EVALUATION OF TWO GENE-SILENCING CONSTRUCTS FOR RESISTANCE TO TOMATO YELLOW LEAF CURL VIRUSES IN *NICOTIANA BENTHAMIANA* PLANTS

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Summary. – Infiltration of *Agrobacterium tumefaciens* into intact plant leaves of *N. benthamiana* was used to test the efficiency of two virus-based silencing constructs conferring resistance to the closely related begomoviruses. The constructs contained the most conserved sequences of the coat protein (CP) gene and replication-associated protein (Rep) gene of Tomato yellow leaf curl Sardinia virus (Sicily strain) (TYLCSV-[Sic]). Both constructs formed a hairpin structure that enhanced the post-transcriptional gene-silencing mechanism. When agro-infiltrated plants were challenged separately with infectious viruses TYLCSV-[Sic] and Tomato yellow leaf curl virus (TYLCV), the plants showed resistance to TYLCSV-[Sic], but not to the related TYLCV.

Key words: agro-infiltration; Nicotiana benthamiana*;* gene silencing; coat protein gene; Rep gene; begomoviruses

Introduction

Tomato production in subtropical and temperate regions is often devastated by the tomato yellow leaf curl disease that is caused by a group of related viruses belonging to the genus *Begomovirus*, the family *Geminiviridae.* The principal members of this group are Tomato yellow leaf curl virus (TYLCV) and Tomato yellow leaf curl Sardinia virus (TYLCSV) (Fauquet and Stanley, 2005). These viruses contain a small genome of 2.8 kb encapsidated in twinned particle (Kheyr-Pour *et al*., 1991; Cohen and Nitzany, 1966). For the first time, TYLCV was described in Israel and this virus proved to be one of the most devastating tomato-infecting viruses (Cohen and Nitzany, 1966). In the host plant, TYLCV spreads faster, is more aggressive, and causes more severe symptoms than TYLCSV. In addition, both viruses were detected on peppers and beans (Anfoka *et al*., 2005; Navas-Castillo *et al*., 1999). So far, only TYLCSV has been detected in the main growing regions in Tunisia (Gorsane *et al*., 2005, 2004; Fekih-Hassen *et al*., 2003). Sequence analysis of the TYLCSV genome, Tunisian isolate (Acc. No. AY736854) showed that the isolate was identical with TYLCSV-[Sic].

Regarding the losses caused by TYLCSV epidemics in the Tunisian tomato growing areas, it is of great importance to develop a resistant tomato hybrid. TYLCV-resistant tomato cultivars were developed in Israel, USA, and Guatemala using genes derived from wild species of *Solanum* spp. (Mejía *et al*., 2005; Scott *et al*., 1995; Zamir *et al*., 1994; Pilowsky and Cohen, 1990). However, Roma-

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Abbreviations: CP = coat protein; CHSA = chalcone synthase; PTGS = post-transcriptional gene silencing; Rep = replication-associated protein; TYLCV = Tomato yellow leaf curl virus; TYLCSV = Tomato yellow leaf curl Sardinia virus; TYLCSV-[Sic] = the Sicily strain of Tomato yellow leaf curl Sardinia virus

type fruit quality of the resistant tomato plants is insufficient for the Tunisian market. Most commercially successful TYLCV-resistant hybrids maintain indeterminate growth and round fruits (Ribeiro *et al*., 1998).

Several studies have focused on the pathogen-derived resistance using partial, entire, or mutated begomovirus Rep genes. Transitory resistance to TYLCSV was produced in *N. benthamiana* plants using the Rep gene with a deletion of 420 nt from the 3'-end. However, the resistance was not extended to an unrelated begomovirus (Noris *et al*., 1996). Other strategies such as the trans-dominant lethal strategy have been developed to engineer plants resistant to viruses. It was successfully applied against the Bean golden yellow mosaic virus (Hanson and Maxwell, 1999).

Furthermore, transgenic tobacco plants expressing anti-sense truncated Rep gene transcript were tested for the virus resistance by agro-inoculation and displayed a reduced virus replication (Bendahmane and Gronenborn, 1997).

