Mutation of Three Cysteine Residues in *Tomato yellow leaf curl virus-China* C2 Protein Causes Dysfunction in Pathogenesis and Posttranscriptional Gene–Silencing Suppression

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The nuclear localized C2 protein of the monopartite begomovirus *Tomato yellow leaf curl virus-China* (TYLCV-C) contributes to viral pathogenicity. Here, we have investigated TYLCV-C C2 protein domains that play a role in the phenotype. Alignment of the C2 protein with 67 homologues from monopartite and bipartite begomoviruses revealed that a putative zinc-finger motif C₃₆-X1-C₃₈-X7-C₄₆-X6-H₅₃-X4-H₅₈C₅₉ and four potential phosphorylation sites (T₅₂, S₆₁, Y₆₈, and S₇₄) are highly conserved. When expressed from a *Potato virus X* (PVX) vector, TYLCV-C C2 protein mutants C2-T52M, C2-H58S, C2-C59S, C2-S61R, and C2-S74D, like the wild-type C2 protein, induced local necrotic ringspots and systemic necrosis in *Nicotiana benthamiana* plants. Mutants C2-H53P and C2-Y68D produced irregular necrotic lesions on inoculated leaves that were distinct from the wild-type phenotype. In contrast, mutants C2-C36R, C2-C38N, and C2-C46I induced chlorosis and mosaic symptoms rather than necrosis. We demonstrate that TYLCV-C C2, like its counterpart in the bipartite begomovirus *African cassava mosaic virus*, mediates suppression of posttranscriptional gene silencing (PTGS). Moreover, the individual mutations C36R, C38N, and C46I abolished the ability of C2 protein to suppress PTGS. These results suggest that the three cysteine residues within the putative zinc-finger motif are essential for C2 protein to induce necrosis and to act as a suppressor of PTGS.

Additional keywords: counter-defense, *Geminiviridae*.

Members of the family *Geminiviridae* are unique small circular single-stranded (ss) plant DNA viruses that replicate in the nucleus via double-stranded (ds) DNA intermediates by a rolling circle mechanism (Hanley-Bowdoin et al. 1999). The monopartite geminivirus *Tomato yellow leaf curl virus-China* (TYLCV-C) belongs to the genus *Begomovirus* (Yin et al. 2001). Similar to *Tomato yellow leaf curl virus-Israel* (TYLCV-Is), *Tomato yellow leaf curl virus-Sardinia* (TYLCV-S), *Tomato leaf curl virus-Australia* (ToLCV-Au), and *Ageratum yellow vein virus*, TYLCV-C has a monopartite genome resembling the bipartite begomovirus DNA A component,

typified by *African cassava mosaic virus* (ACMV) and *Tomato golden mosaic virus* (TGMV) (Dry et al. 1993; Hamilton et al. 1984; Kheyr-Pour et al. 1991; Navot et al. 1991; Stanley and Gay 1983; Tan et al. 1995). Geminiviruses encode several proteins required for viral DNA replication, regulation of gene expression, and virus movement in plants. One of the viral proteins is the transcriptional activator protein (TrAP, also known as AL2 or AC2). TrAP of TGMV and ACMV is involved in the regulation of viral coat protein (CP) and nuclear shuttle protein expression (Brough et al. 1992; Groning et al. 1994; Haley et al. 1992; Hartitz et al. 1997; Hong et al. 1996; Sunter and Bisaro 1991, 1992, 1997). TGMV TrAP is a phosphoprotein and binds zinc to facilitate optimal TrAP–ssDNA interaction. Its C-terminal acidic domain has been implicated in transcriptional activation (Hartitz et al. 1997). ACMV TrAP contributes to viral pathogenicity and acts as an efficient suppressor of posttranscriptional gene silencing (PTGS), a plant defense system against virus attack (Voinnet et al. 1999).

