Physiological and Molecular Plant Pathology 94 (2016) 149–155

Contents lists available at ScienceDirect

Physiological and Molecular Plant Pathology

journal homepage: www.elsevier.com/locate/pmpp

Uncontrolled *Citrus psorosis virus* infection in *Citrus sinensis* transgenic plants expressing a viral 24K-derived hairpin that does not trigger RNA silencing

C.A. Reyes [a, 1], A. De Francesco [a, 1], E.E. Ocolotobiche [a], N. Costa [b], M.L. García [a, *]

[a] Instituto de Biotecnología y Biología Molecular, CCT-La Plata, CONICET - UNLP, calles 47 y 115 (1900), La Plata, Buenos Aires, Argentina
[b] Estación Experimental Agropecuaria, INTA Concordia, Ruta Provincial 22 y vías del Ferrocarril (3200), Concordia, Entre Ríos, Argentina

ARTICLE INFO

Article history:
Received 25 November 2015
Received in revised form
1 April 2016
Accepted 9 May 2016
Available online 10 May 2016

Keywords:
Citrus psorosis virus
Hairpin-RNA
Transgenic sweet orange
Hypersusceptibility

ABSTRACT

Citrus psorosis virus (CPsV) is the causal agent of psorosis disease of citrus. Pineapple sweet orange plants were transformed with a hairpin construct derived from the viral *24k* gene (lines ihp24K). Contrary to expectations, these lines did not trigger efficient RNA silencing, and when infected with CPsV they showed a phenotype of exacerbated symptoms with a persistent and homogeneous infection without the recovery observed in non-transgenic plants. Ihp24K lines did not behave similarly when challenged with Citrus tristeza virus. All these results indicate that hypersusceptibility is likely related to the specific action of 24K-derived hairpin over CPsV multiplication.

© 2016 Elsevier Ltd. All rights reserved.

1. Introduction

Psorosis is a serious and widespread disease affecting most citrus varieties. It is one of the first described citrus diseases [1], reported in many countries around the world, mainly in North and South America and in the Mediterranean Basin [2]. Citrus infected by psorosis are less productive and typical symptoms in the field include bark scaling, pustules and gum accumulation on the trunk; flecking, chlorotic spots, and necrotic shock of young shoots.

The causal agent of psorosis is *Citrus psorosis virus* (CPsV), the type member of the genus *Ophiovirus*, family *Ophioviridae* [3,4]. CPsV is tripartite and its genome consists of three single-stranded RNAs of negative polarity. CPsV RNA 1 is the largest and carries two ORFs; one coding for the RNA-dependent RNA polymerase (RdRp, 280 kDa), and separated by an intergenic region, a 24 kDa polypeptide (24K) that we recently reported that interferes in miRNA biogenesis and function [5]. RNA 2 encodes the movement protein [6], and RNA 3 encodes the coat protein (CP) [7].

RNA silencing is one of the mechanisms that plants use to defend themselves against viruses [8,9]. The phenomenon called recovery was described early as a systemic virus infection with associated symptoms, followed by a decrease and disappearance of symptoms in young leaves [10], and it has been explained as an antiviral defense mechanism based on silencing [11,12].

RNA silencing is triggered by the presence of double-stranded RNA (dsRNA) molecules and causes a sequence-specific shut down in the expression of genes containing sequences identical or highly similar to the initiating dsRNA [13–17]. dsRNA molecules are cleaved by Dicer-like enzymes (DCLs), resulting in small interfering RNA (siRNA) of 21–24 nucleotides which are the effector molecules of the mechanism. Upon processing, one strand of the siRNA duplexes generated by DCLs is incorporated into an Argonaute (AGO)-containing RNA-induced silencing complex (RISC) to guide sequence-specific inactivation of targeted RNA or DNA [18]. The use of dsRNA to trigger RNA silencing can be achieved by transformation with sense and antisense sequences separated by an intron (intron-hairpin constructs). After transcription, the resulting hairpin RNA acts as a strong inducer of RNA silencing [19,20].

We reported previously the development of highly resistant transgenic sweet orange plants (*Citrus sinensis* (L. Osbeck)) expressing hairpin-RNA corresponding to the CPsV coat protein (ihpCP) [21]. We also reported the generation of *C. sinensis* plants expressing hairpin-RNA corresponding to CPsV *24k* gene (ihp24K). In contrast to the ihpCP lines, ihp24K lines did not show RNA

* Corresponding author.
E-mail address: garcia_m@biol.unlp.edu.ar (M.L. García).
[1] Both authors equally contributed to this work.

http://dx.doi.org/10.1016/j.pmpp.2016.05.001
0885-5765/© 2016 Elsevier Ltd. All rights reserved.

silencing of the transgene. Even more, CPsV-inoculated ihp24K plants showed a phenotype of exacerbated symptoms homogeneously distributed in the whole plant, with high viral titer. Here we described the hypersusceptibility phenotype and characterized its specificity.

2. Materials and methods

2.1. Genetic construct, bacterial strains

The genetic construct used in this study was described by Reyes [21]. The length of hairpin 24K (ihp24K) fragment is 312 bp. Briefly, ihp24K fragments were amplified by RT-PCR from CPsV-90-1-1-infected citrus leaves, using primers hp24k1 and hp24k2, and inserted in sense and antisense orientations in the pHANNIBAL vector [22]. The cassette was then sub-cloned into the binary vector pBin19sgfp [23] for plant transformation.

2.2. Plant transformation and regeneration

Pineapple sweet orange seedlings were transformed with *Agrobacterium tumefaciens* EHA105 carrying the ihp24K construct from young internodal stem segments, as previously described [21,24,25]. Transformed shoot tips were grafted *in vitro* on Troyer citrange (*Poncirus trifoliata* (L.) Raf. *x C. sinensis*). After 3–4 weeks, scions were screened for the presence of the transgenes by PCR using primers 35S/ihp24K1, ihp24K1/pdk2 and ihp24K2/tnos (Table 1). Transgenic plantlets were then side-grafted on vigorous 6-month-old seedlings of rough lemon (*Citrus jambhiri* Lush) and grown in a greenhouse maintained at 18–24 °C.

