The EMBO Journal Vol.17 No.22 pp.6739–6746, 1998

Viral pathogenicity determinants are suppressors of transgene silencing in *Nicotiana benthamiana*

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Post-transcriptional gene silencing (PTGS) of a green fluorescent protein (GFP) transgene is suppressed in *Nicotiana benthamiana* plants infected with potato virus Y (PVY) or with cucumber mosaic virus (CMV), but not in plants infected with potato virus X (PVX). By expressing PVY and CMV-encoded proteins in a PVX vector we have shown that the viral suppressors of gene silencing are the HCPro of PVY and the 2b protein of CMV. The HCPro acts by blocking the maintenance of PTGS in tissues where silencing had already been set, whereas the 2b protein prevents initiation of gene silencing at the growing points of the plants. Combined with previous findings that viruses are both activators and targets of PTGS, these data provide compelling evidence that PTGS represents a natural mechanism for plant protection against viruses.
Keywords: cucumber mosaic virus/gene silencing/potato virus X/potato virus Y/virus resistance

Introduction

Post-transcriptional gene silencing (PTGS) in transgenic plants involves sequence-specific degradation of RNA. The targeted RNA species are similar to the transcribed part of a silencer transgene and, in plants exhibiting PTGS, there is only a low level of the transgene RNA even if transcription is at a high level (Depicker and Van Montagu, 1997). In addition, if the silencer transgene is similar to an endogenous gene, there is only a low level of the corresponding endogenous gene RNAs (Matzke and Matzke, 1995). PTGS can also be targeted against viral RNA, (Lindbo *et al*., 1993; Smith *et al*., 1994; Guo and Garcia, 1997) and extrapolating from this finding, it has been proposed that PTGS is a manifestation of a natural virus resistance mechanism in plants (Baulcombe, 1996; Pruss *et al*., 1997). According to this idea, PTGS is activated in plants when the transgene, or its RNA, is perceived as a virus (Ratcliff *et al*., 1997).

In support of the proposed relationship between PTGS and natural virus resistance, it has been shown that tobamo-, potex- and geminiviruses are activators as well as targets of gene silencing, provided they share sequence homology with a nuclear gene (Kumagai *et al*., 1995; English *et al*., 1996; Kjemtrup *et al*., 1998; M.T.Ruiz *et al*., 1998). Furthermore, caulimo- and nepoviruses induce a PTGS-like resistance mechanism even if there is no sequence similarity between the virus and nuclear genes (Covey *et al*., 1997; Ratcliff *et al*., 1997). This mechanism causes the systemically infected leaves to be symptom-free, to have only low levels of the virus and to have RNA sequence-specific resistance against challenge virus infection (Ratcliff *et al*., 1997).

If there is a natural PTGS-like virus resistance in plants, it is likely that viruses would evolve strategies to avoid or suppress this mechanism. This idea was first developed based on the analysis of plants infected with two viruses in which the disease symptoms were more severe than in plants infected with either of the two viruses alone (Pruss *et al*., 1997). In plants infected with a potyvirus this synergism was due to suppression of a host defense mechanism by the P1-HC-protease (P1-HCPro) (Pruss *et al*., 1997). Following from this discovery, it was suggested that P1-HCPro is targeted against a PTGS-like resistance mechanism.

A second candidate suppressor of a PTGS-like resistance mechanism is the 2b protein encoded in cucumber mosaic virus (CMV) (Ding *et al*., 1995). This protein is required for long distance transport of CMV (Ding *et al*., 1995) and is now thought to act by suppressing a host resistance mechanism (L.H.Ji, W.X.Li and S.W.Ding, in preparation). In the absence of this suppressor, the resistance mechanism would prevent entry, translocation or exit of CMV from the phloem of infected plants. It is conceivable that this resistance could rely on a PTGS-like mechanism.

Here, we test the hypothesis that the P1-HCPro and 2b proteins are suppressors of PTGS. Plants exhibiting PTGS of a green fluorescent protein (GFP) transgene were infected with a potyvirus (potato virus Y, PVY) or with CMV. We also infected silenced plants with potato virus X (PVX) and with chimeric constructs carrying coding sequences from PVY and CMV in a PVX vector. If PVY or CMV produce suppressors of a PTGS-like resistance mechanism we predicted that infection by PVY, CMV or the PVX vectors would interfere with PTGS. The outcome of these experiments was consistent with this prediction and reveals that HCPro and the 2b protein suppress different stages of PTGS. The results implicate a PTGS-like mechanism as a limiting factor in the accumulation and spread of PVY, PVX and CMV. Moreover, as these are unrelated viruses, it is likely that this mechanism is a generalized anti-viral defense in plants.

Results

Reversion of GFP silencing by wild-type PVY

From the analysis of plants infected with a potyvirus and a second virus, it had been shown that potyviruses encode

