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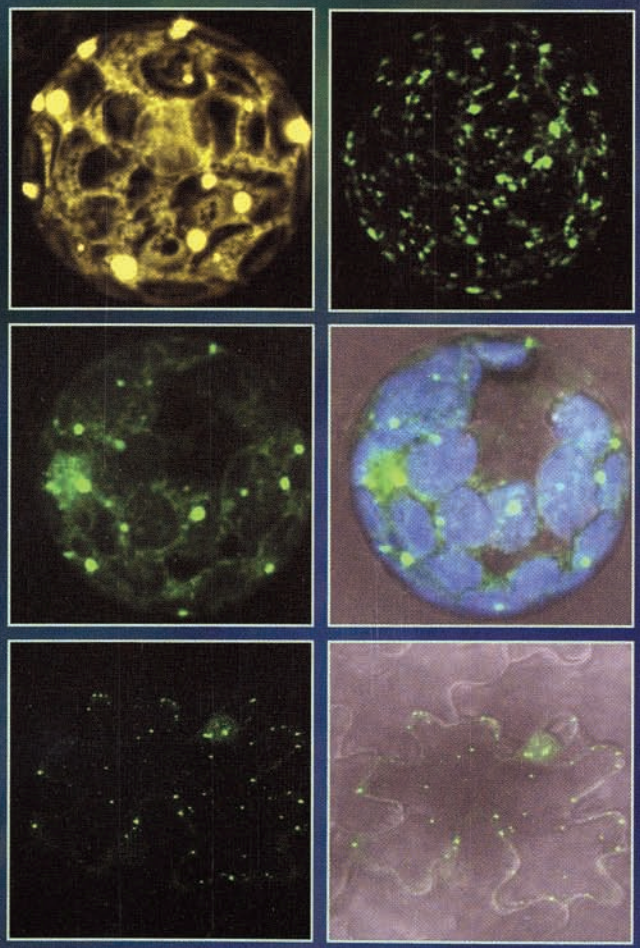

Cover Legend: Sub-cellular localization of TYLCV-Is V2. Please see the article by A. Zrachya et al. in this issue.

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Suppressor of RNA silencing encoded by *Tomato yellow leaf curl virus*-Israel

Avi Zrachya [a], Efrat Glick [a], Yael Levy [a], Tzahi Arazi [a], Vitaly Citovsky [b], Yedidya Gafni [a,*]

[a] *Institute of Plant Sciences, Agricultural Research Organization, Volcani Center, P.O. Box 6, Bet Dagan 50250, Israel*
[b] *Department of Biochemistry and Cell Biology, State University of New York, Stony Brook, NY 11794-5215, USA*

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Abstract

The Israeli isolate of *Tomato yellow leaf curl geminivirus* (TYLCV-Is) is a major tomato pathogen, causing extensive crop losses both in the New and Old World. Surprisingly, however, little is known about the molecular mechanisms of TYLCV-Is interactions with tomato cells. Here, we have identified a TYLCV-Is protein, V2, which acts as a suppressor of RNA silencing and which is unrelated to presently known viral suppressors. Specifically, V2, but not other proteins of TYLCV-Is, inhibited RNA silencing of a reporter transgene, *GFP*. This inhibition elevated the cellular levels of the *GFP* transcript and the GFP protein, but it had no apparent effect on the accumulation of *GFP*-specific short interfering RNAs (siRNAs), suggesting that TYLCV-Is V2 targets a step in the RNA silencing pathway which is subsequent to the Dicer-mediated cleavage of dsRNA. Visualization of the sub-cellular localization of TYLCV-Is V2 in plant protoplasts and tissues showed that this protein is associated with cytoplasmic strands and inclusion bodies in the cortical regions of the cell.

Keywords: Tomato yellow leaf curl virus; RNA silencing suppressor; Sub-cellular localization

