Effect of temperature on RNA silencing of a negative-stranded RNA plant virus: *Citrus psorosis virus*

K. Velázquez[a], A. Renovell[a], M. Comellas[a], P. Serra[a], M. L. García[b], J. A. Pina[a], L. Navarro[a], P. Moreno[a] and J. Guerri[a]*

[a]*Instituto Valenciano de Investigaciones Agrarias (IVIA), Centro de Protección Vegetal y Biotecnología, Cra. Moncada-Náquera Km 4.5, Moncada, 46113-Valencia, Spain; and* [b]*Instituto de Bioquímica y Biología Molecular (IBBM), Universidad Nacional de La Plata, Calles 47 y 115, 1900-La Plata, Argentina*

Citrus psorosis virus (CPsV), genus *Ophiovirus*, causes a bark scaling disease of citrus. CPsV virions are kinked filaments with three negative-stranded RNA molecules (vRNA) and a 48 kDa coat protein. The effect of temperature on symptom expression, virus accumulation and RNA silencing was examined in sweet orange seedlings (*Citrus sinensis*) graft-inoculated with three different CPsV isolates and grown in a glasshouse at 26/18°C or 32/26°C (day/night). Most plants kept in the cooler glasshouse showed a shock reaction in the first flush with shoot necrosis, and then moderate to intense chlorotic fleck-ing and spotting in young leaves, whereas plants incubated at 32/26°C did not exhibit shoot necrosis, and young leaf symp-toms were milder. Virus titre estimated by ELISA and by northern and dot blot hybridization paralleled symptom intensity, with significantly higher virus accumulation in plants incubated at 26/18°C. The amount of CPsV-derived small RNAs (CPsV-sRNAs) slightly increased at 32/26°C, with the ratio of CPsV-sRNA/vRNA being higher at 32/26°C than at 26/18°C. These results suggest that (i) CPsV infection induces RNA silencing in citrus plants, (ii) symptom intensity is associ-ated with virus accumulation, and (iii) temperature increase enhances the RNA silencing response of citrus plants and decreases virus accumulation.

Keywords: bark scaling disease of citrus, *Citrus sinensis*, siRNA, viral symptomatology, virus titre

Introduction

Virus infection in a host plant activates a defence mecha-nism related to post-transcriptional gene silencing (PTGS) that causes degradation of viral RNA and slows down or limits virus accumulation and systemic infec-tion. This process is triggered by double-stranded RNA (dsRNA), produced by replicative intermediates of sin-gle-stranded RNA (ssRNA) viruses, or by genomic or defective viral ssRNAs with extensive secondary struc-ture, which is cleaved by Dicer-like enzymes to produce 21–24 nucleotide (nt) fragments called small interfering RNAs (siRNAs) (Baulcombe, 2004). The siRNAs are incorporated into a large ribonucleoprotein complex (the RNA inducing silencing complex, RISC) that guides sequence-specific cleavage of the target RNA. siRNAs may also play a role in amplification of dsRNA by a plant encoded RNA-dependent RNA polymerase (RdRp), that in turn is degraded by Dicer-like enzymes to produce sec-ondary siRNAs (Baulcombe, 2004). As a counter

*E-mail: jguerri@ivia.es

defence, plant viruses encode proteins that suppress RNA silencing to overcome this defence mechanism (Diaz-Pendón & Ding, 2008).

Temperature affects plant-pathogen interactions, and a higher growth temperature may either increase or decrease disease resistance, thus reflecting a differential influence of the same temperature variation on different plant-pathogen systems (Wang *et al.*, 2009). Symptoms induced by many plant viruses are attenuated when plants are grown at high temperature, a phenomenon that might be related to different efficiency of the host defence system. Indeed it has been observed that RNA silencing is weaker when plants are grown at cool temperatures and stronger when grown at high temperatures (Szittya *et al.*, 2003; Chellappan *et al.*, 2005). However, these studies were limited to a few DNA or positive-stranded RNA plant viruses. This work examines the effect of tempera-ture on RNA silencing of a negative-stranded RNA virus (*Citrus psorosis virus*, CPsV) infecting citrus, its natural woody host.

CPsV, the type species of the genus *Ophiovirus* (Vaira *et al.*, 2005), is the presumed causal agent of citrus psorosis, an economically important graft-trans-missible disease (Martín *et al.*, 2004). CPsV virions

are kinked filaments, 3–4 nm in diameter, that separate into two components in a sucrose gradient (Derrick *et al.*, 1988; García *et al.*, 1994). They are formed by three single-stranded, negative sense RNAs (vRNA) and a coat protein (CP) of ~48 kDa (Barthe *et al.*, 1998; Sánchez de la Torre *et al.*, 1998; Martín *et al.*, 2005). RNA 1 is ~8184 nt in size and its viral complementary strand (vcRNA) has two ORFs potentially encoding a 24 kDa protein of unknown function and a 280 kDa protein with motifs characteristic of RNA dependent RNA polymerase (RdRp) (Naum-Onganía *et al.*, 2003). vcRNA 2 (~1644 nt) encodes a 54 kDa protein without similarities with other known proteins and vcRNA 3 (~1454 nt) encodes the CP (Sánchez de la Torre *et al.*, 1998, 2002; Martín *et al.*, 2005).

