tomate en el mundo, principalmente en las regiones tropicales y subtropicales. La aplicación de la ingeniería genética y los mecanismos de resistencia derivada del patógeno para obtener tomates resistentes a este patógeno es considerada una alternativa promisoriosa en la práctica corriente de protección contra este virus. Sin embargo, el desarrollo de plantas transgénicas de tomate resistentes a virus es un procedimiento que consume mucho tiempo y recursos, y en la mayoría de las veces resulta impredecible su eficiencia, lo cual dificulta la evaluación de los diseños antivirales. Por ello la evaluación de las estrategias que inhiban la replicación de TYLCV en un sistema previo a la obtención de plantas de tomate transgénicas aseguraría el potencial protector del transgén candidato. En un intento por evadir el proceso de transformación de tomate en el presente trabajo se demostró la factibilidad de utilizar líneas celulares de tabaco para estudiar las consecuencias de la expresión del gen *c1* en antisentido sobre la replicación de TYLCV. Como resultado las líneas celulares de tabaco fueron capaces de producir ARN de interferencia, complementarios a la secuencia del gen *c1*, e inhibieron la multiplicación de TYLCV, previendo lo que sucedería en las plantas transgénicas portadoras de esta estrategia antiviral.

Palabras clave: begomovirus, TYCCU, tabaco, silenciamiento génico post-transcripcional, resistencia derivada del patógeno, tomate

## Introduction

The tomato yellow leaf curl disease is a major threat to tomato production in tropical and subtropical areas of the world [1]. This disease is caused by several closely related begomoviruses, among them the *Tomato yellow leaf curl virus* (TYLCV), *Tomato yellow leaf curl Axarquía virus*, *Tomato yellow leaf curl China virus*, *Tomato yellow leaf curl Guandong virus*, *Tomato yellow leaf curl Kanchanaburi virus*, *Tomato yellow leaf curl Malaga virus*, *Tomato yellow leaf curl Mali virus*, *Tomato yellow leaf curl Sardinia virus* and *Tomato yellow leaf curl Thailand virus*. The use of resistant plants is considered the most reliable and economically practicable strategy to control this whitefly trans-mitted disease [2, 3].

Breeding programs to develop TYLCV resistant varieties have enabled the recognition of plant genes

which are involved in the inhibition or attenuation of the symptoms produced by begomoviruses [2, 4-6]. However, the transfer of resistance gene from wild tomato species to commercial ones is accompanied by changes in the agronomical properties of recipient cultivars, which could limit the recovery of the commercial standards [6, 7]. On the other hand, although the resistant lines produced do not show any symptoms, they are still a source of TYLCV inoculants, since they can not block virus replication. With this resistant phenotype the plants remain in a healthy and optimal physiological state, feeding and increasing the whitefly population. As an alternative to these inconveniences, genetic engineering allows the application of *pathogen derived resistance*, a mechanism described in 1985 [8] that allows the plants carrying

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a specific pathogen-derived genomic sequence to withstand viral infection [9]. However, plant transformation and regeneration methodologies are still not a routine process for many important tomato cultivars, and transgenic plant production is a resource-consuming and time consuming procedure, often with unpredictable efficiency [10], which complicates the evaluation of genetic designs.

The development of simple, reliable and demonstrative systems for assessing virus replication preceding transgenic plant production would facilitate the analysis of the protective potential of a candidate transgene. Certain experimental models have been described, in which suspension cells or protoplasts are inoculated with the genomes of some geminiviruses [11-16]. A large part of previous studies were focused on the replication of the bipartite begomoviruses *Tomato golden mosaic virus* (TGMV), *African cassava mosaic virus* (ACMV) and *Bean golden mosaic virus* (BGMV) in tobacco cell lines.

