

Use of Tomato leaf curl virus (TYLCV) truncated Rep gene sequence to engineer TYLCV resistance in tomato plants

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Summary. – Tomato yellow leaf curl disease causes severe losses in tomato production throughout Mediterranean countries including Tunisia. In order to generate engineered resistance to this disease, an intron-hairpin RNA construct harboring a Tomato yellow leaf curl Sardinia virus (TYLCSV) truncated replication-associated protein (Rep) gene was used to transform genotype of tomato plants. Prepared transgenic plants were agro-inoculated with Tunisian infectious strain of TYLCSV and screened for the resistance to infection. The infected transgenic plants were divided into 3 different groups according to their specific symptoms. Only one of them contained transgenic plants fully resistant to the tomato yellow leaf curl disease.

Keywords: Tomato yellow leaf curl virus; transgenic plants; resistance; Rep gene

Introduction

Tomato yellow leaf curl disease is a whitefly-transmitted tomato infection caused by viruses belonging to the family *Geminiviridae*, the genus *Begomovirus* (Stanley, 1985; Navot *et al.*, 1991). Several virus species have been identified as causal agents of the tomato yellow leaf curl disease corresponding mainly to the TYLCV and TYLCSV viral species (Czosnek *et al.*, 1988; Kheyr-Pour *et al.*, 1991). Both viruses have geminate particle morphology and contain small, circular single-stranded DNA of 2.8 kb. The viral genome contains 6 partially overlapping ORFs, two on the viral sense strand and four on the complementary strand separated by an intergenic region (Navot *et al.*, 1991).

Tomato yellow leaf curl disease causes significant losses in several crops worldwide and is a major constraint to the tomato production in Tunisia. Although suspected in Tunisia since the eighties of the last century, the first molecular data were reported in the year 2000 together with the evidence of TYLCSV spread in tomato, pepper, and bean crops (Cherif and Russo, 1983; Fekih-Hassen *et al.*, 2003; Gorsane *et al.*, 2004). Larger field survey revealed the presence of multiple TYLCV strains in the same crop (Gharsallah Chouchane *et al.*, 2007).

Management of plant viruses by breeding of the plants is difficult and restricted in the crop production. Recently, studies focusing on pathogen-derived resistance involved post-transcriptional gene silencing (PTGS) approaches also termed RNA silencing (Waterhouse *et al.*, 2001; Chellapan *et al.*, 2004; Vanitharani *et al.*, 2005). It is a homology-dependent mechanism of RNA degradation induced by a double-stranded RNA leading to the specific RNA degradation at post transcriptional level (Hannon, 2002). RNA silencing is emerging as a powerful tool for a control of plant virus diseases. Most of the control strategies for genetically engineered TYLC(S)V resistance in plants involve partial, entire, or mutated viral coat protein (CP) or Rep genes. The gene

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Abbreviations: CHSA = chalcone synthase; CP = coat protein; p.i. = post-inoculation; PTGS = post-transcriptional gene silencing; Rep = replication-associated protein; siRNA = small interfering RNA; TYLCV = Tomato yellow leaf curl virus; TYLCSV = Tomato yellow leaf curl Sardinia virus

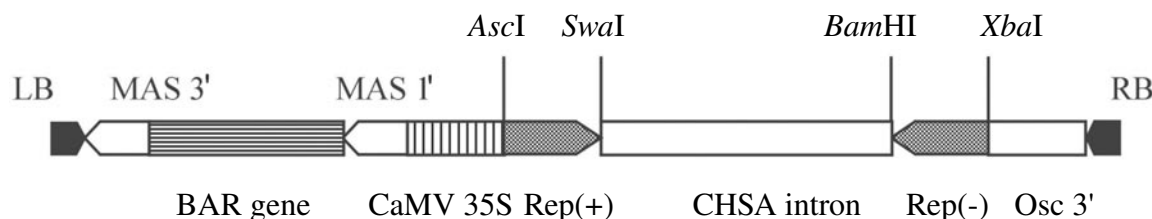


Fig. 1

Schematic representation of the transcription cassette of pFGC5941 vector

LB/RB = left and right borders, MAS 3' = mannopine synthase polyA signal, BAR gene = gene encoding phosphinothricin acetyl transferase conferring resistance to phosphinothricin, MAS 1' = mannopine synthase promoter, CaMV 35S = Cauliflower mosaic virus 35S transcription promoter, Osc 3' = octopine synthase polyA signal. The cassette includes a Rep truncated gene of a Tunisian TYLCSV strain in a sense orientation (+) separated from the reverse complement of the same sequence (-) by a CHSA intron. *AscI*, *SwaI* and *BamHI*, *XbaI* = restriction sites used to clone viral sequences in the sense and antisense orientations, respectively.

encoding intron-spliced RNA seems to be efficient against plant viruses (Smith *et al.*, 2000).