Introduction

Tomato yellow leaf curl virus (TYLCV), a whitefly-transmitted geminivirus, is a major tomato pathogen worldwide (Gafni, 2003; Moriones and Navas-Castillo, 2000; Nakhla and Maxwell, 1997), and its Israeli isolate, TYLCV-Is, causes extensive (up to 100%) crop losses in Israel and in the southeastern U.S. Unlike most other known whitefly-transmitted geminiviruses which divide their genome between two single-stranded (ss) DNA molecules (reviewed by Davies and Stanley, 1989; Gafni, 2003), TYLCV-Is contains only one genomic circular ssDNA molecule (Kheyr-Pour et al., 1991; Navot et al., 1991) which encodes six open reading frames (ORFs), four on the complementary (−) strand (C1, C2, C3 and C4) and two on the viral (+) strand (V1 and V2) (Navot et al., 1991). C1 and C3 are involved in viral replication, C2 is a host-range factor (Wartig et al., 1997) which also can function as an RNA silencing suppressor in a related TYLCV-China virus (Dong et al., 2003) and C4, which induces the disease symptoms (Jupin et al., 1994; Krake et al., 1998; Rigden et al., 1994) and localizes to the host cell periphery and interacts with plasmodesmata (Rojas et al., 2001, 2005), has been proposed to facilitate viral movement (Rojas et al., 2001, 2005). V1 is the capsid protein (CP) likely involved in interaction with viral genomes (Palanichelvam et al., 1998) and in their nucleo-cytoplasmic shuttling within the host cell (Kunik et al., 1998, 1999; Rhee et al., 2000; Rojas et al., 2001) (Fig. 1A). The function of V2–which is essential for TYLCV-Is infection, but is not involved directly in viral replication or movement (Wartig et al., 1997)–remains obscure, and it cannot be predicted because V2 has no homologs among proteins with known biological functions.

One of the fundamental aspects of the virus–plant host interaction is the plant innate defense via RNA silencing and its inhibition by virally encoded RNA silencing suppressor proteins. However, although suppressor proteins that counter the antiviral RNA silencing defense have been identified in many RNA and DNA viruses (e.g., Baulcombe, 2002; Brigneti et al., 1998; Chapman et al., 2004; Guo and Ding, 2002; Moissiard and Voinnet, 2004; Pooggin et al., 2001; Reed et al., 2003; Silhavy et

* Corresponding author. Fax: +1 972 3 968 3471.
E-mail address: ygafni@volcani.agri.gov.il (Y. Gafni).

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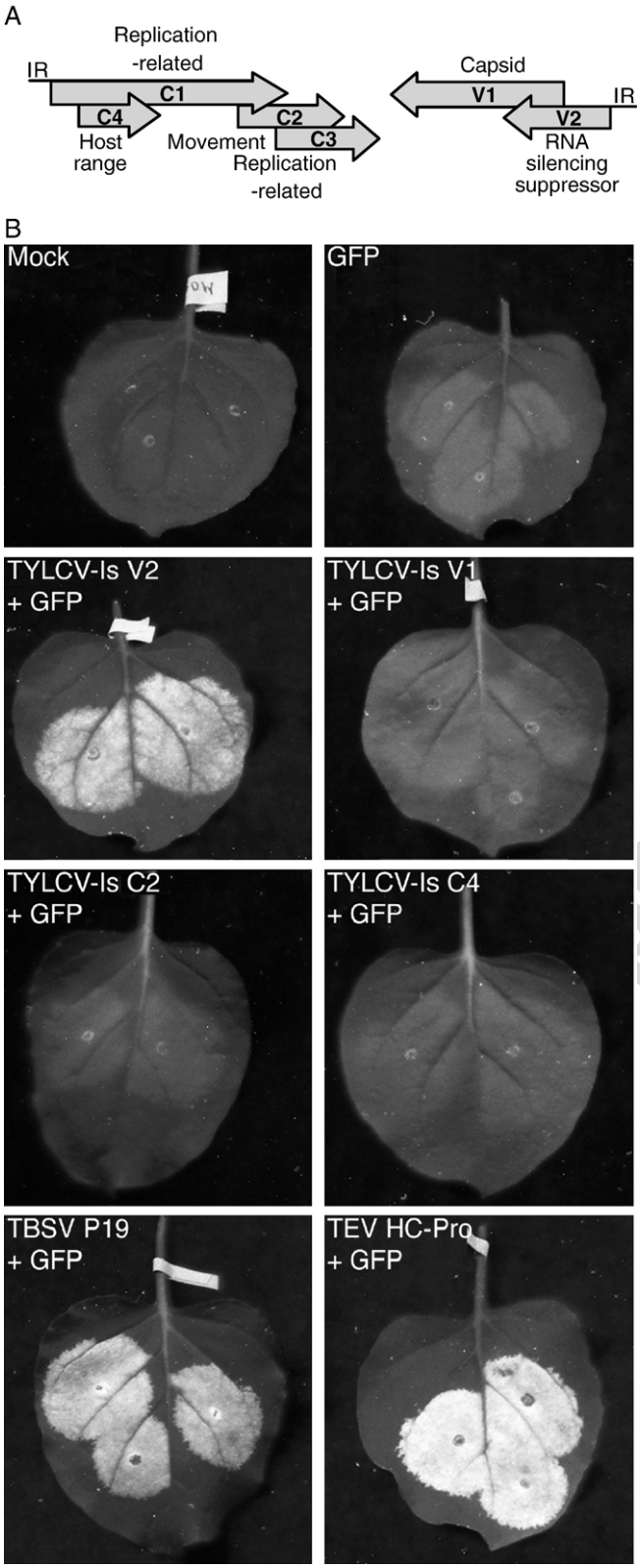