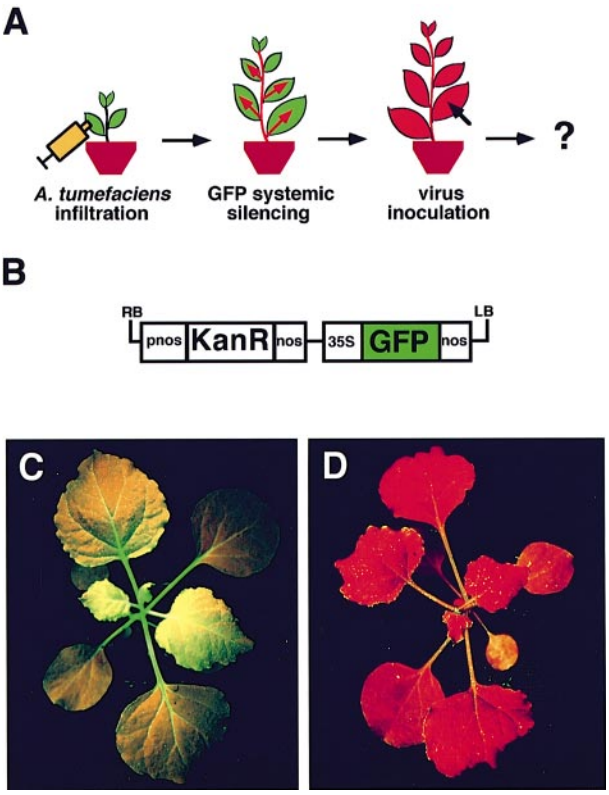

Fig. 1. PTGS of a GFP transgene induced by infiltration with *A.tumefaciens*. (**A**) Schematic representation of the experimental system. GFP silencing was induced in transgenic *N.benthamiana* (line 16c) by infiltration with a hypervirulent strain of *A.tumefaciens* carrying a binary-Ti plasmid shown in (B). Complete GFP silencing was achieved at 10 days post-infiltration and plants appeared uniformly red under UV illumination. It was at this stage that plants were ready for virus inoculation. (**B**) Structure of the binary-Ti plasmid cassette used to generate transgenic *N.benthamiana* plants (line 16c) expressing GFP and to induce GFP silencing in plants from line 16c by *A.tumefaciens* (strain cor308) infiltration. The right and left borders of the T-DNA (RB and LB) flank a kanamycin resistance gene (KanR) in a nos promoter (pnos) and nos terminator (nos) cassette. (**C**) *Nicotiana benthamiana* plant (line 16c) showing high levels of GFP expression under UV illumination. (**D**) *Nicotiana benthamiana* plant (line 16c) after induction of gene silencing by infiltration with a hypervirulent strain of *A.tumefaciens* carrying the binary-Ti plasmid shown in (B). The bright red colour is due to chlorophyll fluorescence under UV illumination.

a suppressor of a host plant defense against virus infection (Pruss *et al*., 1997). To investigate the relationship of this defense mechanism to PTGS we inoculated PVY, the type-member of the potyviridae, to transgenic *Nicotiana benthamiana* exhibiting PTGS of a GFP transgene. We predicted that there would be reversion of GFP silencing in the PVY-infected tissues if the suppressed defense mechanism is related to PTGS.

Our experimental system involves lines of *N.benthamiana* carrying a GFP transgene (Voinnet and Baulcombe, 1997; M.T.Ruiz *et al*., 1998). These plants accumulate a high level of GFP mRNA and appear uniformly green fluorescent under UV illumination (Figure 1C), whereas non-transformed plants appear red due to chlorophyll (M.T.Ruiz *et al*., 1998). PTGS of the GFP transgene (Figure 1A) was induced by infiltration of lower leaves of 3-week-old seedlings with a strain of *Agrobacterium tumefaciens*, as described previously (Voinnet and Baulcombe, 1997). This strain carried a binary-Ti plasmid containing the same GFP expression cassette that was used for plant transformation (Figure 1B). We showed previously that PTGS of GFP is initiated in the infiltrated zone and that a systemic GFP silencing signal spreads through the plant. Eventually the plant appears completely red under UV light (Figure 1C and D) (Voinnet and Baulcombe, 1997). We have also shown that this systemic gene silencing is sequence-specific and acts at the post-transcriptional level (Voinnet *et al*., 1998).

To test for a suppressor of gene silencing, PVY was inoculated two weeks after infiltration when systemic gene silencing was complete in all tissues of the plants (Figure 1A), except in the extreme meristematic zones which always remain non-silenced (Voinnet *et al*., 1998). By 2 weeks post-inoculation, the GFP-silenced plants showed the systemic mild mottle and leaf curling symptoms of PVY, indicating that the virus had spread from the inoculated leaf. Under UV light, there were large regions of GFP fluorescence coinciding with the viral symptoms (Figure 2A–C). Northern analysis of RNA extracted from these plants showed that the effects on GFP fluorescence were parallel to the levels of GFP mRNA. Thus, in mock-inoculated GFP-silenced plants (Figure 2D, track 10) the GFP mRNA levels were below the limit of Northern blot detection, whereas in plants infected with PVY, the levels were similar to those in non-silenced plants (Figure 2D, tracks 5, 6 and 11). One of the samples from a PVY-infected plant had only low level of GFP mRNA (Figure 2D, track 7). However in this sample there was also only a low level of PVY RNA indicating a relationship between the levels of GFP mRNA and of PVY. We could rule out that these increased GFP mRNA levels were due to a non-specific enhancement of transgene expression because PVY infection in non-silenced plants had no effect on the level of GFP mRNA (Figure 2D, track 8) or on GFP fluorescence (data not shown). We could also rule out, based on the effects of PVX, that reversion of silencing was a non-specific effect of virus infection. The symptoms of PVX are a mild mosaic like those of PVY. However the GFP-silenced plants remained red-fluorescent after PVX infection (data not shown) and contained low levels of GFP mRNA (Figure 2D, tracks 1, 2 and 3). Therefore, from the analyses of RNA and GFP fluorescence, these results are consistent with a suppressor of PTGS encoded in the PVY genome.

