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Multiple suppressors of RNA silencing encoded by both genomic RNAs of the crinivirus, *Tomato chlorosis virus*

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Abstract

Viruses express proteins with silencing supression activity to counteract the RNA silencing-mediated defense response of the host. In the family *Closteroviridae,* examples of multiple-component RNA silencing suppression systems have been reported. To ascertain if this is a general strategy in this group of viruses, we have explored the bipartite genome of *Tomato chlorosis virus* (ToCV, genus *Crinivirus*). We have identified the RNA1-encoded p22 protein as an effective silencing suppressor by using a *Agrobacterium* co-infiltration assay. p22 suppressed local RNA silencing induced either by sense RNA or dsRNA very efficiently, but did not interfere with short or long-distance systemic spread of silencing. We have also demonstrated by using the heterologous vector PVX the silencing suppression activity of the RNA-2 encoded coat protein (CP) and minor coat protein (CPm). In this study, we demonstrate an even greater complexity of silencing suppressor activity for a plant virus, and for the first time we show the presence of RNA silencing suppressor genes encoded by both genomic RNA molecules of a bipartite genome in the complex family *Closteroviridae*.

Keywords: *Closteroviridae* / *Crinivirus* / *Tomato chlorosis virus* / RNA silencing suppressor

Introduction

The ability of a plant virus to produce an effective infection in a host is the result of the confrontation between virus multiplication and host defense. Among host defense responses, RNA silencing has emerged as an important natural antiviral mechanism in plants (Ratcliff et al., 1997; Vance and Vaucheret, 2001; Voinnet, 2001, 2002, 2005; Baulcombe, 2004; Ding et al., 2004; Wang and Metzlaff, 2005). RNA silencing is induced by double-stranded RNA (dsRNA), which is processed into small RNA species of 21-24 nucleotides termed small interfering RNA (siRNA) (Hamilton and Baulcombe, 1999). Although it is often assumed that viral replicative forms provide the dsRNA template that triggers RNA silencing, it is likely that highly structured regions of the genomic RNA are also important targets (Szittya et al., 2002; Molnar et al., 2005). The siRNAs produced by the action of a dsRNA-specific RNase called Dicer (or DCL for Dicer-like) are incorporated into an RNA-induced silencing complex (RISC) that initiates the sequence-specific degradation of target RNAs, such as viral RNAs. The presence of these siRNAs is a hallmark of RNA silencing (Hamilton and Baulcombe, 1999). In plants, RNA silencing is locally induced at the single-cell level and a mobile silencing signal is generated (Voinnet and Baulcombe, 1997; Himber et al., 2003). This signal moves from cell to cell through plasmodesmata and systemically via the vascular system (Palauqui et al., 1997).

To counteract antiviral RNA silencing, plant viruses have developed strategies based on the expression of silencing suppressor proteins. Before their identification as silencing suppressors, some of these proteins were identified to also exhibit properties such as the enhancement of viral accumulation and pathogenicity (Ding et al., 1996; Scholthof et al., 1995; Pruss et al., 1997). This dual activity suggests an intrinsic relationship between viral pathogenesis and RNA silencing suppression. Since the

discovery of RNA silencing, many plant viral proteins have been identified as suppressors of RNA silencing (Silhavy and Burgyan 2004; Voinnet, 2005). The great diversity in sequence, structure, and mechanism of action found for these proteins, reinforces the importance of the studies directed to the identification of new RNA silencing suppressors and their modes of action to better understand the basic mechanisms of RNA silencing and virus-host interactions (Voinnet, 2005; Díaz-Pendón and Ding, 2008).