Psorosis disease in field trees is characterized by bark scaling with gum impregnation and wood staining in the stem and main branches (psorosis A), or by rampant bark scaling affecting most branches, that often causes tree death (psorosis B) (Roistacher, 1993; Milne *et al.*, 2003). In the glasshouse, most CPsV isolates induce a shock reaction in sweet orange seedlings (*Citrus sinensis*) that causes leaf shedding and necrosis of young shoots in the first flush (Fig. 1a), and then transitory chlorotic flecking and spotting in young leaves of the following flushes (Fig. 1b), whereas others induce only chlorotic flecking and spotting. Psorosis B additionally causes chlorotic blotching in old leaves with gummy pustules on the leaf underside (Fig. 1c) and blisters on twigs (Fig. 1d) (Fawcett & Klotz, 1938; Roistacher, 1993; Milne *et al.*, 2003). Psorosis symptom expression is affected by temperature, with cool temperatures favouring appearance of the shock reaction in the first flush after inoculation and warm temperatures often inhibiting shock and masking leaf symptoms (Roistacher & Calavan, 1974; Navas-Castillo & Moreno, 1993). However, the underlying molecular mechanism of this temperature effect is presently unknown. This paper studies the effect of temperature on symptom expression and virus accumulation of three different CPsV isolates in sweet orange, and characterises RNA silencing in infected plants by examining CPsV-derived small RNAs (CPsV-sRNAs).

Materials and methods

Virus isolates

The psorosis sources used in this study were isolates P121, PsA-A2 and PsB-B1. Isolate P121 was obtained after shoot-tip grafting *in vitro* a bark scaled clementine tree (*C. clementina*) var. Oroval from Chilches (Castellón), and its genomic RNA was completely sequenced (Martín *et al.*, 2005). Isolates PsA-A2 and PsB-B1 were obtained by graft inoculating sweet orange seedlings with non-scaled or lesion bark patches, respectively, from a psorosis affected field tree from Moncada (Valencia). While P121 and PsA-A2 isolates only induced the psorosis A syndrome, PsB-B1 isolate additionally induced
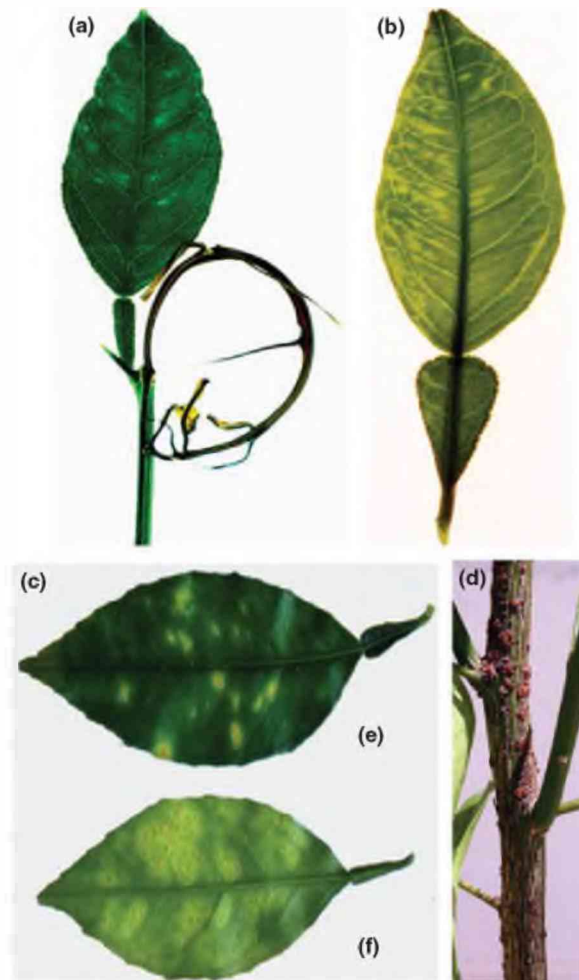

Figure 1 Symptoms induced by *Citrus psorosis virus* on Pineapple sweet orange (*Citrus sinensis*) plants in the glasshouse. (a) Shock reaction with leaf shedding and shoot necrosis in the first flush. (b) Chlorotic flecks and spots in a young leaf. (c) Psorosis B symptoms in an old leaf: chlorotic blotches on the leaf upper-side (e) and gummy pustules on the leaf under-side (f). (d) Psorosis B-induced blisters in a twig.

psorosis B symptoms (chlorotic blotching in old leaves and blisters on twigs).

Biological indexing was performed by graft inoculation of four Pineapple sweet orange seedlings grown in a temperature-controlled glasshouse (26/18°C day/night), without supplemental light, using an artificial potting mix (1:1 sand:peat moss) and a standard fertilizing procedure. All biological experiments were performed between January and October.