Reversion of GFP silencing by PVX–PVY recombinant viruses

The N-terminal P1 and HCPro potyviral proteins have been implicated in suppression of host defense (Pruss *et al*., 1997). In order to test the role of these proteins in the suppression of gene silencing, a series of PVX vectors carrying PVY gene sequences were generated (Figure 3) and inoculated to *N.benthamiana* plants exhibiting systemic PTGS of GFP. The PVX vectors are named according to the PVY-encoded protein produced in the infected plants. Thus, the pTXYHC vector produces HCPro: pTX refers to the backbone of these constructs which is a full-length cDNA of PVX (pTXS; Kavanagh *et al*., 1992) and Y refers to PVY. The plasmids carrying these constructs are pTXY**, where ** identifies the PVY protein. The

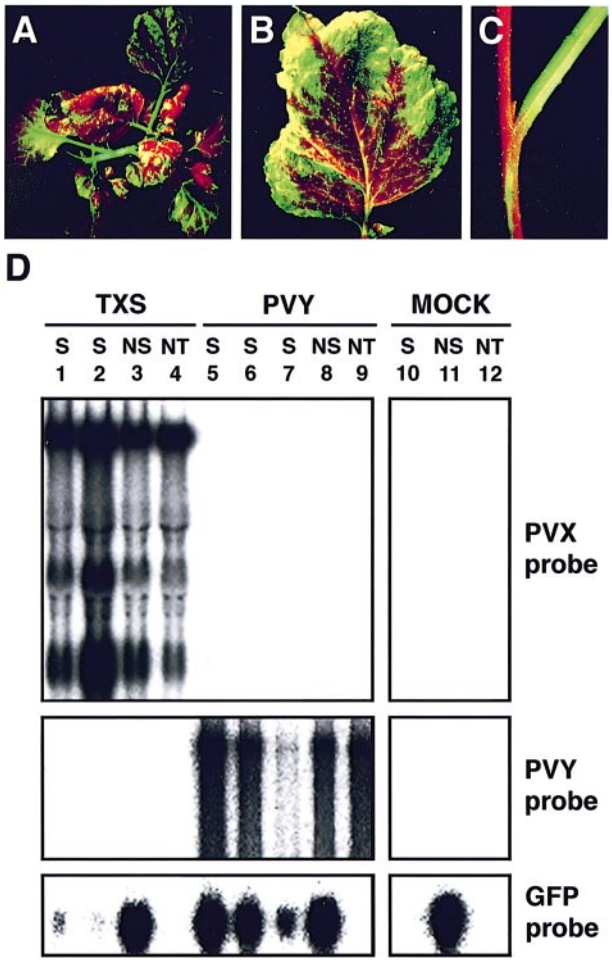

Fig. 2. Suppression of PTGS by PVY. (**A**) GFP-silenced *N.benthamiana* (line 16c) infected with PVY under UV illumination (15 days post-inoculation). The green fluorescence reveals that PTGS of GFP was lost in the PVY-infected tissue. Close-up views of a leaf and stem from the same plant are shown in (**B**) and (**C**), respectively. (**D**) Northern analysis of RNA extracted at 15 days post-inoculation from non-transformed (NT) and 16c *N.benthamiana* inoculated with PVX (TXS), PVY or mock-inoculated. Silenced (S) and non-silenced (NS) plants of line 16c were used in the experiment. Five micrograms of total RNA per sample were fractionated by electrophoresis on 0.9% (w/v) agarose–formaldehyde gels, blotted onto a nylon membrane and hybridized with probes specific for either PVX, PVY or GFP, as indicated.

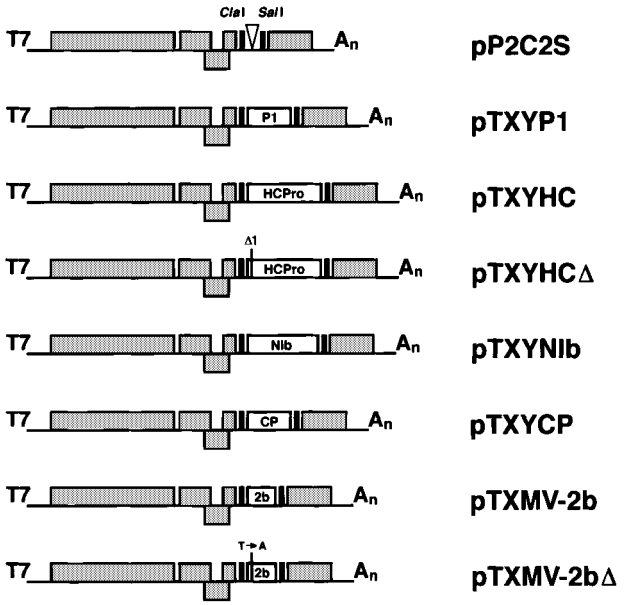

Fig. 3. Schematic representation of the PVX vector and chimeric constructs incorporating PVY and CMV coding sequences. Individual sequences were inserted into the pP2C2S PVX vector using the *Cla*I and *Sal*I cloning sites (Baulcombe *et al*., 1995). Expression of the insert and the PVX coat protein is controlled by duplicated PVX coat protein promoters indicated by a solid bar. The inserted PVY sequences are labeled: P1, 31 kDa N-terminal protein; HCPro, helper component protease; NIb, nuclear inclusion b; CP, coat protein. 2b refers to the CMV RNA 2-encoded protein required for long distance movement of the virus. Mutant versions of the HCPro and the 2b ORFs were also introduced into the PVX vector. In the pTXYHCΔ construct there was a –1 frame shift in the first codon of the HCPro open reading frame (Δ1). The pTXMV-2bΔ construct had a single nucleotide substitution (T→A) that converted the fourth codon (TTG) of the 2b ORF to a stop codon (TAG).

viruses produced when the transcripts of these plasmids were inoculated are simply TXY**.

