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GENETIC TRANSFORMATION AND HYBRIDIZATION

Transgenic resistance to *Citrus tristeza virus* in grapefruit

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Abstract Grapefruit (*Citrus paradisi*) transgenic plants transformed with a variety of constructs derived from the *Citrus tristeza virus* (CTV) genome were tested for their resistance to the virus. Most transgenic lines were susceptible (27 lines), a few were partially resistant (6 lines) and only one line, transformed with the 3′ end of CTV was resistant. Transgene expression levels and siRNA accumulation were determined to identify whether the resistance observed was RNA-mediated. The responses were varied. At least one resistant plant from a partially resistant line showed no steady-state transgene mRNA, siRNA accumulation and no viral RNA, implicating post-transcriptional gene silencing (PTGS) as the mechanism of resistance. The most resistant line showed no transgene mRNA accumulation and promoter methylation of cytosines in all contexts, the hallmark of RNA-directed DNA methylation and transcriptional gene silencing (TGS). The variety of responses, even among clonally propagated plants, is unexplained but is not unique to citrus. The genetics of CTV, host response or other factors may be responsible for this variability.

Keywords *Citrus tristeza virus* · DNA methylation · Plant resistance · RNA interference · RNA silencing · Transformation

Abbreviations

CP	Capsid protein
CPm	Minor capsid protein
CTV	*Citrus tristeza virus*
CU	*Cucumber mosaic virus* (CMV) 5′ untranslated region
DASI-ELISA	Double antibody sandwich indirect-enzyme linked immunosorbent assay
GUS	*β*-Glucoronidase
NptII	Neomycin phosphotransferase II
PTGS	Post-transcriptional gene silencing
RdDM	RNA-mediated DNA methylation
RdRp	RNA directed RNA polymerase
RNAi	RNA interference
SiRNA	Small interfering RNA
TGS	Transcriptional gene silencing
UTR	Untranslated region

Introduction

Commercial citrus, wherever it is grown, is plagued by numerous pathogens. Among those pathogens, *Citrus tristeza virus* (CTV) is the most economically important virus affecting citrus (Bar-Joseph et al. 1989). The production of citrus varieties that are genetically resistant to CTV is the

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most desirable long-term solution to the problems caused by this virus. This is not simple to do using conventional breeding, given the reproductive biology of the genus (i.e., polyembryony, self- and cross-incompatibility and long juvenile periods) and the lack of resistance genes in commercially acceptable citrus types. Therefore, one strategy to overcome these limitations is to engineer citrus plants for pathogen resistance via genetic transformation techniques.

CTV is a member of the genus *Closterovirus* of the *Closteroviridae*, and has a positive-sense single-stranded RNA genome of approximately 20 kb. Different strains of the virus can cause diverse disease syndromes that vary from mild, with weak, scattered vein-clearing; to decline and death of scions grafted on sour orange; to stem pitting of scions (usually grapefruit and sweet orange cultivars) regardless of the rootstock resulting in reduced fruit size and quality. Virions are long flexuous particles encapsidated by two capsid proteins (CP), a major CP of 25 kDa and a minor CP of 27 kDa (CPm) that encapsidates about 5% of the terminal region of the particle (Febres et al. 1996). Including these two genes, the genomic RNA encodes 12 genes (Karasev et al. 1995; Pappu et al. 1994). The virus RNA-dependent RNA polymerase (RdRp) is encoded by gene 1b, in the 5′ half of the genome, and is expressed by a +1 frame shift (Cevik 2001; Karasev et al. 1995). The 3′ half of the genome contains three suppressors of RNA silencing: the major CP, a 20-kDa protein (p20) and a 23-kDa protein (p23, the most 3′ gene of CTV) (Lu et al. 2004).

The 5′ and 3′ untranslated regions (UTR) of the Florida severe strain T36 are 107 and 275 nucleotides long, respectively (Karasev et al. 1995; Pappu et al. 1994) and are necessary for virus replication (Mawassi et al. 2000; Satyanarayana et al. 2002). The 3′ UTR is the most conserved part of the CTV genome, with >95% nucleotide identity (Lopez et al. 1998; Mawassi et al. 1996; Ruiz-Ruiz et al. 2006; Vives et al. 1999). While the sequence divergence of some isolates is relatively uniform throughout the genome, the divergence of other isolates progressively increases toward the 5′ end of the genome to as little as 40% nucleotide identity within the 5′ UTR (Ayllon et al. 2001; Mawassi et al. 1996; Ruiz-Ruiz et al. 2006; Vives et al. 1999; Yang et al. 1999).

Several attempts have been made to produce transgenic CTV-resistant plants using viral sequences, including our own efforts (Batuman et al. 2006; Dominguez et al. 2002a; Febres et al. 2003; Ghorbel et al. 2000; Gutierrez et al. 1997; Herron et al. 2002). Most of these attempts, using a wide diversity of viral constructs, have been unsuccessful. Recently it was shown that in transgenic Mexican lime [*Citrus aurantifolia* (Christ.) Swingle] plants the overexpression of either the CP or p23 gene was capable of conferring resistance (Dominguez et al. 2002a; Fagoaga et al. 2006). However, this resistance was partial (in that only a portion of the plants derived from a particular transgenic line were resistant). Further, few of the transgenic lines expressing p23 showed resistance to CTV. Instead, they spontaneously developed virus-like symptoms.

Virus resistance conferred by a transgene with sequence homology to the virus is, in many cases, RNA-mediated (Lindbo et al. 1993; Ratcliff et al. 1997; Waterhouse et al. 1998). This RNA-mediated resistance, first denoted as gene silencing, is also termed RNA interference, or RNAi. Silencing is initiated in plants by dsRNA that is processed into small interfering RNA (siRNA) of about 21–26 nt. These siRNAs subsequently mediate RNA degradation of complementary sequences in a process known as post-transcriptional gene silencing (PTGS). SiRNAs can also direct methylation of complementary DNA sequences (Mette et al. 2000; Wassenegger et al. 1994). Methylation of the transcribed sequence is associated with PTGS. Methylation of promoter sequences inhibits transcription and is termed transcriptional gene silencing (TGS). RNAi has been proposed to be a natural plant defense mechanism against viruses and transposons and is also involved in the regulation of the expression levels of certain genes (Voinnet 2002; Waterhouse et al. 2001).

In the present study, we describe Duncan grapefruit (*C. paradisi* Macfad) lines transformed with sequences derived from CTV that show various degrees of resistance to the virus. This resistance is stable and is RNA-mediated. Interestingly, TGS was associated with the most resistant line.

Materials and methods

Production of transgenic grapefruit lines

The plasmid vectors and the transformation and regeneration protocol used in this work to produce the transgenic Duncan grapefruit lines have been described previously (Febres et al. 2003). Briefly, the CP constructs contained either the major CP gene from stem pitting-inducing isolate B249 from Venezuela (CP B249), the major CP from mild isolate T30 from Florida (CP T30), the non-translatable version of the major CP from quick decline isolate T36 from Florida (NTCP) or the minor CP (p27) from isolate T36 (CPm). The 3END construct contained the 400 3′-terminal bases, including part of the p23 gene and the 3′ UTR from Florida severe CTV isolate DPI 3800 and the RdRp construct contained the full length gene 1b (with an ATG added at the 5′ end as translation initiation codon) from isolate T36. Lines regenerated from these experiments were tested for GUS activity and presence of the gene of interest by PCR and subsequently established in soil. The transgenic status of each line was determined using Southern analysis (see next). Independent lines confirmed as trasngenic

