

## Genetic transformation of *Citrus sinensis* with *Citrus tristeza virus* (CTV) derived sequences and reaction of transgenic lines to CTV infection

F.R. MUNIZ<sup>1</sup>, A.J. DE SOUZA<sup>1</sup>, L.C.L. STIPP<sup>2</sup>, E. SCHINOR<sup>1</sup>, W. FREITAS, Jr.<sup>1</sup>, R. HARAKAVA<sup>3</sup>, D.R. STACH-MACHADO<sup>4</sup>, J.A.M. REZENDE<sup>1</sup>, F.A.A. MOURÃO FILHO<sup>1</sup> and B.M.J. MENDES<sup>2\*</sup>

*Escola Superior de Agricultura "Luiz de Queiroz", Universidade de São Paulo, 13418-900, Piracicaba, SP, Brazil<sup>1</sup>*  
*Centro de Energia Nuclear na Agricultura, Universidade de São Paulo, 13400-970, Piracicaba, SP, Brazil<sup>2</sup>*  
*Instituto Biológico de São Paulo, 04014-002, São Paulo, SP, Brazil<sup>3</sup>*  
*Instituto de Biologia, Universidade Estadual de Campinas, 13083-970, Campinas, SP, Brazil<sup>4</sup>*

### Abstract

Transgenic *Citrus sinensis* (L.) Osb. plants, cvs. Valencia and Hamlin, expressing *Citrus tristeza virus* (CTV) derived sequences were obtained by genetic transformation. The gene constructs were pCTV-CP containing the 25 kDa major capsid protein gene (CTV-CP), pCTV-dsCP containing the same CTV-CP gene in an intron-spliced hairpin construct, and pCTV-CS containing a 559 nt conserved region of the CTV genome. The transgenic lines were identified by PCR and the transgene integration was confirmed by Southern blot. Transgene mRNA could be detected in most transgenic lines containing pCTV-CP or pCTV-CS transgene. The mRNA of pCTV-dsCP transgene was almost undetectable, with very light bands in most analyzed plants. The transgene transcription appears to be closely linked to the type of gene construct. The virus challenge assays reveals that all transgenic lines were infected. However, it was possible to identify propagated clones of transgenic plants of both cultivars studied with a low virus titer, with values similar to the non-inoculated plants (negative control). These results suggested that the transgenic plants present some level of resistance to virus replication. The higher number of clones with low virus titer and where mRNA could not be detected or was presented in a very light band was found for pCTV-dsCP-derived transgenic lines.

*Additional key words:* disease resistance, hairpin, gene silencing, sweet orange.

*Citrus tristeza virus* (CTV), the causal agent of the most important viral disease affecting citrus, causes quick decline of most citrus species grafted on sour orange. CTV infection may also causes stem pitting, plant stunting, as well as reduction in fruit yield and quality of some scion cultivars regardless of the rootstock (Bar-Joseph *et al.* 1989, Domínguez *et al.* 2000).

Since the first description of citrus genetic transformation, the possibility of obtaining transgenic plants resistant or tolerant to CTV has been evaluated

with the use of different viral gene constructs and citrus genotypes (Dominguez *et al.* 2000). Recently, the studies of RNA-mediated gene silencing and the association of this phenomenon with plant's natural antiviral defense (Waterhouse *et al.* 2001) have stimulated the investigation of the occurrence of transcriptional (TGS) and post-transcriptional gene silencing (PTGS) in transgenic citrus plants containing transgenes that could influence CTV replication (Fagoaga *et al.* 2006, Febres *et al.* 2008). Moreover, it has been found out that gene

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*Abbreviations:* BAP - 6-benzylaminopurine; CTV - *Citrus tristeza virus*.

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\* Corresponding author; fax: (+55) 19 3429 4610, e-mail: beatriz.mendes@pq.cnpq.br

constructs designed to produce self-complementary RNA (hairpin) sequences, which fold into dsRNA structure, are more effective in inducing targeted virus resistance (Waterhouse *et al.* 2001). This fact has encouraged the use of hairpin gene constructs for CTV resistance in citrus (Batuman *et al.* 2006, Ananthakrishnan *et al.* 2007). Although only one citrus transgenic line was identified as resistant to CTV (Febres *et al.* 2008) so far, some propagated clones have shown to impair CTV virus replication (Fagoaga *et al.* 2006, Febres *et al.* 2008) and others have shown a significant delay in virus accumulation and symptom onset (Domínguez *et al.* 2002, Moreno *et al.* 2008).