To assess the effects of temperature on symptom expression and virus and CPsV-sRNA accumulation, each isolate was graft inoculated on 16 Pineapple sweet orange seedlings and the plants were incubated for 15 days in a glasshouse set at 26/18°C (day/night) to facilitate inoculum survival and development of vascular connections. Then the plants were cut back leaving two

buds above the inoculum (about 25 cm above the soil level) and half of the plants inoculated with each isolate were moved to a warm glasshouse (32/26°C day/night), with the other half remaining at 26/18°C. Non-inoculated control plants were also grown at both temperature regimes. After the first flush, CPsV symptoms were evaluated and leaf samples were collected from individual plants for triple antibody sandwich (TAS)-ELISA and northern blot analysis and pooled for dot blot hybridization analysis.

TAS-ELISA

Plant extracts were prepared by blending 0·2 g of tissue in 10 volumes of PBS (8 mM Na_2HPO_4, 1·5 mM KH_2PO_4, 2·7 mM KCl, 0·14 M NaCl, 3 mM NaN_3, pH 7·4) containing 1 mL L^{-1} Tween 20, 20 g L^{-1} polyvinyl pyrrolidone (PVP-10000) and 25 g L^{-1} defatted milk powder, using a Polytron homogenizer (Kinematica). Plate coating, antibody incubations and washing conditions for TAS-ELISA were carried out as described by Martín et al. (2002). Optical density (OD) at 405 nm was measured using a Biorad 3550 Microplate reader (Bio-Rad Laboratories). Samples were tested in duplicate including four wells in each plate with healthy citrus extract as negative control, and two-fold dilutions of an extract prepared from a pool of leaves infected with selected CPsV isolates from the Instituto Valenciano de Investigaciones Agrarias (IVIA) collection as standard positive control. This control based on fresh tissue was necessary due to the labile nature of the CPsV CP that causes rapid decay of ELISA readings of freeze-dried or silica-desiccated tissue, or frozen clarified leaf extracts with or without glycerol. The ELISA readings of the negative control ranged from 0·009 to 0·021, and those of the infected samples ranged from 0·101 to 0·932. A standard curve obtained with the positive control was linear between dilutions 1/2 (OD_{450} 1·074) and 1/16 (OD_{450} 0·095) with a correlation coefficient of 0·983. Mean experimental readings at least three times the mean of the negative controls were considered positive. Final ELISA readings were obtained by subtracting the mean value of the negative control from the mean value of positive samples. The adjusted experimental values were expressed as a percentage of the adjusted value obtained for the 1/4 dilution of the positive standard control in the same plate, thus allowing comparison of ELISA readings from different plates. These normalized readings were subjected to a multifactor analysis of variance (ANOVA) and the Tukey's Studentized Range (HSD) test using the statistical software STATGRAPHICS Plus 5.1 (Statistical Graphics Corp.) to compare virus accumulation of different isolates.

Analysis of vRNA and CPsV-sRNA

Total RNA was extracted from 2 g of young leaves using a standard protocol with two phenol:chloroform:isoamyl alcohol extractions followed by precipitation with 6 M LiCl and RNA re-suspension in 50 µL of RNase-free water (Ancillo et al., 2007). RNA concentration was measured in duplicate in a NanoDrop UV-Vis Spectrophotometer (Thermo Scientific) and then adjusted to approximately 1·5 µg RNA µL^{-1}. Aliquots were stored at −20°C until use.

For northern blot hybridization, total RNA (7·5 µg) was heat-denatured for 18 min at 70°C in 50% formamide, chilled on ice, separated by electrophoresis in formamide-formaldehyde denaturing 0·9% agarose gels in 1× MOPS buffer (5 mM NaAc, 1 mM Na-EDTA, 20 mM Morpholinopropansulphonate, pH 7), and then electroblotted to positively-charged nylon membranes (Roche Diagnostics) using phosphate buffer (25 mM Na_2HPO_4, 25 mM NaH_2PO_4, pH 6·5), and UV cross-linked. The digoxigenin (DIG)-labelled RNA molecular weight marker II (Roche Diagnostics) was included.

To measure the relative vRNA accumulation in plants infected with different CPsV isolates or in plants infected with the same isolate and kept at different temperatures, serial dilutions (1 to 1/64) of total RNA extracts (100 ng µL^{-1}) were used for dot blot hybridization. One microlitre of each total RNA dilution was heat-denatured for 5 min at 95°C in 50% formamide, chilled on ice and spotted onto a nylon membrane.

To obtain preparations enriched in sRNAs, total RNA from 1 g of infected tissue was extracted with TRI-Reagent and 1-bromo-3-chloro-propane (Sigma-Aldrich), precipitated with isopropanol and re-suspended in 150 µL of RNase-free water. High-molecular mass RNAs were precipitated with 1 M NaCl and 10% polyethylene glycol (PEG 8000), and the sRNAs were ethanol precipitated and re-suspended in 50 µL of RNase-free water (Yaegashi et al., 2007). RNA concentration was measured in duplicate in a NanoDrop UV-Vis Spectrophotometer, adjusted to approximately 250 ng RNA µL^{-1} and aliquots were stored at −20°C. sRNAs (5 µg) were separated by electrophoresis in 15% polyacrylamide gels containing 7 M urea in 0·5× TBE (50 mM Tris, 45 mM Boric acid, 0·5 mM EDTA), electroblotted to positively-charged nylon membranes using 0·5× TBE and then UV cross-linked. Oligonucleotides (21 and 24 nt) derived from the CPsV regions selected for probes RNA 1P and RNA 3P (see below) were included in the gel as size markers for CPsV-sRNAs.

