Analysis of Silencing Escape of *Tomato leaf curl virus*: An Evaluation of the Role of DNA Methylation

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RNA silencing is a sequence-specific mechanism regulating gene expression and has been used successfully for antiviral defense against RNA viruses. Similar strategies to develop resistance against DNA containing *Tomato leaf curl virus* (TLCV) and some other geminiviruses have been unsuccessful. To analyze this silencing escape, we transformed tomato plants with a hairpin construct from the TLCV *C2* open reading frame (ORF). The transgenic plants showed a strong RNA silencing response, and following TLCV inoculation, their infection was delayed. However, the viral infection was not prevented and TLCV DNA accumulated to the levels found in nontransgenic plants. To determine the fate of a transgene carrying homology to the virus, we used transgenic plants carrying the TLCV *C4* gene, which induces a distinct phenotype. Upon TLCV infection, the phenotype was abolished and *C4* transcript disappeared. Concurrently, TLCV-specific small interfering RNAs were produced. In situ hybridization showed abundant levels of TLCV DNA in phloem cells of TLCV-infected *C4* transgenic plants. However, the *C4* transcripts were no longer detectable in nonvascular cells. Analysis of the transgene by methylation sequencing revealed a high level of de novo methylation of asymmetric cytosines in both the *C4* ORF and its 35S promoter. A high level of methylation also was found at both symmetric and asymmetric cytosines of the complementary-sense strand of TLCV double-stranded DNA. Given the previous finding that methylated geminiviral DNA is not competent for replication, we provide a model whereby TLCV evades host defense through a population of de novo synthesized unmethylated DNA.

Additional keywords: begomoviruses, gene silencing, transgenic resistance.

Eukaryotic cells suppress foreign genetic elements through a sequence-specific RNA silencing mechanism (Voinnet 2001) known as gene silencing in plants (Napoli et al. 1990; van der Krol et al. 1990), RNA interference in animals, and quelling in

fungi (Cerutti 2003; Pickford and Cogoni 2003). RNA silencing can be triggered by RNA viruses (Burton et al. 2000; Jones et al. 1999), DNA viruses (Al-Kaff et al. 1998; Kjemtrup et al. 1998), antisense transcripts (Nellen and Lichtenstein 1993), highly expressed-sense transcripts (van der Krol et al. 1990), and transgene loci producing double-stranded (ds)RNA (Smith et al. 2000). A key feature of RNA silencing is the presence of small interfering (si)RNA, which acts as an intermediate in the silencing pathway (Hamilton and Baulcombe 1999; Voinnet 2001). Recent studies show that dsRNA is cleaved into siRNAs of approximately 21 to 25 nucleotides (nt) by an RNase III-like enzymes called Dicer (Bernstein et al. 2001; Hamilton and Baulcombe 1999; Zamore et al. 2000). siRNA is used as a guide for the degradation of single-stranded (ss)RNA by an siRNA/ribonuclease complex known as the RNA-induced silencing complex (RISC) (Hammond et al. 2000).

RNA silencing in plants involves RNA-directed DNA methylation (RdDM), in which DNA homologous to a triggering RNA is methylated de novo (Mette et al. 2000; Pelissier et al. 1999). RdDM occurs at both symmetric cytosine sites (CpG and CpNpG, where N is A, T, C, or G) and at asymmetric sites (CpHpH, where H is A, T, or C). Cao and associates (2003) proposed that the initial establishment of RdDM requires the enzyme known as domains rearranged methylase (DRM), which is guided by siRNA to the target sequences. The maintenance of CpG methylation is accomplished by methyltransferase, whereas CpNpG and CpHpH methylation is maintained by chromomethylase and DRM redundantly (Cao et al. 2003).

siRNA homologous to the promoter region of a target gene induces transcriptional gene silencing (TGS), which is associated with promoter methylation. If siRNA is homologous to the coding region of the target gene, it induces posttranscriptional gene silencing (PTGS), which involves sequence-specific RNA degradation and methylation of the coding region. PTGS and TGS are mechanistically related because both involve the production of siRNA (Sijen et al. 2001).

RNA silencing in plants is considered to be an adaptive defense mechanism against viruses (Voinnet 2001; Waterhouse et al. 2001). It is induced in response to viral replicating form RNA, presumably to degrade the invasive viral genomes. Plant endogenous or introduced genes that have homology with an infecting viral RNA also could be silenced (Jones et al. 1999, 2001). This process is known as virus-induced gene silencing (VIGS). It has been found that DNA viruses, including geminiviruses, also are capable of inducing gene silencing (Al-Kaff et al. 1998; Atkinson et al. 1998; Chellappan et al. 2004; Kjemtrup et al. 1998).