To date, no data is available on the behavior of the monopartite begomovirus TYLCV in replication studies on suspension cells as alternatives, which could simulate conditions for the analysis of inhibition and multiplication, avoiding transgenic plant generation. Here, the tobacco NT1 cell line [17] was assessed as a system, to study TYLCV replication. The research was focused on the inhibition of TYLCV multiplication by generating siRNA derived from the expression of the TYLCV replication associated protein gene (*c1*) in antisense orientation. As a result, we demonstrated the feasibility of using tobacco NT1 cell line to simulate conditions for TYLCV replication and to forecast what would happen in transgenic plants harboring this antiviral strategy.

## Materials and methods

### Maintenance of tobacco cell suspension culture

NT1 cell suspension line was maintained as described previously [17], in a liquid Murashige and Skoog (MS) medium [18] supplemented with 2,4-dichlorophenoxyacetic acid (2 mg/L), sub-culturing every week by inoculating 10 mL of the suspension to 100 mL of the fresh medium.

### Gene constructions

To generate a 2 mer TYLCV-[CU] genomic clone, the plasmid pZTYL (1 mer TYLCV-[CU]) previously obtained in our laboratory at CIGB in Cuba, by cloning the TYLCV-[CU] genome into the pZeroTM-2.1 plasmid (Invitrogen, San Diego, CA), was partially digested with *Sac* I for 20 min. At the same time a copy of the TYLCV-[CU] genome was generated by a complete digestion with the same enzyme for one hour. The 6.2 kb product of the partial digestion (pZero + TYLCV-[CU]) was treated with alkaline phosphatase and ligated for 4 hours at room temperature to the 2.8 kb fragment of TYLCV-[CU], obtained from a complete digestion (generation of the plasmid pZ2TYL). After transforming *Escherichia coli* cells, XL1-blue strain, a head to tail clone was selected. The dimer of the TYLCV-[CU] was digested from pZ2TYL by *Hind* III/*Bam* HI enzymes and ligated into the binary vector

pPZP200, previously digested with *Hind*III/*Bam*HI (generation of the p2TYL plasmid).

The TYLCV-[CU] *c1* gene was amplified by polymerase chain reaction (PCR) from the 2 mer TYLCV genomic clone (plasmid p2TYL). Primers were designed to add convenient restriction sites to the end of the amplicons. The *c1* gene was amplified with primers A (TCTCTCGAGTTACGCCCTTATTGGTTTC) and B (CGCGGATCCATGCCTCGTTTTATTAAA) (1 min at 94 °C, 1 min at 46 °C and 3 min at 72 °C, in 35 cycles), digested with *Xho*I-*Bam*HI restriction enzymes and cloned into the pBPÜ8 plasmid [19] digested with *Xho*I and *Bam*HI (generation of the plasmid pBP*c1*<sub>antisense</sub>). The fragment containing the *c1*<sub>antisense</sub> under the 35S promoter and the tNOS terminator was digested from pBP*c1*<sub>antisense</sub> with *Hind* III and cloned into the binary vector pPZP200 digested with *Hind* III (generation of the plasmid pPZP*c1*<sub>antisense</sub>).

### Agroinoculation of tomato plants

Tomato plants were grown up to the four to five leaves stage. Under the temperature of 24 °C and a daylight regime the plants were subjected to a longitudinal incision using a scalpel, at the lower part of the stem. A 100 µL of *Agrobacterium tumefaciens* suspension, strain C58C1 (pGV2260) [20] harboring the p2TYL plasmid and conditioned for tomato transformation as described previously [10], was inoculated within the injured tissues. The agroinoculated plants were grown for several weeks under the same environmental conditions until the TYLCV replication assays were carried out.