In this paper we describe the engineering of the genetically transformed tomato plants using a TYLCSV Rep construct. This silencing construct is harboring a short fragment of the Rep gene cloned as inverted repeats. As was already shown in transient expression system, it efficiently triggered homologous virus resistance through gene silencing (Gharsallah Chouchane *et al.*, 2008). The efficacy of this strategy to control tomato yellow leaf curl disease is explored in transformed tomato plants agro-inoculated with a Tunisian infectious clone of the TYLCSV strain.

Materials and Methods

Virus and agro-inoculation. The 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 *Agrobacterium tumefaciens* GV3101 (Gharsallah Chouchane *et al.*, 2006). Tomato plants were challenged by the agro-inoculation method using the infectious TYLCSV clone.

Silencing construct. The silencing construct used in this study was engineered from a Rep sequence of a Tunisian TYLCSV strain (Gharsallah Chouchane *et al.*, 2008). Thus, a conserved viral fragment within the Rep gene sharing 91% of sequence homology between TYLCSV and TYLCSV was cloned in the pFGC4951 vector in both the sense and anti-sense orientations. The inverted viral RNA was separated by the chalcone synthase (CHSA) intron giving an intron-hairpin RNA construct (Fig. 1).

Tomato transformation. The binary vector containing Rep construct was used to transform *A. tumefaciens* GV3101 strain by an electroporation method. Transformation was performed on the leaves of tomato seedlings of Riogrande stable genotype according to Ellul *et al.* (2003). Regenerated plants were acclimated to the growth chamber and transferred to an insect proof plastic house for a transgene confirmation, resistance screening, and seed production. Then, DNA was extracted from putative R0 tomato

transgenic plants (Dellaporta *et al.*, 1983) and used as a template for PCR reactions.

PCR. The primer set used for transgene detection was: Rep sense: (5'-TGTGGGCCTGGATTGCAGAGGAAGATAGTG-3') and the Rep anti-sense (5'-ATGCCTGGTATAACGTCATTGATGACGTCGA-3'). PCR amplification was carried out with 100 ng of total DNA, 10 μ mol/l of each primer, 10 mmol/l of each dNTP, 1 U of Taq DNA polymerase (Promega), 1x Buffer, 1.5 mmol/l MgCl₂ in a final volume of 25 μ l. The reaction conditions were as follows: 94°C/3 mins, 30 cycles of 94°C/1 min, 56°C/1 min, 72°C/1 min, and 72°C/10 mins. Each PCR assay was run with a negative control (non-transformed tomato plant) and a positive control (Rep gene from the Tunisian TYLCSV isolate) to prevent false-negative and false-positive results. PCR products were loaded on a 2% agarose gel for electrophoretic analysis. The 156 bp-length PCR products were sequenced to confirm both identity and integrity of the transgene.

R1 transgenic plants analysis. The R1 tomato plants were analyzed for the presence of the transgene by molecular hybridization (dot-blot) and validated by PCR test using both the Rep sense and anti-sense primers. We also performed PCR assay using a primer set flanking the Cauliflower mosaic virus 35S transcription promoter (p35S) of the used construct. This set included a p35S sense (5'-CTACTC CAAAATGTCAAAGATACAGTC-3') and a p35S-antisense (5'-GGGCTGTCTCTCCAAATG-3') primers. PCR conditions used for p35S promoter amplification were the same as those used for transgene amplification. Dot-blot was performed under high stringency using the transgene as a probe and following the manufacturer's instructions conditions (Dig-DNA labeling kit, Roche).

Challenge inoculation. The *A. tumefaciens* strain GV3101 transformed by the Tunisian TYLCSV clone was grown in LB medium, in dark, at 28°C for 2 days. Bacterial culture was supplemented with kanamycin (50 mg/l) and gentamycin (15 mg/l) and diluted until A₆₀₀ reached 1. Then, it was supplemented by acetosyringone to the final concentration 375 μ mol/l and inoculated to the transgenic tomato plants at the four-leaf stage using a syringe without needle.