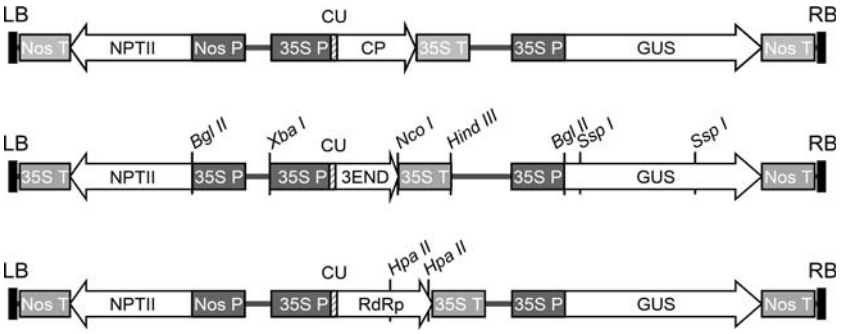

Fig. 1 Schematic representation of the T-DNA region of the binary vectors used for the transformation of grapefruit. *Arrows* indicate the transgene and its orientation. *Boxes* indicate the promoter (*dark gray*) and the termination signals (*light gray*) for each gene. *LB* T-DNA left border; *RB* T-DNA right border; *CP* is either CP B249 (major CP gene from stem pitting-inducing isolate B249 from Venezuela), *CP T30* (major CP from mild isolate T30 from Florida), *NTCP* (non-translatable version of the major CP from quick decline isolate T36 from Florida) or *CPm* (minor CP, p27, from isolate T36); *3END* 400 3′ terminal bases of the CTV genome from the stem pitting isolate DPI3800 from Florida; *RdRp* CTV RNA-dependent RNA polymerase (modified to include a start codon) from isolate T36; *NPTII* neomycin phosphotransferase II; *GUS* β-glucuronidase; *35S P* CaMV 35 S promoter; *CU* CMV 5′ untranslated region (*hatched box*); *35S T* CaMV 35S terminator; *Nos T* nopaline synthase terminator; *Nos P* nopaline synthase promoter. The position of the relevant restriction enzymes is also indicated (*italics*)

(a total of 35) were propagated by grafting and used in the virus challenge experiments described below.

Southern analysis

DNA for Southern analysis was extracted from young, tender leaves of putatively transgenic plants using DNAzol (Molecular Research Center, Cincinnati, OH). Approximately 0.5–1 g of tissue was pulverized in liquid nitrogen and treated according to the manufacturer's instructions. Ten micrograms of DNA were digested overnight with *Ssp*I to cleave a T-DNA site located in the GUS gene, downstream of 3END in the pCAMBIA 2201 vector (Fig. 1). The digested DNA was separated on a 0.8% agarose gel and blotted to a positively charged nylon membrane (Roche, Indianapolis, IN). A 3END probe (full-length gene) was labeled by PCR using DIG-dUTP (Roche) and primers VF9 (ATAGAGCTCCATGGGCCCGTAGGACTGCTAAAGC ATTGTTACCG) and VF10 (ATAGGGCCCATGGAGCT CTGGACCTATGTTGGCCCCCCATG). Plasmid DNA was used as the template in a standard reaction that contained dNTPs at the following final concentrations: 0.2 mM each of dATP, dCTP and dGTP, 0.15 mM dTTP and 0.05 mM DIG-dUTP in a 50-µl PCR reaction. The mixture was subjected to one incubation cycle at 94°C for 2 min; 30 cycles at 94°C for 30 s, 65°C for 30 s and 72°C for 30 s; followed by one last cycle at 72°C for 5 min. The membranes were prehybridized for 1 h with DIG easy hybridization solution (Roche) at 42°C and hybridized overnight with the labeled probe under the same conditions. After probing, the membranes were washed twice each in solutions of 2× SSC, 0.1% SDS at room temperature for 5 min and 0.5× SSC, 0.1% SDS at 65°C for 15 min. The bands on the membranes were visualized using chemiluminescent detection with CSPD (Roche) following the manufacturer's instructions. Hybridization with RdRp and CP probes was previously described (Febres et al. 2003).

Virus challenge

Buds from each of 35 independent and verified transgenic lines (GUS-, PCR- and Southern blot-positive) were grafted on Swingle citrumelo [*Poncirus trifoliata* (L.) Raf., × *C. paradisi* Macfad.]. Each transgenic line was budded in four replicates. After the grafts had taken, the plants were challenged with CTV-severe isolate T66-E (Tsai et al. 2000) by grafting four infected bark segments ('blind' buds) from Mexican lime on the transgenic (or non-transgenic control) scion. The biological characteristics of the CTV isolates used in this study are shown in Table S1. The sequence identity of the CP and 3′END regions, for which information is available, is shown in Tables S2 and S3. The source tissue was confirmed as CTV-infected by ELISA. The grafts were checked for survival after 2 and 4 weeks and re-grafted if the blind bud had died. At the end of a 6-week period at least three to four blind buds were alive on each scion. After 6, 12 and 24 months, the transgenic grapefruit scions were tested by DASI-ELISA (Garnsey and Cambra 1991) using the polyclonal antibody CREC 35 for coating and the monoclonal antibody MCA13 as the secondary antibody. Each sample was tested in duplicate. Bark tissue was used for these assays (CTV is phloem-limited) collected from five different areas of each plant (CTV can have an uneven distribution). The bark was pealed from all five-stem segments, chopped finely as a composite and 0.2 g used for the ELISA assay.

RT-PCR analysis

The plants were tested by RT-PCR for CTV infection and expression of transgenes 36 months after inoculation. Total RNA was extracted from the transgenic plants using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Three micrograms of RNA were treated with 1 U of Turbo DNAase (Ambion, Austin, TX) in a final reaction volume of 10 µl following the manufacturer's instructions. After inactivation of the DNase, 1 µg of the treated RNA was used in a reverse transcription (RT) reaction with 100 U of MMLV-RT, 10 U of RNase inhibitor, 5 µM random decameres, and 0.5 mM dNTPs (all components from Ambion) in a final volume of 20 µl and incubated at 42°C for 1 h. The cDNA generated was used in detecting CTV and transgene expression. For the detection of CTV, 1 µl of the above cDNA was used in a PCR reaction with CTV-specific primers VF259 (GCGTTGGATGATATCCTTCGCTGG) and VF261 (AATTATTCCGCCCAGGACGGAACA) that amplified a 500 bp product from the CTV gene 1a and 1.2 µl of 5 µM 18S universal primer pair and 2.8 µl of 5 µM 18S universal competimers (Ambion) that amplified a 315 bp product in a final volume of 50 µl. The inclusion of the 18S primers allowed the use of ribosomal RNA as a control in the amplification reactions, for both integrity of the RNA and efficiency of the RT-PCR. The reactions were subjected to incubations at 94°C for 2 min; and 35 cycles at 94°C for 30 s, 60°C for 30 s, 72°C for 30 s.

To detect the CTV transgenes' mRNA steady-state levels, we also used RT-PCR from the same cDNA samples described above using primers located in the CU region and the 35S terminator (Fig. 1): VF268 (TGTGGCGTAGA ATTGAGTCGAGTC) and VF270 (CACACATTATTAT AGAGAGAGATAGAT), respectively. The 3END samples were amplified by incubation at 94°C for 2 min; 40 cycles at 94°C for 30 s, 60°C for 30 s, 72°C for 45 s and a final incubation at 72°C for 2 min. The RdRp samples were amplified by incubation at 94°C for 2 min; 40 cycles at 94°C for 30 s, 60°C for 30 s, 72°C for 90 s and a final incubation at 72°C for 2 min.