Tomato chlorosis virus (ToCV; genus *Crinivirus*) is an emerging plant virus that has a large bipartite, single-stranded, positive-sense RNA genome (Wisler et al., 1998; Wintermantel et al., 2005; Lozano et al., 2006, 2007), and belongs to the complex family *Closteroviridae* (Dolja et al., 2006) in which examples of multiple-component RNA silencing suppression systems have been reported (Lu et al., 2004; Kreuze et al., 2005). One of these multiple-component strategies consists of encoding more than one suppressor (Lu et al., 2004). To ascertain if encoding multiple suppressors is a general strategy in members of the family *Closteroviridae,* we have screened the ToCV genome. Our data show that the RNA1-encoded p22 protein suppresses very efficiently RNA silencing, although the mechanism of action seems to be different to that proposed for its ortholog gene in the crinivirus *Sweet potato chlorotic stunt virus* (SPCSV) (Kreuze et al., 2005). Moreover, suppressor activity was also found in the RNA2-encoded coat protein (CP) and minor coat protein (CPm) when they were expressed from a PVX-vector. To date, ToCV is the first crinivirus identified that encodes suppressors of RNA silencing in both of its genomic RNAs.

Results

Identification of p22 as an RNA silencing suppressor encoded by ToCV RNA1.

The ToCV genome consists of two positive single stranded RNA molecules (Wisler et al., 1998; Wintermantel et al., 2005; Lozano et al., 2006, 2007). To identify potential RNA silencing suppressors in the ToCV genome, open reading frames (ORFs) papain-like leader proteinase (L-Pro), RNA-dependent RNA polymerase (RdRp) and p22 encoded by RNA1, and heat shock protein 70 homologue (Hsp70h), p59, capsid protein (CP), minor capsid protein (CPm) and p27 encoded by RNA2 (Figure 1) were tested. The initial screening was carried out by using an *Agrobacterium* co-infiltration assay as described previously (Voinnet et al., 2000). Thus, to assess the suppressor properties of the different ToCV ORFs, *Nicotiana benthamiana* leaves were co-infiltrated with a mixture of 35S-GFP-expressing *Agrobacterium tumefaciens* and a second strain containing the desired ToCV coding sequences under the control of the 35S promoter in a pBIN19 vector. Co-infiltration of 35S-GFP with either the empty pBIN19 vector or a plasmid (35S HCPro) expressing the HCPro suppressor of silencing from Potato virus Y (Brigneti et al., 1998) was used as a negative and a positive control, respectively. Examination of infiltrated leaves 5 days post-infiltration (dpi) showed that in tissues infiltrated with 35S-GFP plus the empty vector, green fluorescence decreased as a consequence of RNA silencing activation (Brigneti et al., 1998; Voinnet et al., 2000). By contrast, in tissues co-infiltrated with 35S-GFP plus 35S-HCPro, intense green fluorescence was observed at 7 dpi (Figure 2A). Among the eight ToCV genes tested, GFP fluorescence was maintained only in presence of the p22 construct (Figure 2A). Thus, even very early after infiltration the leaves infiltrated with a mixture of 35S-GFP plus 35S-p22 exhibited a GFP fluorescence much stronger than that observed in leaves infiltrated with 35S-GFP plus the empty vector. Moreover, the intensity of the fluorescence progressively increased to a very high level by 5 dpi and continued at a such a high level even at 30 dpi, when in HCPro co-infiltrations GFP expression had

been lost. Consistent with these observations, northern blot analysis revealed that at 7 dpi the steady-state levels of GFP mRNA were very low in leaves agroinfiltrated with 35S-GFP plus either empty vector (Figure 2B, top panel) or any ToCV ORFs tested except p22 (data not shown). In contrast, very high level of GFP mRNA accumulation was evident, both at 3dpi and 7dpi, in leaves infiltrated with 35S-GFP plus 35S-p22 and 35S-GFP plus 35S-HCPro (Figure 2B, top panel). Moreover, the northern blot analysis of GFP mRNA accumulation at 30 dpi in the co-infiltrations with 35S-p22 showed similar high levels of mRNA GFP accumulation, indicating that the long sustained fluorescence was due to protection of the mRNA (Figure 2C, top panel). By this time, GFP mRNA was undetectable in leaves co-infiltrated with 35S-HCPro (Figure 2C, top panel).