Most of these TXY** viruses induced mosaic symptoms, like the wild-type PVX (data not shown). However, TXYHC produced symptoms that were much more severe than those of wild-type PVX, inducing necrosis in stems and leaves, in addition to stunting of the infected plants (Figure 4A and C), as described previously for a PVX construct expressing the HCPro of tobacco etch virus (TEV) (Pruss *et al*., 1997).

The TXYP1 and TXYCP viruses were similar to PVX (TXS) in that they had no effect on GFP silencing. Under UV illumination, at 2 weeks post inoculation, GFP-silenced plants infected with these constructs remained red, indicating that there had been no suppression of GFP gene silencing. Correspondingly, the GFP mRNA levels remained low in these plants (Figure 4F, tracks 1–4 and 10–13). However, in TXYHC-infected plants, there were large green fluorescent regions coinciding with the viral symptoms (Figure 4A–E). This effect was evident in all infected tissues, including stems (Figure 4E) and leaves (Figure 4C and D) that were already developed at the time of virus inoculation. Northern blot analysis showed that GFP mRNA levels had increased in these green fluorescent tissues and that the amount of GFP mRNA correlated with the relative amounts of TXYHC present in the sample (Figure 4F, tracks 5–9). We can rule out the possibility that the effect of TXYHC on gene silencing was an RNA-mediated effect because TXYHCΔ, with a frame-shift mutation at the 5′ end of the HCPro insert, did not suppress GFP gene silencing (Figure 4G, tracks 1–4). The symptoms of TXYHCΔ were mild, like those of wild-type PVX.

To rule out that suppression of GFP silencing was a secondary effect of the severe symptoms caused by TXYHC, we inoculated GFP-silenced plants with TXY-NIb. Like TXYHC, TXYNIb is highly necrogenic and causes death of the infected plant by 3 weeks post inoculation (data not shown). Analysis of TXYNIb-infected plants showed that GFP fluorescence and mRNA levels remained low in the severely symptomatic tissues (Figure 4G, tracks 5–7). From this, we conclude that HCPro of PVY is a direct suppressor of gene silencing and host defense in infected plants.

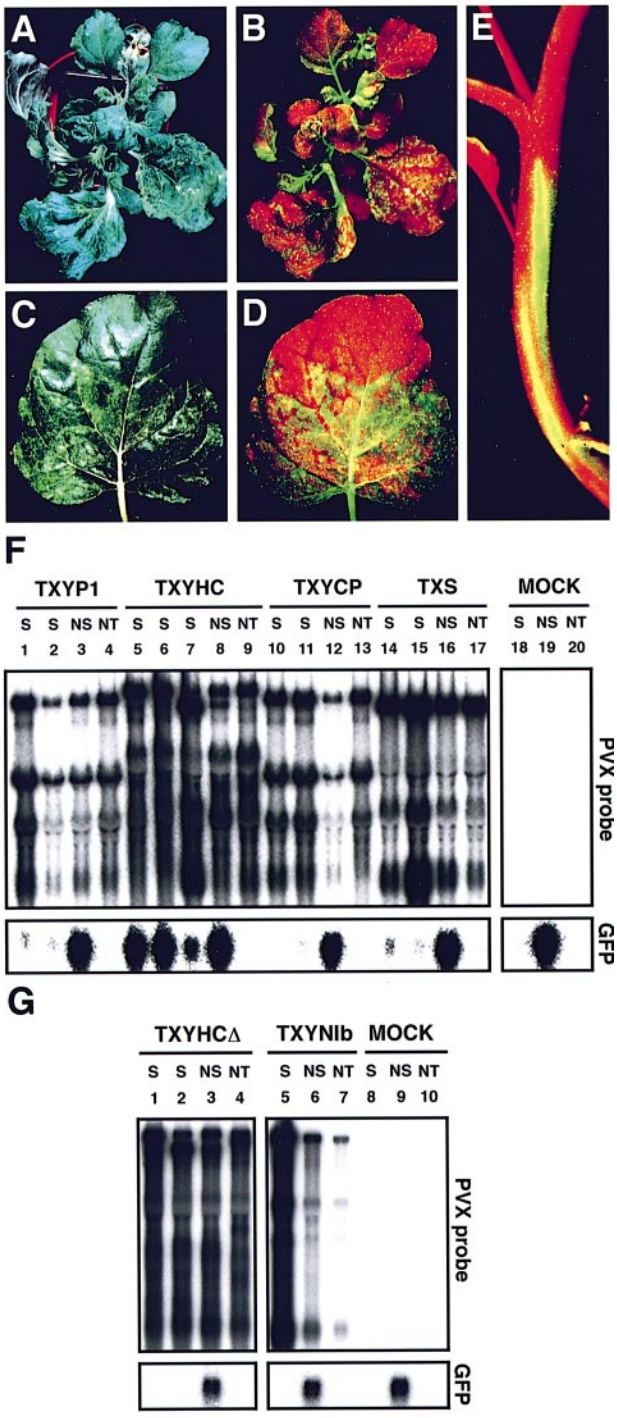

Fig. 4. Suppression of PTGS by TXYHC. (**A**) GFP-silenced *N.benthamiana* (line 16c) under white light showing symptoms of TXYHC infection (15 days post-inoculation). The same plant, shown under UV illumination in (**B**), revealing that PTGS of GFP was suppressed in all symptomatic tissues infected with the chimaeric virus. (**C**) Close-up of a leaf from the same plant under white light. (**D**) Close-up of the same leaf under UV illumination showing the co-localization of GFP expression with viral symptoms. (**E**) Close-up of the stem. (**F** and **G**) Northern analyses of RNA extracted at 15 days post-inoculation from non-transformed (NT) and *N.benthamiana* (line 16c) inoculated with PVX (TXS), TXY** recombinant viruses or mock-inoculated. Silenced (S) and non-silenced (NS) plants of line 16c were used in the experiments. Five micrograms (F) or 1 μg (G) of total RNA per sample were fractionated by electrophoresis on 0.9% (w/v) agarose–formaldehyde gels, blotted onto nylon membranes and hybridized with probes specific for PVX or GFP RNAs, as indicated.