The expression of GUS and NptII was also assayed using RT-PCR. For GUS specific primers GUS Fw (CAACGAA CTGAACTGGCAG) and GUS Rv (CATCACCACGCTT GGGTG) were used with the following conditions: 94°C for 2 min; 40 cycles at 94°C for 30 s, 50°C for 30 s, 72°C for 50 s and a final incubation at 72°C for 2 min. For NptII primers NPTII-1801 (TCACTGAAGCGGGAAGGGACT) and NPTII 2101 (CATCGCCATGGGTCACGACGA) were used under the following conditions: 94°C for 2 min; 40 cycles at 94°C for 30 s, 55°C for 30 s, 72°C for 30 s and a final incubation at 72°C for 2 min.

Detection of siRNAs

Total RNA (10 µg) extracted with the TRIzol procedure described above was precipitated in 0.3 M sodium acetate pH 5.5 and two volumes of ethanol and resuspended in formamide. The samples were denatured (80°C for 5 min) and the siRNAs separated by electrophoresis in 15% polyacrylamide, 7 M urea, 1× TBE gels (Bio-Rad, Hercules, CA), electrotransferred to a positively charged nylon membrane (Roche) and UV cross-linked. Probes for detection were prepared by in vitro transcription using the DIG RNA labeling kit (Roche) following the manufacturer's instructions. The templates used for the transcription reactions were PCR products amplified from plasmid DNA using the following primers that incorporated T7 promoters (lowercase) to both DNA strands: VF247 (taatacgactcactat agggagaGTAGGACTGCTAAAGCATTGTTACCG) and VF248 (taatacgactcactatagggagaTGGACCTATGTTGGCC CCCCATG) for the 3END sequence and VF257 (taata cgactcactatagggagaGTAGTAAGGTCACAAGCAATTCC TCC) and VF258 (taatacgactcactatagggagaGTCGTCGTCA CCAATGATTCTTCTG) for the RdRp (the fragment amplified corresponds to the first 550 bp of this gene). As a result, the probes used were DIG-labeled, intact, double stranded RNA. The membranes were pre-hybridized for 1 h at 40°C with DIG easy hybridization solution (Roche) and subsequently hybridized overnight with the labeled probe under the same conditions. The membranes were washed twice with 2× SSC, 0.2% SDS at 50°C and detected using chemiluminescence with CSPD (Roche).

Bisulfite DNA sequencing

Genomic DNA (1 µg) was first digested with *Xba*I, *Nco*I and *Hin*dIII to release the transgenic insert, purified using Wizard clean up system (Promega, Madison, WI) and subsequently treated with sodium bisulfite, promoting the conversion of unmethylated cytosine to uracil. Bisulfite treatment was performed as described (Paulin et al. 1998). The treated DNA (100 ng) was used in a standard PCR reaction with primers VF278 (AAGATAGTGGAAAAG GAAGGTG) and VF279 (CACAGCACACACACTCTCT AT) which amplified a 310 bp region of the 35S promoter and the CMV 5′ UTR (present only in the 3END and RdRp transgenes and not in the NptII or GUS transgenes). The amplification conditions were: an initial incubation cycle at 94°C for 3 min, followed by 35 cycles at 94°C for 30 s, 55°C for 30 s and 72°C for 30 s, and a final incubation at 72°C for 10 min. Subsequently, the amplified products were cloned into pGEM-T vectors and sequenced.

Results

Transgenic plants and virus challenge

Previously we described the production of grapefruit lines transformed with several CTV sequences (Febres et al. 2003). We used a variety of constructs derived from the CP of mild and severe isolates as translatable and untranslatable genes, the CPm, the 400 terminal bases of the 3′ end (3END) in both sense and anti-sense orientations and a full-length translatable version of the RdRp (Fig. 1). The initial challenge assay used CTV isolate T36 and only lines transgenic with CP constructs or the RdRp. None of the plants were identified as resistant (Febres et al. 2003). Subsequently, we have tested an additional 22 independent lines for their resistance to CTV and re-tested 13 other lines with isolate T66-E. In this report we present the most representative results from this study. All of the transgenic plants analyzed have maintained a normal phenotype throughout the 3-year experimental period. The challenged lines were assayed using Southern blot to determine presence and transgene copy number. Lines 525, 595 and 598 had one copy of the transgene, while line 538 had two copies (data not shown). Untransformed plants (WT) showed no hybridization. Transgene copy numbers in the RdRp lines were previously reported (Febres et al. 2003), and varied from one to three in the lines analyzed here. In particular, line 128 had three copies and line 146 had two copies of the transgene. Four replicates from each analyzed transgenic line were propagated by grafting onto Swingle citrumelo rootstock. Once the grafted plants were established, we challenged them by grafting infected 'blind' buds into the scion with tissue confirmed as CTV-infected. The plants were evaluated for CTV infection using ELISA 6, 12 and 24 months after inoculation and using RT-PCR 36 months after inoculation. Bark tissue from different areas of each plant was combined for these assays since CTV is phloem-limited and can have an uneven distribution in the plant. Most of the transgenic lines displayed replication of CTV by 12 months after inoculation (Table 1). However, in some cases one or two of the replicates of a particular line was consistently resistant, and one transgenic 3END line consistently showed full resistance. To corroborate the efficacy of our inoculation method we tested five non-transgenic Duncan grapefruit plants on Swingle rootstock (Table 1) and 12 additional grapefruit trees (data not shown) all of which were ELISA positive 6 months after inoculation. We selected a smaller number of lines representing the varied responses observed to analyze and compare in more detail. The ELISA results showed three types of response (Fig. 2): (1) susceptible lines (146, 525 and 598), in which all plants were infected;

Table 1 Evaluation of infection with CTV isolate T66-E in grapefruit transgenic plants

Construct	Line	Virus detected/undetected[a]		
		6 months	12 months	24 months
CP B249	159[b]	4/0	4/0	4/0
	160[b]	4/0	4/0	4/0
	165[b]	1/2	3/0	3/0
	169[b]	4/0	3/1	3/1
	182	0/2	1/1	2/0
	185	3/1	3/1	3/1
CP T30	155[b]	1/3	4/0	4/0
	161[b]	4/0	4/0	4/0
	166	4/1[c]	4/0	4/0
	212	4/0	4/0	4/0
	214	NA	4/0	4/0
NTCP	393	3/1	4/0	NA
	432	3/1	3/1	3/0
	465	3/1	4/0	4/0
	488	4/0	4/0	3/0
	493	4/0	4/0	4/0
	505	2/2	2/2	2/2
CPm	263	1/1	2/0	2/0
3END-S	451	4/0	4/0	3/0
	523	3/0	3/0	2/0
	525	4/0	4/0	4/0
	530	2/0	2/0	1/0
	538	3/1	3/1	3/1
	595	0/4	0/4	0/4
	598	4/0	3/0	3/0
3END-AS	454	NA	4/0	4/0
	475	NA	3/0	3/0
	501	NA	4/0	4/0
RdRp	81[b]	4/0	4/0	4/0
	91[b]	4/0	4/0	4/0
	97[b]	4/0	4/0	4/0
	128[b]	NA	3/1	3/1
	146[b]	2/2	2/2	4/0
	153[b]	3/1	4/0	4/0
	453[b]	2/3	4/0	4/0
Control	344	5/0	4/0	4/0

CP B249 major CP gene from isolate B249; *CP T30* major CP from isolate T30; *NTCP* non-translatable version of the major CP from isolate T36; *CPm* minor CP (p27) from isolate T36; *3END-S* 3′ end (400 3′-terminal bases, including part of the p23 gene and the 3′ UTR) in sense orientation from isolate DPI 3800; *3END-AS* 3′ end (400 3′-terminal bases, including part of the p23 gene and the 3′ UTR) in antisense orientation from isolate DPI 3800; *RdRp* RNA-dependent RNA polymerase gene from isolate T36; *control* transgenic line with an empty plasmid; *NA* data not available

[a] Number of CTV challenged plants in which the virus was detected/undetected using DASI-ELISA