Tomato leaf curl virus (TLCV) has an ssDNA genome encoding six open reading frames (ORFs), two (V1 and V2) on

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the virion sense and four (C1 to C4) on the complementary sense strand of the replicative form (RF) of viral DNA (Dry et al. 1993). The TLCV genome replicates in the nucleus of infected cells (Rojas et al. 2005) by a combination of rolling circle replication (RCR) and recombination-dependent replication (RDR) (Jeske et al. 2001). Earlier, we found that TLCV infection induces transcriptional silencing of TLCV promoter-driven β -glucuronidase (GUS) transgenes and causes methylation of the virus-derived promoter (Seemanpillai et al. 2003). Interestingly, the viral replication appeared not to be inhibited by the silencing (Seemanpillai et al. 2003). In line with this observation, it has been shown that expression of a truncated version of *Tomato yellow leaf curl Sardinia virus* (TYLCSV) Rep protein (Rep-210) in transgenic tomato plants produces resistance to TYLCSV infection (Brunetti et al. 1997), but the resistance is overcome due to transgene silencing (Lucioli et al. 2003). To investigate this apparent discrepancy, RNA silencing triggered by C2 hairpin RNA in tomato was used in the current study. Furthermore, transgenic tobacco plants carrying the TLCV C4 ORF driven by *Cauliflower mosaic virus* (CaMV) 35S promoter were used to determine the transgene methylation and expression following TLCV infection. In addition, the methylation levels of RF of TLCV DNA were determined. Our results demonstrate that TLCV infection induces silencing of the virus-derived transgene, but the virus escapes this defense mechanism, possibly due to a differential methylation of the de novo synthesized viral and host plant DNA.

RESULTS

Strong RNA silencing triggered by the C2 hairpin construct delayed but did not prevent TLCV infection.

Highly effective RNA silencing can be triggered by the expression of double-stranded hairpin RNA generated in transgenic plants (Smith et al. 2000). Given that our previous attempts to develop transgenic resistance against TLCV have been unsuccessful, we tested whether this system could provide protection against TLCV. Tomato plants were transformed with the C2 hairpin (hp) construct expressing TLCV C2 RNA in an inverted-repeat form interspaced by an intron. Four independently regenerated lines (C2-B, C2-D, C2-H, and C2-N) were confirmed to carry the transgene by Southern blot analysis (data not shown). To determine whether C2 silencing was triggered in the transgenic lines, the plants were analyzed for

the accumulation of C2-specific siRNA. Northern blotting using a C2 virion-sense RNA probe demonstrated that C2 siRNA accumulated in the transgenic lines C2-B and C2-H (Fig. 1A). We also analyzed siRNA from TLCV-infected tomato and *Nicotiana benthamiana* plants at 21 days postinoculation (dpi). Strong siRNA signals were obtained in samples from *N. benthamiana* and relatively weak signals were detected in tomato (data not shown). siRNA was not detected in the samples taken from healthy tomato and *N. benthamiana* (data not shown).

The C2 hp plants were propagated by tissue culture and nine individuals from each line were transferred to a glasshouse. Six of the nine individual plants from each line were agroinoculated with TLCV; the remaining three served as control. The transgenic lines C2-D and C2-N developed severe symptoms indistinguishable from nontransgenic controls at 14 dpi. The plant lines C2-B and C2-H developed mild symptoms at 20 to 25 dpi. The C2-B and C2-H were observed for a longer period and found to grow higher and produce larger fruit compared with the nontransgenic lines (data not shown).

TLCV DNA accumulation in the six replicates of each transgenic line was determined at 0, 10, 15, 20, 25, and 30 dpi by dot blot analysis. The viral DNA in C2-D and C2-N plants reached a high level, comparable with the levels in nontransgenic tomato plants, at 10 dpi (data not shown). The viral DNA in C2-B (Fig. 1B) and C2-H (data not shown) accumulated at lower levels than those of the nontransgenic plants at 10 and 15 dpi; however, the DNA accumulated to the level of nontransgenic plants at 20, 25, and 30 dpi (Fig. 1C). These results indicate that the induction of silencing, as evidenced by the elevated level of siRNA, could delay but not prevent TLCV replication.

A TLCV-derived transgene is silenced upon the viral infection.

The lack of an effective reduction of TLCV replication in the transgenic lines discussed above is consistent with previous observations in our laboratory that transgenic plants expressing a range of TLCV-derived gene constructs in sense or antisense orientations have failed to protect tobacco or tomato plants from viral infections (I. B. Dry, *unpublished data*). Therefore, we studied the fate of a TLCV-derived transgene after viral infection. Tobacco plants containing the C4 ORF under the control of CaMV 35S promoter (35S:C4) (Krake et al. 1998) were used because they express a severe phenotype that can serve as a marker for monitoring RNA silencing. The 35S:C4 phenotype includes curling of leaf margins, twisting of stems, and

Fig. 1. Transgenic tomato plants (C2 hairpin) delay *Tomato leaf curl virus* (TLCV) infection. **A**, Northern blot analysis showing small interfering (si)RNA in transgenic tomato lines (C2-B and C2-H). RNA samples (5 μ g) were separated by electrophoresis in a 15% denaturing polyacrylamide gel and transferred to a membrane. Hybridization was done with an in vitro transcribed, 32 P-labeled TLCV C2 RNA probe. Ethidium bromide staining of the ribosomal (r)RNA is shown as an indication of loading. The position of 21 mer DNA oligonucleotides is shown. **B**, Dot blot analysis of TLCV DNA extracted from tomato leaves of either nontransgenic or transgenic (C2-B) plants. The samples were taken from six plant replicates at 0, 10, 15, 20, 25, and 30 days postinoculation. **C**, Blots (shown in B) were exposed to a phosphor imager screen and scanned and signals quantified using IQANar V1.2 software (Storm, Amersham Inc.). Levels of TLCV DNA accumulation were determined arbitrarily relative to the highest reading taken as 10.