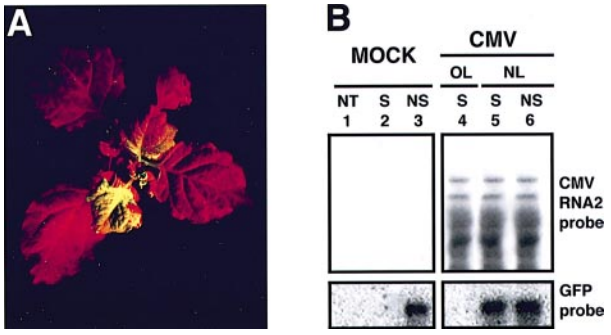

Fig. 5. Suppression of PTGS by CMV. (**A**) GFP-silenced *N.benthamiana* (line 16c) infected with CMV (21 days post-inoculation). GFP expression was restored in the newly emerging tissue after systemic CMV infection had been established. (**B**) Northern analysis of RNA extracted at 15 days post CMV inoculation from silenced (S) and non-silenced (NS) *N.benthamiana* plants of line 16c and from non-transformed plants. These plants were either infected with CMV or were mock inoculated. The RNA samples were taken either from old leaves (OL) that had emerged before systemic CMV infection or from new leaves (NL) emerging after CMV had spread systemically. Five micrograms of total RNA per sample were fractionated by electrophoresis on a 0.9% (w/v) agarose–formaldehyde gel, blotted onto a nylon membrane and hybridized with probes specific for RNA2 of CMV or for GFP RNA, as indicated. The multiple RNA species hybridizing to the CMV probe may represent degraded or subgenomic RNAs and have been described previously (Baulcombe *et al*., 1986).

Reversion of GFP silencing by wild-type CMV

To determine whether viruses other than potyviruses can suppress gene silencing, we carried out experiments similar to those described above but with CMV instead of PVY. CMV was selected for these experiments because, like potyviruses, it encodes a suppressor of host defense (L.H.Ji, W.X.Li and S.W.Ding, in preparation). Three weeks post-inoculation there were mosaic symptoms on the upper leaves of the plants. Under UV light, the symptomatic leaves of the GFP-silenced plants remained red-fluorescent if they had emerged from the growing points before virus infection. However, the leaves emerging from the growing points after systemic spread of the virus became bright green-fluorescent (Figure 5A). There was no effect of CMV on the GFP fluorescence of non-silenced plants (data not shown). Northern analysis revealed that the GFP fluorescence in the CMV-infected plants was correlated with the levels of GFP RNA: the red fluorescent leaves of the silenced plants had GFP RNA levels that were below the limit of detection (Figure 5B, track 4) whereas in the newly emerging tissues, which were green fluorescent, GFP RNA levels were similar to those in non-silenced plants (Figure 5, tracks 5 and 6). The levels of GFP RNA on non-silenced plants were unaffected by CMV infection (Figure 5B, tracks 3 and 6). From these results, we conclude that CMV encodes a suppressor of PTGS. However, because CMV and PVY do not suppress silencing in the same parts of the plant, these viruses must block different stages in the gene silencing mechanism.

Reversion of GFP silencing by PVX–CMV recombinant viruses

The putative CMV-encoded suppressor of host defense is the 2b protein (Ding *et al*., 1995). By analogy with the

potyviral HCPro, we considered that the ability of the 2b protein to suppress a plant defense mechanism could be due to its ability to suppress PTGS. To test this hypothesis, we inoculated a PVX vector expressing the CMV 2b protein (TXMV-2b; Figure 3) to GFP-silenced *N.benthamiana*. We also infected GFP-silenced plants with TXYMV-2bΔ in which a single nucleotide substitution (U to A) converted the fourth codon (UUG) of the 2b open reading frame (ORF) to a stop codon (UAG) (Figure 3).

By 3 weeks post-inoculation, TXMV-2b produced symptoms that were much more severe than those produced by wild-type PVX or by TXMV-2bΔ (Figure 6A). Instead of the normal mild mosaic symptoms of PVX, TXMV-2b induced necrosis on the systemically infected leaves and stem, leading to death of the plants (Figure 6A). However, at 14 days post-inoculation, before the systemic necrosis had developed, the newly emerging leaves of TXMV-2b-infected plants were green fluorescent under UV illumination. As in the CMV-infected plants, the leaves that had emerged prior to virus infection, although symptomatic, remained red fluorescent (Figure 6B–F). The phenotype of TXMV-2b-infected plants was associated with corresponding changes in the levels of GFP RNA. In the older, red-fluorescent leaves, the level of GFP RNA remained below the level of detection as in mock-infected leaves (Figure 6I, tracks 2 and 4). In contrast, in the new green-fluorescent leaves the GFP RNA had increased to the levels of non-silenced plants (Figure 6I, tracks 3, 5 and 6).