These results have motivated the production of *Citrus sinensis* cvs. Valencia and Hamlin transgenic lines containing three different gene constructs, which could influence the host resistance to CTV. The transgenic lines have been challenged with the pathogen in order to investigate the efficiency of each gene construct in controlling virus replication.

*Citrus sinensis* (L.) Osb. cvs. Valencia and Hamlin seeds were extracted from ripe fruits and dried at room temperature (24 h). *In vitro* seeds germination and explants preparation were performed according to Tavano *et al.* (2009).

The CTV 25 kDa major capsid protein gene (*CTV-CP*) was RT-PCR amplified from total RNA of a CTV infected sweet orange tree. For genetic transformation the explants were incubated with *Agrobacterium tumefaciens* (Smith and Towns) Conn-EHA105 suspension ( $5 \times 10^8$  CFU cm<sup>-3</sup>, 20 min), carrying one of the following binary vectors: *pCTV-CP* containing the capsid protein gene cloned in sense orientation between the CaMV 35S promoter and terminator, *pCTV-dsCP* containing the same *CTV-CP* gene in a intron-spliced hairpin construct, and *pCTV-CS* containing a 559 nt conserved region of the CTV genome, cloned in antisense orientation between CaMV 35S promoter and terminator. The explants were blotted dry and transferred to regeneration medium according to Grosser and Gmitter (1990; EME), supplemented with 1.0 mg dm<sup>-3</sup> 6-benzylaminopurine (BAP), for a 3-d co-cultivation period (24 °C, in the dark). Then, explants were transferred to selection and regeneration medium consisted of EME medium supplemented with 1.0 mg dm<sup>-3</sup> BAP, 100 mg dm<sup>-3</sup> kanamycin, and 500 mg dm<sup>-3</sup> cefotaxime. Cultures were incubated in the dark (27 °C) for approximately 4 weeks and then transferred to 16-h photoperiod (irradiance of 63  $\mu\text{mol m}^{-2} \text{s}^{-1}$ , temperature of  $27 \pm 1$  °C).

Genomic DNA was extracted according to Doyle and Doyle (1991) from leaves of *in vitro* shoots and evaluated by PCR. The CP gene fragment (684 bp) of *pCTV-CP* and *pCTV-dsCP* gene constructs was amplified with the primers 5'-GGGCCCATGGACGACGARACAAAG-3' and 5'-CTCGAGTCAACGTGTGTTTAAATTTCC-3' and the reactions were performed under the following conditions: 94 °C 3 min, 39 cycles of 94 °C 15 s, 56 °C

30 s, 72 °C 40 s, and final extension at 72 °C 5 min. The conserved sequence fragment (559 bp) of *pCTV-CS* was amplified with the primers 5'-AGAACGAGG TGGCGCATA-3' and 5'-CTTTGGTTCACGCAT ACGTT3' and the reactions were performed under the following conditions: 94 °C 2 min, 39 cycles of 94 °C 30 s, 60 °C 30 s, 72 °C 30 s, and a final extension at 72 °C 5 min. PCR-positive shoots were carefully excised from the explants and micrografted *in vitro* onto Carrizo citrange [*C. sinensis*  $\times$  *Poncirus trifoliata* (L.) Raf] seedlings. Well-developed plants were acclimatized in a greenhouse. The transformation efficiency was expressed as the percentage of PCR-positive plants over to the total number of explants exposed to *A. tumefaciens*. Genetic transformation was confirmed by Southern blot analysis.

The transgene transcription was studied by reverse transcriptase RT-PCR. Total RNA was extracted from leaves (200 mg) of transgenic lines and a non-transgenic control line. Genomic DNA was removed using *RQ1* RNase-free DNase (*Promega*, Madison, USA) and RNA was quantified using *Qubit* fluorometer (*Invitrogen*, Carlsbad, USA). First strand cDNA synthesis was carried out with *SuperScript II* reverse transcriptase (*Invitrogen*).