Fig. 1. (A) Map of the TYLCV-Is genome. The open reading frames are shown as boxes, and their potential roles in the virus infection cycle are indicated. Non-coding intergenic region (IR) is shown as lines. For clarity, the circular molecule of the viral genomic DNA is presented in a linear form. (B) *In planta* assay for RNA silencing suppression. GFP fluorescence was visualized at 7 dpi. Mock, mock infiltration with infiltration solution alone.

al., 2002; Vance and Vaucheret, 2001; Voinnet et al., 2000; Zamore, 2004), no such protein activity has been described for TYLCV-Is. Thus, we hypothesized that V2, one of the few TYLCV-Is proteins with as yet unassigned function, may act as an RNA silencing suppressor. Here, we substantiated this hypothesis by showing that TYLCV-Is V2, but not other proteins of this virus, inhibited RNA silencing of a reporter transgene, *GFP*. This inhibition elevated the cellular levels of the *GFP* transcript and the GFP protein, but it had no effect on the accumulation of *GFP*-specific short interfering RNAs (siRNAs). TYLCV-Is V2 partitioned between cytoplasmic strands and cortical bodies in the peripheral areas of plant cells.

Results

The TYLCV-Is V2 protein inhibits RNA silencing in plants

To identify the potential RNA silencing suppressor of TYLCV-Is, we tested all six proteins encoded by the genome of this virus (Navot et al., 1991) (Fig. 1A) for their ability to prevent silencing of a *GFP* reporter gene in plant tissues. Technically, we followed the protocol developed for studies of several other viral suppressors (Bucher et al., 2003, 2004; Johansen and Carrington, 2001). In this approach, two strains of *Agrobacterium* are infiltrated into leaves of a wild-type *Nicotiana benthamiana* plant; one strain carries the RNA silencing initiator *GFP* reporter gene and the other the candidate silencing suppressor gene. If the candidate gene possesses silencing suppression activity, it will induce a visible increase in GFP expression in the infiltrated leaf area (Bucher et al., 2003, 2004; Johansen and Carrington, 2001). Besides detecting the RNA silencing suppressor activity, this experimental system focuses on early events in gene silencing, as opposed to RNA silencing in systems which carry a stably integrated transgene (e.g., Brigneti et al., 1998; Dong et al., 2003).

Strong expression of GFP observed 2 days post infiltration (dpi) (not shown) was almost completely silenced at 7 dpi as assessed by observations of GFP fluorescence (Fig. 1B). This silencing of *GFP* was not affected by co-expression of TYLCV-Is proteins V1, C2, C4 (Fig. 1B), C1 and C3 (not shown). Only one TYLCV-Is protein, V2 (see Fig. 1A), suppressed silencing, substantially elevating the levels of GFP expression. In positive controls, *GFP* silencing was prevented by co-expression of known viral RNA silencing suppressors, TBSV P19 and TEV HC-Pro, which promoted even higher levels of GFP accumulation (Fig. 1B).

These results were confirmed by RT-PCR analyses of *GFP* transcripts (Fig. 2A) and Western analyses of the GFP protein (Fig. 2B). Fig. 2A shows that, at 2 dpi, PCR products corresponding to *GFP*-specific transcripts were detected in tissues transformed with GFP alone or in combination with TYLCV-Is C2, TYLCV-Is V2, or TBSV P19. At 7 dpi, however, expression of *GFP* alone or in the presence of TYLCV-Is C2 was reduced to almost undetectable levels, whereas the amount of *GFP* transcripts remained high. In a positive control, co-expression of TBSV P19 promoted accumulation of even higher amounts of the *GFP* transcript (Fig. 2A). Equal efficiency of all RT-PCR reactions was confirmed using a constitutively expressed