Detection of systemic infection. The efficiency of the agro-inoculation method was tested by PCR amplification of a viral

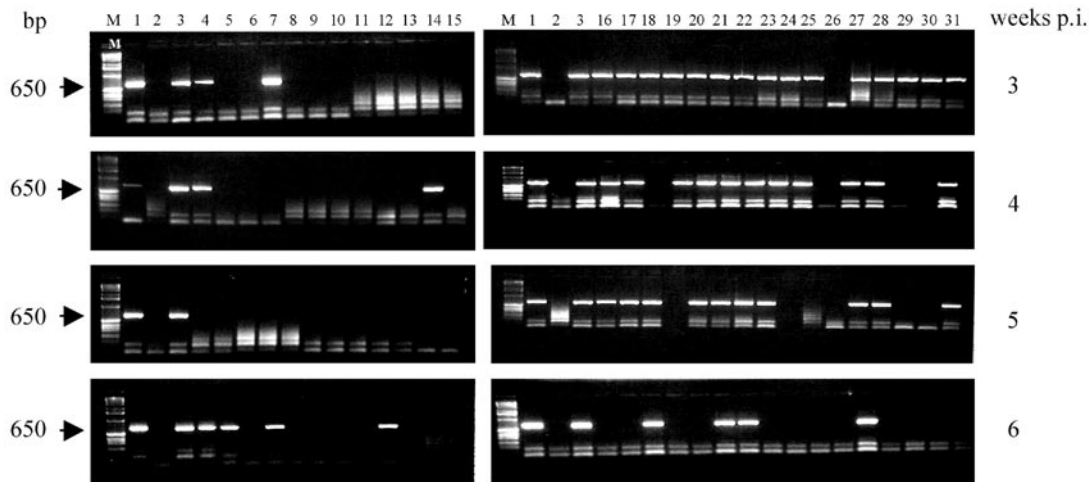


Fig. 2

PCR amplification of CP gene in transgenic tomato plants inoculated by the Tunisian TYLCSV infectious clone from 3rd to 6th week p.i.

DNA size markers 1kb ladder (Invitrogen) (lanes M); positive controls corresponding to the non-transgenic plant inoculated with the infectious clone and CP gene from the Tunisian TYLCSV isolate (lanes 1 and 3, respectively); non-inoculated transgenic plant used as a negative control (lane 2). R1 tomato plants: resistant (lanes 6, 8, 9, 10, 11, 13, 15, and 26), delayed infection (lanes 5 and 12), recovery phenotype (lanes 14, 16, 17, 19, 20, 23, 24, 25, 28, 29, 30, and 31), susceptible (lanes 4, 7, 18, 21, 22, and 27).

fragment within the CP using TY369v primer (5'-AAGAGATTTTT TAAAATTAATACCCATGTAG-3') and TY1023c primer (5'-AGCATGAGTACAAGCCATATACAATAACAAG-3') (Nakhla *et al.*, 1993). DNA was extracted from the inoculated leaves 1 week post-inoculation (p.i.) as well as from the upper new leaves at the 3rd, 4th, 5th, and 6th week p.i. The DNA was used as a matrix in PCR tests for plant resistance screening. Each PCR assay was run with a negative control (non-inoculated transgenic plant) and two positive controls (non-transgenic plant inoculated with the infectious clone and the CP gene from the Tunisian TYLCSV isolate) to prevent false-negative and false-positive results.

Results

Molecular evaluation of transgenic tomato plants

26 independent R0 transgenic tomato lines transformed with the binary vector containing the silencing Rep construct were obtained. These plants were subjected to preliminary molecular tests and evaluated by PCR amplification of the transgene and sequencing of the obtained PCR amplicons. Evidence of the transgene integration into the plant genome was obtained (data not shown).

Following self-fertilization of R0 plants, the R1 progeny derived from different R0 lines was tested. The presence of the integrated intron-hairpin construction in the selected tomato transgenic lines was assessed by a dot-blot and PCR using 2 sets of primers (data not shown). The first set was

used to amplify the transgene and the second set targeted a fragment within the p35S promoter. Out of 39 R1 plants tested, 33 plants were genetically transformed as proved by the 3 molecular methods (dot-blot hybridization, Rep and p35S PCR amplifications). The evidence of the stability of the integrated construction and the transfer of the transgene from R0 to the R1 generation was provided.

Screening of R1 plants for resistance against infection

Agro-inoculation of the Tunisian TYLCSV infectious clone was performed on the R1 generation of tomato plants as well as on non-transformed plants. The efficiency of this experiment was assessed by the DNA extraction and PCR amplification of the CP gene of TYLCSV using specific primers.

R1 generation plants had been screened for the yellow leaf curl typical symptoms from the 3rd week p.i. and continuing weekly until plants were discarded. Inoculated plants displayed different symptoms, what allowed us to divide them into three different groups.

The first group containing 6 plants showed systemic infection as soon as from the 3rd week p.i. These plants were consequently considered as susceptible. The second group contained 8 tomato plants having normal phenotype with asymptomatic leaves showing the resistance behaviour. The last group included 14 tomato plants showing either a delay in virus multiplication or a recovery phenotype.