Six to nine transgenic lines containing each gene construct and a non-transgenic control line of each cultivar studied were propagated for pathogen resistance evaluation. Five well-developed buds of each transgenic and non-transgenic line were grafted onto 5-month-old seedlings of Rangpur lime (*C. limonia* Osb.) rootstock plants. After a 4-month incubation period, when the new shoots were 30 - 40 cm long, three plants from each transgenic and control lines were selected for pathogen challenge assays. Each plant was graft-inoculated with two buds collected from a mild strain CTV infected sweet orange cv. Pera (CTV-IAC), at Centro APTA Citros Sylvio Moreira, IAC, Cordeirópolis/SP, Brazil. The inoculation was performed in the rootstock 1 - 2 cm below the bud union. After 4 - 5 weeks, virus infection and/or accumulation in leaves of the first flush were monitored by indirect double antibody sandwich enzyme linked immunosorbent assays (DASI-ELISA).

Total proteins were extracted from leaf tissue (0.4 g) collected from transgenic and non-transgenic inoculated plants (positive control), and from non-transgenic non-inoculated plants (negative control) using phosphate buffer saline (2 cm<sup>-3</sup>) + *Tween 20* (0.05 %; PBS-T), supplemented with polyvinylpyrrolidone (2 %). The leaves were randomly collected from different regions of the plants. All DASI-ELISA (Clark *et al.* 1986, Gamsey and Cambra 1991) were performed in flat bottomed-96 wells plates (*Nunc*, Rochester, USA), using three wells per sample. Briefly, the plates were coated with 100 mm<sup>-3</sup> well<sup>-1</sup> of #1006 rabbit polyclonal immunoglobulin in a dilution of 1:1 000 (Baptista *et al.* 1996) in sodium carbonate buffer (0.05 M, pH 9.6, 4 °C, overnight). The wells were washed in PBS-T (3 $\times$ ) and incubated with bovine serum albumin (BSA; 2 %, 1 h, 37 °C). After

washing, the wells were incubated with 100  $\text{mm}^{-3}$  of purified recombinant proteins (CB-22 or CB-104) or plant extracts (2  $\text{ng cm}^{-3}$ , 4 h, 37 °C). After rinsing with PBS-T (3 $\times$ ), CTV-IAC strain monoclonal antibodies 30.G.02 (100  $\text{mm}^{-3}$ , 2  $\text{ng cm}^{-3}$ ), was added to each well (2 h, 37 °C). Later, the wells were rinsed again and a 1:30 000 dilution of goat anti-mouse antibody conjugated with alkaline phosphatase (2 h, 37 °C, *Sigma*, St. Louis, USA) was added. Finally, after another PBS-T wash, the reaction was determined by adding *p*-nitro-phenyl-phosphate (pNPP; 1  $\text{mg cm}^{-3}$ ) in diethanolamine buffer (10 %, pH 9.8, room temperature, 1 h). The absorbance (A) was measured at 405 nm using an *Expert Plus* Multiplate plate reader (*ASYYS HITECH*, Eugendorf, Austria). A plant was considered infected when the absorbance values (mean of 3 measures) were at least twice those of non-inoculated control plants.

Putative transgenic shoots were identified by PCR. The shoots were *in vitro* grafted onto Carrizo citrange seedlings and developed into 15, 13, and 16 plants of cv. Valencia containing the *pCTV-CP*, *pCTV-dsCP*, and *pCTV-CS* transgenes, respectively. For cv. Hamlin 18, 22, and 17 plants were obtained containing the *pCTV-CP*, *pCTV-dsCP*, and *pCTV-CS* transgenes, respectively. These results correspond to a genetic transformation efficiency of 1.5 % for *pCTV-CP*, 1.5 % for *pCTV-dsCP*,

and 2.0 % for *pCTV-CS* gene construct for cv. Valencia, and 1 % for *pCTV-CP*, 2.4 % for *pCTV-dsCP*, and 0.67 % for *pCTV-CS* gene construct for cv. Hamlin. The plants were acclimatized and cultivated in greenhouse. Genetic transformation was confirmed by Southern blot analyses (data not shown). Most of the transgenic lines displayed 1 - 2 integration events. Only the transgenic line 6 of *pCTV-CP* in cv. Valencia showed 4 integration events. The Southern blot profile indicated that the transgenic lines came from different transformation events.