It has been suggested that the presence of artificially expressed dsRNA activated the plant post-transcriptional machinery even in the absence of pathogen as a defense mechanism. Namely, the constructs expressing both sense and anti-sense RNA and forming a dsRNA hairpin loop were effective in viral gene silencing and often induced immunity (Wesley *et al*., 2001; Wang *et al*., 2000; Smith *et al*., 2000).

In the present study, we report the engineering of two intron-containing binary vectors based on the TYLCSV-[Sic] Tunisian isolate sequence. We evaluated their ability to induce resistance to TYLCV and TYLCSV in *N. benthamiana* plants in the *Agrobacterium*-mediated transient assay.

Materials and Methods

Choice of the viral sequences for gene silencing constructs. TYLCV Dominican isolate (Acc. No. AF024715) and TYLCSV-[Sic] Tunisian isolate (Acc. No. AY736854) complete sequences were compared using DNAMAN software (Lynnon Corporation). The most conserved regions of the CP and Rep genes were selected. A 110-bp fragment (GSCP) sharing 83% sequence identity in the CP gene and a 156-bp fragment (GSRep) sharing 90% sequence identity in the Rep gene of the TYLCSV-[Sic] were selected (Table 1). *Asc*I, *Bam*HI, *Swa*I, and *Xba*I restriction enzymes did not cut these two sequences. PCR primers were designed to amplify the GSCP and GSRep fragments from the full-length clone of the TYLCSV-[Sic] Tunisian isolate. Specific restriction endonuclease sites were introduced in the primer sequences, *Xba*I and *Asc*I sites in the sequences of both forward sense primers, *Bam*HI and *Swa*I sites in the sequences of the reverse sense primers (Table 2).

Table 1. Sequence homology of CP and Rep sequences of TYLCV isolates

TYLCV isolate (Acc. No.)	Construct	
	GS Rep	GS CP
Dominican (AF024715)	90.45%	83.78%
Mild (X76319)	91.72%	83.78%
Sardinia (X61153)	93.63%	94.59%
Sicily (AY736854)	100.00%	100.00%

The PCR reactions amplifying the GSCP and GSRep fragments were performed in a final volume of 25 µl, in Perking-Elmer thermalcycler PTC-200 (MJ Research Inc.). PCR amplification was carried out with 100 ng of total DNA and 10 µmol/l of each primer, 10 mmol/l of each dNTP, 0.2 µl of Taq DNA polymerase (Promega), 1x Buffer, 1.5 mmol/l $MgCl_2$. PCR conditions were initiated with 1 cycle at 94°C for 3 mins, followed by 30 cycles consisting of denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min, primer extension at 72°C for 1 min, and a final extension step at 72°C for 10 mins. PCR products were loaded onto 2% agarose gel for electrophoretic analysis.

The GSCP and GSRep PCR products were cloned in the pGEM-T Easy vector (Promega) according to the manufacturer's instructions using competent XL1 Blue *Escherichia coli*. Transformed colonies were selected in media with added ampicillin and tetracyclin (Sigma-Aldrich). The recombinant plasmids pGEMGSCP and pGEMGSRep were sequenced. All the sequencing reactions were performed using the Big Dye sequencing kit as described by the manufacturer (Applied Biosystems) and sent to the Biotechnology Centre (Madison, USA).

Description of the intron-hairpin construction. Considering the enzyme restriction sites of multi-cloning sites, the pFGC5941 binary vector (Acc. No. AY310901) was chosen for cloning selected viral sequences. The multicloning sites were separated by the chalcone synthase (CHSA) intron from *Petunia hybrida* that conferred to the construct a hairpin-structure after transcription in plant cells (Abhary *et al*., 2006).

Table 2. Primers used to amplify viral sequences cloned in the gene silencing vector pFGC4951

		Primer sequence
Forward	Rep	5'-*TCTAGA*GGCGCGCCTGTGGGCCTGGATTGCAGAGGAAGATAGTG-3'
	CP	5'-*TCTAGA*GGCGCGCCAAGAGATTTTTTAAAATTAATACCCATGTAG-3'
Reverse	Rep	5'-***GGATCC*ATTTAAAT**ATGCCTGGTATAACGTCATTGATGACGTCGA-3'
	CP	5'-***GGATCC*ATTTAAAT**AGCATGAGTACAAGCCATATACAATAACAAG-3'

*Xba*I restriction site (italics); *Asc*I restriction site (underlined); *Bam*HI restriction site (italics and boldface); *Swa*I restriction site (boldface).