The monopartite begomovirus C2 protein, similar to its homologue TrAP, possesses an N-terminal basic domain, a C-terminal acidic domain, and an intervening core region containing a zinc-finger motif. An in vitro DNA binding assay indicated that TYLCV-S C2 protein, like TrAP, exhibited sequence-nonspecific ssDNA and dsDNA binding activity (Hartitz et al. 1997; Noris et al. 1996; Sung and Coutts 1996). On the other hand, genetic analysis suggested that TYLCV-S C2 protein plays a role in virus systemic spread and ToLCV-Au C2 protein regulates CP expression in planta, although the mechanisms remain to be established (Dry et al. 2000; Wartig et al. 1997). Recently, we showed that TYLCV-C C2 protein is localized in nuclei and is a pathogenicity determinant. Expression of TYLCV-C C2 protein in *Nicotiana benthamiana* plants from a *Potato virus X* (PVX) vector induced necrotic ringspots on inoculated leaves (van Wezel et al. 2001). Here, using PVX-mediated expression of TYLCV-C C2 protein mutants, we have identified amino acid residues that are required for viral pathogenesis and suppression of PTGS.

RESULTS

PVX-based constructs for expression of C2 protein and its mutant derivatives in plants.

TYLCV-C gene C2 encodes a small protein consisting of 134 amino acids. When compared to the TrAP homologues of

TGMV and TYLCV-S (Hartitz et al. 1997; Noris et al. 1996), three putative functional domains were assigned to the amino acid sequence of the TYLCV-C C2 protein (Fig. 1). Amino acids 1 to 31 represent the N-terminal basic domain with a putative nuclear localization signal, amino acids 32 to 104 are the central core which may have DNA binding activity, and amino acids 105 to 134 may represent the C-terminal acidic domain responsible for transcriptional activation of gene expression. Alignment of the amino acid sequences of the TYLCV-C C2 protein and 67 homologues from monopartite and bipartite begomoviruses revealed that C2 protein has a highly conserved putative zinc-finger motif C₃₆-X₁-C₃₈-X₇-C₄₆-X₆-H₅₃-X₄-H₅₈-C₅₉ and four highly conserved potential phosphorylation sites (T₅₂, S₆₁, Y₆₈, and S₇₄) (Fig. 1). These amino acid residues were targeted for mutagenesis analysis to test whether or not

they are involved in TYLCV-C C2 protein-mediated pathogenesis in plants. To achieve this, 10 C2 protein mutants, each having a single mutated amino acid, were produced (Tables 1 and 2).

Effects of individual amino acid replacement on C2 protein-mediated induction of necrosis in *N. benthamiana*.

RNA transcripts produced by in vitro transcription from each of the PVX constructs were infectious in *N. benthamiana*. The accumulation of viral RNAs reached a similar level in infected plants as shown by northern blot analysis (Fig. 2). Viral genomic (6.9 kb) and subgenomic RNAs (2.6 and 1.4 kb) containing either wild-type or mutant gene C2 were detected in systemically infected tissues using either PVX- or C2 gene-specific probes (Fig. 2A and B). As expected, the PVX CP subgenomic RNA (0.9 kb) was only detectable using a PVX-specific probe (Fig. 2B). These results suggested that the C2 gene and its mutant derivatives were maintained during PVX infection, and that wild-type and mutant C2 proteins should be expressed to comparable levels from the 1.4-kb subgenomic RNA under the control of the duplicated PVX CP promoter.

The biological effect of expression of wild-type and mutant C2 proteins was assessed by symptom development in plants infected with the PVX recombinants. Local lesions developed 3 to 5 days postinoculation (dpi) and systemic symptoms 6 to 7 dpi. Typical local symptoms on leaves inoculated with either PVX/C2, PVX/mC2, or PVX expressing C2 proteins containing single amino acid substitutions are shown in Figure 3. As reported previously, the wild-type C2 protein expressed from PVX/C2 induced unusual necrotic ringspots on inoculated leaves and necrotic vein banding on systemically infected leaves, whereas the nonsense mutant expressed from PVX/mC2 produced typical PVX local chlorotic lesions and systemic chlorosis and mosaic symptoms (van Wezel et al. 2001). The point mutations T52M, H58S, C59S, S61R, and S74D had little effect on C2 protein-mediated necrosis. Although expression of missense mutants C2-H53P and C2-Y68D induced necrosis, these two mutants produced irregular local necrotic lesions rather than the typical necrotic ringspots. Infection of plants by PVX/C2-C36R, PVX/C2-C38N, or PVX/C2-C46I did not produce a necrotic phenotype but induced chlorotic lesions on the inoculated leaves and systemic chlorosis and mosaic symptoms.