The transgene expression was investigated by RT-PCR in non-inoculated transgenic lines (Fig. 1). The mRNA accumulation of *pCTV-CP* transgene was found in 8 out of 9 Valencia and in all 6 Hamlin transgenic lines analyzed. The mRNA of *pCTV-dsCP* transgene (hairpin) was undetectable in most Valencia transgenic lines evaluated. In fact, only 1 Valencia transgenic line (line 3) showed a very light band. For transgenic lines of Hamlin the mRNA of *pCTV-dsCP* transgene could be detected in all lines analyzed, however the bands were very light, almost undetectable in some plants (lines 4, 5 and 6). This result is probably related with a lower amount of mRNA in these lines. The mRNA of the *pCTV-CS* transgene was present in all Valencia and in 8 out of 9 Hamlin transgenic lines analyzed. The transgene

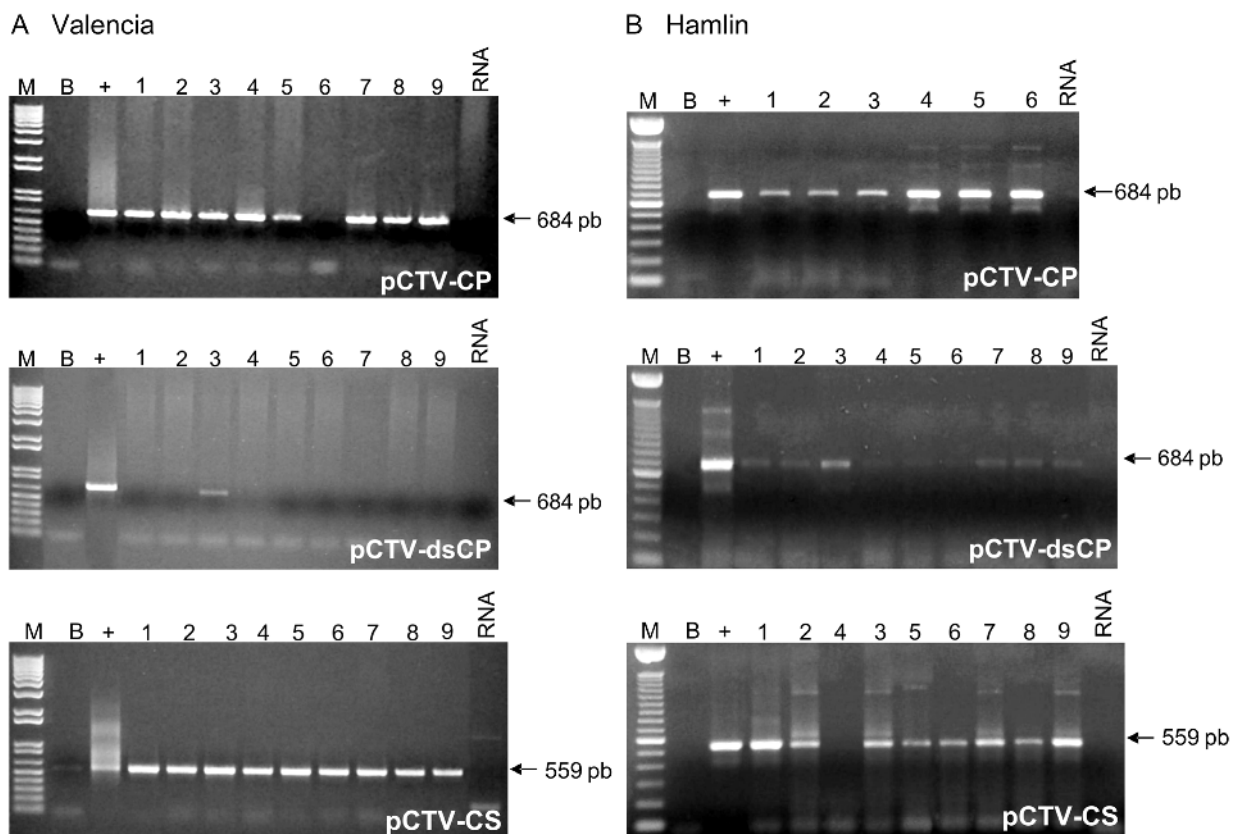


Fig. 1. RT-PCR analyses of *C. sinensis* cvs. Valencia (A) and Hamlin (B). Lanes 1-9: transgenic lines, +: positive control (consisted of DNA from a transgenic plant); B: blank (water); RNA: RNA sample treated with DNase.

transcription appears to be closely linked to the type of gene construct.