### Transformation of tobacco cells

The NT1 cell line was transformed using the gene bombardment system designed by *Finer et al.* [21]. Cells at the exponential growth state (4<sup>th</sup> day after the sub-culture) were harvested in a number of 5 x 10<sup>5</sup> units and plated on Whatman #1 discs with the aid of a Buchner funnel. Before the bombardment, discs were incubated in the dark for 6 hours at 24 °C on the MS medium [18] containing sorbitol and mannitol 0.2 M. Gold particles (1-3 µm) carrying each plasmid were prepared according the procedure described by *Sanford* [22]. Particle mixtures for bombardment contained 50 µL of gold particles (60 mg/mL), 10 mL of the plasmid (3 µg/µL), 50 µL CaCl<sub>2</sub> (2.5 M), and 20 µL spermidine (0.1 M). After incubation for 10 min at room temperature, the particles were rinsed in 140 µL of ethanol 95% and resuspended in 25 µL of ethanol 95%. For bombardment, 3 µL of the mixtures were used per shot. After shots, samples were incubated for two days in a liquid NT1 medium. To generate transgenic cell lines, the bombarded discs were placed on a solid NT1 medium (solidified with Phytigel 2 g/L) containing kanamycin 25 mg/L, and 20 days later, antibiotic concentration was adjusted to 150 mg/L. Kanamycin resistant calluses were propagated in the same medium or disaggregated in the liquid NT1 medium for 15-20 days to develop the cell suspension.

### Analysis of TYLCV-[CU] replication in tobacco cells

After the first two days of bombardment, total DNA of the NT1 line cells was extracted by grinding in

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liquid nitrogen, using 500  $\mu$ L of the extraction buffer containing urea 7 M, NaCl 5 M, Tris-HCl 1 M pH 8, ethylenediaminetetra-acetic acid (EDTA) 0.5 M, Sarkosyl 0.7%. Extracts were incubated for 30 min at room temperature and shaken periodically. Later, mixtures were treated with phenol/chloroform (v/v) and total DNA was obtained after precipitation in isopropanol.

The DNA digested with the *Mung Bean* or *MboI* nucleases was analyzed by Southern blot. Briefly, fifteen micrograms of DNA were fractionated in 0.8% agarose gel and transferred onto Hybond N+ membranes (Amersham Pharmacia Biotech, USA) using a TE 80, Vacuum Blotting Unit (Amersham Pharmacia Biotech, USA). DNA was cross-linked to the membrane using an Ultraviolet Crosslinker device (Amersham Pharmacia Biotech, USA). Hybridizations were done at 60 °C using the [ $\alpha$ - $^{32}$ P]dATP-labelled TYLCV *c2* gene (capsid protein gene) or the entire viral genome.

#### Extraction of RNA from transgenic tobacco cells and siRNA detection

For total RNA extraction, 0.5 g of NT1 cells were ground in the presence of liquid N<sub>2</sub>. After the addition of the extraction buffer (LiCl 0.1 M, Tris-HCl 100 mM pH 8, EDTA 10 mM and sodium dodecyl sulfate (SDS) 1%) and saturated phenol pH 4.4, the mixtures were homogenized by vortex for 30 s and 250 mL of chloroform-isoamylalcohol (24:1, v/v) was added. The water phase was removed after centrifugation and a volume of LiCl 4 M was added. RNAs were allowed to precipitate at -20 °C and later collected by centrifugation. The pellets were dissolved in water and precipitated in the presence of NaOAc 3 M pH 5.2 and 2 volumes of cold 95% ethanol. Later, the pellets were washed with 75% ethanol and resuspended in water. High molecular weight RNA was discarded after the precipitation of 10  $\mu$ g of total RNA with polyethylene glycol (MW 8000) 5% and NaCl 0.5 M. Low molecular weight RNAs were precipitated in the presence of 0.1 volume of NaOAc 3 M pH 5.2 and 3 volumes of ethanol 95%. For blotting, both RNAs were denatured at 65 °C and fractionated in denatured conditions. Fractionation of low molecular weight RNA was performed on 15% polyacrylamide gel containing 7 M urea and transferred to the Hybond N+ membrane (Amersham Pharmacia Biotech, USA) using a TE 80, Vacuum Blotting Unit (Amersham Pharmacia Biotech, USA). In the case of high molecular weight RNA, the fractionation was carried out in 1% agarose gels containing 6.84% formaldehyde. RNAs were cross-linked to the membrane using an ultraviolet Crosslinker device (Amersham Pharmacia Biotech, USA). Hybridizations were done at 50 °C using the [ $\alpha$ - $^{32}$ P]dATP-labelled *c1* gene as the probe, prepared by the random primer procedure according to the Prime-a-Gene Labeling System (Promega, USA) instructions.