Northern and dot blot hybridization were performed with DIG-labelled riboprobes specific for the genomic RNA segments between nucleotide positions 4594 and 5053 of the RNA 1 (probe RNA 1P), 1205 and 1546 of the RNA 2 (probe RNA 2P), and 719 and 1318 of the RNA 3 (probe RNA 3P). To clone these segments, cDNA of the CPsV isolate PsA-A2 was obtained by RT-PCR using specific primers (Table 1). The PCR products were electrophoresed in a 2% agarose gel and DNA fragments of the expected size were ligated into the pGEM-T vector (Promega Corp.) and cloned. Plasmid DNA was purified using a commercial kit (Roche Diagnostics) and DNA concentration determined in a NanoDrop UV-Vis Spectrophotometer. The nucleotide sequence of DNA inserts was determined with an ABI PRISM DNA Sequencer

Table 1 Primers used for cDNA synthesis and PCR amplification of DNA fragments used to prepare *Citrus psorosis virus*-specific riboprobes to the three genomic RNAs

Probes	Primer	Polarity	Primer sequence (5′ to 3′)	Positions[a]
RNA 1P	Ps 44	−	GACAGAGATTTAGAAATAGA	4594–4613
	Ps 45	+	TACATCACTATCTAATCCTAT	5053–5035
RNA 2P	Ps 62	−	AAAGATGTTTTCATGTTCTCT	1205–1226
	Ps 63	+	TATTTAAAGCGAAACATGAT	1546–1526
RNA 3P	CPV1	−	GCTTCCTGGAAAAGCTGATG	719–738
	CPV2	+	TCTGTTTTTGTCAACACACTCC	1318–1297

[a]Nucleotide positions of the primers in the viral complementary RNAs 1 (probe RNA 1P), 2 (probe RNA 2P) and 3 (probe RNA 3P). Primers Ps 44, Ps 45, Ps 62 and Ps 63 are based on the sequence of isolate P121 (Martín *et al.*, 2005) and primers CPV1 and CPV2 on the sequence of isolate CPV4 (Barthe *et al.*, 1998).

3100 (Applied Biosystems). The plasmids were linearized by restriction with *Sal*I endonuclease (Promega Corp.) and purified using the Ultra Clean™ 15 DNA purification Kit (MO BIO). DIG-labelled positive-stranded RNA transcripts were synthesized from the cDNA clones by incorporation of DIG-UTP using the T7 RNA polymerase (Roche Diagnostics) according to the manufacturer's instructions. Ten-fold serial dilutions from 1 μg to 1 pg of each plasmid DNA were used to equalize the riboprobes.

The membranes were pre-hybridized in 0·02% sodium dodecyl sulphate (SDS), 50% formamide, 5× SSC (750 mM NaCl, 75 mM sodium citrate, pH 7), 2% blocking reagent (Roche Diagnostics) and 0·1% lauryl sarcosine for 1 h at 42°C. Hybridization with the DIG-riboprobes was carried out in ULTRAhyb™ Hybridization Buffer (Applied Biosystems) overnight at 68°C for vRNA or at 42°C for CPsV-sRNA analysis. Membrane washing (10 min) was done with 2× SSC and 0·1% SDS at room temperature, followed by 0·1× SSC and 0·1% SDS at 65°C (vRNA) or 50°C (CPsV-sRNA). Hybridization was detected with the DIG Nucleic Acid Detection kit and revealed with the chemiluminiscent substrate CPD-Star (Roche Diagnostics) according to the manufacturer's instructions.

Results

Virus derived sRNAs

To assess the ability of CPsV to induce RNA silencing in citrus plants, total RNA and sRNA-rich extracts from a pool of young sweet orange leaves healthy or infected with the CPsV isolate P121 were analysed by northern blot using probes RNA 1P, RNA 2P and RNA 3P, individually or mixed. Hybridization of individual probes with total RNA showed a strong reaction of genomic RNAs 2 and 3 with probes RNA 2P and RNA 3P, respectively, whereas hybridization of RNA 1 with the RNA 1P probe only yielded a faint signal (Fig. 2a). Additional minor bands were sometimes observed with probes RNA 2P and RNA 3P whose size did not correspond to any predictable vRNA. No hybridization signal was observed
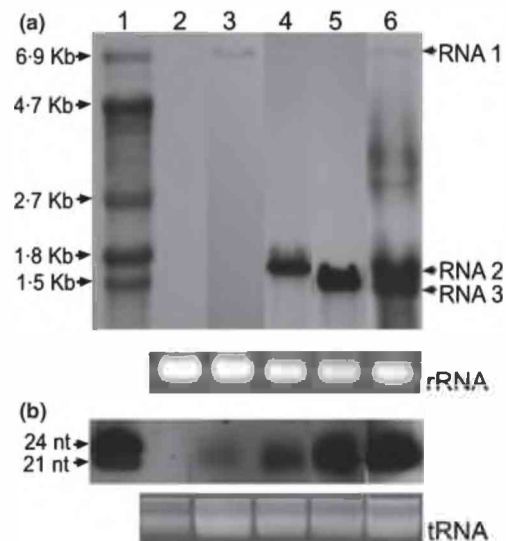