From the symptoms of TXMV-2bΔ, we ruled out the possibility that the suppression of GFP silencing was an RNA-mediated effect of the 2b sequence. GFP silenced plants infected with this construct remained totally red-fluorescent (Figure 6G and H) and contained low levels of GFP mRNA as in the GFP-silenced plants that had been mock-inoculated (Figure 6I, tracks 2 and 7–9). We also ruled out the possibility that the suppression of GFP silencing was due to a non-specific enhancement of transgene expression by the 2b protein by showing that TXMV-2b had no effect on GFP RNA or the green fluorescence of the non-silenced GFP lines (data not shown). Therefore, from the similarity of the CMV and TXMV-2b effects, we conclude that the 2b protein is the major suppressor of gene silencing encoded in the CMV genome.

Discussion

In this study, we identified two viral suppressors of PTGS in CMV and PVY. Our findings confirm the previously made suggestion that PTGS in plants is a natural protection mechanism against viruses (Baulcombe, 1996; Pruss *et al*., 1997; M.T.Ruiz *et al*., 1998). Furthermore, by implicating PTGS in resistance against diverse types of RNA virus, our findings indicate that this mechanism has general significance in plant–virus interactions.

Suppressors at different stages of PTGS

The two viral suppressors of PTGS in plants identified in this work are HCPro encoded by the PVY genome and

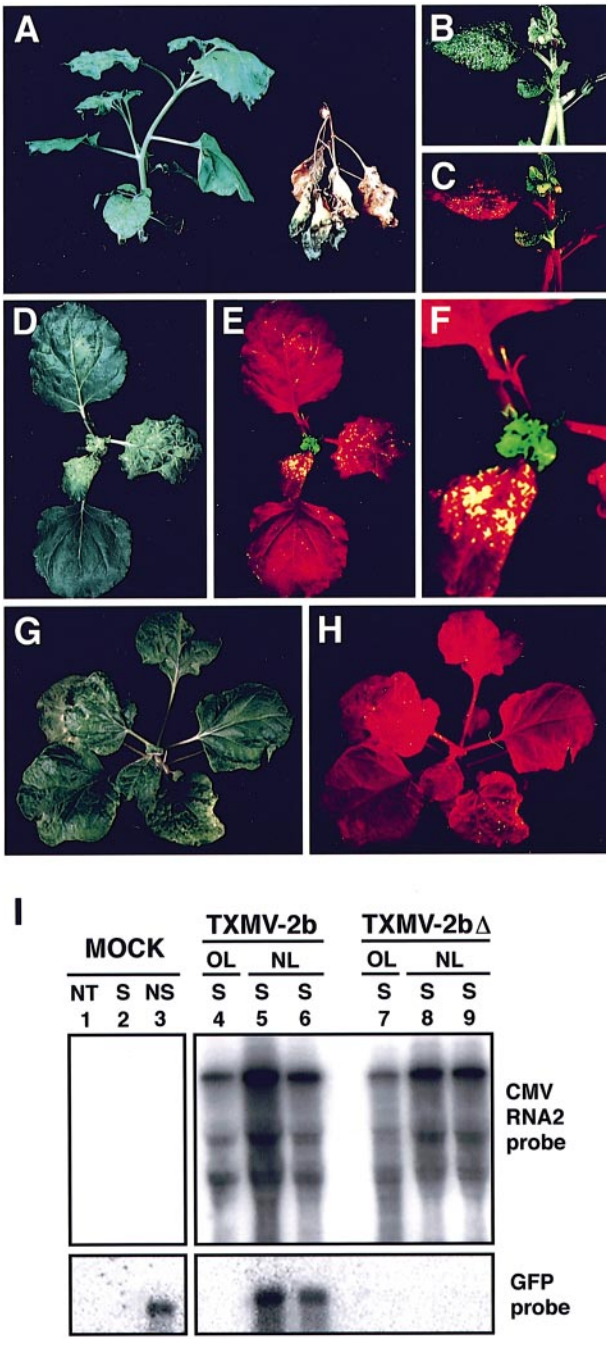

Fig. 6. Suppression of PTGS by the 2b protein of CMV (**A**) Non-transformed *N.benthamiana* plants inoculated with TXMV-2bΔ (left) and TXMV-2b (right). The photograph was taken at 21 days post-inoculation and both plants were the same age when inoculated. (**B**) GFP-silenced *N.benthamiana* (line 16c) showing symptoms of TXMV-2b infection at 21 days post-inoculation. (**C**) The same plant shown under UV illumination revealing the PTGS of GFP persisted in symptomatic leaves that had emerged from meristems before systemic infection but that it is suppressed in the post emergence leaves. (**D** and **E**) Aerial views of the plant shown in (B) under white light and UV illumination. (**F**) The apical zone from the image in (E). (**G** and **H**) GFP-silenced *N.benthamiana* (line 16c) showing symptoms of TXMV-2bΔ under white light (G) and UV illumination (H). (**I**) Northern analysis of RNA extracted at 15 days post-inoculation from either NT, non-silenced (NS) or silenced (S) *N.benthamiana* (line 16c) inoculated with TXMV-2b and TXMV-2bΔ. The RNA samples were taken either from old leaves (OL) that had emerged before systemic virus infection or from new leaves (NL) emerging after the virus had spread systemically. Five micrograms of total RNA per sample were fractionated by electrophoresis on a 0.9% (w/v) agarose–formaldehyde gel, blotted onto a nylon membrane and hybridized with probes specific for RNA2 of CMV or for GFP RNA as indicated.

the 2b protein encoded by the RNA 2 of CMV. Expression of either of these proteins from a PVX vector suppressed PTGS of a GFP transgene (Figures 4 and 6). This effect was clearly protein- rather than RNA-mediated because there was no suppression of PTGS when the PVX vector carried modified forms of the HCPro and the 2b coding sequences (Figures 4G and 6G). We can rule out that suppression of PTGS was due to a non-specific stimulation of the GFP transgene expression because GFP fluorescence and RNA levels remained unaffected in a non-silenced line infected with PVY, TXYHC or CMV (Figures 3D, 4F and 5B). We can also rule out the possibility that the results obtained are an artefact associated with a virus vector because P1-HCPro of TEV expressed in transgenic plants is also a suppressor of PTGS (Anandalakshmi *et al*., 1998; Kasschau and Carrington, 1998).