## Results

To demonstrate the replicating capacity of the NT1 tobacco cell line for TYLCV, the 2-mer TYLCV-[CU] construction (p2TYL, Figure 1) was transfected into cells applying a particle bombardment procedure. Previously, replication activity of the generated p2TYL was confirmed by agroinoculation into tomato plants.

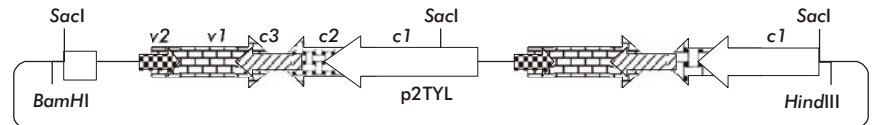


Figure 1. Representation of gene construction p2TYL. *c1*, *c2*, *c3*, *v1* and *v2*: open reading frames (ORFs) in the TYLCV-[CU] genome. *V* represents virion sense genes, and *c* represents complementary sense genes.

The agroinoculated tomato plants were able to produce double and single strand viral genome DNA corresponding to 2.8 and 1.6 kb respectively, associated to replicative intermediate and viral genome DNA of the new synthesis (lanes 1-5 and 7-10; Figure 2).

The time course process of viral replication was followed by Southern blot hybridization on total DNA extracted from transfected NT1 cells. As observed on X-ray film, a band of approximately 1.6 kb corresponding to the TYLCV single strand genomic DNA was synthesized after four days of tobacco cell incubation (lanes 3, 4 and 5; Figure 3). Concentrations of TYLCV-[CU] single strand and double strand viral DNA increased daily, and reached the maximum level at the last day of the trial (lane 5; Figure 3).

To verify that the 1.6 kb signal corresponds to the TYLCV-[CU] single strand genome, and to evaluate it as an evidence of the new viral genome replicated from the 2 mer construction, total DNAs extracted from bombarded cells were digested with *Mung Bean* nuclease before the Southern blot hybridization. Since

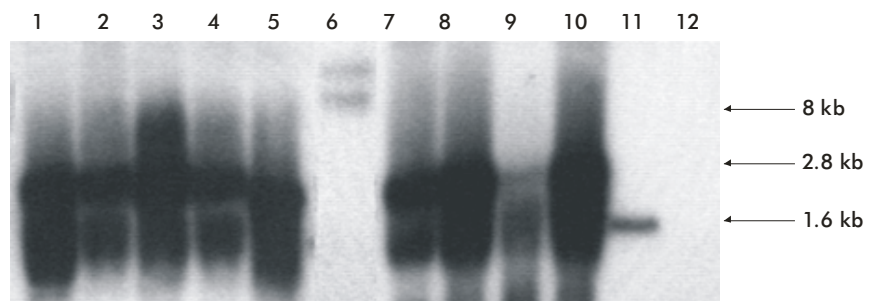


Figure 2. Radiography on Southern blot hybridization for the TYLCV-[CU] genome detection. Lanes 1-5 and 7-10: total DNA from agroinoculated tomato plants with p2TYL construction; lane 6: DNA construction p2TYL; lane 11: total DNA from tomato plant infected with *Bemisia tabaci*; lane 12: total DNA from non-infected plant. The 700 bp fragment of *c2* gene was used as a probe for hybridization.