2.3. Viral isolates

For the infection assays two viruses were used: CPsV isolate 90-1-1 and *Citrus tristeza virus* (CTV) isolate 268-2, both belonging to INTA-Concordia collection (Entre Ríos, Argentina). Citrus transgenic lines were infected by graft inoculation. Non-transgenic plants of Pineapple sweet orange (*Citrus sinensis* (L. Osbeck)) and Key Lime (*Citrus aurantiifolia* (Christm. Swingle)) were inoculated as infected controls.

2.4. Propagation, graft-inoculation, and symptom observation

For challenge assays, 2 sets of 12 propagations were made for each line and non transgenic control, grafting one bud from sweet orange (transgenic 6117, 6119 or non-tg) onto 10-12-month-old rough lemon seedlings. After 10–12 months, replicates of one set were graft-inoculated on the scion with one piece of infected bark carrying the CPsV 90-1-1 isolate and the other set with CTV 268-2 isolate. Grafted inoculum piece remained throughout the challenge period. Symptoms were evaluated on young leaves of CPsV-inoculated plants for three successive flushes (every 30–40 days), or adult leaves of CTV-inoculated plants for two successive flushes (150–180 days for each flush).

A symptom-severity scale for CPsV was defined ranging from 1 to 10. Maximum value was given to the death of all shoots (no shoots), which is the most severe symptom finally leading to plant death. Lower scores were assigned to different kinds and intensities of leaf chlorosis. The scores assigned in the scale were: Mild flecking, mild variegated and mild spots = 1; moderate flecking, moderate variegated and moderate spots = 2; severe flecking, severe variegated and severe spots = 3; mild shock = 4; moderate shock = 5 and severe shock = 6; no shoot (dead shoot) = 10. In the last case was assigned a value of 10 instead of 7 (which would be the consecutive number on the scale) in order to ponder this state of the plant, which is much more drastic than the onset of symptoms. The overall sum was made for each group of plants. No mean calculation was needed given the same number of individuals assayed in each group (12). After each observation, leaf tissue was collected for molecular analysis.

Table 1
Primers used.

Primer	Sequence
hp24k1	5′ AGAATTCGGATCCATGGCTGAATATATAGAAG 3′
hp24k2	5′ AGGGTACCAAGCTTCTGCGTCACTGCCATCTG 3′
24Ks-arg1	5′ ATGGCTGAATATATAGAAGT 3′
24Kas-arg1	5′ TCCAAATTCCTATTATCCTGG 3′
CP1c	5′GTTTCAAGATGGAGCAAGTTGATGG 3′
CP3	5′GAGACCCTTGTGTAAAAACCAGCAC 3′
ubqt 1	5′ CACCTCGTGCTTCGTCTCCG 3′
ubqt 2	5′ GCGTCCTTCCATCCTCCAGC 3′
35S	5′ CTATCCTTCGCAAGACCCTT 3′
pdk2	5′ AATATACAAAGCGCAAGATC 3′
tnos	5′GACACCGCGCGCGATAATTTATCC 3′

2.5. CPsV TAS-ELISA-HRP assay

Triple sandwich immunoassay-horseradish peroxidase (TAS-ELISA-HRP, hereinafter TAS-ELISA) was carried out essentially as described [26–28], with A376 polyclonal antiserum at 1/7500 dilution, monoclonal antibody MAbs 13C5 (IgG) at 1/10,000 dilution, and conjugated antibody, goat anti-mouse-HRP IgG (H+L) – Peroxidase (Jackson Immuno Research Laboratories, Inc., West Grove, PA, USA) at a dilution of 1/2500. Each sample was analysed in triplicate of the same extract. Samples of infected plants, healthy, non-transgenic plants, and extraction buffer were taken as positive and negative controls, respectively. OD values 3 times the average of the healthy control are considered as positive for detection of CPsV and diagnosis [26–29]. Data were statistically analysed using Student's *t*-test.

2.6. CTV TAS-ELISA-AP assay

This procedure is similar to the described for CPsV but using polyclonal antibody G 604 at a dilution of 1/1000 in carbonate-coating buffer and incubated overnight at 4 °C; 100 μl of sample extraction and incubated overnight again at 4 °C; 100 μl of a 1/2000 dilution of a mix of monoclonal antibodies (IgG) 3CA5 + DF1 [30] for 2 h at 37 °C; conjugated antibody goat anti-mouse–alkaline phosphatase enzyme (Agdia, Inc., Elkhart, IN, USA); and detected with PNP substrate according to the steps indicated by the manufacturer. Optical density was measured at 405 nm. TAS-ELISA values were expressed as a ratio of the OD of inoculated replicates (I) to the healthy, non-inoculated controls (H). Samples are considered positive when OD values are twice the average of the healthy control [30,31]. Data were statistically analysed using Student's *t*-test.

2.7. RNA preparation

Total RNA was prepared from 200 mg of fresh leaf ground tissue with 1 ml of TRI Reagent (Molecular Research Center, Cincinnati, OH, USA) and processed according to the manufacturer's instructions. RNA extracts were resuspended in 25 μl of RNase-free water and quantified using a spectrophotometer at 260 nm.

2.8. siRNA isolation and detection

The abundance of siRNA was monitored by Northern blot analysis. Total RNA was extracted from 1 g of citrus leaves pooled

from all replicates. One hundred micrograms of RNA from each sample were separated in 17% polyacrylamide gels containing 7 M urea and 0.5X TBE buffer, transferred to a positively charged nylon membrane (Roche Diagnostics Corporation, Indianapolis, IN, USA) by a Bio-Rad (Hercules CA, USA) transfer unit and chemically fixated according to Pall and Hamilton [32]. AmpliScribe™ T7-Flash™ Transcription Kit (Epicentre Biotechnologies, Madison, WI, USA) was used to generate the ^{32}P-labelled riboprobe from the hairpin construct ihp24K complementary to negative viral RNA. Hybridization was performed at 45 °C overnight and signals were detected by autoradiography.