mosaic patterns (Fig. 2A, panel 1). These are distinct from the mild symptoms caused by TLCV in tobacco (Fig. 2A, panel 4). After inoculation of the 35S:C4 plants in triplicate with TLCV, the typical 35S:C4 phenotypes disappeared gradually in all the plants. At 60 dpi, TLCV-infected 35S:C4 plants (Fig. 2A, panel 2) showed typical mild symptoms present on TLCV-infected nontransgenic plants (Fig. 2A, panel 4). The TLCV symptoms persisted and developed on the newly growing parts of transgenic plants. The presence of TLCV in transgenic and nontransgenic tobacco plants was confirmed by Southern analysis (Fig. 2B). These results suggested that expression of the virus-derived 35S:C4 transgene might have been silenced following TLCV infection.

To examine whether the change in 35S:C4 phenotype was due to RNA silencing, we analyzed the levels of *C4* transcript expressed from the 35S:C4 transgene before and after infection of the plants with TLCV. Northern blot analysis was carried out with a poly A⁺ RNA preparation to avoid hybridization interference by TLCV DNA in infected samples. The *C4* transcript was readily detectable in noninoculated 35S:C4 transgenic plants (Fig. 2C, lane 1). In contrast, the transcript was not detected in the 35S:C4 transgenic plants at 60 dpi with TLCV (Fig. 2C, lane 2) or in healthy nontransgenic tobacco (Fig. 2C, lane 3). It was noted that the *C4* transcript expressed from replicating TLCV in nontransgenic tobacco plants was below the level of detection by Northern hybridization technique (Fig. 2C, lane 4). These results indicate that the 35S:C4 transgene

was silenced following TLCV infection. Previously, we have shown, by nuclear run on assays, that the silencing of the transgene carrying homology to TLCV DNA is caused by virus-induced TGS (Seemanpillai et al. 2003). The silencing of *C4* transgene also is likely to be due to TGS.

Tissue-specific replication of TLCV induces systemic silencing of the 35S:C4 transgene.

To investigate virus-induced gene silencing at a cellular level, we carried out in situ hybridization experiments with noninoculated and TLCV-infected 35S:C4 transgenic tobacco leaf tissues, using fluorescent probes. Following hybridization with the *C4* virion-sense probe (C4-v), strong fluorescent output was observed in all cell types, including phloem and mesophyll of noninoculated 35S:C4 transgenic plants (Fig. 3B), indicating constitutive expression of the *C4* transcripts from the 35S:C4 gene. When the sections of the TLCV-infected 35S:C4 transgenic plants were hybridized with the same probe, fluorescent signals were observed only in phloem-associated cells and were restricted to nuclei (Fig. 3C). These complementary-sense signals presumably represent the replicative form of TLCV DNA in the nucleus or *C4* transcripts, to which the C4-v probe can hybridize. The presence of viral nucleic acids in these cells was confirmed by probing the serial sections immediately adjacent to that shown in Figure 3C with a digoxigenin-labeled complementary-sense probe from the V2 ORF (V2-c). Strong colorimetric signals developed in the vas-

Fig. 2. Tomato leaf curl virus (TLCV) infection results in the silencing of a transgene homologous to the viral DNA. **A**, Showing the *C4* phenotype: panel 1, healthy 35S:C4; panel 2, TLCV-infected *C4* transgenic; panel 3, healthy nontransgenic; and panel 4, TLCV-infected nontransgenic tobacco plants. Photographs were taken at 60 days postinoculation (dpi). **B**, Southern blot analysis of DNA extracted from healthy (H) or TLCV-inoculated (I) 35S:C4 transgenic and nontransgenic tobacco plants. The positions of replicative form (RF) and single-stranded (ss)DNA are shown. In the bottom panel, genomic DNA is shown as an indication of the loading levels. **C**, Northern blot analysis of mRNA extracted from *C4* transgenic and nontransgenic tobacco leaves. Messenger RNA was extracted from either healthy (H) or TLCV-infected (I) leaves at 60 dpi. In the bottom panel, the membrane was stripped and reprobed for ubiquitin (Ubi) mRNA to provide an indication of RNA loading levels.

cular cells, indicating the presence of virion-sense DNA and transcripts (Fig. 3D) in both cytoplasm and the nucleus. In the same experiment, hybridization signals were not detected in healthy, nontransgenic leaves of tobacco (Fig. 3A). Moreover, vascular-associated accumulation of viral DNA and transcripts in nontransgenic tobacco (Fig. 3E) was found to be similar to that of transgenic tobacco (Fig. 3D). These results confirm that TLCV is phloem specific and causes a systemic silencing response in nonvascular cells. The uninterrupted replication of TLCV in vascular cells indicates that functional viral transcripts are produced in TLCV-infected phloem cells of silenced plants.

The *C4* gene in transgenic plants and in TLCV RF DNA is heavily methylated.