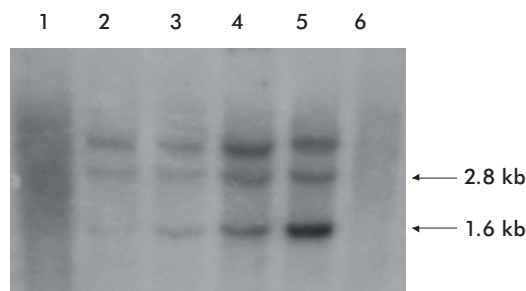


Figure 3. Radiography on Southern blot hybridization for TYLCV-[CU] genome detection in tobacco suspension cells. Lanes 1-5: total DNA from tobacco cells collected at 2-6 days respectively, after bombardment with p2TYL; lane 6: total DNA from non-bombarded cells. The 2.8 kb TYLCV-[CU] genome was used as a probe.

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the activity of this enzyme is specific for single strand DNA, the 1.6 Kb signal in treated cell DNA extracts was missing from the autoradiography (lane 1 and 2; Figure 4). In parallel, to confirm the *de novo* synthesis of the double TYLCV-[CU] strand, total DNAs from inoculated NT1 cells were digested with *Mbo*I nuclease. *Mbo*I restriction analysis allows to identify, in a general way, the organism that is the source of a given DNA. *Mbo*I-recognized DNA sequences harboring a GATC palindrome which is also recognized by Dam methylase in *E. coli*. The DNA used to transform NT1 tobacco cells was purified from *Escherichia coli* XL-1 Blue strain which is characterized by Dam methylation [23]. Hence, the cloned TYLCV-[CU] genome from *E. coli* is methylated at *Mbo*I restriction sites and could not be digested by this enzyme (lanes 5 and 6; Figure 4). In contrast, the *de novo* TYLCV-[CU] genome synthesized in NT1 cells is *Mbo*I susceptible (lanes 3 and 4; Figure 4). Hence, it was demonstrated that the hydrolyzed double strand TYLCV-[CU] genome molecule has been replicated in NT1 tobacco cells.

Once the replication of the TYLCV-[CU] genome in the NT1 cell line was established, the next goal of this investigation was to develop transgenic cell lines harboring the antiviral strategy. We chose the construction carrying the *c1* antisense sequence (pPZPc1<sup>antisense</sup>; Figure 5) to induce post-transcriptional gene silencing against TYLCV-[CU] in NT1 cells. The development of transgenic lines was performed by particle bombardment. More than 10 transgenic lines were established in the selective NT1 medium, and all of them were bombarded with p2TYL. Among the transfected transgenic lines there were two that were not able to replicate TYLCV-[CU] (lanes 5-7 and 8-10, corresponding to three replicates of transgenic cell lines Ant3 and Ant4 respectively; Figure 6). Whereas, the rest of the cell lines were able to produce both the double (2.8 kb) and single (1.6 kb) strand viral DNA (lanes