Figure 2 Northern blot analysis of viral RNA (vRNA) (a) and *Citrus psorosis virus*-derived small RNAs (CPsV-sRNA) (b) in Pineapple sweet orange (*Citrus sinensis*) plants healthy (Lane 2) or infected with the CPsV isolate P121 (Lanes 3–6) using equalized DIG-labelled riboprobes specific for RNA 1 (Lane 3), RNA 2 (Lane 4) and RNA 3 (Lane 5), or a mix of the three riboprobes (Lanes 2 and 6). Lane 1: DIG-labelled RNA molecular weight marker II (a) or 21 and 24-nt DNA oligonucleotide markers (b). Ethidium bromide staining of rRNA (a) and tRNA (b) were used as loading control for vRNA and CPsV-sRNA, respectively.

with equivalent extracts from healthy plants. Under the electrophoresis conditions RNAs 2 and 3 were not always clearly separated and a single wide band was detected after hybridization with a mix of the three probes (Fig. 2a). Since the probes were previously equalized these results indicate that the vRNAs 2 and 3 accumulate to a higher level than RNA 1.

The presence of CPsV-sRNAs was examined as an indication of RNA silencing. Northern blot hybridization of sRNA-rich extracts with CPsV-specific riboprobes revealed the accumulation of 21–24 nt CPsV-sRNAs in infected plants, but not in equivalent extracts from healthy plants, suggesting that CPsV induces and is the

target of RNA silencing in citrus plants. Again a stronger reaction was observed with probes RNA 2P and RNA 3P than with probe RNA 1P (Fig. 2b), suggesting that CPsV-sRNA accumulation correlates with the amount of the cognate vRNA.

Symptom intensity and CPsV accumulation

To assess if the intensity of symptoms is related to virus accumulation in infected tissues, both parameters were evaluated in young flush of Pineapple sweet orange plants inoculated with CPsV isolates P121, PsA-A2 and PsB-B1 and kept in a temperature-controlled glasshouse at 26/18°C (day/night). Symptoms induced by the three isolates were different: P121 induced severe shock and necrosis in all shoots of the first flush after inoculation, and intense chlorotic flecking and mottling in young leaves of the following flushes; plants infected with PsA-A2 displayed mild to moderate flecking and mottling in young leaves but only occasionally shock in some new shoots; and plants infected with PsB-B1 showed shock in about half of the shoots of the first flush and intense mottling and flecking in young leaves of surviving shoots. The latter isolate additionally induced chlorotic blotching and gummy pustules in old leaves and twigs, characteristic of psorosis B isolates. After symptom onset a pool of young leaves, from individual inoculated and non-inoculated plants, was analysed for virus accumulation by TAS-ELISA. The highest CPsV accumulation was found in plants inoculated with isolate P-121, followed by those inoculated with PsB-B1 and PsA-A2, with mean normalized ELISA values ($\pm$SE) of 132·7 ± 1·6, 109·3 ± 9·6 and 79·6 ± 2·7, respectively. ANOVA of these values revealed significant differences between isolates (P-value = 0·0004 < 0·05). However, Tukey's Studentized Range (HSD) test to compare mean pairs indicated differences between P121 and PsA-A2 isolates (P-value = 0·0001 < 0·05), but not between PsA-A2 and PsB-B1 (P-value = 0·0876 > 0·05) or between P121 and PsB-B1 isolates (P-value = 0·1672 > 0·05).

Northern blot hybridization of vRNA with the three riboprobes also yielded a more intense signal with P121 and PsB-B1 isolates than with PsA-A2 (Fig. 3a). To confirm these results, a leaf pool from the four plants inoculated with each CPsV isolate was used to prepare serial two-fold dilutions of total RNA ($\sim$100 to 1·56 ng μL^{-1}) that were analysed by dot blot hybridization with a mix of the three riboprobes. Positive hybridization was observed with the 1/32 dilution ($\sim$3·12 ng total RNA μL^{-1}) of the P121 and PsB-B1 extracts, whereas the limit dilution for detection of the PsA-A2 isolate was 1/16 ($\sim$6·25 ng total RNA μL^{-1}). No hybridization was observed with similar extracts from healthy plants (Fig. 4).