2.9. RT-qPCR assay

For RT-qPCR, we synthesized first-strand cDNA from total DNAse treated RNA by using oligodT (Promega, Madison, WI, USA) and M-MLV Reverse Transcriptase (Promega) for transgene transcript determination or *cp* mRNA accumulation. cDNA was used as template for PCR. PCR was performed in the Fluorescent Quantitative Detection System FQD- 48A (BIOER, Hangzhou, China), in a total volume of 20 μl using 5x HOT FIREPol® EvaGreen® qPCR Mix (SOLIS Biodyne, Tartu, Estonia) and the corresponding pair of primers. The conditions used for PCR were 95 °C for 5 min; 44 cycles of 20 s at 95 °C, 30 s at 55 °C, and 20 s at 72 °C; followed by 10 min at 72 °C. Gene-specific primers are 24Ks-arg1/24Kas-arg1 for hairpin transcript and CP1c/CP3 for *cp* mRNA. Their sequences are indicated in Table 1. The presence of a unique product of the expected size was verified on ethidium-bromide-stained agarose gels. The absence of contaminant genomic DNA was confirmed in reactions with DNAse-treated RNA as the template. Control cDNA synthesis without primers was also performed. Ubiquitin from *C. sinensis* (GU362416.1) was used as an internal control to normalize the amount of template cDNA [33,34], which was previously tested and resulted stable under CPsV infection [28]. Each sample was treated in technical triplicate and their fluorescence values were averaged. Data were statistically analysed using Student's *t*-test.

3. Results

3.1. Sweet orange plants expressing ihp24K inoculated with CPsV manifested a hypersusceptible phenotype without recovery

Two transgenic lines of sweet orange (*Citrus sinensis*) expressing ihp24K, designated 6117 and 6119, were previously challenged with CPsV in two independent assays (Reyes et al., 2011). Although both non-infected transgenic lines were phenotypically indistinguishable from non-transgenic sweet orange, the inoculated replicates showed more severe symptoms with respect to the inoculated non-transgenic (non tg) plants in the first two flushes analysed [21].

A set of 12 new propagations of both transgenic lines and the non tg control were generated. Ten of each group (6117, 6119, and non tg) were challenged by grafting with the Argentine CPsV 90-1-1 isolate and two individuals were not inoculated (non-inoculated controls). After each of three successive flushes, characteristic symptoms were observed. Observations spanned a range of symptoms from chlorosis parallel to the leaf veins (flecking), variegated chlorosis and spots (Fig. 1a) to necrosis of very young shoots (shock) (Fig. 1b). The intensity of each symptom was markedly higher in the transgenic lines than in the non tg control plants (Fig. 1). A greater number of spots and larger flecking areas were manifested in 6117 and 6119 inoculated propagations. Shock reactions were found in almost all of the new ihp24K shoots and maintained throughout three flushes. Non tg plants showed shock only in the first flush of some shoots and recovered later. Normally, systemic psorosis infection is clearly observed in the first flush (young leaves). However, in the second flush and even more notably in the third flush, the non tg plant recovers, showing mild symptoms, and lower virus titers. Besides, the CPsV distribution is heterogeneous, with some branches expressing marked psorosis symptoms while others remain completely asymptomatic, even in seedlings under greenhouse conditions as previously reported [35]. These transgenic ihp24K lines showed increased intensity and expansion of symptoms and also showed a more homogeneous symptom distribution throughout the whole plant. The different distribution between transgenic and non-transgenic plants was observed for each kind of symptoms and in every replicate plants (See Fig. 1b for shock distribution and Supplementary Fig. 1 for foliar symptom distribution). Table 2 shows the symptoms observed in the transgenic and non tg plants. To compare the overall symptom behavior of these lines a symptom-severity scale was applied to each individual of transgenic and non tg plant described in Table 2. The overall sum was made and the results are shown in Fig. 2.

Recovery from infection was also evaluated as an indicator of plant response [36]. Recovery rate was determined, using the ratio between the score of the first flush and the second or third flush. Non tg plants showed symptom scores of 30 for the first, 14 for second and 11 for the third flush, displaying a recovery rate of 2.1 and 2.7 for second and third flushes respectively, thus the plants recovered from infection (Fig. 2). Line 6117 was the most drastically affected in the three flushes (symptom scores of 59, 65 and 53 respectively) compared to line 6119 (42, 34 and 39 for the three flushes respectively). Both showed more severe symptoms than the infected non tg control and did not recover (recovery ratios about 1) (Fig. 2).

3.2. ihp24K lines showed higher CPsV titers

To obtain quantitative measures of CPsV multiplication in new leaves, CP protein and *cp* mRNA levels were determined. CP accumulation was quantified by TAS-ELISA in every replicate of the two transgenic lines during two successive flushes separated by two months. TAS-ELISA values were expressed as a ratio of the optical density (OD) obtained for inoculated replicates (I) to healthy, non-inoculated controls (H). The average of the replicates for each transgenic line for the two flushes are shown in Fig. 3a and b. OD ratios for the 6117 line in the first flush was about 6 and 4 in the second flush. For line 6119, OD ratios were lower than 6117 for both flushes (about 5 in the first and less than 4 in the second). Both lines showed viral accumulation higher than the non tg control. It was significantly different in the first and second flush for 6117 and in the second flush for 6119. These results correlated with the previously described symptom scores.

In order to confirm these results and determine if there is also an alteration in the accumulation of viral mRNA, RT-qPCR for *cp* mRNA in transgenic and non-transgenic plants were performed using specific primers and ubiquitin as an internal control (Fig. 4). An increment in *cp* mRNA was observed in both transgenic lines with significant differences compared to non tg controls, correlating with CP accumulation and symptom scores.

3.3. ihp24K lines did not show exacerbated CTV infection

To characterize the specificity of the hypersusceptibility phenomenon observed in the sweet orange ihp24K plants, another set of 12 propagations of each line (6117 and 6119) and non tg control were generated and challenged with CTV. Citrus species infected with CTV do not always produce visible symptoms since expression depends on virus strain, citrus cultivar and environmental