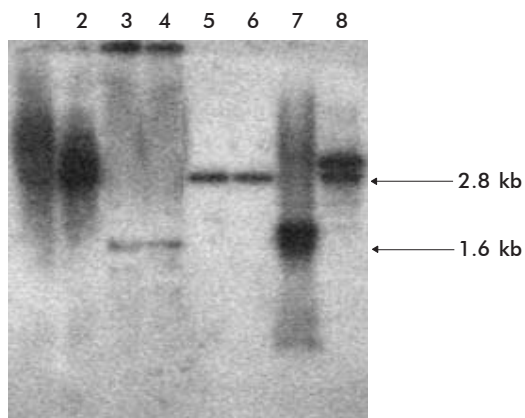


Figure 4. Radiography on Southern blot hybridization for detection of TYLCV-[CU] replicated in NT1 cells. Lanes 1-4: total DNA from NT1 cells bombarded with p2TYL; lanes 1-2: total DNA from bombarded cells digested with *Mung Bean* nuclease; lanes 3-4: total DNA from bombarded cells, digested with *Mbo*I. Lane 5: DNA from 1 mer TYLCV-[CU] construction purified from *Escherichia coli* XL-1 Blue cells digested with *Sac*I; lane 6: DNA from 1 mer TYLCV-[CU] construction digested with *Sac*I and *Mbo*I. Lane 7: total DNA from TYLCV-[CU] infected tomato plant digested with *Mbo*I; lane 8: total DNA from TYLCV-[CU] infected tomato plant digested with *Mung Bean* nuclease. The 2.8 kb genome of TYLCV-[CU] was used as a probe.

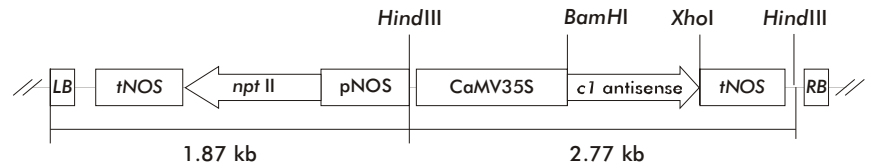


Figure 5. Physical map of the *c1* antisense expression cassette from pPZPc1<sup>antisense</sup>; CaMV 35S: 35S genomic promoter of *Cauliflower Mosaic Virus* (CaMV); LB: T-DNA left border; RB: T-DNA right border; tNOS: nopaline synthase terminator; *nptII*: neomycin phosphotransferase II gene; pNOS: nopaline synthase promoter.

1-4 and 11-13; Figure 6), resembling what had occurred in non transgenic NT1 cells bombarded with the p2TYL construction (lanes 14 and 15; Figure 6). Northern hybridization for the detection of small RNA specific to the *c1* sequence evidenced the production of siRNA in the two resistant lines Ant3 and Ant4 (lanes 3 and 4; Figure 7).

## Discussion

### Tobacco NT1 cell line allows TYLCV replication

The NT1 cell line was selected as a model to analyze the consequences of the TYLCV *c1* antisense transgene expression on TYLCV replication due to several practical features: high propagation rate at a low cost, little demand for *in vitro* manipulation, possibility of being transformed by direct and indirect methods and the short time required to establish transgenic cell lines [22, 24, 25]. Probably, the high reproductive capacity of the NT1 cell line favors its competency for its genetic transformation, similar to that suggested for several plant species when they were transformed via *Agrobacterium tumefaciens* [26-28]. As NT1 cells can grow continuously, they should be a good target

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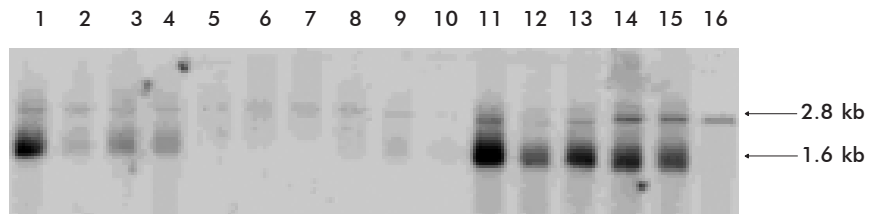


Figure 6. Radiography on Southern blot hybridization applied to cell lines transformed with pPZPc1<sup>antisense</sup> (cell lines Ant) and bombarded with p2TYL. Lanes 1-4: replicates of transgenic cell line Ant2; lanes 5-7: replicates of transgenic cell line Ant3; lanes 8-10: replicates of transgenic cell line Ant4; lanes 11-13: replicates of transgenic cell line Ant5; lanes 14 and 15: non transgenic cells; lane 16: 1 mer TYLCV-[CU] of 2.8 kb size, digested with *Sac*I. The 2.8 kb probe corresponding to the genome of TYLCV-[CU] was used for hybridization.