Consistent with northern blot analysis, gene C2 RNAs were detectable in infected plants by reverse transcriptase-poly-

Table 1. Primers used for mutagenesis

Primer	Sequence (5' – 3') ^a	Modification introduced
PP47	GGCTGTAatcgATGCGATCTTCG ^b	Clal ^c
PP48	GGCTGTAatcgATGtGATCTTCG ^b	Clal ^d
PP49	ATTGGcgccgAATACTCTTAAGAAATG	EagI
PP54	GCTGCA GgacGTc GTTCCTCTGTG	AatII
PP55	GGAACgAC gtc CTGCAGCTCAA ^b	AatII
PP57	GTTCCTCT ATG CaTGAATCCATGATTG	NsiI
PP58	GATTC AtG CA At AGGGGAACACTCA ^b	NsiI
PP59	ATTCTCTGAT Tctaga GCAGTGAGTAG	XbaI
PP60	CACTGC tctag AATCAGAGAATGGC ^b	XbaI
PP61	GTCCAG ATc TACACGCCATTCTCTG	BglII
PP62	TGGCGTGT AGAT CTCTGGACGGTC ^b	BglII
PP63	ATACAG Gatcc TTTTGACCGTCCAG	BamHI
PP64	GGTCAAAA ggat CCCTGTATTTCAAG ^b	BamHI
PP65	CAAC Ct AGGTAGATCAACCCG	AvrII
PP66	TGATCTAC TaGg GGTTGTCTTAC ^b	AvrII
PP67	GTAAGA Aatc CCACAAGGTAG	EcoRI
PP68	GTGG gaaT CTTACTTCGTTG ^b	EcoRI
PP69	ATTGT Gaat CTAAAGCAACGAAG	EcoRI
PP70	GCTTTAG Gaat TCACAATCATG ^b	EcoRI
PP71	TAGTTCCCT Tag GCGTGAATCC	AvrII
PP72	TCACG Cct AGGGGAACACTC ^b	AvrII
PP73	CTGATTGAGCT cg AGTGAGTAGTTC	XhoI
PP74	CTACTCA Tcg AGCTCAATCAGAGAAT ^b	XhoI

^a Introduced restriction endonuclease sites are underlined, modified nucleotides are in lower case and mutated codons are in bold font.

^b Coding strand primers; others are complementary to the coding strand.

^c C2 start codon ATG italicised.

^d Stop codon TGA introduced immediately downstream of C2 start codon.

Fig. 1. Amino acid sequence and potential functional domains of *Tomato yellow leaf curl virus-China* C2 protein. The putative nuclear localization signal is underlined. The putative zinc-finger motif is in bold. The aspartic acid and glutamic acid rich region is italicized. Conserved cysteine and histidine residues are indicated by ▲. Potential phosphorylation sites are indicated by arrows.

merase chain reaction (RT-PCR). Primers PP47 and PP49 allowed the amplification of a 424-bp fragment specific to gene C2 using RNA extracts from plants infected with PVX/C2, PVX/mC2, or PVX expressing C2 missense mutants (Fig. 4A). Specific restriction endonuclease digestion of each RT-PCR product yielded DNA fragments with the predicted sizes (Fig. 4B and C). Sequence analysis of RT-PCR products confirmed that neither reversion nor random spontaneous mutations had occurred in gene C2 during infection. These analyses showed that wild-type and mutant C2 proteins expressed in plants from PVX-based vectors had maintained their integrity. Our results demonstrate that the three cysteine residues, C₃₆, C₃₈, and C₄₆, impose a significant influence on C2 protein-mediated pathogenesis and amino acids H₅₃ and Y₆₈ may also contribute to this process.

C2 protein is a suppressor of PTGS.