In a previous analysis of GFP silencing, we showed that initiation, systemic spread of a silencing signal and maintenance are separate stages of the PTGS mechanism (Voinnet *et al*., 1998). Here, from the GFP phenotype in virus-infected plants, we have shown that these stages are differentially targeted by the two viral suppressors. In plants infected with PVY or TXYHC there was suppression of PTGS in all of the symptomatic tissues, including the older leaves that would have received the systemic signal and initiated PTGS before the viruses had established infection (Figures 2 and 4). Based on these observations, we conclude that HCPro must be blocking at least the maintenance stage of PTGS, although our data do not rule out the possibility that initiation and systemic spread are also affected. The extent of suppression of PTGS was correlated with the level of PVY or TXYHC RNA (Figure 2D, lane 7), which suggests that high levels of the HCPro are required for suppression of PTGS.

Suppression of PTGS in CMV- or PVX-2b-infected plants was unlike the HCPro-mediated effect, because the only affected leaves were those that emerged from the growing points after the viruses had spread systemically (Figures 5 and 6). From this pattern, we conclude that the 2b protein affects the cells in, or near to, the growing points of the plants. The growing points do not normally exhibit PTGS, even when there is extensive silencing in the rest of the plant (Voinnet *et al*., 1998). Therefore, it is possible that the 2b protein prevents entry of the gene silencing signal to the cells emerging from the growing points. Alternatively, it could be that the signal enters these cells but that the 2b protein prevents initiation of the PTGS mechanism.

Viral symptom determinants and suppressors of gene silencing

From the results presented here and elsewhere, it is shown that the HCPro and the 2b proteins have several activities. First, these proteins are pathogenicity factors of the respective viruses (Cronin *et al*., 1995; Ding *et al*., 1995). They also enhance the accumulation and symptoms of PVX when they are expressed from PVX vectors (Pruss *et al*., 1997) and, as shown here, they suppress PTGS. In addition, in protoplasts from transgenic plants expressing HCPro, there is enhanced accumulation of PVX, TMV or CMV (Pruss *et al*., 1997).

Although it remains formally possible that these are unrelated effects of the two proteins, a more likely explanation is that they are all associated with the suppression of a PTGS-like resistance mechanism. From the findings that PVY, CMV and the various PVX constructs are all affected by this mechanism, we propose that it is activated or given specificity by different types of RNA virus. We envisage that this mechanism could target degradation of RNA species that are similar to the inoculated virus. The effect of the mechanism would be to restrict viral RNA accumulation in infected cells. In addition, as shown in PTGS (Palauqui *et al*., 1997; Voinnet and Baulcombe, 1997), we consider it possible that there is a sequence-specific signal molecule that spreads away from the cells in which the process is initiated. The potential to transmit a signal out of the initially infected cell could mean that this PTGS-like resistance mechanism has the ability to activate viral RNA degradation in non-infected cells located beyond the front of infection. Consequently, the PTGS-like mechanism could be responsible for suppression of virus movement as well as restriction of viral accumulation in infected cells.

It is unlikely that an effect of a PTGS-like resistance mechanism is specific to the potex, cucumo- and potyviral groups. These groups represent extremes in the evolution of positive strand RNA viruses having either monopartite or multipartite genome organization and similarity to either the picorna- or sindbis-virus groups (Matthews, 1991). Therefore, the shared property of these viruses as activators of the PTGS-like resistance mechanism is probably common to most groups of positive strand RNA viruses in plants. Moreover, if a PTGS-like mechanism is implicated in virus resistance, it is expected that other virus-encoded proteins will function as suppressors of gene silencing. From the results presented here, we predict that many viral proteins that were originally identified as pathogenicity determinants, like the 2b protein or HCPro, will eventually be identified as suppressors of PTGS.

The lack of an effect of PVX on the GFP silenced plants shows that this virus does not produce a suppressor of PTGS. However, the increase in symptoms and virus accumulation in plants infected with TXYHC and PTXMV-2b implies that PVX does activate the PTGS-like resistance. To reconcile these observations, we propose that PVX has the potential to partially evade rather than to suppress the PTGS-like resistance mechanism. Evasion might involve very rapid replication and spread of the virus so that the resistance mechanism is out-competed. Alternatively, PVX might be compartmentalized, so that it avoids the PTGS-like mechanism.

Several virological phenomena could be explained by the proposed involvement of a PTGS-like resistance mechanism in plants and the corresponding suppressors of gene silencing encoded in different viruses. Most obviously, there are various synergistic interactions between viruses resulting in disease that is much more severe in plants infected with two viruses than in plants infected with either virus alone (Pio-Riberio *et al*., 1978; Vance, 1991; Murphy and Kyle, 1995). As with the PVX–PVY synergism, these interactions could be explained by the production of a suppressor of gene silencing by either or both of the interacting viruses.