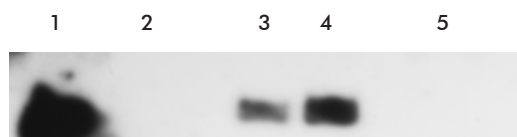


Figure 7. Radiography on Northern blot hybridization applied to NT1 transgenic cell lines without inoculating with TYLCV-[CU]. Detection of siRNA complementary to the *c1* gene in transgenic NT1 cell lines; lane 1: 50 ng of the 26-base oligonucleotide corresponding to the *c1* sequence; lane 2: transgenic cell line Ant2; lane 3: transgenic cell line Ant3; lane 4: transgenic cell line Ant4; lane 5: non transgenic cells. A 700 pb probe corresponding to the *c1* gene was used for hybridization.

for systems requiring high transformation frequencies such as the case of our study.

The first step in begomovirus replication consists of an intermediary double stranded DNA (dsDNA) synthesis from a single stranded (SSDNA) genome [29, 30]. This process is entirely executed by host enzymes. Afterwards, the start of the rolling cycle replication requires a joint action between viral Rep and REN proteins and cell factors to produce new dsDNAs and ssDNA progenies [31]. For several geminiviruses the amount of intermediary ds DNA has been correlated with the occurrence of the S-phase [32-34], and the activation of mitosis and the S-phase entry of the cell is due to the release of the E2F transcription factor. E2F is released through hyperphosphorylation and subsequent inactivation of the retinoblastoma related protein (RBR) that represses it [35]. Actually, two geminiviral proteins, Rep and RepA, inactivate the cell RBR, which promotes cell DNA replication necessary for viral genome multiplication [36]. Probably the NT1 cell line carries all the necessary elements for the replication of TYLCV by its perennial division state and it could be predicted that no interactions between the geminivirus Rep protein and the repressor of E2F are required. As a supporting fact for this idea other authors have demonstrated the replication of bipartite begomoviruses *Tomato golden mosaic virus* (TGMV), *African cassava mosaic virus* (ACMV), *Bean golden mosaic virus* (BGMV) [13, 14, 16, 37], and monopartite mastrevirus *Bean yellow dwarf virus* (BeYDV) [38] in tobacco plant cells.

Correspondingly, the NT1 cell line resulted attractive for the establishment of the TYLCV replication model. Therefore, after a two-day incubation period the transfected NT1 cells with the p2TYL construction supported TYLCV replication, which was evidenced by the fact that the dsDNA and ssDNA corresponding signals from the bombarded cells were intensified (Figures 3 and 4).

As a conclusion we can state that the tobacco NT1 cells contain the necessary elements for TYLCV replication and are therefore an appropriate host for an *in vitro* model for TYLCV multiplication assays.

#### Virus challenge: transgenic NT1 tobacco cell line carrying *c1* antisense sequence inhibited TYLCV replication

As soon as the NT1 ability for TYLCV replication was demonstrated, we proceeded to assess its capacity to be used in studies on the inhibition of TYLCV multiplication. The cells carrying the *c1* antisense construction (Figure 5) were bombarded with 2 mer TYLCV-[CU]. With the aim of detecting the *de novo* synthesis of the viral genome, total DNA from the bombarded cells was analyzed by Southern hybridization, using the TYLCV genome as the probe.

Two transgenic lines out of ten grown on NT1 solid medium supplemented with kanamycin 150 mg/L showed the capacity to inhibit TYLCV replication; the cell lines Ant3 and Ant4 (Figure 6). This result is evidenced by the absence of autoradiographic signals corresponding to viral dsDNA and ssDNA in comparison to non transgenic lines.