Previously, TLCV infection has been found to induce methylation of its homologous promoter sequence in transgenic plants (Seemanpillai et al. 2003). We questioned whether the differential silencing of the *C4* gene encoded by the transgene and by the virus was linked to DNA methylation. Methylation status of the 35S:C4 transgene following TLCV infection and the *C4* region in TLCV RF DNA was determined by bisulfite sequencing (Clark and Frommer 1997). Total genomic DNA was extracted from noninoculated 35S:C4 transgenic, TLCV-infected 35S:C4 transgenic, and TLCV-infected nontransgenic tobacco leaves at 60 dpi and subjected to methylation sequencing. In the procedure, bisulfite treatment converts unmethylated cytosines to uracil while methylated cytosine remains unchanged. The converted DNA then is cloned and subjected to

sequencing. To ascertain DNA conversion, we determined the methylation status of the tobacco 5S rRNA gene (Fulnecek and Matyasek 1998) in bisulfite-treated samples. The three samples were found to have very similar methylation status in the 5S rRNA gene: all the 11 symmetric cytosines were methylated and all the 15 asymmetric cytosines were unmethylated (data not shown). Thus, a total of 42% cytosines were methylated, a rate consistent with the previous report of tobacco 5S rDNA methylation (Fulnecek and Matyasek 1998).

The region of the 35S promoter and *C4* ORF was cloned using bisulfite-treated total DNA from TLCV-inoculated and noninoculated 35S:C4 transgenic plants. Five individual clones from each sample were sequenced and the sequences were aligned (Fig. 4A). An example of sequencing pattern is shown in Figure 4C. A high level (86 to 92%) of symmetric cytosine methylation was observed in both TLCV-infected and noninfected 35S:C4 transgenic plants (Table 1; Fig. 4B). The difference in the levels of symmetric cytosine methylation between infected and healthy transgenic plants was not statistically significant (Fig. 4B). In contrast to the levels of symmetric cytosine methylation, there was a statistically significant ($P = 0.032$) increase in asymmetric cytosine methylation of the transgene after TLCV infection. In the noninoculated plants, only 6.4% of asymmetric cytosines were methylated (Table 1; Fig. 4B); whereas, in infected 35S:C4 transgenic plants, 39.3% of asymmetric cytosines were methylated (Table 1; Fig. 4B). Cytosine methylation was observed not only in the *C4* coding region which has homology with TLCV DNA but also in the 35S promoter region with no homology to the viral genome (Table 1).

Fig. 3. In situ hybridization showing *Tomato leaf curl virus* (TLCV)-induced silencing of the 35S:C4 transgene. The sections were derived from **A**, healthy, nontransgenic tobacco, **B**, healthy transgenic tobacco, **C** and **D**, TLCV-infected transgenic tobacco, and **E**, TLCV-infected nontransgenic tobacco plants. The probes in **A** through **C** were fluorescence-labeled, virion-sense single-stranded (ss)RNA from the *C4* open reading frame (ORF) and in **D** and **E** were digoxigenin-labeled, complementary-sense ssRNA from the V2 ORF. The sections were examined using differential interference contrast optics. Hybridization signals are indicated by arrows. Bars = 100 µm, M = mesophyll, X = xylem, and P = phloem.

A

B

C

Fig. 4. Analysis of cytosine methylation of the 35S:C4 transgene. Genomic DNA extracted from either healthy or *Tomato leaf curl virus* (TLCV)-infected transgenic plants at 60 days postinoculation was treated with bisulfite, amplified by polymerase chain reaction (PCR), and cloned. Five individual clones were sequenced for each treatment. **A**, The 35S:C4 sequence shown includes the complete C4 open reading frame (ORF), nucleotides -1 to -98 of the 35S promoter, and nucleotides 1 to 45 of the NOS terminator. The initiation and termination codons are boxed. The positions of PCR primers used for cloning are underlined. Methylated cytosines in TLCV-infected (I) and healthy (H) transgenic plants are shown above and below the 35S:C4 sequence, respectively. Methylated symmetric cytosines are represented by capital C, whereas methylated asymmetric cytosines are in bold italic C. Dots represent other nucleotides, including unmethylated cytosines. The numbers to the right of the figure represent individual clones analyzed. **B**, The percentage of methylated symmetric and asymmetric cytosines in the 35S promoter and the C4 ORF was calculated separately for each individual clone. The averages and standard deviation calculated from the five independent clones analyzed are shown. **C**, Part of a chromatogram showing the quality of sequencing data for one of the clones is shown as an example.

Given that *Geminivirus* replication occurs in the host cell nuclei (Rojas et al. 2005), we questioned whether TLCV DNA also was subject to methylation by the host. A fragment of DNA containing *C4* ORF was amplified by polymerase chain reaction (PCR) from the bisulfite-treated genomic DNA of TLCV-infected 35S:C4 transgenic and nontransgenic tobacco plants. The use of TLCV-specific primers outside the region present in the transgene ensured that the transgene was not amplified. PCR products were cloned and four individual clones were sequenced from each sample and aligned (Fig. 5A and C). Interestingly, the TLCV DNA region sequenced contained methylated cytosines at both symmetric and asymmetric positions (Fig. 5A). The levels of cytosine methylation at either symmetric or asymmetric sites of TLCV-infected transgenic and nontransgenic plants ranged between 36 to 54% (Table 1; Fig. 5B). These differences are not statistically significant. These results indicated that TLCV DNA was methylated during its infection cycle, although at significantly lowers levels compared with the transgene, and that the methylation of TLCV RF DNA was independent of the presence of 35S:C4 transgene. We also noted that the level of cytosine methylation in a 50-nt segment of the noncoding intergenic region, which was present in the methylation sequencing data, was close to the methylation level of *C4* ORF (48 and 53%, respectively). Therefore, we have no evidence to suggest that TLCV DNA methylation was preferentially directed to either the coding region or noncoding region.