[b] Lines previously tested (Febres et al. 2003) using isolate T36

[c] Variations in the total number of plants between the evaluation periods are due to losses by unrelated causes

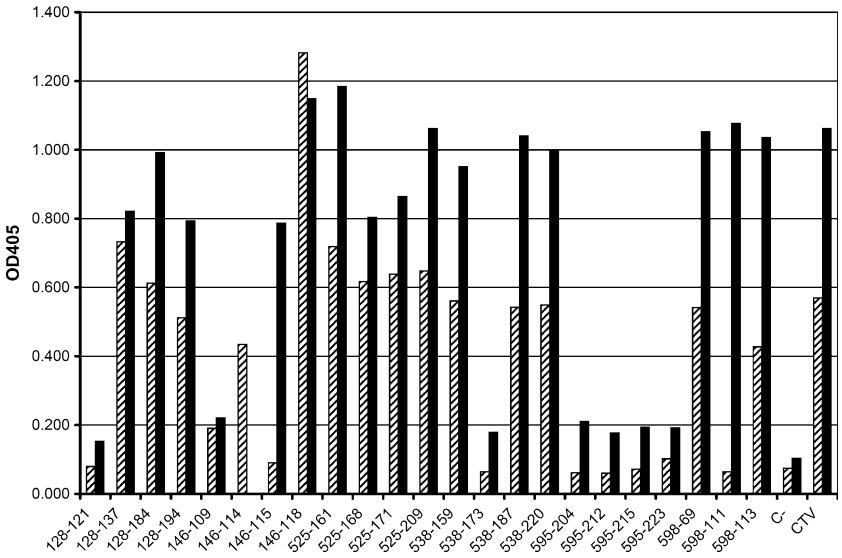

Fig. 2 DASI-ELISA evaluation of CTV infection in grapefruit transgenic plants over a 2-year period. *Hatched bars* 12 months, *solid bars* 24 months. The *numbers below* indicate the plant line-individual plant number. The constructs were: 128 and 146, RdRp; 525, 538, 595 and 598, 3END in sense orientation. *C–* is non-inoculated wild type (negative control); *CTV* is inoculated wild type (positive control). A plant was considered infected when the OD 405 value was at least twice that of C–

(2) partially resistant (128 and 538), in which some of the plants were infected and others remained uninfected; and resistant (595), in which all tested plants remained uninfected.

Resistance, transgene expression and siRNA accumulation

Three years after inoculation we simultaneously evaluated the lines for CTV infection and transgene expression by RT-PCR as well as siRNA accumulation using northern blots to test for any relation among these parameters. Total RNA was extracted from each plant and incorporated into an RT reaction to produce cDNA that was subsequently used in detecting CTV infection. We employed PCR amplification with primers specific to the CTV gene 1a. This target gene was not part of any of the transgenes and allowed using one set of primers to detect infection in all samples while precluding the amplification of any transgenic mRNA. For the 3END transgenic plants (Fig. 3a) lines 525 and 598 were considered susceptible (CTV infection was detected in all of the plants), line 538 was partially resistant (CTV was undetected in plant 173 but detected in 187 and 220) and line 595 was resistant (CTV was not detected in any of the plants). These results were consistent with the ELISA results (Fig. 2). In order to allow quantitative comparison, the same cDNA was used to determine the steady-state level of the 3END transgene mRNA (Fig. 3b). We chose RT-PCR instead of northern blot to specifically detect the transgene mRNA and not any of the homologous CTV RNAs. The forward primer used was located in the CU region (Fig. 1) which is present exclusively in the CTV transgene and not in the GUS or NptII transgenes. The reverse primer was located in the 35S terminator. Our results showed that the most resistant line, 595, had undetectable levels of transgene mRNA, whereas the partially resistant line 538 and susceptible line 598 had high mRNA accumulation. Susceptible line 525 had low mRNA accumulation. PCR of the RNA without RT did not produce any bands (data not shown), indicating that the products did not derive from genomic DNA. Subsequently we determined that siRNAs (Fig. 3c) accumulated to various levels only in the CTV infected plants (161, 168, 171, 187, 220, 69, 111, 113 and more abundantly in CTV) but not in the uninfected plants (173, 204, 212, 215, 223 and C–). The origin of the siRNAs (transgene or virus) was not further investigated as they could have formed from the degradation of either one. For instance abundant siRNA presence in infected wild type plants (CTV) suggests that virus degradation contributed at least in some cases.

For the RdRp transgenic lines the CTV RT-PCR (Fig. 3d) showed that line 128 was partially resistant (only plant 121 was resistant) and line 146 was susceptible, also in agreement with the ELISA results (Fig. 2). Steady-state levels of the RdRp transgenic mRNA (Fig. 3e) showed that two susceptible plants (137 and 184) accumulated the transgene while the resistant plant (121) and the susceptible plants 194, 109, 114, 115 and 118 showed no accumulation of the transgene mRNA. Again, PCR amplification from the RNA without an RT step did not produce any bands (data not shown). Susceptible plant 109 showed a smaller than expected product (~500 bp instead of ~1,700 bp). However, amplification of the transgene from genomic DNA using the same set of primers, produced only the expected ~1,700 bp product (data not shown), suggesting that the transgene was full-length. We did not investigate the origin of this unexpected band any further. SiRNAs (Fig. 3f) accumulated to higher levels in the susceptible 137, 184, 194, 115, 118 and wild type (CTV) plants. Very low, almost

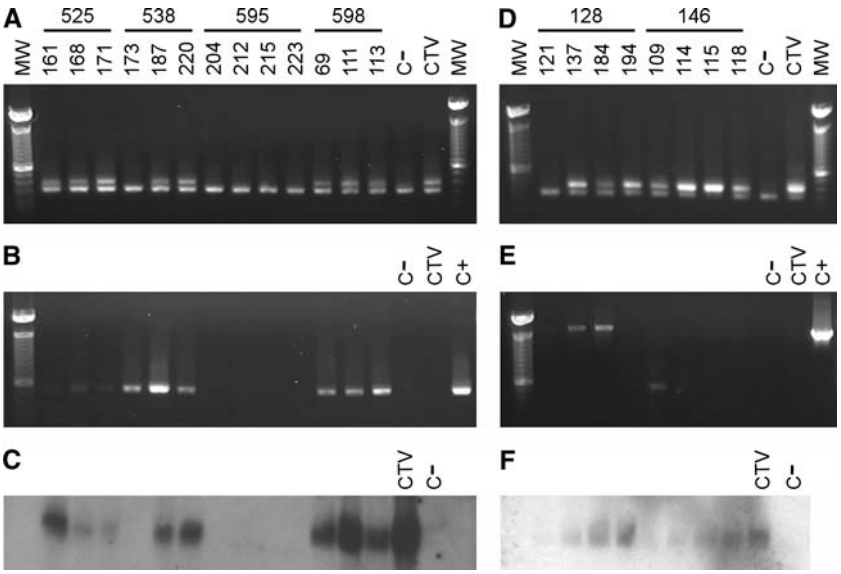

Fig. 3 Relation between infection, transgene expression levels and accumulation of siRNA in the 3END (*left*) and RdRp (*right*) transgenic plants challenged with CTV. The *numbers above the horizontal lines* indicate the transgenic line; *numbers below the horizontal line* indicate the individual plants of each transgenic line; *MW* molecular weight marker (100 bp ladder), *C*– uninfected wild type plant, *CTV* infected wild type plant, *C*+ positive control (plasmid DNA). RT-PCR was used to detect CTV in the 3END (**a**) and RdRp (**d**) transgenic plants. The *upper band* is a CTV-specific product amplified from ORF1a (not a part of any of the transgenes) and indicates infection; the *lower band* is an RT control (18S rRNA) that shows the integrity of the RNA and the efficiency of the RT and PCR. The expression levels of the 3END (**b**) and RdRp (**e**) transgene mRNAs were detected by RT-PCR using the same cDNA as above and specific primers (unique CU region of the promoter and 35S T). No 18S control primers were included in this reaction since the previous step established the integrity and equality in cDNA amounts thus allowing quantitative comparison between reactions. Accumulation of siRNA (23–24 bp) in the 3END (**c**) and RdRp (**f**) transgenic lines was determined by Northern blot. Small RNA (from 10 μg of total RNA) was separated in a denaturing 15% polyacrylamide gel and subsequently hybridized with DIG-labeled dsRNA probes homologous to the respective transgene RNAs (and CTV genome)

undetectable levels of siRNAs were observed in the resistant plant 121 and the susceptible plants 109 and 114.