Previously, an induced post-transcriptional gene silencing (PTGS) against the Rep transcripts aimed to

inhibit the ACMV replication in a protoplast system was described [16]. For that purpose, the authors introduced specific Small interfering RNA (siRNA) molecules from replication associated gene *ac1* sequence into protoplasts of tobacco BY-2 cells, in the presence of ACMV. As a result, partial inhibition of ACMV replication in protoplast, co-transfected with the mixture of siRNA was obtained [16]. Although this system has the advantage of giving a rapid demonstration of the suitability of PTGS to inhibit ACMV replication in a protoplast system (in 3-4 days), only a partial effect on ACMV genome multiplication was evidenced in comparison to the transfected samples without siRNA. In addition, ACMV replication still took place, which seems to be controversial since the Rep protein is required for virus replication, although the authors were not able to detect the mRNA specific for Rep by Northern hybridization. In our study the cell transgenic lines Ant3 and Ant4 totally inhibited the TYLCV genome replication (Figure 6), which could prevent the multiplication of TYLCV.

The genetic design to provoke *c1* gene silencing was focused on the promotion of double strand RNA (dsRNA) which is expected to induce post-transcriptional gene silencing against the target sequence. To obtain this design the *c1* antisense sequence was ligated downstream of the 35S promoter of the *Cauliflower mosaic virus* (CaMV) and upstream to the nopaline synthetase terminator of the *Agrobacterium tumefaciens*. This construction, known for its capacity to diminish TYLCV infection in *Nicotiana benthamiana* transgenic plants [39], should originate complementary transcripts to those produced from the *c1* gene of the TYLCV genome. Complementary transcripts could form dsRNA molecules which would constitute the substrate for RNase type III (Dicer) specific for dsRNA. As a result, fragments of 21 to 25 bp siRNA should be produced [40].

Consistently, transgenic cell lines Ant3 and Ant4 are those producing siRNA complementary to the *c1* gene (Figure 7). The inhibition of TYLCV replication in these two lines reflects the capacity of the PTGS mechanism, directed against the Rep, to diminish TYLCV multiplication, and, on the other hand, the capacity of tobacco NT1 cell line to develop the PTGS against the *c1* gene.

Nevertheless, transgenic cell lines Ant3 and Ant4 produced siRNA that was complementary to the *c1* gene sequence even when not inoculated with TYLCV. The PTGS process in these two lines could be started from a single aberrant strand of RNA which are converted to dsRNA by the action of cell RNA dependent RNA polymerase (RdRP) [41, 42]. The siRNA complementary to the *c1* gene may be derived from transcripts without the 5' cap structure or poly A tail at the 3' end, originated under a high 35S promoter activity [43, 44], as a substrate for RdRP. Also, multiple copies of the *c1* antisense sequences may have occurred at high frequencies after the particle bombardment procedure [45, 46] (not demonstrated in the present study) triggering, through an excess of mRNAs, the formation of secondary structures which could be diced into siRNA molecules [47]. Consequently, the advantage of the system proposed in the present work consists of the stable production, *a priori*, of the siRNA mole-

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cules complementary to the *c1* sequence as the consequence of the transgene integration. Therefore, the synthesis of the Rep protein once the virus is inside the cell is unlikely to occur, which could be explained by the immediate degradation of the viral *c1* transcript. In this way, our system closely resembles what could occur in plants carrying this antiviral design. Besides, our model circumvents the lack of the stability evaluation of the silencing effect through successive cell generations in transiently evaluated antiviral designs.

In conclusion, transgenic tobacco NT1 cell lines capable to stably generate siRNA molecules specific for the *c1* sequence inhibited the replication of TYLCV. This fact was confirmed later in transgenic tomato

lines that harbour double strand RNA forming construction design specific for *c1*, which became immune with TYLCV inoculation under high whitefly population conditions [48].

The proposed transgenic system of NT1 tobacco cells demonstrated its suitability for replication studies in TYLCV. In addition, the capacity of these cells to generate siRNA and, subsequently, to induce PTGS against viral genes make them an attractive system for studies on viral functions.

### Acknowledgments

This work was funded by the State Council of the Republic of Cuba.

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*Received in March, 2009. Accepted for publication in June, 2009.*