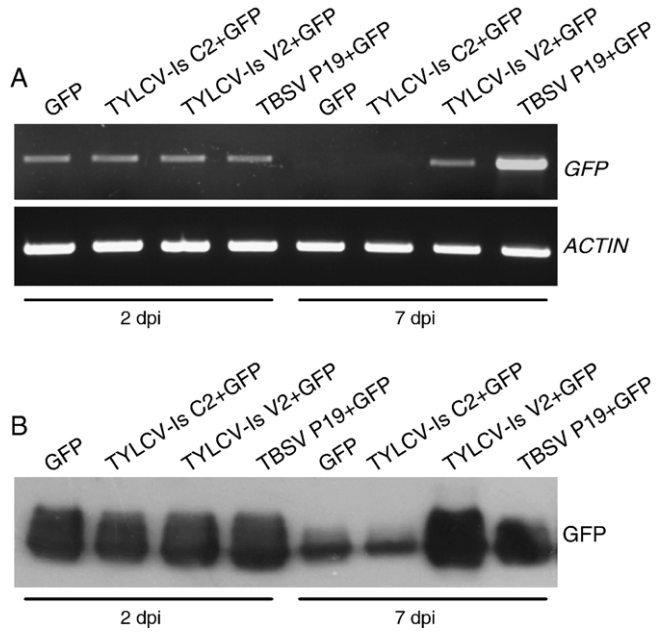

Fig. 2. (A) RT-PCR assay for the *GFP* mRNA. RT-PCR products specific for the *GFP* and *ACTIN* mRNA are shown in the top and bottom panels, respectively; dpi, days post infiltration. (B) Western blot assay for the GFP protein.

ACTIN gene (Fig. 2A). The changes in the levels of *GFP* transcripts closely correlated the total amounts of the GFP proteins accumulated in the transformed tissues and detected by Western blot analysis using anti-GFP antibodies (Fig. 2B), Western blotting detected relatively low amounts of GFP even in the tissues in which virtually no GFP mRNA-specific RT-PCR products were observed (Fig. 2), suggesting that the GFP protein may be somewhat more stable than its transcript. Taken together, these data suggest that TYLCV-Is V2 functions as a silencing suppressor. Interestingly, because TYLCV-Is V2 is unrelated in its sequence to all other known viral RNA silencing suppressors, it may represent a novel type of such suppressor.

TYLCV-Is V2 is a cytoplasmic protein

Sub-cellular localization of TYLCV-Is V2 was examined using autofluorescent tags GFP and its yellow spectral variant, YFP. To control for the position of the tag relative to TYLCV-Is V2, YFP was fused to the N-terminus of V2 or GFP was fused to the C-terminus of V2 and expressed in tobacco protoplasts and *N. benthamiana* leaf tissues. Fig. 3 shows that, in protoplasts, both YFP-V2 (panel A) and V2-GFP (panels B–D) were distributed in the cell cytoplasm, accumulating in inclusion bodies or aggregates which were observed mainly at the cell periphery. Similar sub-cellular localization patterns were observed following expression in leaf epidermis (Figs. 3E, F). This association of TYLCV-Is V2 with cytoplasmic strands and punctate bodies is reminiscent of the sub-cellular localization of a V2 homolog from the Dominican Republic isolate of TYLCV, TYLCV-DO (Rojas et al., 2001); however, unlike the TYLCV-DO homolog (Rojas et al., 2001), TYLCV-Is V2 did not move to neighboring cells (Figs. 3E, F).

TYLCV-Is V2 does not suppress accumulation of siRNAs

To obtain a better insight into how TYLCV-Is V2 suppresses RNA silencing, we examined whether or not it suppresses accumulation of siRNAs. Fig. 4A shows that, at 2 dpi, no GFP-specific siRNA was detected, consistent with strong GFP expression in these tissues (see Fig. 2). At 7 dpi, when the RNA silencing becomes established (see Figs. 1 and 2), 21–24-nt siRNAs have accumulated in the transformed tissues, and their amounts were unaffected by co-expression of the C2 and V2 proteins of TYLCV-Is. Consistent with previous observations (Reed et al., 2003), TEV HC-Pro also did not affect siRNA accumulation (Fig. 4A). Equal loading of all samples was confirmed by visualizing their tRNA content (Fig. 4A). Thus, TYLCV-Is V2 likely targets a step in the RNA silencing pathway which is subsequent to generation of siRNAs.

Interestingly, the RNA silencing suppressor activity of TYLCV-Is V2 was virus-specific because a V2 protein from a