Northern blot analysis of the sRNA-rich extracts showed that accumulation of CPsV-sRNAs in the infected plants was also higher for isolates P121 and PsB-B1 than for isolate PsA-A2 (Fig. 3b), suggesting that in
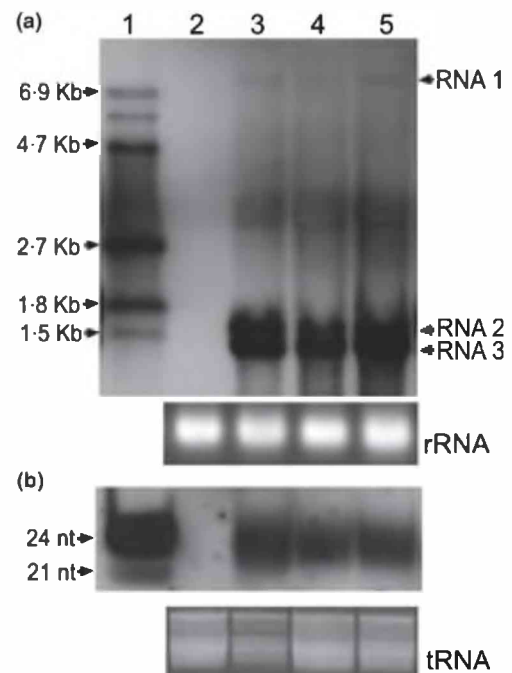

Figure 3 Northern blot analysis of viral RNA (vRNA) (a) and *Citrus psorosis virus*-derived small RNAs (CPsV-sRNA) (b) in Pineapple sweet orange (*Citrus sinensis*) plants healthy (Lane 2) or infected with CPsV isolates P121 (Lane 3), PsA-A2 (Lane 4) or PsB-B1 (Lane 5), using a mix of equalized DIG-labelled riboprobes to the three viral RNAs. Lane 1: DIG-labelled RNA molecular weight marker II (a), or 21 and 24-nt DNA oligonucleotide markers (b). Ethidium bromide-stained rRNA and tRNA were used as loading control for vRNA and CPsV-sRNA, respectively.

plants incubated at 26/18°C the titre of CPsV-sRNAs followed the trend of the vRNAs.

Effect of temperature on symptom intensity and CPsV accumulation

The effects of temperature on symptom intensity and virus accumulation were evaluated in Pineapple sweet orange plants inoculated with CPsV isolates P121, PsA-A2 or PsB-B1, and incubated at 26/18°C or 32/26°C (day/night). For all isolates, symptoms were milder in plants incubated at 32/26°C than in plants incubated at 26/18°C. In particular, the shock reaction with shoot necrosis was not observed with any isolate, except for $\sim$10% of the new shoots in plants infected with P121 and kept at the warmer temperature, which were twisted, as in the first stage of a shock reaction. In plants infected with the other isolates only mild chlorotic flecking and/or mottling was observed.

Since symptom attenuation in plants grown at 32/26°C in comparison with those grown at 26/18°C could be due to reduced virus accumulation, equivalent new shoots were collected from individual plants grown under both temperature regimes and analysed for virus load by TAS-ELISA and by northern blot hybridization
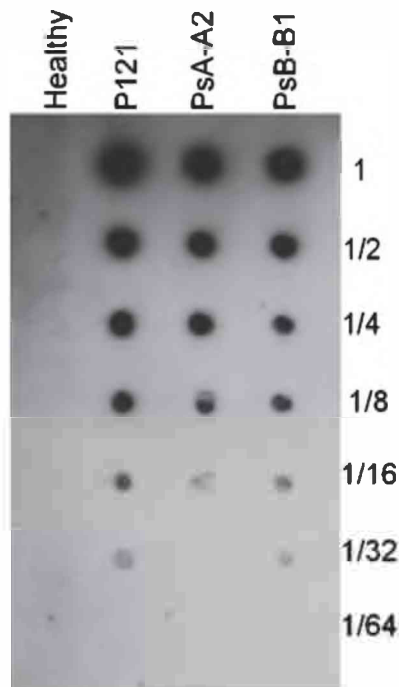
Figure 4 Dot blot hybridization of serial dilutions of total RNA extracts obtained from Pineapple sweet orange (*Citrus sinensis*) plants healthy or infected with different *Citrus psorosis virus* isolates, using a mix of equalized DIG-labelled riboprobes to the three viral RNAs.

Table 2 Virus accumulation in sweet orange (*Citrus sinensis*) plants infected with different *Citrus psorosis virus* isolates and kept at two temperature regimes, as detected by TAS ELISA[a]

Isolate	Temperature (°C)	
	26/18 (day/night)	32/26 (day/night)
P121	122·8 ± 4·4	83·0 ± 5·9
PsA-A2	89·4 ± 10·5	41·3 ± 7·7
PsB-B1	113·0 ± 5·5	49·7 ± 6·1

[a]Samples with ELISA readings more than three times the mean value of the healthy control in the same plate were considered positive and used to calculate the values in the table. ELISA values from healthy plants ranged from 0·009 to 0·021. In each plate the mean value of the healthy control was subtracted from the mean value of each sample and these adjusted values were expressed as a percentage of the adjusted positive control. Each value in the table was the average of eight different plants ± SE.