We also studied the mRNA levels of the contiguous transgenes (GUS and NptII) to determine if their expression was affected. The same cDNA produced to analyze the lines for CTV and transgene mRNA accumulation was also used in this case to reduce inconsistencies and allow comparison. In the 3END transgenic lines, GUS expression (Fig. 4a) was similar in all of the transgenic plants, except plant 113, with lower levels and was undetectable in wild type plants lacking the transgene (C– and CTV). NptII expression (Fig. 4b) was similar in all of the transgenic plants except the silenced line 595 where the mRNA levels were low (204 and 212) or almost undetectable (215 and 223). For the RdRp lines, GUS (Fig. 4d) and NptII (Fig. 4e) expression was similar in all of the transgenic plants. Only plant 115 (susceptible) showed lowered levels of GUS mRNA.

Transgene expression and promoter methylation

Because we were not able to detect mRNA or siRNAs in any of the plants from the most resistant line 595, we decided to determine whether this was linked to DNA cytosine methylation. RNAi has been shown to induce changes in the chromatin structure such as methylation. To investigate the methylation status of the transgene promoter in detail the bisulfite genomic sequencing technique was employed. We compared two plants, one resistant and silenced (595-223) and another susceptible and highly transcribed (598-113). Both lines have only one copy of the transgene as determined in the Southern analysis. This is important because all of the sequences generated from the PCR are then derived from only one locus in each plant. Because the bisulfite treatment modifies the primary structure of the DNA, the primers used were designed to amplify the sense-strand. Also, plasmid DNA was used as a control to establish full conversion (data not shown). The promoter from the highly transcribed plant (598-113) showed no cytosine symmetric methylation and only 0.25% of asymmetric cytosines were methylated (Fig. 5). In contrast, silenced plant 595-223 had 85% of the symmetric cytosines and 61% of the asymmetric cytosines methylated (Fig. 5). We also compared the methylation levels of plants from the partially resistant line 128 (Figure S4) which contains three copies of the transgene. In this case the resistant, silenced and susceptible, not silenced plants showed equally low levels of cytosine methylation as compared with plants from the highly resistant, silenced line 595

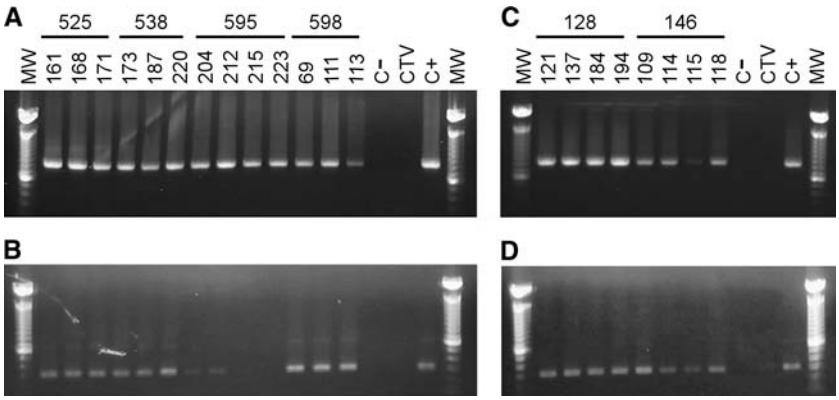

Fig. 4 Expression of GUS and NptII mRNAs in the 3END (*left*) and RdRp (right) transgenic plants. RT-PCR was used to determine the accumulation of transgenic mRNA with GUS (**a**, **c**) and NptII (**b**, **d**) specific primers. The *numbers above the horizontal lines* indicate the transgenic line; *numbers below the horizontal line* indicate the individual plants of each transgenic line; *MW* molecular weight marker (100 bp ladder); *C*– wild type uninfected plant; *CTV* wild type infected plant; *C*+ positive control (plasmid DNA)

Discussion

Because the production of citrus transgenic plants and the assaying of their resistance to CTV is a long term effort, we started testing a variety of virus-derived sequences for their potential to induce resistance. The constructs and transgenic lines described here were produced well before the mechanisms of RNAi were elucidated. We now know that

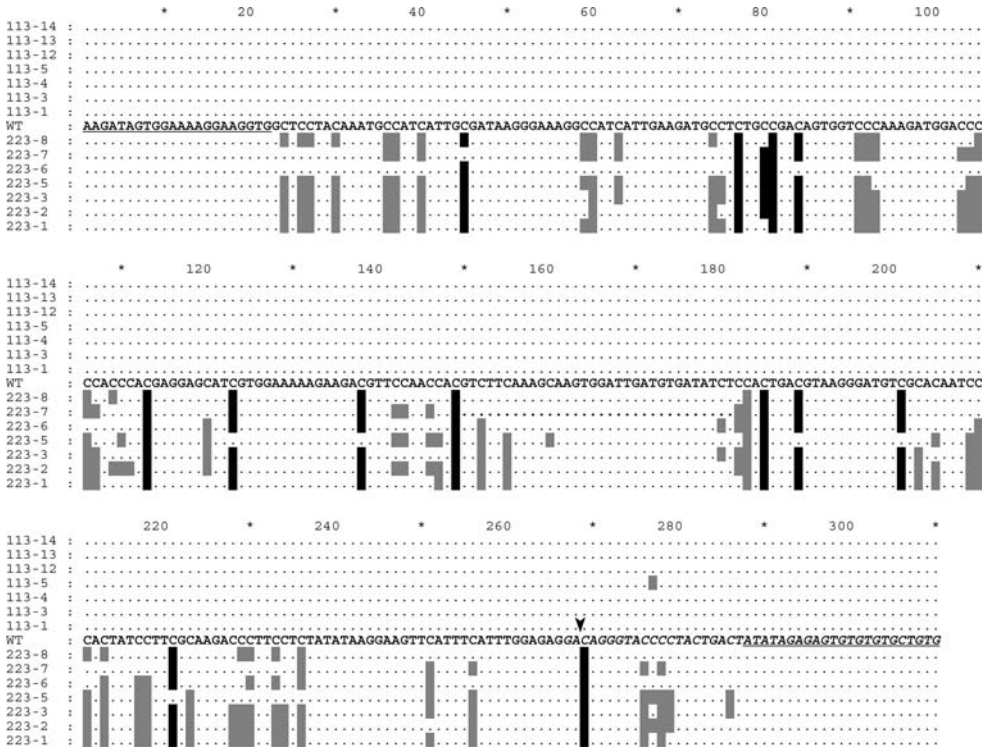

Fig. 5 Cytosine methylation in the sense strand of the promoter regions of two 3END transgenes. Genomic DNA extracted from a susceptible, highly transcribed (598-113) and a resistant, silenced (595-223) plant was first digested with *Xba*I, *Nco*I and *Hin*dIII to release the transgenic insert and subsequently treated with sodium bisulfite, promoting the conversion of unmethylated cytosine to uracil. This leads to a primary sequence change allowing the distinction of cytosine from 5-methylcytosine. The treated DNA was amplified with specific primers (*underlined*), cloned and sequenced. The region studied comprises 310 bp of the 35S promoter and the CU (present only in the 3END transgene and not in the NptII or GUS transgenes). Seven independent clones from each plant are shown (plant-clone number indicated). *Black boxes* indicate methylated symmetric cytosine residues (CpG and CpHpG where H is A, C or T); *gray boxes* denote methylated asymmetric cytosine residues (CpHpH); the wild type (*WT*) sequence is represented in *black bold cases*. The transcription initiation site is indicated in with an *arrowhead*