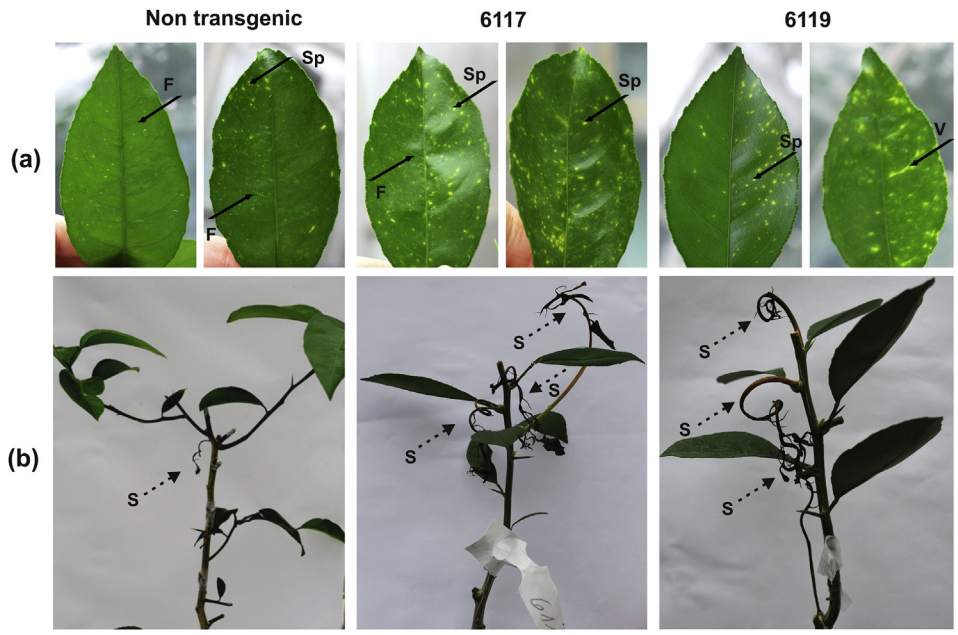

Fig. 1. Symptoms observed in representative leaves of inoculated *Citrus sinensis* ihp24K transgenic lines (6117 and 6119) and control (non transgenic) plants. (a) flecking (F), variegated chlorosis (V) and spots (Sp). (b) necrosis of very young shoots (shock; S).

conditions. Ten individuals of each group were graft-inoculated with the Argentinian CTV isolate 268-2 [37], which was previously described as inducing vein-clearing in Pineapple sweet orange (N. Costa, INTA Concordia, personal communication). Two individuals were the non-inoculated controls. Key Lime plants (*Citrus aurantiifolia*), were also included as CTV indicator and as inoculum control. Characteristic tristeza symptoms were observed after two successive flushes. Inoculated 6117 and 6119 lines and non tg inoculated controls showed very mild vein clearing, with no difference between transgenic and controls. Key Lime showed characteristic tristeza symptoms including vein-clearing and leaf cupping.

CTV accumulation was also evaluated by TAS-ELISA in new leaves of every replicate after two flushes of six months. Averages of the replicates for each transgenic line for the two flushes are shown in Fig. 5a and b. Neither line 6117 nor line 6119 showed significant differences in virus accumulation with respect to the non-tg inoculated sweet orange plants.

Table 2
Symptom observation in transgenic lines 6117 and 6119 and non transgenic (Non tg) control plants during three successive flushes. Numbers correspond to individual plants.

Plant#	First flush			Second flush			Third flush		
	Non tg	6117	6119	Non tg	6117	6119	Non tg	6117	6119
1	F+	S+	S+++	F+	S+	ns	F+	S+	ns
2	F++ V++	F+ V++ S+	F++ Sp++	V+	V+	S++	F+	V+	F+
3	V++	F++ S+	Sp+ S++	V+ Sp+	F+ S+	Sp+	Sp+	F+	Sp+
4	V++ S++	F+ S++	F+ V++	F+ Sp+	S+	F++	Sp+	Sp+ S+	V++
5	V++	S++	S++	Sp+	F+ Sp+ S++	Sp+	Sp+	ns	Sp+
6	F++ V++	ns	F++ Sp+	F++	ns	Sp+	Sp++	ns	V++
7	F++ V++	S++	F++ Sp+ S+	F++	ns	Sp+	Sp+	Sp+ S++	V+
8	F+ V++	S+	V++	Sp+	ns	F++	Sp+	Sp+	ns
9	V++ S++	S+	F+	Sp+	Sp+	ns	Sp+	Sp+ S+	ns
10	V++	S++	F+ V+	F+	ns	F+	Sp+	ns	Sp+
11*	H	H	H	H	H	H	H	H	H
12*	H	H	H	H	H	H	H	H	H

F: flecking; V: variegated; Sp: spotted; S: shock; H: healthy; Ns: no shoot (dead shoot); * non inoculated control. Symptom Intensity: +: mild; ++: moderate; +++: severe.

3.4. ihp24K lines did not show detectable RNA silencing of the transgene

Hairpin transcripts are well-known as strong inducers of RNA

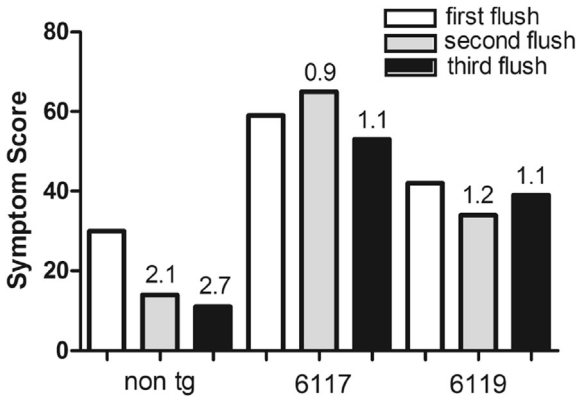

Fig. 2. Symptom scores and recovery ratios of transgenic 6117 and 6119 lines and non transgenic (non tg) control plants. Each individual plant was scored along three successive flushes. Symptom-severity scale: Mild flecking, mild variegated and mild spots = 1; moderate flecking, moderate variegated and moderate spots = 2; severe flecking, severe variegated and severe spots = 3; mild shock = 4; moderate shock = 5 and severe shock = 6; no shoot (dead shoot) = 10. Numbers above bars indicate recovery ratios of second or third flushes with respect to the first flush.

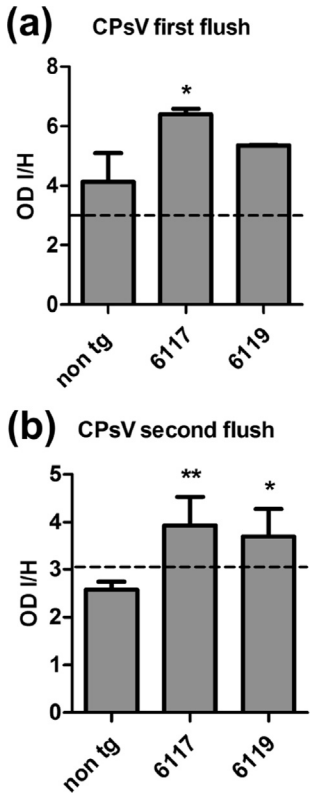

Fig. 3. ELISA evaluations of transgenic lines (6117 and 6119) and non transgenic control (non tg) challenged with CPsV in two successive flushes (a) and (b). The ELISA ratio plotted is the average of the ELISA readings of triplicates of all the inoculated individuals divided by the average ELISA reading of the healthy control (I/H). A plant is considered CPsV infected when the OD is 3 times that of the healthy control. *, ** indicates significant differences with the non tg sample at $P < 0.05$ and $P < 0.01$ respectively using a two-tailed paired *t*-test.