To test whether it acts as a suppressor of PTGS, the TYLCV-C C2 protein was expressed as a green fluorescent protein (GFP) fusion protein from the PVX-based vector in transgenic *N. benthamiana* line 16c. *N. benthamiana* line 16c carries a single copy of the GFP gene under the control of the 35S promoter. Plants challenged with PVX developed typical local chlorotic lesions, and mosaic and chlorotic symptoms on systemically infected leaves. PVX infection had no effect on GFP expression in these plants (Fig. 5) which, like mock-inoculated plants, showed green fluorescence under long-wavelength UV light. However, plants inoculated with either PVX/GFP or PVX/mC2-GFP developed systemic symptoms by 7 dpi, but later recovered and showed only a very mild mosaic phenotype on young leaves. Silencing of GFP expression in these plants was first apparent approximately 7 dpi and was almost complete by 15 dpi. Under the same experimental conditions, plants infected with PVX/C2-GFP developed necrotic ringspots on the inoculated leaves and systemic necrosis. No recovery of infection was observed in these plants, and silencing did not occur (Fig. 5). In view of the dramatic effect of the three cysteine mutations on C2 protein-mediated pathogenesis, we also investigated whether these missense mutant proteins had altered silencing properties. Plants inoculated with PVX/C2-C36R-GFP, PVX/C2-C38N-GFP, and PVX/C2-C46I-GFP, like those infected with PVX/GFP and PVX/mC2-GFP, showed significant recovery from infection, and suppression of *gfp* gene silencing did not occur (Fig. 5).

The level of viral RNAs and *gfp* transgene mRNA in infected plants was investigated by northern blot analysis using C2-, PVX- and *gfp*-specific probes (Fig. 6). Consistent with the recovery phenotype, recombinant PVX RNAs were almost undetectable in plants challenged with PVX/mC2-GFP,

PVX/C2-C36R-GFP, PVX/C2-C38N-GFP, and PVX/C2-C46I-GFP. However, considerable amounts of PVX genomic and subgenomic RNAs were detected in plants infected with PVX/C2-GFP (Fig. 6). Indeed, a similar amount of viral RNAs was detected in plants showing no silencing and silencing suppression, associated with PVX and PVX/C2-GFP infection, respectively. Moreover, transgene *gfp* mRNA was only detected in mock-inoculated plants and in plants infected with either PVX or PVX/C2-GFP (Fig. 6C). Taken together, our data demonstrate that TYLCV-C C2 protein can suppress PTGS of both the *gfp* transgene and the *gfp* gene inserted into the PVX genome. The three cysteine missense mutations in the putative zinc-binding motif, described above, abolished the ability of C2 protein to suppress PTGS.

DISCUSSION

TrAP encoded by bipartite begomoviruses activates virion-sense gene expression at the level of transcription, contributes to viral pathogenicity and suppresses PTGS (Hanley-Bowdoin et al. 1999; Hong et al. 1997; van Wezel et al. 2001; Voinnet et al. 1999). TrAP, a zinc-binding phosphoprotein, interacts with ssDNA in preference to dsDNA in a sequence-nonspecific manner (Hartitz et al. 1997; Noris et al. 1996; Sung and Coutts 1996). However, lack of correlation between TrAP biochemical and biological properties confounds our understanding of the mechanisms by which it carries out these apparently distinct functions. We have initiated the functional analysis of gene C2 of the monopartite begomovirus TYLCV-C that expresses a protein homologue of bipartite begomovirus TrAP. We have demonstrated that none of the four highly conserved potential phosphorylation sites (T₅₂, S₆₁, Y₆₈, and S₇₄) is essential for C2 protein-mediated necrosis, although mutation of Y₆₈ produced a different necrotic phenotype, which may imply that phosphorylation at this position occurs in plants. Although TGMV TrAP is known to be a phosphoprotein (Hartitz et al. 1997), it may be that phosphorylation is not a prerequisite for TrAP-mediated pathogenesis but is required for other functions involving the control of viral or possibly host gene expression. Additionally, TrAP may be phosphorylated at less conserved amino acids rather than those mutated here. Indeed, amino acid sequence comparisons have shown that there were several potential phosphorylation sites present in New World begomovirus TrAPs that are absent in their Old World begomovirus counterparts, and vice versa (unpublished data).