dsRNA-inducing constructs (such as hairpin structures or inverted repeats) are more effective than single genes in inducing silencing and virus resistance, although, this strategy has not worked for CTV (Batuman et al. 2006). Still, durable resistance has been obtained with single gene constructs in other perennial species (Gonsalves 1998; Hily et al. 2004) so we have continued our efforts to identify resistance in our transgenic plants. For this purpose we propagated the transgenic grapefruits on Swingle citrumelo, a rootstock that is resistant to many CTV strains, including the one we used, T66E. We proceeded to inoculate the plants using blind buds grafted onto the scion. This challenge was expected to be much more severe than any encountered in nature. Using this procedure we tested 35 transgenic lines, 13 of which had been previously tested (Febres et al. 2003) with a different isolate (T36). Most of these lines were either completely susceptible (CTV was detected in all replicates) or only partially resistant (CTV was detected in some but not all replicates). This is similar to what has been observed by other authors working with citrus and CTV (Batuman et al. 2006; Dominguez et al. 2002a; Fagoaga et al. 2006). We did observe a delay of infection in some of the transgenic plants. For instance, plants from lines 182 and 146 remained uninfected 12 months after inoculation but became infected 24 months after inoculation (Table 1). Other susceptible transgenic plants were infected earlier and control plants were all infected 6 months after inoculation. There was one case of recovery (line 169) in which CTV was detected in a plant 6 months after inoculation but was not subsequently detected. Most significantly, line 595, transformed with the 3′ end of CTV, was completely resistant throughout the experiment. All transgenic plants showed normal phenotype. Of the 13 transgenic lines previously found to be susceptible two lines, 169 and 128, were partially resistant in the present experiment. However, still in agreement with findings from us and others that a portion of the clones can be resistant only one plant from the four replicates of each line was resistant. Sequence identity between the transgene and the challenging isolate influences the outcome of the interaction. The CP constructs (some of which were tested with two isolates, Table 1) had identities of 91–98% with isolate T66 and 92–100% with isolate T36 (Table S2). The 3END constructs were 94–96% identical to T66 (Table S3), the only isolate to which they were tested. We do not have sequence information between the RdRp transgene and T66 but it was 100% identical to isolate T36. Previous reports indicate that identities of 89% or higher are sufficient to induce resistance in other systems with better control the higher the identity (Holzberg et al. 2002; Jones et al. 1998a; Lindbo and Dougherty 1992b; Mueller et al. 1995). Thus we expected enough sequence homology between the transgenes used and the challenging strains to induce RNA-mediated resistance.

We did not find an association between resistance and transgene copy number. The transgenic lines had between 1 and 3 copies of the transgene. Susceptible lines 525 and 598 had one copy and so did resistant line 595. The line with the most copies (128) was only partially resistant. Previous reports have found a direct correlation between transgene copy number and silencing/resistance using single gene constructs (Lindbo and Dougherty 1992a, b; Waterhouse et al. 1998). However, unique copy inserts can also induce TGS and PTGS (Kooter et al. 1999).

From all of the lines initially assayed we decided to further characterize a smaller number to determine whether RNA-mediated mechanisms were associated with the resistance observed and if there were any differences in this response between the lines/constructs. We used RT-PCR to confirm the infection status of the plants and the results corroborated those of the ELISA assay. We then estimated the steady-state levels of CTV transgene mRNA and the siRNA accumulation in plants from susceptible and partially resistant lines and from the resistant line. Line 598 accumulated high levels of transgene mRNA and was susceptible to CTV, indicating that although RNAi was triggered (high levels of siRNA were produced) it did not stop virus infection or transgene accumulation. Line 525 also accumulated transgene mRNA, although to much lower levels than 598. While we did not investigate the origin of the siRNAs, it is possible that in this particular case RNAi was triggered against the transgene (lowering its steady-state level) but failed to prevent virus infection. Alternatively, transgene expression may be low due to the position of the insertion and thus not sufficient to trigger silencing. Susceptible line 146 also showed low or no accumulation of the transgene mRNA. In this case it is also possible that RNAi triggered against the transgene failed to control the virus. In the partially resistant line 128 the susceptible plants (137 and 184) accumulated transgene mRNA but resistant plant 121 did not. This is an indication that PTGS was probably the cause of resistance in plant 121. In support of this, siRNA accumulated in plant 121 (despite no viral infection) and cytosines in the 35S promoter sequences of this plant that were analyzed were not methylated. Line 128 has three copies of the transgene and we do not know which ones contributed clones we sequenced. However, because we observed low cytosine methylation the results indicate that at least one of the transgene copies is not transcriptionally silenced. The other partially resistant line (538) showed transgene expression in both resistant (173) and susceptible (187 and 220) plants, indicating that neither PTGS nor TGS was established. Specifically, plant 173 did not accumulate siRNAs. Although unlikely, it is possible that the inoculation failed to infect plant 173. Alternatively, the silenced state may have reverted in plant 173 once the virus was eliminated

from the plant. Overall, all susceptible plants accumulated siRNAs, regardless of the steady-state levels of the transgene mRNA. It is likely that at least some of these small RNAs were the product of CTV degradation by the RNAi machinery since even WT plants accumulated the siRNAs to high levels. CTV carries three suppressors of silencing, however many of these proteins have been found in other viruses to bind the siRNAs and disrupt their incorporation into the RISC complex rather than preventing their synthesis (Lakatos et al. 2006).

Plants from the resistant line 595 showed no transgene mRNA or siRNA accumulation. In addition to causing sequence specific RNA degradation or PTGS, RNAi can also cause the hypermethylation of gene promoters and the suppression of gene transcription (i.e., TGS). A unique characteristic of RNA-directed DNA methylation (RdDM) is that all cytosine residues (symmetric and asymmetric) are targeted for de novo methylation (Pelissier et al. 1999). Our results show that all plants derived from line 595 were transcriptionally silenced, with hypermethylation of cytosines in all contexts in the 35S promoter, pointing to RdDM. Some reports have shown that spontaneous transgene silencing as well as virus-induced transgene silencing and subsequent transgene methylation is limited to regions of homology to the transcribed sequence (Jones et al. 1998b, 1999; Mette et al. 2000; Mourrain et al. 2007) and methylation does not spread much beyond this region of homology (Pelissier et al. 1999; Wassenegger 2000). Other reports, however; associate transgenic derived virus resistance (for example, in plum to *Plum pox virus*) with low or no transgene expression and promoter methylation (Hily et al. 2004), although the extent of the promoter methylation was not studied in detail. Spontaneous promoter methylation was also observed in transgenic citrus (Dominguez et al. 2002b) although again the extent of the methylation was not studied in detail. This is what we observed in line 595. Heavy methylation was present beyond the transcription initiation site. Further, steady state levels of the NptII gene (adjacent to the 3END gene but in reverse orientation) but not the GUS gene (downstream of the 3END gene) were also reduced or undetectable. This suggests that methylation likely extended much farther than the sequence analyzed. All three genes were driven by the 35S promoter (although only the 3END transgene has the UC region) indicating that the 35S promoters were not specifically targets for TGS but rather the RdDM was associated with the silencing of the 3END transgene. Additionally, line 595 had only one copy of the transgene and therefore no T-DNA inverted repeats were present that would produce read-through transcripts of the entire 35S promoter region that could trigger methylation of the region beyond the natural point of transcription initiation. Alternatively, or in addition, it is possible that in line 595 a process similar to RNAi-mediated chromatin formation was induced. This mechanism is also accompanied by DNA methylation but can spread over hundreds of bases (Wassenegger 2000, 2005).