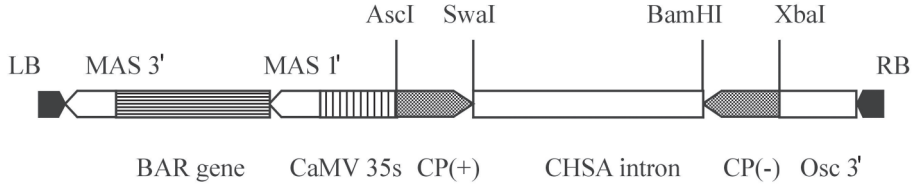

Fig. 1

Map of the T-DNA fragment carrying the TYLCSV-[Sic] CP sequence inverted repeats

T-DNA left border (LB), T-DNA right border (RB), mannopine synthase polyA signal (MAS3'), gene encoding phosphinothricin acetyl transferase conferring resistance to phosphinothricin (BAR gene), mannopine synthase promoter (MAS1'), CaMV promoter (CaMV35S), chalcone synthase A gene intron (CHSA intron), octopin synthase polyA signal (Osc3'), inverted viral sequences CP(+) and CP(-), restriction sites used to clone viral sequences *Asc*I, *Swa*I, *Bam*HI, *Xba*I.

Plasmid constructs were prepared using standard cloning techniques. Anti-sense and sense orientations of the same fragment were cloned on the both sides of the CHSA intron. The pGEMGSCP and pGEMGSRep recombinant plasmids were digested with *Swa*I and *Asc*I restriction enzymes and the digestion products were loaded onto 1% agarose gel for electrophoretic analysis. The respective 110-bp and 156-bp bands were extracted from the gel using the gene clean kit (Qiagen) according to the manufacturer's instructions. Both gene-cleaned fragments were ligated to the empty pFGC5941 double-digested with the same *Swa*I and *Asc*I restriction enzymes using the T4 DNA ligase (Promega). The ligation product was used to transform competent XL1 Blue *E. coli*. After selection on media containing tetracyclin and kanamycin (Sigma), the recombinant GSCPS and GSRepS plasmids containing the gene-silencing fragments in the sense orientation were sequenced. Then pGEMGSCP, pGEMGSRep, GSCPS, and GSRepS were digested with *Xba*I and *Bam*HI restriction enzymes (New England BioLabs). The respective 110-bp, 156-bp, 11-kb and 11-kb fragments were extracted from agarose gels. Ligation reactions of the gene-silencing fragments GSCP and GSRep to the digested respective silencing plasmids GSCPS and GSRepS were performed using the T4 DNA ligase. The ligation products were used to transform competent XL1 Blue *E. coli* and the transformed bacteria were selected using tetracyclin and kanamycin. The resulting recombinant plasmids pFGSCP and pFGSRep containing the gene-silencing constructs in both sense and antisense orientations were sequenced and used to transform the competent *A. tumefaciens* GV3101 (Fig. 1). The transformed bacteria were selected in the medium containing kanamycin and gentamycin (Sigma).

Virus resistance assays. N. benthamiana plants were grown in growth chambers (28°C, 16 hrs light, 8 hrs darkness). The plants at their four-leaf stage were inoculated with the gene-silencing construct using a syringe without a needle by pressing the tip of the syringe against the lower surface of the leaf and applying gentle pressure. The 2/3 of the leaf was inoculated with the *Agrobacterium* suspension. The bacteria were grown for 24 hrs in a shaker at 28°C and 250 rpm in dark in Luria Bertani (LB) broth media containing kanamycin and gentamycin. Cells were centrifuged for 15 mins at 5,000 rpm at 4°C and the pellet was resuspended in Murashige and Skoog medium (Sigma) with 375 µmol/l of acetosyringon (Sigma) to the $OD_{600} = 1$. The challenge with the TYLCV and TYLCSV-[Sic] infectious clones was done after 1 week in the same leaves in the same way to get overlapping areas. After each agro-inoculation, the plants were sprayed with water and covered with plastic bags for 24 hrs. The experiment was repeated twice.