Our results demonstrate that the motif C₃₆-X₁-C₃₈-X₇-C₄₆-X₆-H₅₃, which is conserved in TrAP homologues of all begomoviruses sequenced so far, may be biologically functional in C2 protein-mediated pathogenesis. The three cysteine residues

Table 2. Construction of C2 protein mutants

Construct	Primer pair ^a	Cloning sites ^b	Amino acid mutated
PVX/C2	PP47/PP49	<i>ClavEagI</i>	wild type
PVX/mC2	PP48/PP49	<i>ClavEagI</i>	nonsense mutant
PVX/C2-C36R	PP47/PP65 + PP66/PP49	<i>ClavAvrII</i> + <i>AvrII/EagI</i>	Cys → Arg
PVX/C2-C38N	PP47/PP67 + PP68/PP49	<i>ClavEcoRI</i> + <i>EcoRI/EagI</i>	Cys → Asn
PVX/C2-C46I	PP47/PP69 + PP70/PP49	<i>ClavEcoRI</i> + <i>EcoRI/EagI</i>	Cys → Ile
PVX/C2-T52M	PP47/PP57 + PP58/PP49	<i>ClavNsiI</i> + <i>NsiI/EagI</i>	Thr → Met
PVX/C2-H53P	PP47/PP71 + PP72/PP49	<i>ClavAvrII</i> + <i>AvrII/EagI</i>	His → Pro
PVX/C2-H58S	PP47/PP54 + PP55/PP49	<i>ClavAatII</i> + <i>AatII/EagI</i>	His → Ser
PVX/C2-C59S	PP47/PP73 + PP74/PP49	<i>ClavXhoI</i> + <i>XhoI/EagI</i>	Cys → Ser
PVX/C2-S61R	PP47/PP59 + PP60/PP49	<i>ClavXbaI</i> + <i>XbaI/EagI</i>	Ser → Arg
PVX/C2-Y68D	PP47/PP61 + PP62/PP49	<i>ClavBglII</i> + <i>BglII/EagI</i>	Tyr → Asp
PVX/C2-S74D	PP47/PP63 + PP64/PP49	<i>ClavBamHI</i> + <i>BamHI/EagI</i>	Ser → Asp

^a Either PP47 with the indicated noncoding strand primer or PP49 with the indicated coding strand primer were used for PCR amplification of the 5' and 3' regions of TYLCV-C gene C2.

^b The PCR products of the 5' and 3' region of gene C2 were digested with either *ClavI* or *EagI* and the indicated restriction endonuclease, and cloned into the *ClavEagI* sites of p45P46.

C₃₆, C₃₈, and C₄₆ are essential for this function whereas mutation of H₅₃ has a slight effect on the necrotic phenotype. Although extremely conserved, amino acids H₅₈ and C₅₉ exerted no obvious effect on the phenotype. Furthermore, we have provided experimental evidence that the C2 protein, like ACMV TrAP, is a suppressor of PTGS. Moreover, cysteine residues C₃₆, C₃₈, and C₄₆ in the putative zinc-finger motif are essential for C2 protein to suppress PTGS. Investigation of the contribution of the C₃₆-X1-C₃₈-X7-C₄₆-X6-H₅₃ motif to zinc binding and whether the three cysteine mutations affect zinc-

Fig. 2. Northern blot analysis of RNAs extracted 7 days postinoculation from mock-inoculated (lane 1) and virus-infected (lanes 2–14) plants. Lane 2, *Potato virus X* (PVX); lane 3, PVX/C2; lane 4, PVX/mC2; lane 5, PVX/C2-C36R; lane 6, PVX/C2-C38N; lane 7, PVX/C2-C46I; lane 8, PVX/C2-T52M; lane 9, PVX/C2-H53P; lane 10, PVX/C2-H58S; lane 11, PVX/C2-C59S; lane 12, PVX/C2-S61R; lane 13, PVX/C2-Y68D; lane 14, PVX/C2-S74D. Blots were hybridized with digoxigenin-labeled probes specific to **A**, PVX or **B**, gene C2 sequences. The positions and sizes (kb) of the genomic and subgenomic RNAs are indicated at the right.