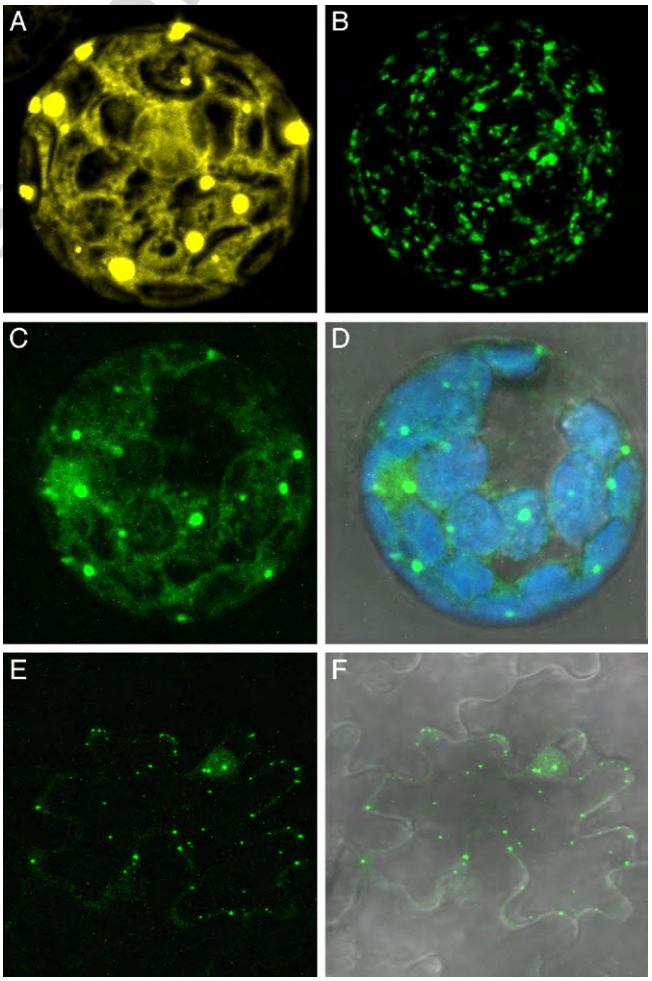

Fig. 3. Sub-cellular localization of TYLCV-Is V2. (A) YFP-V2 in tobacco protoplast. (B, C) V2-GFP in tobacco protoplast. (D) The GFP fluorescence image in panel B superimposed on plastid autofluorescence and the DIC image of the same cell. YFP signal is in yellow, GFP signal is in green, and plastid autofluorescence is in blue. (E) V2-GFP in *N. benthamiana* leaf epidermis. (D) The GFP fluorescence image in panel E superimposed on the DIC image of the same cell. GFP signal is in green, and plastid autofluorescence was filtered out. Fluorescence images are single confocal sections.

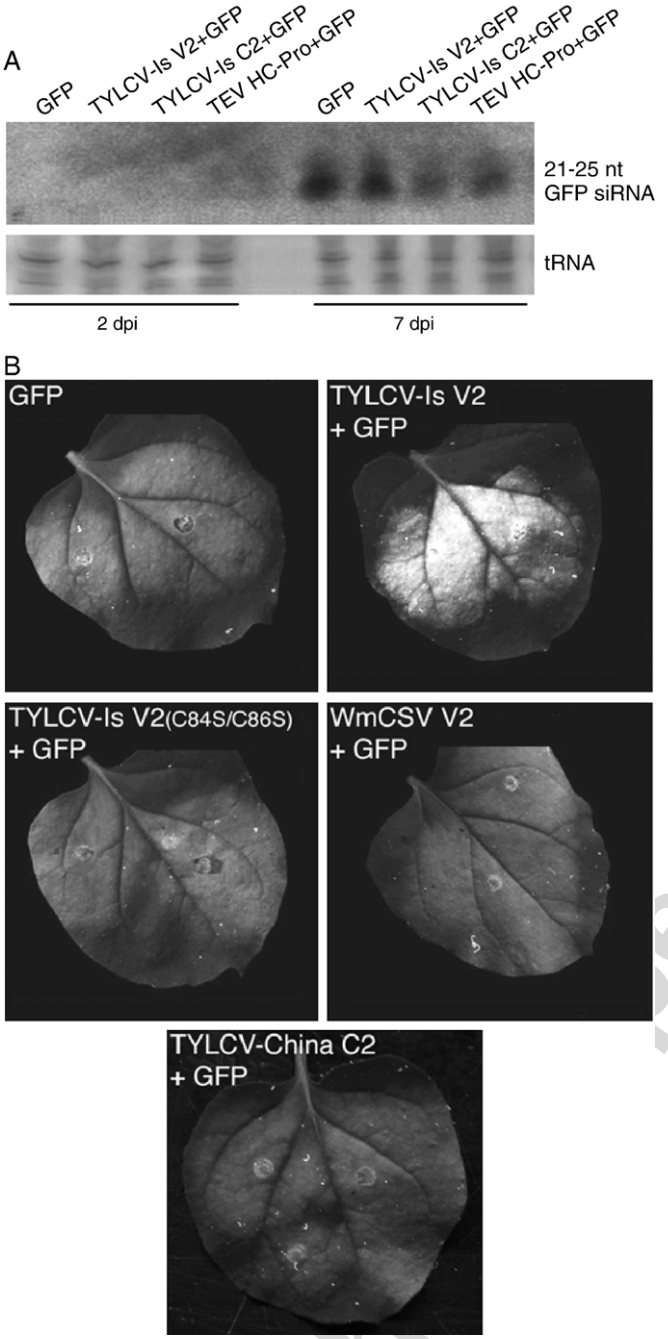

Fig. 4. (A) Northern blot analysis of *GFP*-specific siRNA. Autoradiogram detecting siRNA bands and ethidium-bromide-stained tRNA content of the same samples are shown in the top and bottom panels, respectively; dpi, days post infiltration. (B) The TYLCV-Is V2 (C84S/C86S) mutant, WmCSV V2 and TYLCV-China C2 are unable to suppress RNA silencing of *GFP in planta*.