TYLCV and TYLCSV infectious clones. The TYLCV infectious clone presents a dimer of the full-length genome of the Israeli isolate cloned in the pBin302 binary vector (Acc. No. AY594174) (Abhary *et al*., 2006). This construction was introduced in *A. tumefaciens* LB4404.

The TYLCSV-[Sic] infectious clone is a 1.7-mer of the TYLCSV Tunisian isolate (Acc. No. AY736854) clustered in the Sicily strain cloned in the pCB301. This construction was introduced in *A. tumefaciens* GV3101 (Gharsallah Chouchane *et al*., 2006).

Detection of systemic infections by dot blot hybridization. One week after the challenge with the infectious clone, the DNA was extracted from one inoculated leaf of each plant using the Dellaporta heat extraction protocol (Potter *et al*., 2003). 400 mg of fresh tissue were ground with a Kontes^{R} pestle in 100 µl of Dellaporta buffer (10 mmol/l ß-mercaptoethanol, 100 mmol/l Tris, pH 8, 50 mmol/l EDTA, 500 mmol/l NaCl). The suspension was incubated at 65°C for 5 mins and centrifuged at 14,000 rpm for 10 mins at room temperature. The supernatant was kept at -20°C until use. The second and the third week after the challenge with the infectious clones, DNA was extracted from the upper new leaves of each plant using the same protocol.

Virus detection in the inoculated and upper new leaves was also performed by dot-blot hybridization. 5 µl of each extracted DNA were blotted on a Biodyne B Membrane (Pall Corporation). The membrane was allowed to dry, denatured (0.5 mol/l NaOH, 1 mol/l Tris, pH 7.4, 2x SSC, 95% ethanol) and cross-linked (Stratagene). A probe spanning the CP gene able to hybridize either TYLCV or TYLCSV was labeled as described by the Alkaline Phosphatase Direct Hybridization kit (Amersham Pharmacia) manufacturer's instructions and used under low stringency conditions (55°C). The membrane was exposed to a Kodak Biomax MR film and developed. The presented data represented the mean of two experiments; each treatment consisted of 10 replicates.

Results

Choice of viral sequences for engineering intron-hairpin constructs

Two viral sequences were selected to be cloned and used for engineering gene-silencing constructs (Table 2). The first one of 110 bp was located in the CP gene starting from nt 900–1010. Its sequence shared 90% of sequence homology with the TYLCV and did not contain any restriction sites used to introduce the selected fragment in the binary vector. The second sequence of 156 bp long (nt 1694–1850) was selected in the Rep gene sequence following the same criteria and shared 83% of sequence homology with the TYLCV.

Cloning of viral sequences in the binary vector pFGC5941

The two selected viral sequences were introduced in the pFGC5941 *Agrobacterium* binary vector. The multiple cloning sites in pFGC5941 facilitated insertion of target sequences in both anti-sense and sense orientations separated by an intron creating a hairpin inverted and repeated structure. The resulting plasmid constructs (pFGSCP and pFGSRep) were introduced into *Agrobacterium* GV3101 and used for the transient silencing experiments in *N. benthamiana* plants.

Plant inoculations

The natural host plant tomato (*Solanum lycopersicum*) of TYLCV was not used in this study, since the symptoms developed more intensely in *N. benthamiana* plants. Moreover, it was easier to infiltrate agro-inoculated *N. benthamiana* leaves with the pFGSCP and pFGSRep constructs at the 4-leaf stage. Two types of negative controls were used, e.g. plants inoculated with *Agrobacterium* containing binary vector without any viral sequence (empty pFGC5941) and the mock-inoculated plants.

After one week, the construct-inoculated plants were challenged with the infectious virus to examine plants for a possible resistance. Each category of construct-inoculated plants was divided into two groups. One group was challenged with the TYLCV dimer, the other one with the TYLCSV-[Sic] Tunisian 1.7-mer. Susceptibility to virus infection was scored on the basis of the virus detection by hybridization and symptom development.