Accordingly, a positive correlation between TYLCSV symptoms present on R1 tomato leaves and amplification of CP gene by the PCR assays was determined (Fig. 2). We were able to amplify CP gene of TYLCSV in all plants belonging to the susceptible group, while the amplification was nearly negative in plants of the second resistant group. In the last group, some tomato plants showed positive CP amplification within the 3rd week of the infection. However, this amplification was not maintained in the later weeks of the infection indicating the recovery behavior. Other plants showed CP amplification starting from the 6th week of the infection pointing to a delay in the virus multiplication.

Discussion

Tomato yellow leaf curl disease is one of the most limiting diseases affecting tomato production in many countries. However, current tomato cultivars are not fully resistant to the infection with TYLCSV. A new approach based on the virus-derived transgene mediated by PTGS mechanism has been shown as effective (Lindbo *et al.*, 1993; Pooggin *et al.*, 2003). It was shown that plants transformed by the viral genes allowing production of small interfering RNAs (siRNAs) were resistant against DNA and RNA viruses (Baulcombe, 1996; Asad *et al.*, 2003). Comparative studies involving separately silencing strategies demonstrated that hairpin-RNA strategy was the most efficient (Chuang and Meyerowitz, 2000; Wesley *et al.*, 2001). Since the plant transformation has become an essential tool to engineer virus resistance, this work focuses on the use of a hairpin-RNA construct involving a Rep truncated gene to transform a tomato genotype. In this process, sense and anti-sense RNAs generated from an inverted repeated sequences anneal together to form dsRNA that is cleaved into siRNAs (Hamilton and Baulcombe, 1999; Xie *et al.*, 2004). One strand of the siRNA duplex is incorporated into a nuclease complex known as the RISC (RNA induced silencing complex) containing AGO protein and guiding to the binding and the cleavage of the homologous target RNA (Hammond *et al.*, 2000; Hannon, 2002). We previously demonstrated the ability of the Rep-truncated construct to give effective resistance against TYLCSV in *Nicotiana benthamiana* transient transformation experiments (Gharsallah Chouchane *et al.*, 2008). In the current study, we used a stable tomato Riogrande genotype that is well-appreciated and predominantly cultivated in Tunisia, as a model system to study the efficiency of hairpin-RNA strategy to control the viral disease. Riogrande tomato cultivar, known as susceptible to the infection with TYLCSV, was transformed using an improved *Agrobacterium*-mediated protocol. The resulting generations R0 and R1 of transformed tomato plants were

submitted to the molecular analysis to confirm the integration of the transgene. R1-transgenic tomato plants were challenged by the Tunisian TYLCSV infectious clone and screened for the resistance to the homologous virus. On the basis of their symptoms after virus inoculation, transgenic tomato plants could be divided into 3 different groups. Different resistance levels reflected the differences in Rep expression driven by the p35S promoter. The first group involved susceptible tomato plants. One hypothesis that could account for the absence of the resistance anticipated that the amount of siRNA molecules derived from the transgene-dsRNA were too low. This hypothesis suggested that a certain level of the transgene expression was necessary to confer a resistance (Brunetti *et al.*, 1997). However, RNA-mediated DNA methylation of the construct might also be a reason for the lack of resistance (Mathieu and Bender, 2004). The second group of transgenic plants did not show the typical disease symptoms p.i. and consequently, it was considered as resistant to the TYLCSV infection. As expected, PCR failed to detect viral DNA suggesting that these plants are immune. These findings are consistent with other studies involving a TYLCSV derived hairpin-RNA construct (Smith *et al.*, 2000; Yang *et al.*, 2004; Abhary *et al.*, 2006). A strategy based on gene encoding intron-spliced RNA induced PTGS directed against viruses with 100% efficiency (Smith *et al.*, 2000).

The third group included the tomato plants showing a delay in symptom expression. It was possible that viral suppressors were involved in the delayed symptom appearance, but it could be difficult to explain why silencing did happen in some lines and not in others. It might occur at low levels or being specific to some types of cells. This group also included the tomato plants with a recovery phenotype. One explanation assumed that the recovery of sensitivity to the viral infection was a result of virus-host interaction. Although it is considered as unusual for geminiviruses, symptom recovery has been observed within two isolates of Sri Lanka cassava mosaic virus (Chellapan *et al.*, 2004). The resistance levels achieved in the different transgenic plants can also be explained by the difference in the integration site of the transgene that can induce a difference in the production of transcripts causing an insufficient amount of siRNA able to confer the resistance (Gallie, 1998).

Taken together, it was demonstrated that a hairpin RNA construct harboring a short sequence of the TYLCSV Rep gene was able to activate PTGS mechanism against the homologous virus in the transgenic tomato plants. The integrated transgene was efficient in preventing systemic infection after TYLCSV agro-inoculation in a fraction of transgenic plants. Also, the progeny of these plants will be used for further characterization of a correlation between resistance and accumulation of TYLCSV-specific siRNA.

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