of vRNA with a mix of the three probes. Sampling was done when new shoots of infected plants grown in the cool glasshouse began twisting, a symptom that precedes the shock reaction. ELISA readings (Table 2) were significantly higher (P-value = 0·0001 < 0·05) in plants incubated at 26/18°C than in those kept at 32/26°C. The interaction temperature × isolate was not significant (P-value = 0·2451 > 0·05), indicating that the effect of temperature was similar for the three isolates. Northern
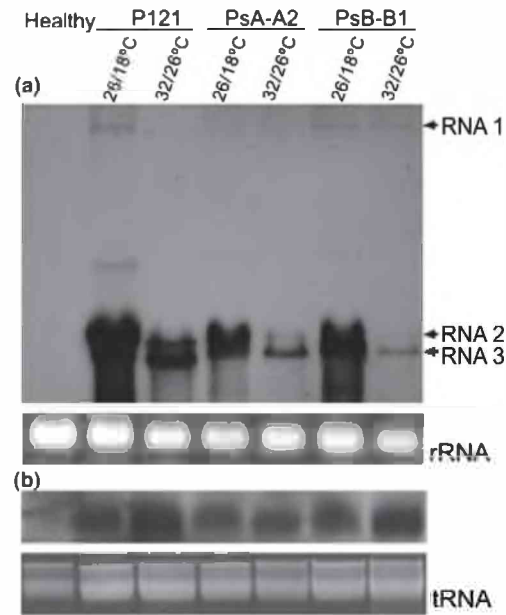

Figure 5 Northern blot analysis of viral RNA (vRNA) (a) and *Citrus psorosis virus*-derived small RNAs (CPsV-sRNA) (b) in Pineapple sweet orange (*Citrus sinensis*) plants healthy or infected with CPsV isolates P121, PsA-A2 or PsB-B1 and incubated at 26/18°C or 32/26°C (day/night), using a mix of equalized DIG-labelled riboprobes to the three viral RNAs. Ethidium bromide stained rRNA and tRNA were used as loading control for vRNA and CPsV-sRNA, respectively.

blot hybridization also showed that vRNA accumulation in plants infected with each isolate was lower at the warmer temperature (Fig. 5a). To confirm the above results, leaves from the eight plants inoculated with each CPsV isolate under each temperature regime were pooled and serial two-fold dilutions (1 to 1/64) of total RNA extracts from each pool (100 ng μL^{-1}) were analysed by dot blot hybridization using a mix of the three riboprobes. For the three isolates the dilution end point to observe a positive hybridization signal was more than two fold higher in plants kept at 26/18°C than in plants kept at 32/26°C (Fig. 6). No hybridization was observed in similar extracts from healthy plants.

Finally, CPsV-sRNA accumulation was examined by northern blot hybridization using sRNA-rich extracts from the same shoots and the same riboprobes. In contrast to vRNA, accumulation of CPsV-sRNA was slightly higher for each isolate at 32/26°C than at 26/18°C, and therefore the ratio between CPsV-sRNA and vRNA was clearly higher at the warmer temperature (Fig. 5b). These findings suggest that CPsV-induced RNA silencing is temperature-dependent.

Discussion

The effect of temperature on symptom expression and virus accumulation of three CPsV isolates inoculated in Pineapple sweet orange plants was examined. Consistent
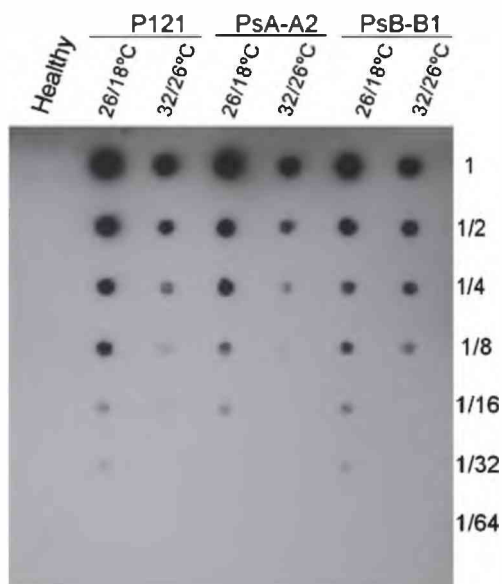
Figure 6 Dot blot hybridization of serial dilutions of total RNA from Pineapple sweet orange (*Citrus sinensis*) plants healthy or infected with *Citrus psorosis virus* isolates P121, PsA-A2 or PsB-B1 incubated at 26/18°C or 32/26°C (day/night), using a mix of equalized DIG-labelled riboprobes to the three viral RNAs.

with previous results (Roistacher & Calavan, 1974), it was found that cool temperature favours the appearance of a shock reaction in young shoots and intense chlorotic flecks and spots in young leaves, whereas warm temperature tends to inhibit shock and mask leaf symptoms. The attenuated symptoms were also found to be associated with reduced virus accumulation, consistent with the observation that virus concentration was higher in plants infected with isolates inducing more intense symptoms. These results suggest that expression of CPsV symptoms is associated with virus accumulation and that temperature plays a role in regulating the virus load.