bipartite geminivirus, Watermelon chlorotic stunt virus (WmCSV), was unable to suppress silencing of *GFP* in plant tissues (Fig. 4B). Furthermore, a C84S/C86S amino acid substitution mutant of TYLCV-Is V2 lost its ability to suppress silencing (Fig. 4B); a previous study showed that this mutation in the V2 homolog of the *Tomato leaf curl virus* (ToLCV) attenuated viral infection (Padidam et al., 1996), suggesting correlation between the RNA silencing suppressor activity of V2 and viral infectivity. In addition, the lack of the RNA suppression activity of the C84S/C86S mutant makes it a valuable negative control for the TYLCV-Is V2 activity in our assays.

Finally, we examined whether or not the TYLCV-China C2 protein, shown to act as a suppressor of long-term RNA silencing events (Dong et al., 2003), i.e., in plants that carry a stably integrated target transgene (Brigneti et al., 1998), also functions in our experimental system, i.e., transient expression of the target transgene, which focuses on earlier stages of silencing. Fig. 4B shows that TYLCV-China C2 had no suppressor activity in our assay, suggesting that this protein may indeed affect a stage of RNA silencing different from that targeted by the TYLCV-Is V2 protein.

Discussion

Antiviral RNA silencing response is thought to initiate with the production of the viral double-stranded (ds) RNA or partially dsRNA (Baulcombe, 2002, 2004; Molnár et al., 2005). In the case of TYLCV-Is, a monopartite DNA geminivirus, its transcripts produced in the host cell may trigger RNA silencing (reviewed by Bisaro, 2006); indeed, the strong fold-back structure of geminiviral mRNAs has been proposed to induce RNA silencing (Vanitharani et al., 2005), and a plant virus-derived siRNAs have been shown to originate mainly from secondary structures in viral ssRNAs (Molnár et al., 2005). Furthermore, abundant CP transcripts of monopartite geminiviruses that may serve as templates for the host RNA-dependent RNA polymerases (Vanitharani et al., 2005). To counteract this host defense, TYLCV-Is must encode an RNA silencing suppressor, but this functional activity has not been identified in the TYLCV-Is genome.

While bipartite geminiviruses have been shown to encode two types of suppressor proteins, AC2 or transcriptional activator protein, TrAP (Moissiard and Voinnet, 2004), and AC4 (Vanitharani et al., 2004, 2005), our knowledge about suppressors encoded by monopartite geminiviruses is more limited. To date, most studies focused on TYLCV-China, in which silencing suppression has been attributed to a TrAP homolog, the C2 protein (Dong et al., 2003; van Wezel et al., 2002; van Wezel et al., 2003). However, virally encoded silencing suppressors often do not share homology in their sequence or in other functions during the viral life cycle (Vanitharani et al., 2005), and we did not detect the suppressor activity in TYLCV-Is C2. Instead, having tested all TYLCV-Is proteins for suppression of RNA silencing, we observed this activity with the V2 protein. However, it is important to emphasize that our assay detected early evens in establishing the RNA silencing, whereas the experimental system used to study TYLCV-China C2 (Dong et al., 2003) was better suited for detection of the more long-term silencing events, such as chromatin remodeling (reviewed by Bisaro, 2006). Thus, it is tempting to speculate that TYLCV encodes two types of RNA silencing suppressors, V2 for earlier silencing events and C2 for later silencing events. The idea that V2 and C2 may act at different stages of virus–plant interactions is lent further support by the lack of the suppressor activity of TYLCV-

China C2 in our assay system. Alternatively, the silencing suppression function may have evolved independently and differently in these two isolates of TYLCV, i.e., TYLCV-Is has evolved to utilize its V2 protein as suppressor whereas TYLCV-China uses C2 as suppressor, potentially even at different stages of the silencing pathway; this possibility, however, is somewhat offset by a relatively high degree of identity of TYLCV-Is C2 and V2 to their TYLCV-China homologs, i.e., 64.4% and 70.7%, respectively, as calculated using the global alignment function of the LALIGN program (http://www.ch.embnet.org/software/LALIGN_form.html).