The most remarkable characteristic that our experiments and those of others show in the transgenic citrus/CTV interaction is the variability in the responses, even among clonally propagated plants. Transgene expression levels, siRNA accumulation and resistance levels were varied in the plants we studied. Only plants from line 595 consistently showed resistance that was also associated with TGS and promoter methylation. Other works in perennial species (plum and citrus) have studied transgene methylation, but mostly in the transcribed region and not in detail. Therefore it is unknown whether the promoters of resistant plants in these studies were also highly methylated, beyond the homology region with the transcript. It is possible the three distinct CTV suppressors of silencing operate in tandem, each serving as a 'backup' if the other fails and this, at least in part, is responsible for the varied responses observed. Other researchers have suggested that targeting RNAi to the viral suppressors of silencing may be a more successful strategy to obtain durable resistance (Di Nicola-Negri et al. 2005; Roy et al. 2006; Savenkov and Valkonen 2002) and perhaps targeting all three CTV suppressors of silencing at once, would be more effective in producing citrus plants resistant to this economically important virus (Batuman et al. 2006). Other factors regarding the sequences targeted for silencing may also influence the outcome of the interaction. Highly structured regions of the viral RNA are more prone to degradation by the DCL enzymes (Molnar et al. 2005) but these target regions vary in different hosts (Ribeiro et al. 2007). Even though we used three different CTV regions in our constructs, they may not be the ideal target sequences for controlling this virus.

Because the number of replicates in this experiment was relatively small we need to validate the resistance observed in line 595 and also determine whether it can be replicated under field conditions. For this purpose we are currently preparing a field experiment of this and other lines grafted on sour orange rootstock. This will allow determining whether long-term resistance to decline strains of CTV as well as stem pitting strains has been achieved. There is some evidence that environmental factors such as low temperatures can interfere with the silencing pathway in certain plant species (Sos-Hegedus et al. 2005; Szittya et al. 2003). We also know that in WT citrus plants CTV levels are higher during cooler months. Whether this phenomenon is associated with any variation in the efficiency of silencing in citrus is not known, but this needs to be studied in our transgenic plants. Our plants were kept under greenhouse conditions and although they experienced some temperature variations these were not as extreme as those undergone by

plants grown in the field. Another major difference between our conditions and natural field conditions is the way plants are infected in the field: by aphids, most likely repeatedly and with a mixture of strains. Potentially, all of these factors can have an effect on the response of the transgenic plants and they will be evaluated.

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References

Ayllon MA, Lopez C, Navas-Castillo J, Garnsey SM, Guerri J, Flores R, Moreno P (2001) Polymorphism of the 5′ terminal region of *Citrus tristeza virus* (CTV) RNA: incidence of three sequence types in isolates of different origin and pathogenicity. Arch Virol 146:27–40

Bar-Joseph M, Marcus R, Lee RF (1989) The continuous challenge of *Citrus tristeza virus* control. Annu Rev Phytopathol 27:291–316

Batuman O, Mawassi M, Bar-Joseph M (2006) Transgenes consisting of a dsRNA of an RNAi suppressor plus the 3′ UTR provide resistance to *Citrus tristeza virus* sequences in *Nicotiana benthamiana* but not in citrus. Virus Genes 33:319–327

Cevik B (2001) Characterization of the RNA-dependent RNA polymerase gene of *Citrus tristeza closterovirus*. Plant Pathology Department. University of Florida, Gainesville, p 138

Di Nicola-Negri E, Brunetti A, Tavazza M, Ilardi V (2005) Hairpin RNA-mediated silencing of *Plum pox virus* P1 and HC-Pro genes for efficient and predictable resistance to the virus. Transgenic Res 14:989–994

Dominguez A, de Mendoza AH, Guerri J, Cambra M, Navarro L, Moreno P, Pena L (2002a) Pathogen-derived resistance to *Citrus tristeza virus* (CTV) in transgenic Mexican lime (*Citrus aurantifolia* (Christ.) Swing.) plants expressing its p25 coat protein gene. Mol Breed 10:1–10

Dominguez A, Fagoaga C, Navarro L, Moreno P, Pena L (2002b) Regeneration of transgenic citrus plants under non selective conditions results in high-frequency recovery of plants with silenced transgenes. Mol Genet Genomics 267:544–556

Fagoaga C, Lopez C, de Mendoza AH, Moreno P, Navarro L, Flores R, Pena L (2006) Post-transcriptional gene silencing of the p23 silencing suppressor of *Citrus tristeza virus* confers resistance to the virus in transgenic Mexican lime. Plant Mol Biol 60:153–165

Febres VJ, Ashoulin L, Mawassi M, Frank A, BarJoseph M, Manjunath KL, Lee RF, Niblett CL (1996) The p27 protein is present at one end of *Citrus tristeza virus* particles. Phytopathology 86:1331–1335

Febres VJ, Niblett CL, Lee RF, Moore GA (2003) Characterization of grapefruit plants (*Citrus paradisi* Macf.) transformed with *Citrus tristeza closterovirus* genes. Plant Cell Rep 21:421–428

Garnsey SM, Cambra M (1991) Enzyme linked immunosorbent assay for citrus pathogens. In: Roistacher CN (ed) Graft transmissible diseases of citrus: handbook for detection and diagnosis. IOCV, FAO, Rome, pp 193–216

Ghorbel R, Dominguez A, Navarro L, Pena L (2000) High efficiency genetic transformation of sour orange (*Citrus aurantium*) and production of transgenic trees containing the coat protein gene of *Citrus tristeza virus*. Tree Physiol 20:1183–1189

Gonsalves D (1998) Control of *Papaya ringspot virus* in papaya: a case study. Annu Rev Phytopathol 36:415–437

Gutierrez MA, Luth D, Moore GA (1997) Factors affecting *Agrobacterium*-mediated transformation in *Citrus* and production of sour orange (*Citrus aurantium* L.) plants expressing the coat protein gene of *Citrus tristeza virus*. Plant Cell Rep 16: 745–753

Herron CM, Yang ZN, Molina JJ, Da Graca JV, van Vuuren SP, Mirkov TE (2002) Preliminary evaluation of *uncp* transgenic Rio Red grapefruit scions for resistance to *Citrus tristeza virus*. In: Duran-Vila N, Milne RG, Da Graca JV (eds) Proceedings of the 15th conference IOCV. IOCV, Riverside, p 434

Hily JM, Scorza R, Malinowski T, Zawadzka B, Ravelonandro M (2004) Stability of gene silencing-based resistance to *Plum pox virus* in transgenic plum (*Prunus domestica* L.) under field conditions. Transgenic Res 13:427–436

Holzberg S, Brosio P, Gross C, Pogue GP (2002) Barley stripe mosaic virus-induced gene silencing in a monocot plant. Plant J 30: 315–327

Jones AL, Johansen IE, Bean SJ, Bach I, Maule AJ (1998a) Specificity of resistance to pea seed-borne mosaic potyvirus in transgenic peas expressing the viral replicase (Nlb) gene. J Gen Virol 79:3129–3137

Jones AL, Thomas CL, Maule AJ (1998b) De novo methylation and co-suppression induced by a cytoplasmically replicating plant RNA virus. EMBO J 17:6385–6393