Susceptibility to TYLCSV-[Sic] and TYLCV

Resistance to TYLCSV-[Sic] infection was monitored weekly for 1 month. One week after the challenge with the TYLCSV-[Sic] 1.7-mer, the viral genome was detected in the infiltrated leaves from all inoculated plants. Two weeks after inoculation, hybridization was completed in DNA extracted from the upper new leaves. The virus was detected

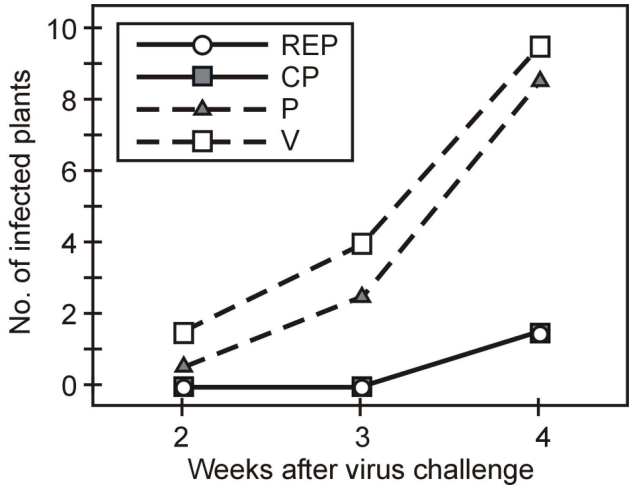

Fig. 2

Number of plants systemically infected with TYLCSV-[Sic] detected by dot-blot hybridization

Prior infection the plants were inoculated with Rep construct (Rep), CP construct (CP), empty plasmid (P), no plasmid (V).

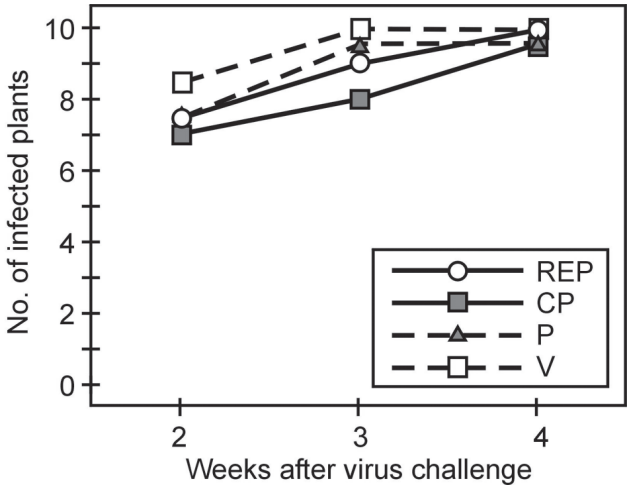

Fig. 3

Number of plants systemically infected with TYLCV detected by dot-blot hybridization

Prior infection the plants were inoculated with Rep construct (Rep), CP construct (CP), empty plasmid (P), no plasmid (V).

in some plants mock-inoculated by the construct as well as in some plants inoculated with the empty binary pFGC5941. None of the plants infiltrated with the gene-silencing constructs were systemically infected by the virus (Fig. 2).

Three weeks after virus inoculation, few plants inoculated with the Rep construct were systemically infected by the TYLCSV-[Sic]. A similar result was observed with the CP construct, whereas the controls were systemically infected. These results were confirmed by virus detection completed 4 weeks after virus inoculation (Fig. 2). This delay of infection observed with the construct-inoculated plants compared to the controls proved the efficiency of the gene-silencing constructs on triggering TYLCSV-[Sic] resistance mechanism. This observation was confirmed by the fact that 6 weeks after virus infiltration, the construct-inoculated plants were phenotypically immune. On the control plants, the symptoms started to appear 4 weeks post virus inoculation.

No infection delay was observed on the construct-inoculated plants compared to the control plants for TYLCV-inoculated plants (Fig. 3). Symptoms appeared on the controls and on the construct-inoculated plants at the same time, e.g. 3 weeks after virus inoculation. These results showed that no resistance to TYLCV infection was triggered by the two engineered silencing TYLCSV-[Sic]-derived constructs under the experimental conditions used.