That V2 and C2 may target different steps of the RNA silencing pathway is consistent with the different sub-cellular localization patterns of these proteins: C2 is nuclear (Dong et al., 2003; van Wezel et al., 2001) whereas V2 is cytoplasmic. The TYLCV-Is V2 distribution within the cell cytoplasm was similar to that of TYLCV-DO V2 (Rojas et al., 2001) and of the p21 RNA silencing suppressor of *Beet yellow virus* (BYV) (Reed et al., 2003). In this regard, TYLCV-Is V2 was also similar to other viral suppressors exemplified by P19 of TBSV, HC-Pro of *Cowpea aphid-borne mosaic virus* (CABMV) and γb of *Poa semilatent virus* (PSLV) which reside outside of the host nucleus (Mlotshwa et al., 2002; Uhrig et al., 2004; Yelina et al., 2005), but unlike such suppressors as *Cucumber mosaic virus* (CMV) 2b, *Mungbean yellow mosaic virus-Vigna* (MYMV) AC2 and begomoviral DNAβ-encoded βC1 which are nuclear proteins (Cui et al., 2005; Lucy et al., 2000; Mayers et al., 2000; Trinks et al., 2005; Wang et al., 2004). The detailed functional significance of these differences in sub-cellular distribution of different viral silencing suppressors remains to be determined.

Collectively, our observations identify V2 as a viral factor likely responsible for counteracting the anti-viral RNA silencing defense of the host plants against the TYLCV-Is infection. TYLCV-Is V2 may exert this suppressor effect by targeting a step in the RNA silencing pathway which occurs after siRNA production by the Dicer-mediated cleavage of dsRNA. Besides helping us to better understand the process of the of TYLCV-Is–host plant interaction, this knowledge may help future development of new strategies to attenuate TYLCV-Is infection by interfering with the viral suppression of the host defense.

Materials and methods

Plasmid construction

For RNA silencing assays, each of the TYLCV-Is genes was PCR-amplified from the plasmid TOPO-TYLCV-Is (obtained from Dr. M. Lapidot, Volcani Center) and cloned into the *Bam*HI–*Not*I sites of the binary vector pBin19 (GenBank accession number U09365). A similar strategy was used to subclone the genes encoding WmCSV V2, TYLCV-China C2, TBSV P19 and TEV HC-Pro. All PCR reactions were performed using a high fidelity Pfu DNA polymerase (Promega) and their products were verified by sequencing. GFP-expressing binary plasmid pBin19-GFP was obtained from Dr. B. Epel (Tel Aviv University).

For sub-cellular localization studies, TYLCV-Is V2 was tagged either at its C-terminus with GFP or at its N-terminus with YFP by cloning a PCR-amplified *V2* gene into the *Bam*HI–*Sal*I sites of pBin-GFP and into the *Eco*RI–*Bgl*I I sites of pSAT6A-EGFP-C1 (Tzfira et al., 2005) or into the *Bam*HI–*Sal*I sites of pSAT6-EYFP-N1 (Tzfira et al., 2005), respectively. The pBin-based construct was used for agroinfiltration, and the pSAT6-based constructs were used for electroporation (see below).

To produce a C84S/C86S amino acid substitution mutant of TYLCV-Is V2, TYLCV-Is V2 mutagenesis, a silent point mutation was produced to create a recognition site for *Xho*I to cleave TYLCV-Is V2 at the nucleotide in position 201. This V2 derivative was inserted into the *Bam*HI–*Xho*I sites of pBin19 and into the *Xho*I–*Not*I sites of pGEM-T Easy (Promega). Using the mutagenic forward primer 5′-CTCGAGGGTTCGCCGAAGGCTGAACTTCGACAGCCCATACAGCAGCCGAGCTGCAGTCCCCATTGTCCAAGGCACAAACAAGCGACG-3′ and the wild-type reverse primer 5′-ATTGCGGCCGCTCAGGGCTTCGATACATTCTG-3′, the mutant sequence was generated from the pGEM-T Easy-based construct by PCR and subcloned into the *Xho*I–*Not*I sites of the pBin19-based construct.

Agroinfiltration of tissues and electroporation of protoplasts

For agroinfiltration, all binary plasmids were transferred to *Agrobacterium tumefaciens* (strain EHA105) by heat shock transformation, and the resulting bacteria were cultured overnight at 28 °C in LB medium containing 50 μg/ml kanamycin. Then, bacterial cultures were diluted 1:100 in fresh LB medium without antibiotics, grown to an optical density $A_{600}=0.5$, resuspended in 20 ml of an infiltration solution containing 10 mM $MgCl_2$, 10 mM MES pH 5.6 and 150 μM acetosyringone. For co-expression of GFP with different viral proteins, the corresponding bacterial cultures were mixed in 1:1 v/v ratio before agroinfiltration which was achieved by pressure infiltration of the bacteria into young leaves of 3-week-old greenhouse-grown *N. benthamiana* plants.