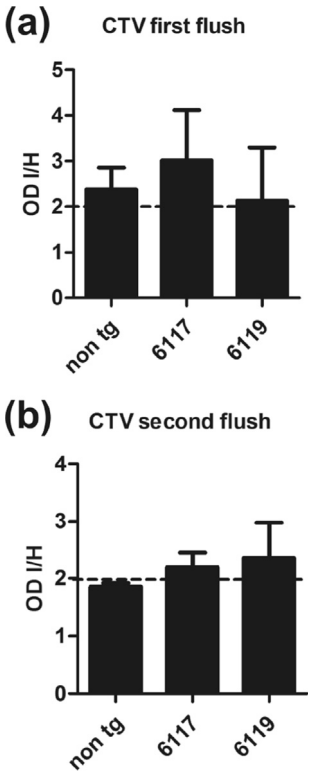

Fig. 5. ELISA evaluations of transgenic lines (6117 and 6119) and non transgenic control (non tg) challenged with CTV in two successive flushes (a) and (b). The ELISA ratio plotted is the average of the ELISA readings of triplicates of all the inoculated individuals divided by the average ELISA reading of the healthy control (I/H). A plant is considered CPsV infected when the OD is twice that of the healthy control.

silencing. To determine whether RNA silencing was triggered in these ihp24K transgenic lines, the accumulation of ihp24K-transcript and ihp24K-derived siRNAs were analysed. Total RNA was extracted from a pool of leaves collected from all the replicates of non tg, 6117 and 6119 lines. To assess the accumulation of the ihp24K-transcript, polyadenylated ihp24K-mRNA was evaluated by RT-qPCR, as shown in Fig. 6a. This transcript was detectable in both lines, with a greater accumulation in line 6119 than in 6117.

siRNAs derived from the ihp24K-transcript were analysed by Northern blot in both the transgenic lines and non tg control (Fig. 6b). None of the lines accumulated detectable levels of siRNA derived from the transgene, even though very low accumulation cannot be excluded. This fact together with the mentioned accumulation of the full-length ihp24K-transcript indicate that the 24K hairpin is not efficiently processed nor was RNA silencing triggered in these lines. When lines 6117 and 6119 were inoculated, a high level of virus-derived small RNAs (vsRNAs) was detected. Furthermore, these transgenic lines showed higher vsRNA accumulation

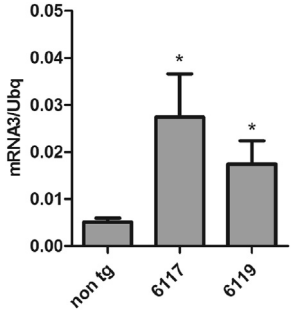

Fig. 4. Evaluation of accumulation of CPsV *cp* mRNA in transgenic 6117 and 6119 lines and non transgenic (non tg) control by RT-qPCR. Ubiquitin transcript was used as internal control. *, indicates significant differences with the non tg sample at $P < 0.05$ using a two-tailed paired *t*-test.

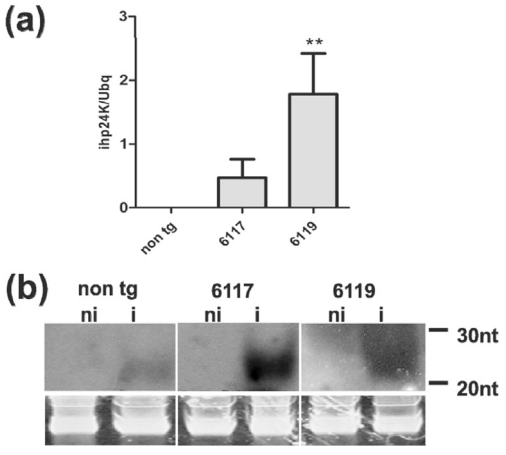

Fig. 6. Molecular analysis of ihp24K transgenic lines and non transgenic control (non tg). (a) ihp24K transcript accumulation by RT-qPCR. Ubiquitin transcript was used as internal control. (b) siRNA accumulation by Northern blot. RNA was extracted from CPsV-inoculated (i) and non inoculated (ni) ihp24K lines and control (non tg). DNA oligomers (20 mer and 30 mer) used as size markers are indicated. Ethidium-bromide-stained rRNA is shown as a loading control.

than the inoculated non tg control (Fig. 6b), in agreement with the increased viral accumulation shown by TAS-ELISA and RT-qPCR assays and the exacerbated symptoms.

4. Discussion

Transgenic plants expressing a hairpin derived from CPsV sequences did not produce resistance to the virus as expected but manifested a hypersusceptibility phenotype. Ihp24K lines did not show efficient RNA silencing since siRNAs were not detected in non-inoculated plants whereas the ihp24K-transcript did accumulate in both lines, displaying no degradation.

The kind of symptoms spanned in the hypersusceptibility phenotype was not different from the normal infection, but symptom intensity and distribution in the plant did change. Instead of the heterogeneous symptom distribution throughout the non-transgenic citrus plants, the virus could invade the whole ihp24K plants. The heterogeneous distribution of CPsV, particularly of psorosis A isolates (CPsV 90-1-1), in non-transgenic plants was extensively described [2,26,38]. Virus distribution of this kind of psorosis isolates covers only main branches. Symptoms in ihp24K infected lines were distributed in main and secondary branches covering almost the whole plant and including all leaves. In addition, ihp24K lines did not show symptom recovery as do the non tg orange plants. The persistent shock reaction in the second and third flushes, which is only observed in the first flush in non tg plants, is also a strong indication of the no recovery phenotype of these lines, and how sustained and invasive is CPsV infection.