Comparisons of methylation data revealed a high level of standard deviation associated with TLCV-infection (Figs. 4B and 5B). Possible significance of this observation is discussed below.

DISCUSSION

RNA silencing is an effective defense tool against RNA viruses (Hamilton and Baulcombe 1999; Voinnet 2001). However, application of this strategy against geminiviruses has produced inconsistent results. This is probably the reason why numerous attempts have been made to develop transgenic resistance against geminiviruses (Covey and Al-Kaff 2000). For example, resistance against TYLCV considered originally to be mediated by truncated *Rep* (Brunetti et al. 1997; Noris et al. 1996) was found to involve RNA silencing (Lucioli et al. 2003) and to be overcome by the virus (Noris et al. 2004). More recently, several variations of the TYLCV *Rep* gene constructs were introduced into the tomato genome (Yang et al. 2004). The plants produced a range of responses to the virus, from susceptibility with some constructs to resistance with others. Within transgenic lines showing resistance, a proportion of the plants became infected despite the presence of the transgene.

More successful results have been reported with *African cassava mosaic virus* (ACMV), where *Rep* gene constructs produced variable but significant resistance to the virus. The resistance was suggested to involve RNA silencing rather than protein interference although it was effective against distinct virus species infecting cassava (Chellappan et al. 2004).

Given that our attempts to develop resistance against TLCV using a range of gene constructs have been unsuccessful, we questioned whether inducing an effective silencing by a hairpin construct provides protection against the virus. The *C2* hp transgenic plants produced a significant level of siRNA; nevertheless, they exhibited only delayed symptoms and were overcome by TLCV infection (Fig. 1).

Evidence supporting the ability of geminiviruses to escape RNA silencing also comes from the studies of these viruses as silencing vectors. A number of virus–host systems have been used successfully to demonstrate the silencing of introduced or endogenous plant genes by episomal replication of *Geminivirus*

vectors (Atkinson et al. 1998; Fofana et al. 2004; Peele et al. 2001; Tang and Leisner 1998; Tao and Zhou 2004; Turnage et al. 2002). These plant virus systems share a common feature with those targeted for virus-derived resistance. In both cases, the host plants share DNA sequence homology with the virus involved. In the cases of *Geminivirus*-induced gene silencing where virus replication in the silenced plant has been determined (Atkinson et al. 1998; Kjemtrup et al. 1998; Peele et al. 2001), the silencing of a plant gene does not prevent the replication of homologous virus. In line with these findings, we have observed that TLCV promoter-driven GUS transgenes become silenced when the plants are infected with the homologous virus, whereas the replication of TLCV itself is not affected significantly (Seemanpillai et al. 2003). Likewise, a drastic phenotype induced by the β C1 protein of a geminiviral satellite DNA was completely silenced upon inoculation of the plants with the satellite and the helper virus, while virus replication continued (Saeed et al. 2005).

The inconsistencies in reports of transgenic resistance and our lack of success with the *C2* hp transgenic plants led us to speculate that a TLCV-derived transgene may become silenced upon virus infection. To examine this possibility, we analyzed the fate of a 35S promoter-driven TLCV *C4* transgene following infection.

Analysis of 35S:C4 transgenic plants revealed high levels of symmetric cytosine methylation of the transgene, irrespective of whether these plants were infected with TLCV or were healthy. This is in agreement with our previous results (Seemanpillai et al. 2003), where TLCV promoter-driven GUS transgenes have high levels methylation of symmetric cytosines, and with the report that approximately 80% of symmetric cytosines in higher plant genomic DNA are methylated (Gruenbaum et al. 1981). It has been suggested that methylation of symmetric cytosines may be independent of their transcriptional activity (Pena et al. 2004).

Contrary to the methylation of symmetric cytosines, methylation of asymmetric cytosines in the 35S:C4 transgene was quite low but increased significantly following TLCV infection of the plants. Consistent with this finding, VIGS by both an RNA virus (Jones et al. 2001) and a DNA virus (Rodman et al. 2002) has been reported to be associated with asymmetric cytosines methylation, a process linked to gene suppression (Dieguez et al. 1997). Therefore, it appears likely that the observed silencing of the *C4* transgene is linked to the enhanced

Table 1. Rates of methylation of 35S:C4 transgene and *Tomato leaf curl virus* (TLCV) *C1*-intergenic region

Regions ^a	Plants ^b	Cytosine methylation (%)	
		Symmetric	Asymmetric
Transgene			
35S Promoter	I	94.3	36.7
	H	89.7	7
<i>C4</i> ORF	I	80	46.9
	H	64	5.2
Combined	I	92.5	39.3
	H	86.5	6.4
TLCV RF			
<i>C1</i> -IR	T	52.6	48.9
	NT	31.9	33.7
<i>C4</i> ORF	T	64.3	46.2
	NT	53.6	58.2
Combined	T	54.9	47.5
	NT	36.1	45.9

^a ORF = open reading frame, RF = replicative form, and *C1*-IR = a segment of *C1* and intergenic region containing the entire overlapping *C4* ORF.