Reduced virus accumulation at 32/26°C could result from slow virus replication due to loss of replicase activity, or from increased RNA breakdown due to temperature. However, it is unlikely that these factors play a major role in low CPsV accumulation at 32/26°C. Previous experiments with a tobamovirus and a bromovirus showed that viral replicase activity was lost after prolonged incubation at 40°C but not at 35°C (Dawson, 1976, 1978; Dawson *et al.*, 1978). These authors observed that immediately after a set-up shift from 25 to 40°C, ssRNA synthesis was drastically reduced, but synthesis of dsRNA replicative forms or intermediates was little affected until ssRNA synthesis had stopped. The higher ratio of dsRNA after temperature increase could trigger PTGS. Furthermore, RNA synthesized at 25°C broke down when incubated at 40°C. Assuming that thermal stability of the replicase and the ssRNA of this negative-stranded virus were similar to those of positive-stranded viruses, reduced CPsV accumulation in plants

incubated at 32/26°C might be due to temperature-induced enhancement of the RNA silencing pathway (Kalantidis *et al.*, 2002; Szittya *et al.*, 2003).

While incubation at 32/26°C caused a substantial reduction in the amount of vRNA in comparison with plants incubated at 26/18°C, accumulation of 21–24-nt CPsV-sRNAs in the former condition was only slightly higher than in the latter. The presence of siRNAs in infected tissues is considered a hallmark of the antiviral defence of the plant (Kalantidis *et al.*, 2002; Szittya *et al.*, 2003). However, the abundance of siRNAs in the host may not be a reliable indicator of the intensity of RNA silencing. Thus virus-specific siRNAs are more abundant in the presence than in the absence of functional silencing suppressors (Voinnet *et al.*, 2000; Qu & Morris, 2002; Szittya *et al.*, 2002), but suppressor-deficient viral mutants accumulate less viral RNA, suggesting that the amount of virus-specific siRNAs may be related to viral RNA accumulation in the infected cells. Since CPsV-infected plants grown at 32/26°C had reduced levels of vRNA but slightly increased CPsV-sRNA accumulation, it is suggested that decrease in virus accumulation may be due in part to temperature-induced enhancement of RNA silencing. Inhibited RNA silencing and high susceptibility to *Cymbium ringspot virus* was observed at low temperature, whereas raising temperature activated RNA silencing and increased siRNA accumulation (Szittya *et al.*, 2003). Similarly, RNA silencing of five distinct cassava geminiviruses in *Nicotiana benthamiana* and cassava (*Manihot esculenta*) was enhanced at higher temperatures (Chellappan *et al.*, 2005). Temperature increase might alter or inhibit activity of virus-encoded RNA silencing suppressors. Thus activity of the *Potato virus Y* silencing suppressor HCPro expressed in transgenic tobacco plants on infection by several viruses was different at 25°C and at 33°C (Shams-Bakhsh *et al.*, 2007). Similarly, *Tobacco ringspot virus* systemically infected *N. tabacum* plants at 25°C but at 29°C infection was limited to the inoculated leaves, with the effect of several viral silencing suppressors being different at both temperatures (Siddiqui *et al.*, 2008).

Symptom attenuation induced by high temperature was less important in plants inoculated with CPsV isolate P121 than in those inoculated with isolates PsA-A2 or PsB-B1. Thus although shoot necrosis was not observed at 32/26°C in plants inoculated with P121, some shoots of the first flush were twisted as in the first stage of the shock reaction and young leaves showed moderate chlorotic leaf flecking and mottling. Navas-Castillo & Moreno (1993) also found that the effect of temperature on psorosis symptom expression depends on the host-isolate combination.

The finding that CPsV likely induces and is the target of RNA silencing in infected citrus plants, and that this reaction may be enhanced by high temperature, helps to explain biological observations on psorosis disease that were empirically used for diagnosis and control procedures. Psorosis has been diagnosed for years by biological indexing on sensitive indicator plants, usually sweet

orange seedlings grown in a temperature-controlled (26/18°C) glasshouse (Roistacher, 1993). Since chlorotic leaf flecking and spotting in young leaves are also induced by other graft-transmissible diseases, specific diagnosis of psorosis additionally required a cross protection test using psorosis B (Wallace, 1957; Roistacher, 1993; Martin *et al.*, 2002, 2004). In this test, healthy sweet orange seedlings inoculated with psorosis B show the characteristic psorosis B symptoms, whereas plants previously infected with a candidate psorosis A isolate are protected and do not develop psorosis B symptoms. In line with the accepted model of PTGS as the most likely mechanism to explain cross protection between closely related virus strains (Ratcliff *et al.*, 1999; Baulcombe, 2004), the finding that CPsV may be targeted for silencing in citrus plants leads to the hypothesis that CPsV-sRNAs derived from the candidate psorosis A isolate could prevent subsequent invasion by psorosis B isolate by sequence-specific degradation of its vRNA.

Increased silencing and reduced virus accumulation at high temperature is likely to be behind the recovery of CPsV-free buds from infected citrus plants by heat therapy (Calavan *et al.*, 1972). It may also explain the increased ratio of CPsV-free shoot-tip grafted plants obtained when shoot tips are taken from infected plants previously defoliated and forced to produce new flush in a warm glasshouse or in a growth chamber at 32°C (Navarro, 1993). Finally, CPsV-induced RNA silencing might be used to induce pathogen-derived resistance in transgenic citrus plants (Zanek *et al.*, 2008) in areas where natural dispersion of CPsV has been observed (Timmer & Garnsey, 1980; Beñatena & Portillo, 1984).

Acknowledgements

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