It has been extensively reported that downregulation of plant genes coding for components of gene silencing machinery such as DCLs, AGO1, RDRs and SDE5 is associated with viral hypersusceptibility phenomenon [39–41]. The fact that only CPsV- and not CTV-inoculated plants showed the increase in viral titers and symptoms compared to non tg control suggests that some 24K-derived siRNAs do not operate over plant transcripts in a so-called nonspecific *off-targeting* against the silencing machinery but it is a specific CPsV mechanism. Moreover ihp24k sequence was verified *in sílico* for putative siRNA generation and off-targeting against *C. sinensis* databases (http://citrus.hzau.edu.cn/orange/ and http://www.citrusgenomedb.org/species/sinensis) using two siRNAs prediction tools (siRNA design (http://jura.wi.mit.edu/bioc/siRNAext/)) and (Block-iT™ RNAi designer (https://rnaidesigner.invitrogen.com/rnaiexpress/)). No relevant match of ihp24k sequence to any component of the silencing machinery was found (data not shown).

The use of dsRNA to trigger RNA silencing has been generally successful to trigger viral resistance through transgenic expression. However, there are some reports where hairpin transcription does not necessarily lead to efficient triggering of the RNA silencing pathway [42–44]. Parameters, such as the integration locus of a transgene as well as inherent characteristics of the transcript, transgene localization, its intermediate processing and interaction with various proteins of competing processing and degradation machineries may be critical in determining the fate of hairpin transcripts and efficient triggering of RNA silencing [42]. A transgene localization (position effect) of the ihp24K transgene leading to a defective defense phenotype would be unlikely since both lines were independent transformation events. Inherent characteristics of the hairpin are the same for ihpCP, which efficiently triggers RNA silencing [21], and ihp24K. Therefore, this is not likely to be the reason for unsuccessful RNA silencing and siRNA accumulation. ihp24K-transcript, may contain structural features or recalcitrant intermediate processing intermediates that make it less suitable as a substrate for DCL proteins and that could be one of the reasons for non-detectable levels of siRNAs.

Besides, we demonstrate that ihp24K transcript accumulate in transgenic plants. Transcription is indeed essential but not sufficient for triggering RNA silencing and siRNA accumulation. It is likely that transcription exceeding a certain threshold level is necessary for efficient induction of RNA silencing [45]. In addition, both exo- and endo-nucleolytic RNA degradation pathways exist in plants. These RNA decay processes must be under tight control to enable a dynamic regulation of the RNA silencing threshold. Perhaps, ihp24K did not reach that threshold and it is degraded before inducing efficient RNA silencing.

The 5′ and 3′-terminal regions of viral RNAs play a key role in their translation and replication processes as well as pathogenicity and symptom expression [46]. Petty et al. [47], observed a clear correlation between alterations in the 5′-terminal region and viral movement, which significantly conditioned the pathogenic process. There is also evidence that the 3′-terminal region plays an important role in viral pathogenicity as shown for *Tobacco vein mottling virus* [48], and *Melon necrotic spot virus* [49]. In addition, Albiach-marti et al. [50], reported that pathogenicity determinant of CTV maps at the 3′-terminal region of the viral genome. As CTV p23 is a suppressor of RNA-mediated gene silencing, it potentially could disrupt micro-RNA metabolism [51,52], thus inducing symptoms. Besides, several viral suppressors of RNA mediated gene silencing have been identified as pathogenicity determinants. This could be the case of CPsV, since 24K sequence is also located at the 3′-terminal region of genomic RNA1 and is involved in silencing suppression and miRNA misregulation [5]. Therefore, overexpression of part of 24K transcript in hairpin construct could, someway favors virus replication and/or symptom expression.

A wide variety of RNA elements that regulate fundamental processes of viral life cycles were reported in viral genomes. These elements function as promoters, enhancers, and repressors of viral translation, transcription, genome replication, and encapsidation [53,54]. Viral RNA can adopt different conformations in the infected cell, which can be affected by cis or trans-acting elements present in the genome sequence. As an example, Villordo et al. [54], found that cyclization of the positive strand RNA of dengue virus was facilitated by hybridization with a sequence involved in a local dumbbell structure at the viral 3′ untranslated region. The author demonstrates that this cis-acting interaction differentially enhances viral replication in mosquito and mammalian cells. Regulation mechanisms of negative-strand viral genomes are less characterized, and particularly the way CPsV controls its genome replication or expression is still largely unknown. We can hypothesize that the presence of the ihp24K hairpin-transcript in the plant cell could alter the viral genome expression or its replication mediated by RNA-RNA interactions with its homologous 24K sequence or with another CPsV genome region, leading to the hypersusceptibility phenotype.

Acknowledgements

We thank Fabián Ramos (EEA-Concordia) for their observations and plant maintenance in the greenhouse. We thank L.W. Timmer for helpful comments on the manuscript and editing. This work has been supported by Agencia Nacional de Promoción Científica y Tecnológica (ANPCYT) PICT 2010-1726, PIP 445 Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Universidad Nacional de La Plata (UNLP) X692, and Instituto Nacional de Tecnología Agropecuaria (INTA) (PNFRU-1172; 11721; ERIOS-630081).

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.pmpp.2016.05.001.