^b I = TLCV-infected tobacco plants, H = healthy tobacco plants, T = 35S:C4 transgenic tobacco plants, and NT = nontransgenic tobacco plants.

level of asymmetric cytosine methylation following TLCV infection. The asymmetric cytosine methylation may be a host response to TLCV infection which targets both the viral RF DNA (discussed below) and its transgene homologue.

Interestingly, asymmetric cytosine hypermethylation of the *C4* coding region of transgenic plants infected with TLCV extended to the 35S promoter (98 bp analyzed) (Fig. 4). Spread of methylation to transcribed sequences not homologous to the viral genome has been reported upon RNA virus infection (Jones et al. 1999; Van Houdt et al. 2003). Because our transgene contained a tandem-repeat of the 35S promoter, it is possible that a population of the transcripts was initiated from the first promoter element and, therefore, contained the promoter sequences.

We also observed a high level methylation of both symmetric and asymmetric cytosines in TLCV RF DNA. In contrast to this finding, methylation analysis of TGMV DNA by methylation-sensitive restriction enzymes has shown that the viral dsDNA is not appreciably methylated at 13 restriction sites tested in the entire DNA molecule (Brough et al. 1992). The reason for this difference remains unknown but may relate to the analytical approaches utilized. The bisulfite DNA sequencing used in our study reveals methylated positions across the entire target sequences rather than only at restriction sites. With respect to the levels of methylation, TLCV DNA resembles the host plant genome, where high levels of most symmetric cytosines are methylated (Gruenbaum et al. 1981). Like the plant DNA, geminiviral DNA replicates in the cell nuclei and

is organized in the form of minichromosomes (Pilartz and Jeske 1992). Therefore, it is possible that TLCV DNA is subjected to the same process that results in high levels of symmetric cytosines methylation in plants.

The replication of TLCV in silenced plants indicates that the viral genes, including complementary-sense genes, which are indispensable for the viral DNA replication, are expressed. Various possibilities can be envisioned to explain how TLCV gene expression can take place in a silenced background. The silencing process, although highly effective against the transgene (Fig. 2C), may be incomplete, leaving a small pool of

Table 2. Oligonucleotide primers

Name	Sequence (5' to 3') ^a
P1	G GG AATTCAGCAAGCGACCAGCAGAT
P2	G GGG ATCCTTAATTCTGAATCGA
P3	T TGA ATTCATGAGAATGGGGAGCCTC
P4	G TGG ATCCATTCCCTAAGGACGTTA
P5	tAgGTtTTtAAAGtAAGTGGATTGATGTGAtAtTtAt
P6	CCCTTATCccAAAACtACTCACACATTATTATAAaa
P7	CACATTATTATaaAaAAAATAaAaAaATAaATTtTA
P8	ACATAATCCTTAaaaaCTAATTCCCTAAaaACcTTAAaAa
P9	TTAttAAAATGttATAgGGTGtTtAAtTATAAAATAAGatt

^a Restriction sites are shown in bold type. Lowercase g and c denote bases complementary to methylated C in the DNA strand, sequence or in its complementary-sense strand, respectively. Lowercase t and a denote bases complementary to unmethylated C in the DNA strand, sequence or in its complementary strand, respectively.

Fig. 6. Model depicting methylation status of *Tomato leaf curl virus* TLCV DNA produced by **A**, the rolling circle replication (RCR) and **B**, the recombination-dependent replication (RDR). In RCR, double-stranded (ds)DNA is methylated in one strand (solid line) and is unmethylated in the other strand (broken line). Both DNA strands produced by RDR are unmethylated (broken lines). Roman numerals specify sequential steps in DNA replication. The figure has been redrawn from the published data (Jeske et al. 2001; Rigden et al. 1996).

viral transcripts that may be sufficient for TLCV replication. Alternatively, the silencing process may not be operating in the phloem cells where TLCV replication occurs (Fig. 3C). Such a possibility is plausible (Noris et al. 2004) because TLCV, like other begomoviruses, encodes a suppressor of RNA silencing (Selth et al. 2003). With TGMV, it has been found that the RNA silencing suppressor is present only in the virus infected cells (Wang et al. 2003). Here, we have shown that TLCV is restricted to phloem cells but the silencing of 35S:C4 transgene spreads systemically. This suggests that a silencing signal is produced in TLCV-infected cells and then spreads to other tissues. Therefore, the silencing process is functional in noninfected tissues where the TLCV-encoded suppressor protein is likely to be absent. Given the presence of various forms of viral DNA and viral transcripts in TLCV infected cells, we were unable to establish whether the transgene homologue was expressed from TLCV DNA in infected cells.