Discussion

RNA silencing serves as a defense mechanism against invading viruses and is known to control the developmental regulation of plant gene expression through the production of microRNA (Bartel and Bartel, 2003; Voinnet, 2001). To investigate the efficiency in triggering resistance to TYLCSV and TYLCV of the two gene-silencing constructs based on highly conserved viral sequences, *N. benthamiana* plants were used in a transient expression assay for each construct. The effects of the expression of these constructs on viral replication were determined. Monitoring of viral infection each week showed that both Rep-based and CP-based constructs effectively reduced viral replication for TYLCSV, but not for TYLCV. Thus, the gene-silencing mechanism was not dependent on the specific viral sequences, either CP or Rep gene. That was consistent with results obtained from studies using non-coding begomovirus sequences (Abhary *et al*., 2006; Rezk *et al*., 2006; Pooggin *et al*., 2003). Furthermore, protein expression did not appear to be an important part of this mechanism. Whatever the targeted viral sequence, virus inhibition was operating by viral RNA degradation resulting in a greatly reduced accumulation of viral DNA.

The two silencing constructs were based on the TYLCSV, the only begomovirus detected in Tunisia until TYLCV was identified (Gharsallah Chouchane *et al*., 2007). Surprisingly, even sequences sharing 91% of identity with the TYLCV for the Rep-based construct did not diminish TYLCV DNA accumulation in challenged *N. benthamiana* plants. This was not in accordance with previous Post-Transcriptional Gene Silencing (PTGS) experiments showing specific elimination of viral sequences sharing more than 90% homology (Dougherty *et al*., 1994; Smith *et al*., 1994; Lindbo *et al*., 1993). The presence and concentration of siRNA immediately prior to the challenge by the virus played a decisive role for plant resistance to the viral nucleic acid accumulation. Namely, plants producing high levels of siRNA were resistant to the inoculations with highly infectious extracts of Cucumber mosaic virus (Kalantidis *et al*., 2002).

The difference of behavior between the plants challenged by the TYLCV and TYLCSV can be explained by difference in the virulence of both viruses. Namely, the TYLCV replicates faster in the plant cells than the TYLCSV (Gharsallah Chouchane *et al*., 2006). The level of siRNA reached a week after construct delivery is the same in both kinds of plants and this level seems to be efficient against TYLCSV but not against TYLCV. Recently, Abhary *et al.* (2006) showed that a time period of 12 days between infiltrations of the silencing constructs and infectious clone is necessary to induce efficiently resistance in *N. benthamiana* plants against TYLCV. The virus may evade the RNA-mediated mechanism by replicating rapidly. By the time the defense mechanism becomes active, the virus may have already accumulated at high level, whereas the siRNA concentration is insufficient. Increasing this time period may lead to effective resistance by the TYLCSV constructs against TYLCV. Furthermore, our experiments demonstrated that a 7-days period is enough to efficiently induce resistance to the homologous TYLCSV with TYLCSV-based silencing constructs.

Another hypothesis could explain why the virus may escape the plant defense mechanism by expressing a gene silencing suppressor that would be absent from the TYLCSV genome. As a response to PTGS, viruses have evolved or adapted genes that suppress PTGS (Vance and Vaucheret, 2001). Indeed, gene silencing suppressors as AC2 and AC4 proteins have already been identified in the begomovirus genome. The absence of detectable viral DNA by hybridization in silenced TYLCSV-inoculated plants indicated that the resistance may represent immunity. That is consistent with the previous reports of gene silencing experiments induced by double-stranded RNA hairpin loops often generating immunity (Abhary *et al*., 2006; Wesley *et al*., 2001; Smith *et al*., 2000; Wang *et al*., 2000).

The TYLCSV gene-silencing constructs can be used to transform the tomato plants that are expected to be resistant at least to TYLCSV (Brunetti *et al*., 2001). Furthermore, it would be noteworthy to test the resistance of transgenic plants against related viruses and also against other viruses affecting tomato crops in Tunisia.

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