Fig. 3. Phenotypes on inoculated *Nicotiana benthamiana* leaves. The panels show necrotic ringspots on leaves inoculated with *Potato virus X* (PVX)/C2 (C2), PVX/C2-T52M (T52M), PVX/C2-H58S (H58S), PVX/C2-C59S (C59S), PVX/C2-S61R (S61R), and PVX/C2-S74D (S74D); irregular necrotic lesions on leaves inoculated with PVX/C2-H53P (H53P) and PVX/C2-Y68D (Y68D); and chlorotic lesions on leaves inoculated with PVX/mC2 (mC2), PVX/C2-C36R (C36R), PVX/C2-C38N (C38N), and PVX/C2-C46I (C46I). Leaves were photographed 7 days postinoculation.

binding activity and the optimal interaction of the C2 protein with dsDNA and ssDNA is currently ongoing. It also will be interesting to examine whether this motif plays a direct role in the regulation of virion-sense gene expression.

PTGS occurs through homology-dependent degradation of RNA and is regarded as a fundamental process related to a wide range of epigenetic phenomena. In plants, PTGS represents an effective defense system against virus infection. On the other hand, plant RNA and DNA viruses have evolved a counter-defense mechanism by encoding suppressors of PTGS, which often contribute to viral pathogenicity. PTGS suppressors characterized so far include 2b, HC-Pro, P1, and P19 proteins from cucumoviruses, potyviruses, sobemoviruses, tombusviruses, PVX p25 movement protein, ACMV AC2 (TrAP) (Carrington et al. 2001; Voinnet 2001), and TYLCV-C C2 protein described here. There is much variation in the extent of suppression of PTGS by different viruses (Voinnet et al. 1999). Indeed, such proteins mediate suppression by targeting different steps in the PTGS pathway (Anandalakshmi et al. 2000; Voinnet et al. 2000). Considering the biochemical properties, nuclear localization, and biological functions involved in specific modulation of gene expression, the mechanism of suppressing PTGS triggered by TYLCV-C C2 protein and its ACMV homologue may be distinct and remains to be elucidated.

MATERIALS AND METHODS

Plasmid constructs.

Ten individual C2 protein mutants, each of which had a single amino acid replaced with another of unrelated physicochemical properties, were constructed. The primers shown in

Fig. 4. Detection of C2 protein mutations by reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. RNA was extracted 7 days postinoculation from mock-inoculated (lane 1, negative control) and virus-infected (lanes 2–14) plants. Lane 2, *Potato virus X* (PVX); lane 3, PVX/C2; lane 4, PVX/mC2; lane 5, PVX/C2-C36R; lane 6, PVX/C2-C38N; lane 7, PVX/C2-C46I; lane 8, PVX/C2-T52M; lane 9, PVX/C2-H53P; lane 10, PVX/C2-H58S; lane 11, PVX/C2-C59S; lane 12, PVX/C2-S61R; lane 13, PVX/C2-Y68D; lane 14, PVX/C2-S74D; Lane M, 1-kb DNA ladder (Invitrogen, Groningen, The Netherlands). **A**, 1.4% agarose gel electrophoresis of C2 gene specific RT-PCR products. The size (bp) of RT-PCR products is indicated. **B** and **C**, Restriction endonuclease analysis of RT-PCR products. The positions and sizes of DNA markers (bp) are indicated to the left of each gel.