References

[1] H.S. Fawcett, New angles on treatment of bark diseases of citrus, Calif. Citogr. 17 (1932) 406–408.
[2] C.N. Roistacher, Psorosis- a review, in: P. Moreno, J.V. da Graça, L.W. Timmer (Eds.), Proceedings of the 12th International Conference of the Organization of Citrus Virologists, IOCV, California, USA, 1993, pp. 139–154.
[3] M.L. García, E. Dal Bó, O. Grau, R.G. Milne, The closely related citrus ringspot and citrus psorosis viruses have particles of novel filamentous morphology, J. General Virol. 75 (1994) 3585–3590.
[4] R.G. Milne, M.L. García, O. Grau, Genus ophiovirus, in: M.V.H. van Regenmortel, C.M. Fauquet, D.H.L. Bishop, E.B. Carstens, M.K. Estes (Eds.), Virus Taxonomy, Sevent Report of the International Committee on Taxonomy of Viruses, Elsevier/Academic, London, UK, 2000, pp. 626–631.
[5] C.A. Reyes, E.E. Ocolotobiche, F.E. Marmisollé, G. Robles Luna, M.B. Borniego, A.A. Bazzini, S. Asurmendi, M.L. García, *Citrus psorosis virus* 24K protein interacts with citrus miRNA precursors, affects their processing and subsequent miRNA accumulation and target expression, Mol. Plant Pathol. (2015), http://dx.doi.org/10.1111/mpp.12282.
[6] G. Robles Luna, E.J. Peña, M.B. Borniego, M. Heinlein, M.L. García, Ophioviruses CPsV and MiLBVV movement protein is encoded in RNA 2 and interacts with the coat protein, Virology 441 (2013) 152–161.
[7] M.E. Sánchez de la Torre, O. Riva, R. Zandomeni, O. Grau, M.L. García, The top component of *Citrus psorosis virus* contains two ssRNAs, the smaller encodes the coat protein, Mol. Plant Pathol. Online (1998). http://www.bspp.org.uk/mppol/1998/1019sanchez.
[8] M.J. Axtell, Classification and comparison of small RNAs from plants, Annu. Rev. Plant Biol. 64 (2013) 137–159.
[9] A. Molnar, C.W. Melnyk, A. Bassett, T.J. Hardcastle, R. Dunn, D.C. Baulcombe, Small silencing RNAs in plants are mobile and direct epigenetic modification in recipient cells, Science 328 (2010) 872–875.
[10] R. MacDiarmid, RNA silencing in productive virus infections, Annu. Rev. Phytopathol. 43 (2005) 523–544.
[11] F. Li, S.W. Ding, Virus counterdefense: diverse strategies for evading the RNA-silencing immunity, Annu. Rev. Microbiol. 60 (2006) 503–531.
[12] X. Ma, M.C. Nicole, L.V. Meteignier, N. Hong, G. Wang, P. Moffett, Different roles for RNA silencing and RNA processing components in virus recovery and virus-induced gene silencing in plants, J. Exp. Bot. 66 (2014) 919–932.
[13] R. Almeida, R.C. Allshire, RNA silencing and genome regulation, Trends Cell Biol. 15 (2005) 251–258.
[14] D. Baulcombe, RNA silencing in plants, Nature 431 (2004) 356–363.
[15] T. Gura, A silence that speaks volumes, Nature 404 (2000) 804–808.
[16] P. Susi, M. Hohkuri, T. Wahlroos, N.J. Kilby, Characteristics of RNA silencing in plants: similarities and differences across kingdoms, Plant Mol. Biol. 54 (2004) 157–174.
[17] A. Takeda, M. Tsukuda, H. Mizumoto, K. Okamoto, M. Kaido, K. Mise, T. Okuno, A plant RNA virus suppresses RNA silencing through viral RNA replication, EMBO J. 24 (2005) 3147–3157.
[18] H. Vaucheret, Plant ARGONAUTES, Trends Plant Sci. 13 (2008) 350–358.
[19] A. Fire, RNA-triggered gene silencing, Trends Genet. 15 (1999) 358–363.
[20] N.A. Smith, S.P. Singh, M.B. Wang, P.A. Stoutjesdijk, A.G. Green, P.M. Waterhouse, Total silencing by intron-spliced hairpin RNAs, Nature 407 (2000) 319–320.
[21] C.A. Reyes, A. De Francesco, E.J. Pena, N. Costa, M.I. Plata, L. Sendin, A.P. Castagnaro, M.L. García, Resistance to *Citrus psorosis virus* in transgenic sweet orange plants is triggered by coat protein-RNA silencing, J. Biotechnol. 151 (2011) 151–158.
[22] S.V. Wesley, C.A. Helliwell, N.A. Smith, M.B. Wang, D.T. Rouse, Q. Liu, P.S. Gooding, S.P. Singh, D. Abbott, P.A. Stoutjesdijk, S.P. Robinson, A.P. Gleave, A.G. Green, P.M. Waterhouse, Construct design for efficient, effective and high-throughput gene silencing in plants, Plant J. 27 (2001) 581–590.
[23] W. Chiu, Y. Niwa, W. Zeng, T. Hirano, H. Kobayashi, J. Sheen, Engineered GFP as a vital reporter in plants, Curr. Biol. 6 (1996) 325–330.
[24] L. Peña, M. Cervera, J. Juárez, A. Navarro, J.A. Pina, N. Durán-Vila, L. Navarro, Agrobacterium-mediated transformation of sweet orange and regeneration of transgenic plants, Plant Cell Rep. 14 (1995) 616–619.
[25] M.C. Zanek, C.A. Reyes, M. Cervera, E.J. Peña, K. Velazquez, N. Costa, M.I. Plata, O. Grau, L. Peña, M.L. García, Genetic transformation of sweet orange with the coat protein gene of *Citrus psorosis virus* and evaluation of resistance against the virus, Plant Cell Rep. 27 (2008) 57–66.
[26] D. Alioto, M. Gangemi, S. Deaglio, S. Sposato, E. Noris, E. Luisoni, R.G. Milne, Improved detection of *Citrus psorosis virus* using polyclonal and monoclonal antibodies, Plant Pathol. 48 (1999) 735–741.
[27] M.C. Zanek, E. Peña, C.A. Reyes, J. Figueroa, B. Stein, O. Grau, Detection of *Citrus psorosis virus* in the northwestern citrus production area of Argentina by using an improved TAS-ELISA, J. Virol. Methods 137 (2006) 245–251.
[28] A. De Francesco, N. Costa, M.I. Plata, M.L. García, Improved detection of *Citrus psorosis virus* and coat protein-derived transgenes in citrus plants: comparison between RT-qPCR and TAS-ELISA, J. Phytopathol. (2015), http://dx.doi.org/10.1111/jph.12392.
[29] S. Martín, D. Alioto, R.G. Milne, S.M. Garnsey, M.L. García, O. Grau, J. Guerri, P. Moreno, Detection of *Citrus psorosis virus* by ELISA, molecular hybridization, RT-PCR and immunosorbent electron microscopy and its association with citrus psorosis disease, Eur. J. Plant Pathol. 110 (2004) 747–757.