The phenomenon of cross protection (Matthews, 1991) could also be explained, at least in part, by the involvement of a PTGS-like resistance mechanism. Cross protection is

an induced resistance mechanism in virus-infected plants that affects a second virus (the challenge strain) inoculated days or weeks after the initial inoculation. This resistance is specific for challenge strains that are similar to the inducing strain. Perhaps the inducing strain would initiate or provide the specificity determinant for the PTGS-like resistance mechanism so that the challenge strain, having sequence similarity to the inducer, would be suppressed. We have already demonstrated that cross protection associated with nepoviral recovery is similar to PTGS (Ratcliff *et al*., 1997). However, based on our findings that diverse viruses may activate a PTGS-like resistance mechanism, it now appears likely that many other examples of cross protection may also fit into the same category.

Viral adaptations to gene silencing

The general principles of suppression and evasion of the PTGS-like resistance mechanism will probably apply to many plant–virus interactions. Evasive or strongly suppressive viruses will be highly pathogenic, whereas the others will be weak or non-pathogens. The probable central role of this mechanism in plant virus interactions means that there will be strong selective pressures on the virus to evade or suppress the mechanism. Similarly, there will be corresponding selective pressures on the plant side to ensure that the mechanism is effective against many viruses. These selective pressures will probably cause a high level of polymorphism in both the plant and the viral genes involved in the mechanism. Clearly, identification of other viral suppressors and further investigation of this proposed adaptive mechanism will certainly provide support for a previously uncharacterized, generalized virus resistance mechanism in plants. In addition, it is hoped that identification of viral suppressors of PTGS will provide an experimental handle for the characterization of host components involved in PTGS. It may also be possible to use the suppressors of PTGS to investigate the relationship of superficially similar gene silencing phenomena in plants, fungi, ciliates and nematodes (Cogoni and Macino, 1997; Fire *et al*., 1998; F.Ruiz *et al*., 1998).

Materials and methods

Plant material

Transgenic *N.benthamiana* plants carrying the GFP ORF were described previously (M.T.Ruiz *et al*., 1998). The line used in this study, 16c, carries one copy of the transgene at a single locus in homozygous conditions (M.T.Ruiz *et al*., 1998).

Agrobacterium induction of post-transcriptional gene silencing

GFP-expressing seedlings from line 16c were infiltrated with an hypervirulent strain of *A.tumefaciens* (strain cor308) (Hamilton *et al*., 1995) carrying a binary-Ti plasmid vector into which a functional 35S-GFP cassette had been inserted. This cassette is the same as the one used for plant transformation (Figure 2B). Infiltration of *A.tumefaciens* was based on a previously described method (English *et al*., 1997).

Wild-type and recombinant viruses

A PVY isolate belonging to the necrotic strain group was maintained in *Nicotiana clevelandii* plants. Wild-type PVX (pTXS) (Kavanagh *et al*., 1992) and the PVX vector (pP2C2S) (Baulcombe *et al*., 1995) have been described previously. pP2C2S was used to deliver PVY proteins into plant cells (Figure 1). PVY cDNA fragments encoding the 31 kDa protein (P1), helper component (HC), nuclear inclusion b (NIb) and coat protein (CP) were PCR-amplified from cDNA and individually cloned, in-frame, into the PVX vector under the control of a duplicated PVX CP promoter. An additional recombinant virus that carried a frameshift version of the HC coding sequence was also generated and termed pTXYHCΔ. The mutation in this construct consisted of the deletion of the first nucleotide of the HCPro coding sequence, creating a –1 frameshift. The sequence of the PVY mature proteins and the primer design for cloning purposes were based mainly in the published full-length sequences of the virus (Robaglia *et al*., 1989; Thole *et al*., 1993).

Wild-type CMV was maintained in *N.benthamiana* plants. As described above, the PVX vector (pP2C2S) was used to deliver CMV sequences into plant cells (Figure 1). pTXMV-2b contained a 500 bp DNA fragment derived from nucleotides 2410–2908 of CMV RNA 2 and thus the complete 2b coding sequence (CMV RNA 2, nucleotides 2410–2712; Ding *et al*., 1995). This inserted CMV sequence was rendered non-coding in pTXYMV-2bΔ by a single nucleotide substitution (T to A) that converted the fourth codon (TTG) of the 2b ORF to a stop codon (TAG).

In vitro transcription

In vitro transcription reactions to produce infectious PVX and PVX derivatives RNAs were performed as described previously (Chapman *et al*., 1992).

Northern blot analysis

RNA was extracted from systemically infected leaves 15 to 21 days post-inoculation as described previously (Mueller *et al*., 1995). RNA samples (1–5 μg) were electrophoresed on a 0.9% (w/v) agarose formaldehyde gels, transferred to Hybond-N membranes and hybridized with ^{32}P-labeled RNA or cDNA probes specific for the respective viruses or for the GFP transgene RNA.

GFP imaging

Visual detection of GFP fluorescence in whole plant was performed using a 100 W hand-held long-wave ultraviolet lamp (UV products, Upland, CA, Black Ray model B 100AP). Plants were photographed with a Kodak Ektachrome Panther (400 ASA) film through a Wratten 8 filter. Exposure times varied up to 70 s depending on the intensity of the fluorescence and the distance of the camera and lamp from the plant.

Acknowledgements

We are grateful to the Gatsby Charitable Foundation for continuing support. We are also grateful to Vicki Vance, James Carrington, Andrew Maule and our colleagues in the Sainsbury Laboratory for helpful discussions.

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Received August 21, 1998; revised and accepted September 24, 1998