TLCV DNA has been found to replicate by a combination of RCR and RDR (Alberter et al. 2005). RCR is a semi-conservative process resulting in the production of RF DNA (Saunders et al. 1991) consisting of a de novo unmethylated strand and a preexisting methylated strand (Fig. 6). In eukaryotic cells, the pattern of methylation is copied rapidly from the preexisting to the de novo strand by methyltransferases that recognize methylated CG dinucleotides (Holliday and Pugh 1975; Smith et al. 1992). By contrast, both DNA strands produced by RDR are synthesized de novo and initially lack methylation. Although TLCV DNA showed a significant overall methylation, replication of TLCV DNA by RDR (Alberter et al. 2005) may give rise to a population of unmethylated, transcriptionally active DNA (Fig. 6B) that may become methylated subsequently. Consistent with this idea, in vitro methylated TGMV (Brough et al. 1992) and ACMV (Ermak et al. 1993) DNA have been reported to replicate with lower efficiencies in transfected protoplasts and to produce demethylated progenies (Brough et al. 1992; Ermak et al. 1993).

We observed a high level of variation in the methylation levels of TLCV RF DNA, as indicated by relatively large standard deviation (Fig. 5). For example, clone NT-1 (Fig. 5A) has only four methylated asymmetric cytosines in the C4 region compared with clone NT-4, which has 40 methylated asymmetric cytosines (Fig. 5A). Furthermore, the rate of symmetric cytosine methylation of TLCV RF DNA was considerably lower compared with the transgene (compare Figs. 4B and 5B). This is consistent with the suggestion that a proportion of TLCV DNA produced by RDR (Alberter et al. 2005) may not be efficient target for symmetric cytosine methylation by methyltransferases. Data in Figure 4B indicate that asymmetric cytosine methylation of the transgene also was variable in TLCV-infected plants. The reason for this variation remains unknown, although it could be speculated that this variation is the consequence of a sequence-specific process (e.g., siRNA mediated) that affects viral DNA methylation.

Assuming that the ability of TLCV to replicate in silenced plants is mediated by the lack of DNA methylation, it could be argued that this process is regulated by the viral C2 protein, which is a suppressor of gene silencing (Selth et al. 2003; Vanitharani et al. 2004; van Wezel et al. 2002; Voinnet et al. 1999). Wang and associates (2003) have demonstrated that AL2 (also designated AC2 and C2) of geminiviruses interacts with adenosine kinase (ADK) and have suggested that AL2 inhibits ADK activity by interfering with DNA methylation. ADK activity has been shown to play an important role in S-adenosylmethionine-dependent transmethylation (Lecoq et al. 2001). Our finding that TLCV RF DNA is highly methylated indicates that a C2-induced inhibition of methylation is not occurring in infected cells. It remains possible that a C2- or

ADK-mediated inhibition of methylation would contribute to the generation of a small pool of unmethylated TLCV RF sufficient for viral transcription.

We noted a high level of TLCV-specific siRNA in the virus-infected *N. benthamiana* plants (data not shown). Therefore, it would appear that TLCV has evolved the mechanisms to efficiently overcome the host silencing response. siRNA is known to mediate gene silencing through methylation of target sequences (Cao et al. 2003). It remains unclear whether TLCV DNA methylation is linked to the presence of virus-specific siRNA.

The detection of *Geminivirus*-specific siRNAs in infected hosts suggests that an RNA-silencing process is triggered in response to DNA virus infection. Our results indicate that TLCV has evolved the means of circumventing this silencing pathway. This may explain why the development of a virus-derived resistance against geminiviruses has provided inconsistent results, whereas these viruses continue to pose a significant challenge to global agriculture.

MATERIALS AND METHODS

Plant materials.

Tobacco (*N. tabacum* cv. Samsun), tomato (*Solanum lycopersicon* L. var. Money Maker), and *N. benthamiana* were grown under glasshouse conditions at 25 to 30°C. Transgenic tobacco lines described previously (Krake et al. 1998), carrying a CaMV 35S promoter-driven C4 ORF cassette, were used under containment.

TLCV infectivity assays.

Plants at four- to five-leaf stage were inoculated with an overnight culture of *Agrobacterium tumefaciens* (strain C58) containing a TLCV dimeric construct in pBin19 (Dry et al. 1993). Developing leaves from individual plants (50 to 100 mg) were sampled and homogenized with 0.5 M NaOH at a ratio of 4 µl of buffer per 1 mg of tissue. The presence of TLCV DNA was tested by dot-blot analysis (Stonor et al. 2003).

Preparation of TLCV-specific RNA probes.

TLCV primers corresponding to the V2 (Table 2, P1 and P2) and C4 (Table 2, P3 and P4) ORFs were used to amplify DNA fragments by PCR. The DNA was ligated into a pGEM-T Easy vector (Promega Corp., Madison, WI, U.S.A.). The clones were linearized by suitable restriction enzymes and used as templates for transcription using T7 or SP6 DNA-dependent RNA polymerase (Promega Corp.) in the presence of $\alpha^{32}\text{P}$ -UTP according to the manufacturer's instruction. RNA probes used for in situ hybridization were labeled with fluorescein-12-dUTP (for the probe from the complementary-sense gene) and digoxigenin-11-dUTP (for the probe from the virion-sense gene) using Fluorescein or DIG RNA Labeling Mix, respectively (Roche Diagnostics, Castle Hill, Australia). These probes were digested to approximately 200 bp by carbonate hydrolysis as described (Guerin et al. 2000).