Jones L, Hamilton AJ, Voinnet O, Thomas CL, Maule AJ, Baulcombe DC (1999) RNA–DNA interactions and DNA methylation in post-transcriptional gene silencing. Plant Cell 11:2291–2301

Karasev AV, Boyko VP, Gowda S, Nikolaeva OV, Hilf ME, Koonin EV, Niblett CL, Cline K, Gumpf DJ, Lee RF (1995) Complete sequence of the *Citrus tristeza virus* RNA genome. Virology 208:511–520

Kooter JM, Matzke MA, Meyer P (1999) Listening to the silent genes: transgene silencing, gene regulation and pathogen control. Trends Plant Sci 4:340–347

Lakatos L, Csorba T, Pantaleo V, Chapman EJ, Carrington JC, Liu YP, Dolja VV, Calvino LF, Lopez-Moya JJ, Burgyan J (2006) Small RNA binding is a common strategy to suppress RNA silencing by several viral suppressors. EMBO J 25:2768–2780

Lindbo JA, Dougherty WG (1992a) Pathogen-derived resistance to a potyvirus—immune and resistant phenotypes in transgenic tobacco expressing altered forms of a potyvirus coat protein nucleotide-sequence. Mol Plant Microbe Interact 5:144–153

Lindbo JA, Dougherty WG (1992b) Untranslatable transcripts of the *Tobacco etch virus* coat protein gene sequence can interfere with *Tobacco etch virus*-replication in transgenic plants and protoplasts. Virology 189:725–733

Lindbo JA, Silvarosales L, Proebsting WM, Dougherty WG (1993) Induction of a highly specific antiviral state in transgenic plants—implications for regulation of gene-expression and virus-resistance. Plant Cell 5:1749–1759

Lopez C, Ayllon MA, Navas-Castillo J, Guerri J, Moreno P, Flores R (1998) Molecular variability of the 5′- and 3′-terminal regions of *Citrus tristeza virus* RNA. Phytopathology 88:685–691

Lu R, Folimonov A, Shintaku M, Li W-X, Falk BW, Dawson WO, Ding S-W (2004) Three distinct suppressors of RNA silencing encoded by a 20-kb viral RNA genome. Proc Natl Acad Sci USA 101:15742–15747

Mawassi M, Mietkiewska E, Gofman R, Yang G, Bar-Joseph M (1996) Unusual sequence relationships between two isolates of *Citrus tristeza virus*. J Gen Virol 77:2359–2364

Mawassi M, Satyanarayana T, Albiach-Marti MR, Gowda S, Ayllon MA, Robertson C, Dawson WO (2000) The fitness of *Citrus tristeza virus* defective RNAs is affected by the lengths of their 5′- and 3′-termini and by the coding capacity. Virology 275: 42–56

Mette MF, Aufsatz W, van der Winden J, Matzke MA, Matzke AJ (2000) Transcriptional silencing and promoter methylation triggered by double-stranded RNA. EMBO J 19:5194–5201

Molnar A, Csorba T, Lakatos U, Varallyay E, Lacomme C, Burgyan J (2005) Plant virus-derived small interfering RNAs originate predominantly from highly structured single-stranded viral RNAs. J Virol 79:7812–7818

Mourrain P, van Blokland R, Kooter JM, Vaucheret H (2007) A single transgene locus triggers both transcriptional and post-transcriptional silencing through double-stranded RNA production. Planta 225:365–379

Mueller E, Gilbert J, Davemport G, Brigneti G, Baulcombe D (1995) Homology-dependent resistance: transgenic virus resistance in plants related to homology-dependent gene silencing. Plant J 7:1001–1013

Pappu HR, Karasev AV, Anderson EJ, Pappu SS, Hilf ME, Febres VJ, Eckloff RM, McCaffery M, Boyko V, Gowda S (1994) Nucleotide sequence and organization of eight 3′ open reading frames of the *Citrus tristeza closterovirus* genome. Virology 199:35–46

Paulin R, Grigg GW, Davey MW, Piper AA (1998) Urea improves efficiency of bisulphite-mediated sequencing of 5′-methylcytosine in genomic DNA. Nucleic Acids Res 26:5009–5010

Pelissier T, Thalmeir S, Kempe D, Sanger HL, Wassenegger M (1999) Heavy de novo methylation at symmetrical and non-symmetrical sites is a hallmark of RNA-directed DNA methylation. Nucleic Acids Res 27:1625–1634

Ratcliff F, Harrison B, Baulcombe D (1997) A similarity between viral defense and gene silencing in plants. Science 276:1558

Ribeiro SG, Lohuis H, Goldbach R, Prins M (2007) Tomato chlorotic mottle virus is a target of RNA silencing but the presence of specific short interfering RNAs does not guarantee resistance in transgenic plants. J Virol 81:1563–1573

Roy G, Sudarshana MR, Ullman DE, Ding SW, Dandekar AM, Falk BW (2006) Chimeric cDNA sequences from *Citrus tristeza virus* confer RNA silencing-mediated resistance in transgenic *Nicotiana benthamiana* plants. Phytopathology 96:819–827

Ruiz-Ruiz S, Moreno P, Guerri J, Ambros S (2006) The complete nucleotide sequence of a severe stem pitting isolate of *Citrus tristeza virus* from Spain: comparison with isolates from different origins. Arch Virol 151:387–398

Satyanarayana T, Gowda S, Ayllon MA, Albiach-Marti MR, Dawson WO (2002) Mutational analysis of the replication signals in the 3′-nontranslated region of *Citrus tristeza virus*. Virology 300:140–152

Savenkov EI, Valkonen JP (2002) Silencing of a viral RNA silencing suppressor in transgenic plants. J Gen Virol 83:2325–2335

Sos-Hegedus A, Lovas A, Kondrak M, Kovacs G, Banfalvi Z (2005) Active RNA silencing at low temperature indicates distinct pathways for antisense-mediated gene-silencing in potato. Plant Mol Biol 59:595–602

Szittya G, Silhavy D, Molnar A, Havelda Z, Lovas A, Lakatos L, Banfalvi Z, Burgyan J (2003) Low temperature inhibits RNA silencing-mediated defence by the control of siRNA generation. EMBO J 22:633–640

Tsai JH, Liu YH, Wang JJ, Lee RF (2000) Recovery of orange stem pitting strains of *Citrus tristeza virus* (CTV) following single aphid transmissions with *Toxoptera citricida* from a Florida decline isolate of CTV. Proc Fla State Hortic Soc 113:75–78

Vives MC, Rubio L, Lopez C, Navas-Castillo J, Albiach-Marti MR, Dawson WO, Guerri J, Flores R, Moreno P (1999) The complete genome sequence of the major component of a mild *Citrus tristeza virus* isolate. J Gen Virol 80:811–816

Voinnet O (2002) RNA silencing: small RNAs as ubiquitous regulators of gene expression. Curr Opin Plant Biol 5:444–451

Wassenegger M (2000) RNA-directed DNA methylation. Plant Mol Biol 43:203–220

Wassenegger M (2005) The role of the RNAi machinery in heterochromatin formation. Cell 122:13–16

Wassenegger M, Heimes S, Riedel L, Sanger HL (1994) RNA-directed de novo methylation of genomic sequences in plants. Cell 76:567–576

Waterhouse PM, Graham HW, Wang MB (1998) Virus resistance and gene silencing in plants can be induced by simultaneous expression of sense and antisense RNA. Proc Natl Acad Sci USA 95:13959–13964

Waterhouse PM, Wang MB, Lough T (2001) Gene silencing as an adaptive defence against viruses. Nature 411:834–842

Yang ZN, Mathews DM, Dodds JA, Mirkov TE (1999) Molecular characterization of an isolate of *Citrus tristeza virus* that causes severe symptoms in sweet orange. Virus Genes 19:131–142