Table 1 were designed to introduce specific restriction endonuclease sites in the C2 protein coding sequence in order to modify selected amino acids. Sequences corresponding to 5' and 3' regions of the C2 gene were amplified by PCR using either PP47 and the appropriate noncoding strand primer or PP49 and the appropriate coding strand primer. PCR products corresponding to the two halves of the gene were digested with either *Cla*I or *Eag*I, together with the restriction endonuclease appropriate to the introduced site, and were cloned into *Cla*I/*Eag*I-digested PVX-based vector p45P46 (van Wezel et al. 2001) to produce the mutants described in Table 2. Each of the mutations was confirmed by restriction endonuclease analysis and nucleotide sequencing. The wild-type C2 protein coding sequence and a nonsense mutant derivative were PCR-amplified using primer pairs PP47/PP49 and PP48/PP49, respectively, and were cloned as *Cla*I/*Eag*I fragments into p45P46 as previously described to produce PVX/C2 and PVX/mC2 (van Wezel et al. 2001).

Virus infection and plant maintenance.

RNA transcripts were produced by in vitro transcription from each of the PVX constructs after linearization with *Spe*I, and mechanically inoculated to *N. benthamiana* plants as described (Chapman et al. 1992). Plants were maintained in an

insect-free growth room at 25°C with continuous lighting to give a 12-h photoperiod. Local and systemic symptom development in inoculated plants was assessed on a daily basis and photographically recorded using a Nikon Digital Camera Coolpix990.

RNA extraction and northern blot analysis.

Total RNAs were extracted from leaf tissues using an RNeasy plant mini kit (Qiagen, Hilden, Germany). RNA aliquots (2.5 µg) were fractionated on a 1% formaldehyde agarose gel, transferred to nylon membrane, hybridized with digoxigenin (DIG)-labeled probes specific to either PVX, C2, or *gfp* gene sequences, and immunodetected using a DIG DNA labeling and detection kit (Roche Diagnostics, Mannheim, Germany).

PTGS suppression assay.

The GFP coding sequence was isolated from TXS.GFP-CP (Santa Cruz et al. 1996) as an *Eag*I/*Bsp*EI fragment and fused in-frame to the coding sequences of C2-C36R, C2-C38N, and C2-C46I in PVX/C2-C36R, PVX/C2-C38N, and PVX/C2-C46I, to produce PVX/C2-C36R-GFP, PVX/C2-C38N-GFP, and PVX/C2-C46I-GFP, respectively. PVX/GFP, PVX/C2-GFP, and PVX/mC2-GFP were constructed as described previously (van Wezel et al. 2001). Seedlings of transgenic *N. benthamiana* line 16c carrying the *gfp* gene (Brigneti et al. 1998) were mechanically inoculated with RNA transcripts produced

Fig. 5. Suppression of posttranscriptional gene silencing in green fluorescent protein (GFP) transgenic *Nicotiana benthamiana* plants by *Potato yellow leaf curl virus-China* C2 protein. Plants were observed under long-wavelength UV illumination through a Wratten 58 (green) filter at 15 days postinoculation. Green fluorescence occurs in mock inoculated and *Potato virus X* (PVX)- and PVX/C2-GFP-infected transgenic plants. Silencing of *gfp* expression occurs in transgenic plants infected with PVX/GFP, PVX/mC2-GFP, PVX/C2-C36R-GFP, PVX/C2-C38N-GFP, and PVX/C2-C46I-GFP.

Fig. 6. Northern blot analysis of total RNA extracted at 15 days postinoculation from green fluorescent protein (GFP) transgenic *Nicotiana benthamiana* plants. Plants were either mock inoculated or infected with *Potato virus X* (PVX), PVX/GFP, PVX/C2-GFP, PVX/mC2-GFP, PVX/C2-C36R-GFP, PVX/C2-C38N-GFP, and PVX/C2-C46I-GFP. Blots were hybridized with digoxigenin-labeled probes specific to **A**, the *Potato yellow leaf curl virus-China* C2 gene **B**, PVX, and **C**, the *gfp* gene. The positions of the genomic (g) and subgenomic (sg) RNAs as well as the *gfp* transgene mRNA are indicated at the right. CP refers to coat protein.

by in vitro transcription from the *SpeI*-linearized plasmids. PTGS of GFP and suppression of PTGS was routinely examined under long-wavelength UV light after systemic symptoms appeared. Leaf samples were taken for RNA extraction and northern blot analysis as described above.

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