Based on DAS-ELISA, all transgenic lines as well as the non-transgenic controls were infected by the mild strain of CTV. The  $A_{405nm}$  values ranged from -0.050 to 1.510 for Valencia transgenic lines and from 0.000 to 2.926 for Hamlin transgenic lines. The  $A_{405nm}$  for the non-transgenic positive and negative controls were 1.111 and -0.021 for Valencia and 1.663 and 0.025 for Hamlin. However, it was possible to identify propagated clones of particular transgenic lines in both cultivars studied, with a very low virus titer (Table 1). For Valencia, it was possible to identify 1 clone with low virus titer for transgenic lines 2, 3, and 9 containing the *pCTV-CP* ( $A_{405nm}$  -0.016 to -0.003), for transgenic lines 1, 5, and 8 containing the *pCTV-dsCP* ( $A_{405nm}$  -0.050 to -0.016), and for transgenic lines 7, 8, and 9 containing the *pCTV-CS* ( $A_{405nm}$  -0.019 to -0.015). In addition, transgenic line 6 containing *pCTV-dsCP* presented 2 clones with very low

virus titer ( $A_{405nm}$  -0.045 and -0.028). Considering Hamlin transgenic lines 3 and 5 containing the *pCTV-CP*, and transgenic lines 1, 2, 6, 8 and 9 containing the *pCTV-CS* only 1 clone was with very low virus titer ( $A_{405nm}$  0.051 to 0.268). The transgenic lines 1, 3, 6 and 7 containing the *pCTV-dsCP* presented 2 clones with low virus titer ( $A_{405nm}$  0.000 to 0.008).

It was not possible to identify any transgenic line, for both cultivars studied, having the 3 propagated clones with low virus titer. However transgenic lines containing the *pCTV-dsCP* gene construct showed a higher number of propagated clones with low virus titer ( $A_{405nm}$  values similar to non-inoculated control) and where mRNA was undetectable or presented in a very light band (Fig. 1, Table 1). This variable response among propagated cloned plants has also been described for *C. aurantifolia*/CTV pathosystem (Dominguez *et al.* 2002) with 25 - 33 % of clonal plants from the same transgenic line not developing symptoms and virus replication not being detected by DAS-ELISA. Fagoaga *et al.* (2006) and Febres *et al.* (2008) also reported that propagations from the same transgenic line showed different responses to CTV in *C. aurantifolia* and *C. paradise*. Such response is not fully understood, but it may be related to either characteristics of the inoculation process, such as virus dosing or different viral populations (Dominguez *et al.* 2002), or to other abiotic factors that may interfere with the silencing pathway (Febres *et al.* 2008, Moreno *et al.* 2008). Our results indicate that the transgenic lines containing *pCTV-dsCP* gene construct showed the highest number of propagated clones exhibiting resistance to virus replication, based on the very low mean absorbance values obtained in DAS-ELISA.

To the best of our knowledge, this is the first report of pathogen derived resistance (Sanford and Johnston 1985) use in *C. sinensis*/CTV pathosystem. Efforts are underway in order to challenge the transgenic plants with a more severe CTV strain by vector (*Toxoptera citricida* Kirk.) inoculation, which is a natural way of virus dissemination. The resistant clones will also be checked for siRNA accumulation.

Table 1. Evaluation of CTV replication by DAS-ELISA in *Citrus sinensis* cvs. Valencia and Hamlin transgenic lines, containing the gene constructs *pCTV-CP*, *pCTV-dsCP* and *pCTV-CS*. C<sup>-</sup> - negative control (non-transgenic non-inoculated plants); C<sup>+</sup> - positive control (non-transgenic inoculated plants).

Lines	Infected plants/inoculated plants					
	<i>pCTV-CP</i>	<i>pCTV-dsCP</i>	<i>pCTV-CS</i>			
	Valencia	Hamlin	Valencia	Hamlin	Valencia	Hamlin
1	3/3	3/3	2/3	1/3	3/3	2/3
2	2/3	3/3	3/3	3/3	3/3	2/3
3	2/3	2/3	3/3	1/3	3/3	3/3
4	3/3	3/3	3/3	3/3	3/3	3/3
5	3/3	2/3	2/3	3/3	3/3	3/3
6	3/3	3/3	1/3	1/3	3/3	2/3
7	3/3	-	3/3	1/3	2/3	3/3
8	3/3	-	2/3	3/3	2/3	2/3
9	2/3	-	3/3	3/3	2/3	2/3
C <sup>-</sup>	0/3	0/3	0/3	0/3	0/3	0/3
C <sup>+</sup>	3/3	3/3	3/3	3/3	3/3	3/3

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