To test if the reduced levels of GFP mRNA were indeed the result of RNA silencing, the relative levels of the GFP-specific siRNAs was assessed (Hamilton et al., 1999). The accumulation of GFP siRNAs of ~21 nt and ~25 increased remarkably from 3 dpi to 7 dpi in leaves infiltrated with 35S-GFP plus the empty vector (Figure 2B), and in leaves infiltrated with 35S-GFP plus each of the other ToCV ORFs (data not shown) except p22. However, this accumulation was drastically reduced in samples infiltrated with 35S-GFP plus 35S-p22, or 35S-GFP plus 35S- HCPro, both at 3 dpi and 7dpi (Figure 2B). Interestingly, by 30 dpi, the low levels of GFP siRNAs were maintained in leaves co-infiltrated with 35S-p22 in contrast to the increased accumulation observed in leaves co-infiltrated with 35S-HCPro (Figure 2C). By 30 dpi, low lewels of GFP siRNAs were also observed in leaves co-infiltrated with the empty vector. This could be explained by the lack of the inducer of silencing 35 S-GFP by this time, due to the total degradation of GFP mRNA in the absence of a suppressor of RNA silencing.

Taken together, these results indicate that the encoded ToCV p22 is an efficient and strong RNA silencing suppressor.

ToCV p22 supresses RNA silencing triggered by both sense RNA and dsRNA.

The above described transient co-infiltrations assays showed that p22 could suppress local GFP silencing triggered by positive-sense RNA. Agroinfiltration with an inverted repeat GFP construct (GF-IR) leads to expression of hairpin GFP RNAs that are considered strong inducers of RNA silencing since they are rapidly processed into siRNAs (Johansen and Carrington, 2001). To test whether p22 can suppress hairpin-induced silencing, we carried out a dsRNA-triggered silencing assay by co-infiltrating leaves of *N. benthamiana* with *A. tumefaciens* strains harbouring 35S-GFP (sense GFP RNA), 35S-GF-IR (inverted repeat generating GFP dsRNA), and empty vector, 35S-p22 or 35S-HCPro. Only a moderate enhancement of GFP fluorescence was visible at 3 dpi in the co-infiltrations with the empty vector, compared to the fluorescence observed at this same time post-infiltration when the strong inducer was not present. In contrast, a clearly increased fluorescence was observed in patches infiltrated with a mixture of 35S-GFP + 35S-GF-IR and 35S-p22 or 35S-HCPro, either at 3 and 7 dpi (Figure 3A), which was maintained even at 30 dpi in the case of p22 (not shown). These results were confirmed by northern blot analysis. Thus, leaves infiltrated with 35S-GFP + 35S-GF-IR and 35S-p22 or 35S-HCPro exhibited high accumulation of GFP mRNAs and reduced accumulation of siRNA at either 3 and 7 dpi, being the siRNA reduction at 7 dpi especially marked in leaves co-infiltrated with 35S-p22 (Figure 3B). It is interesting to note that, even in the presence of a strong inducer of RNA silencing like dsRNA, by 30 dpi, in the co-infiltrations with 35S-p22 the high levels of mRNA GFP accumulation

were linked to a drastic reduction of GFP siRNAs (see the right lane of the northern blot showed in Figure 2C).

Therefore, the results of the dsRNA co-infiltration assays indicate that p22 interferes with silencing downstream of dsRNA production.

The action of ToCV p22 does not prevent cell-to cell or long distance spread of RNA silencing.

To determine whether p22 could interfere with cell-to-cell or long-distance spread of RNA silencing, we agroinfiltrated GFP-expressing transgenic *N. benthamiana* line 16c (Brigneti et al., 1998) with 35S-GFP plus either the empty vector, 35S-p22 or 35S-HCPro. As in non-transgenic *N. benthamiana* plants, local RNA silencing did not take place when 35S-GFP was co-infiltrated with 35S-p22 or 35S-HCPro at 7 dpi. In contrast, no GFP fluorescence was observed in patches infiltrated with 35S-GFP plus the empty vector, indicating that the infiltrated GFP and the endogenous GFP transgene had been locally silenced.The GFP fluorescence was maintained in leaves infiltrated with 35S-GFP plus 35S-p22 even at 30 dpi as before but not in co-infiltrations with HCPro. Accumulation of GFP mRNA and siRNAs was consistent with the fluorescence assay results (not shown).