For protoplast transformation, leaf mesophyll protoplasts were isolated from *Nicotiana tabacum* L. cv. *Samsun* NN as previously described (Draper et al., 1988), and a mixture of 5 μg plasmid DNA and 15 μg calf thymus DNA was used for electroporation of 0.5 ml of protoplast solution as described (Fromm et al., 1985). Transformed protoplasts were incubated in the dark for 46–48 h at 27 °C prior to imaging.

Fluorescence imaging

Visual detection of GFP fluorescence in plant leaves was performed using a 100 W hand-held long-wave ultraviolet lamp (Black Ray model B 100AP, UV products, Upland, CA). Plants were photographed with a digital camera Canon EOS 20D. Photographic images were prepared using Adobe Photoshop version 9.0 (Adobe Systems, San Jose, CA).

For confocal laser-scanning microscopy, protoplasts or plant tissues were viewed directly under an Olympus IX81/FV500

microscope equipped with an argon laser (488 nm), a green helium/neon laser (543 nm) and the PLAPO60×oil/NA 1.4 WD 0.15 mm or PLAPO60×W/LSM/NA1.00 WD 0.15 mm objectives, as well as with a Nomarski differential interference contrast (DIC) lens for capturing transmitted light images. Image analysis was carried out using the MICA software (Cytoview, Israel).

RT-PCR

RNA (1 μg), extracted from frozen and ground infiltrated zones of leaves with TRI-reagent (Sigma-Aldrich) according to the manufacturer's instructions, was reverse-transcribed using Reverse-iTTM 1st Strand Synthesis Kit (ABgene) and PCR-amplified using primers specific for *GFP* (forward 5′-ATGGTGAGCAAGGGCGAGGAG-3′, reverse 5′-TTACTTGTACAGCTCGTCCATGCCG-3′ with 1 μg template and 20 cycles) and *ACTIN* (forward 5′-CAATGAGCTTCGTGTTGCACCC-3′, reverse 5′-CCGGTGCCCTGAGGTCCTTTTCC-3′ with 0.25 μg template and 30 cycles) which generated 780-bp and 540-bp products from the corresponding transcripts. RT-PCR products were resolved on a 1.5% agarose gel and detected by ethidium bromide staining. The specificity of the PCR products was verified by DNA sequencing.

Western blot analysis

Infiltrated leaf zones (1 g) were homogenized in 1 ml of 8 M urea, 4.5% SDS, 4.0% BME and 75 mM Tris–HCl pH 6.5. The resulting homogenate was centrifuged at 14,000 rpm for 10 min at 4 °C, mixed with sample buffer (Laemmli, 1970), boiled for 5 min and its protein content (30 μg) was resolved on a 15% SDS polyacrylamide gel (Laemmli, 1970) followed by electro-transfer to a nitrocellulose membrane. The membrane was blocked with 5% dry milk in phosphate-buffered saline (PBS) and incubated for overnight at room temperature with the anti-GFP primary antibody (Santa-Cruz Biotechnology, Inc.) in PBS. Immunoreactive bands were visualized using goat anti-rabbit IgG conjugated to horseradish peroxidase (Bio-Rad) and the ECL Western blotting detection kit (Amersham Biosciences).

Detection of 21–25-nt RNAs

Total RNA was extracted from plant tissue using a TRI reagent (Sigma-Aldrich). Total RNA (30 μg) was resuspended in 50% formamide and denatured for 10 min at 65 °C. After adding a one-third volume of 5× loading solution [4× TBE buffer (1× TBE is 45 mM Tris–borate, pH 8.0, 1 mM EDTA), 0.08% w/v bromophenol blue], samples were separated on a 15% polyacrylamide gel. The upper part of the gel was removed, stained with ethidium bromide and photographed to verify RNA separation and equal loading of samples. The lower part of the gel was blotted onto a Hybond N^+ membrane (Roche). To prepare the riboprobe, pGEM-T/GFP plasmid was linearized at its *Not*I site, and the *GFP* gene was transcribed *in vitro* from the T7 promoter in the presence of [α-^{32}P]UTP (3000 Ci/mmol) (Amersham) using the T7 Transcription Kit (MBI, Fermentas) according to the manufacturer's instructions. Hybridization was performed overnight at 30 °C in 50% formamide buffer followed by 30-min post-hybridization washes performed sequentially at 50 °C with 2× sodium chloride-sodium citrate buffer (SSC), 0.5× SSC and 0.2× SSC supplemented with 0.1% SDS followed by autoradiography on X-ray films at −80 °C.

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