[30] S.M. Garnsey, M. Cambra, Enzyme-linked immunosorbent assay (ELISA) for citrus pathogens, in: C.N. Roistacher (Ed.), Graft-transmissible Diseases of Citrus. Handbook for Detection and Diagnosis, FAO, Rome, 1991, pp. 193–216.
[31] C. Vela, M. Cambra, E. Cortés, P. Moreno, J.G. Miguet, C. Pérez de San Román, A. Sánz, Production and characterization of monoclonal antibodies specific for *Citrus tristeza virus* and their use for diagnosis, J. General Virol. 67 (1986) 91–96.
[32] G.S. Pall, A.J. Hamilton, Improved northern blot method for enhanced detection of small RNA, Nat. Protoc. 3 (2008) 1077–1084.
[33] L.P. Boava, M. Cristofani-Yaly, V.S. Mafra, K. Kubo, L.T. Kishi, M.A. Takita, M. Ribeiro-Alves, M.A. Machado, Global gene expression of *Poncirus trifoliata, Citrus sunki* and their hybrids under infection of Phytophthora parasitica, BMC Genom. 12 (2011) 39.
[34] V. Mafra, K.S. Kubo, M. Alves-Ferreira, M. Ribeiro-Alves, R.M. Stuart, L.P. Boava, C.M. Rodrigues, M.A. Machado, Reference genes for accurate transcript normalization in citrus genotypes under different experimental conditions, PLoS One 7 (2) (2012) e31263, http://dx.doi.org/10.1371/journal.pone.0031263.
[35] D. Alioto, J. Guerri, P. Moreno, R.G. Milne, Citrus psorosis virus, in: G.P. Rao, A. Myrta, K.S. Ling (Eds.), Characterization, Diagnosis and Management of Plant Viruses, vol. 2, Studium Press LC, Houston, USA, 2008, pp. 69–96.
[36] B. Ghoshal, H. Sanfaçon, Symptom recovery in virus-infected plants: revisiting the role of RNA silencing mechanisms, Virology 479 (2015) 167–179.
[37] N.G. Iglesias, S.P. Gago-Zachert, G. Robledo, N. Costa, M.I. Plata, O. Vera, Population structure of *Citrus tristeza virus* from field Argentinean isolates, Virus Genes 36 (2008) 199–207.
[38] J.M. Wallace, A half century of research on psorosis, in: J.M. Wallace (Ed.), Proceedings of the Conference on Citrus Virus Diseases, University of California, Division of Agricultural Sciences, Riverside, USA, 1959, pp. 5–21.
[39] T. Dalmay, A. Hamilton, S. Rudd, S. Angell, D.C. Baulcombe, An RNA-dependent RNA polymerase gene in *Arabidopsis* is required for posttranscriptional gene silencing mediated by a transgene but not by a virus, Cell 101 (2000) 543–553.
[40] I. Hernandez-Pinzon, N.E. Yelina, F. Schwach, D.J. Studholme, D. Baulcombe, T. Dalmay, SDE5, the putative homologue of a human mRNA export factor, is required for transgene silencing and accumulation of trans-acting endogenous siRNA, Plant J. 50 (2007) 140–148.
[41] J.B. Morel, C. Godon, P. Mourrain, C. Béclin, S. Boutet, F. Feuerbach, F. Proux, H. Vaucheret, Fertile hypomorphic ARGONAUTE (ago1) mutants impaired in post-transcriptional gene silencing and virus resistance, Plant Cell 14 (2002) 629–639.
[42] A. Dalakouras, M. Tzanopoulou, M. Tsagris, M. Wassenegger, K. Kalantidis, Hairpin transcription does not necessarily lead to efficient triggering of the RNAi pathway, Transgenic Res. 20 (2011) 293–304.
[43] K. Kalantidis, S. Psaradakis, M. Tabler, M. Tsagris, The occurrence of CMV-specific short RNAs in transgenic tobacco expressing virus-derived double-stranded RNA is indicative of resistance to the virus, Mol. Plant Microbe Interact. 15 (2002) 826–833.
[44] H. Vanderschuren, A. Alder, P. Zhang, W. Gruissem, Dose-dependent RNAi-mediated geminivirus resistance in the tropical root crop cassava, Plant Mol. Biol. 70 (2009) 265–272.
[45] K. Kalantidis, M. Tsagris, M. Tabler, Spontaneous short-range silencing of a GFP transgene in Nicotiana benthamiana is possibly mediated by small quantities of siRNA that do not trigger systemic silencing, Plant J. 45 (2006) 1006–1016.
[46] V. Pallas, J.A. García, How do plant viruses induce disease? Interactions and interference with host components, J. Gen. Virol. 92 (12) (2011) 2691–2705.
[47] I.T.D. Petty, M.C. Edwards, A.O. Jackson, Systemic movement of an RNA plant virus determined by a point substitution in a 5′ leader sequence, Proc. Natl. Acad. Sci. U. S. A. 87 (1990) 8894–8897.
[48] E. Rodríguez-Cerezo, P.G. Klein, J.G. Shaw, A determinant of disease symptom severity is located in the 39-terminal noncoding region of the RNA of a plant virus, Proc. Natl. Acad. Sci. U. S. A. 88 (1991) 9863–9867.
[49] J.A. Díaz, C. Nieto, E. Moriones, V. Truniger, M.A. Aranda, Molecular characterization of a melon necrotic spot virus strain that overcomes the resistance in melon and nonhost plants, Mol. Plant Microbe Interact. 17 (2004) 668–675.
[50] M.R. Albiach-Marti, C. Robertson, S. Gowda, S. Tatineni, B. Belliure, S.M. Garnsey, S.Y. Folimonova, P. Moreno, W.O. Dawson, The pathogenicity determinant of Citrus tristeza virus causing the seedling yellows syndrome maps at the 39-terminal region of the viral genome, Mol. Plant Pathol. 11 (2010) 55–67.
[51] E.J. Chapman, A.I. Prokhnevsky, K. Gopinath, V.V. Dolja, J.C. Carrington, Viral RNA silencing suppressors inhibit the microRNA pathway at an intermediate step, Genes Dev. 18 (2004) 1179–1186.
[52] P. Dunoyer, O. Voinnet, The complex interplay between plant viruses and host RNA-silencing pathways, Curr. Opin. Plant Biol. 8 (2005) 415–423.
[53] B. Wu, J. Pogany, H. Na, B.L. Nicholson, P.D. Nagy, K.A. White, A discontinuous RNA platform mediates RNA virus replication: building an integrated model for RNA-based regulation of viral processes, PLoS Pathog. 5 (3) (2009) e1000323.
[54] S.M. Villordo, D.E. Alvarez, A.V. Gamarnik, A balance between circular and linear forms of the dengue virus genome is crucial for viral replication, RNA 16 (2010) 2325–2335.