Gel blot analysis.

Total RNA was extracted from expanding leaves (100 mg) using the TRIzol reagent (Invitrogen, Carlsbad, CA, U.S.A.) according to the manufacturer's instructions. mRNA was isolated from the total RNA by a poly-A mRNA extraction kit (Promega Corp.). mRNA obtained from 20 µg of total RNA was analyzed in a 1.2% agarose MOPS gel containing 2 M formaldehyde (Selth et al. 2003). siRNA analysis of total RNA (5 µg) was carried out by electrophoresis in a denaturing 15% polyacrylamide, 7 M urea gel using the Mini-PROTEAN 2 electrophoresis system (BioRad, Hercules, CA, U.S.A.) (Wang

et al. 2001). The nucleic acids were blotted onto Hybond⁺ membrane (Amersham Biosciences, Piscataway, NJ, U.S.A.) and hybridized with ³²P-labeled TLCV RNA probes at 42°C overnight in 50% formamide, 7% sodium dodecyl sulfate, 250 mM NaCl, and 125 mM NaH₂PO₄. The membrane was washed as described (Wang et al. 2001) and hybridization signals were detected using Kodak X-ray film. Southern blot analysis was carried out as described (Dry et al. 1993).

Plant transformation.

The C2 hp construct under the control of CaMV 35S promoter was made by insertion of TLCV DNA sequence spanning coordinates 1,627 to 1,223 in sense and antisense orientations into the pHannibal vector (Wesley et al. 2001). The integrity of the inserts was checked by nucleotide sequencing. The *NorI* fragment containing the TLCV inserts was cloned into the pBS389 binary vector (Wesley et al. 2001). The plasmid then was introduced into *A. tumefaciens* (LBA4404). Transformation of tomato with the C2 hp construct was performed as described (Frary and Earle 1996).

In situ hybridization.

Leaf samples were collected from TLCV-inoculated (at 60 dpi) and noninoculated 35S:C4 transgenic and nontransgenic plants. The tissues were fixed as described (Nadeau et al. 1996) and sections, 6 to 8 µm thick, were placed on silane-coated slides (ProSciTech, Thuringowa Central, Australia). Prehybridization, hybridization, and posthybridization were performed as described (Guerin et al. 2000). Probes were detected using Fast Red (Roche Diagnostics) or Western Blue (Promega Corp.) substrates. The slides were dehydrated, mounted in entellan mounting medium, and observed under a light microscope (Axioscope2; Carl Zeiss, Jena, Germany).

Bisulfite DNA sequencing.

Bisulfite modification and sequencing were performed as described (Grunau et al. 2000). The 5S rRNA genes in the treated samples were sequenced as a control using the primers reported (Fulnecek and Matyasek 1998) to monitor bisulfite conversion rate. It was assumed that the cytosines in symmetric (CpG and CpNpG) sites were methylated and cytosines in asymmetric (CpHpH) sites unmethylated. The PCR primers were selected following the guidelines described (Clark and Frommer 1997). In selecting PCR primers, regions containing symmetric sites were avoided and, when this was not possible, a G residue was used instead of the C in the primers. The primers were targeted against unmethylated DNA, so that a lack of methylation in the result would not be attributed to the failure of bisulfite conversion. Amplification of the 35S:C4 transgene, including 98 bp of the 35S promoter and complete C4 ORF, was carried out by nested PCR in two consecutive reactions. The primer pair P5 and P6 (Table 2) was used in the first reaction and the primer pair P5 and P7 (Table 2) was used in the second reaction. The primer pair P8 and P9 (Table 2) was used to amplify a part of the intergenic region (TLCV coordinates from 2,706 to 2,614 bp) and a part of C1 ORF (TLCV coordinates from 2,614 to 2,140 bp) overlapping the complete sequence of C4 ORF (TLCV coordinates from 2,464 to 2,156 bp). PCR was carried out as described (Grunau et al. 2000). PCR products were separated by electrophoresis in a 1% agarose gel, purified using the QIAquick gel extraction kit (Qiagen, Clifton, Australia), ligated into pGEM-T-Easy (Promega Corp.), and introduced into *Escherichia coli* XL1-Blue cells. Plasmid DNA was prepared from *E. coli* using a Qiagen Spin miniprep kit (QIAgene) and cloned DNA sequenced using ABI Prism BigDye (Perkin Elmer, Wellesley, MA, U.S.A.) with T7 or SP6 primers.

Statistical analysis.

Statistical comparisons of methylation data from 35S:C4 transgene and TLCV C4 coding region and upstream noncoding region (nucleotides 2,706 to 2,465 of the intergenic region) were done by individual unpaired *t* tests using the Graphpad Instant statistical software package. Due to unequal variances in asymmetric cytosine methylation in TLCV infected and healthy 35S:C4 transgenic plants, *t* tests were performed on the log-transformed data.

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