In the co-infiltrations with 35S-p22, although the amount of GFP siRNAs was significantly reduced, they were not completely eliminated. Therefore, we studied if p22 could interfere with cell-to-cell spread of RNA silencing by monitoring GFP expression in the cells neighboring the agroinfiltrated patches. If the silencing signal exits from the agroinfiltrated area, the cell to cell movement should cause shutting down of GFP expression, which is evidenced by a narrow red ring around the infiltrated patch

(Himber et al., 2003). In plants infiltrated with 35S-GFP plus empty vector a red ring could be observed at 6 dpi (not shown). Similarly, when 35S-p22 or 35S-HCPro were co-infiltrated with 35S-GFP, a red ring could also be observed in all the agroinfiltrated patches, although delayed at 10 dpi (Figure 4A). We also monitored GFP expression in upper noninfiltrated leaves to determine whether p22 could interfere with systemic RNA silencing. Systemic silencing was observed in plants infiltrated with 35S-GFP plus empty vector at 10 dpi. Although delayed (13-15 dpi), a similar systemic RNA silencing was observed when p22 or HCPro were co-infiltrated with 35S-GFP, in most of the infiltrated plants. By 30 dpi, although the spread of systemic silencing was complete in plants co-infiltrated with 35S-GFP plus 35S-p22, while the infiltrated patches still exhibited bright GFP fluorescence due to local silencing suppression activity of ToCV p22 (Figure 4B). By this time, the patch co-infiltrated with HCPro had lost the fluorescence. Therefore, these results demonstrated that although p22 can very efficiently suppress local silencing, it is not able to block the short or long distance spread of the RNA silencing signal.

Expression of ToCV encoded proteins from the heterologous vector PVX identifies CP and CPm as RNA silencing suppressors.

A *Potato virus X* (PVX) derived vector was used to express the different ToCV proteins *in planta*, reasoning that suppression ability of proteins with a weak intracellular suppression activity could be overlooked if the transient expression under a 35S promoter does not allow expression at sufficient levels to see their effect in the infiltrated patch. Then, the same ToCV coding sequences from RNA1 and RNA2 tested in the co-infiltration assay described above, were expressed from the PVX derived vector pGR107 and tested individually in *N. benthamiana* leaves in co-infiltration

assays with a 35S-GFP-expressing *A. tumefaciens*. Co-infiltration of 35S-GFP with either the empty PVX vector or a recombinant PVX virus expressing the NSs suppressor of silencing, from *Tomato spotted wilt virus* (Takeda et al., 2002; Bucher et al., 2003) were used as a negative and a positive control, respectively. In the case of the co-inoculations with the recombinant PVX virus expressing the suppressor NSs (PVX.NSs), a fluorescence that lasted for at least 6 days was observed in the infiltrated area, whereas no such fluorescence was observed for co-inoculations with the empty PVX vector. Unexpectedly, no sustained fluorescence was observed in the co-inoculations with PVX expressing the ToCV p22. Interestingly, for the other ToCV genes expressed from the PVX vector, the fluorescence was maintained in inoculated patches even at 6 dpi when the coat protein (CP) or the minor coat protein (CPm) were expressed (Figure 5A). In contrast, a weak or absence of GFP fluorescence at 5-6 dpi similar to that observed for co-infiltration of 35S-GFP with the empty PVX vector was observed for any of the other ToCV ORFs (exemplified in Figure 5A for empty PVX). Persistence of the GFP fluorescence in co-infiltrations with CP, CPm and NSs expressed from PVX correlated with high steady state levels of GFP mRNA, in contrast to the marked reduction observed in leaves co-infiltrated with the empty PVX vector (Figure 5B, upper panel) or with recombinant PVX expressing other ToCV ORFs (data not shown). The differences in size observed for genomic PVX RNA of each construct, indicated that the ToCV (CP, CPm) and TSWV (NSs) ORFs cloned were retained in the viral progeny (Figure 5B, middle panel). The analysis of GFP-specific siRNAs in the infiltrated patches showed a markedly reduced accumulation at 3 dpi in co-infiltrations with any of the recombinant PVX viruses expressing CP, CPm or NSs (Figure 5B, bottom panel). At 6 dpi, the GFP siRNAs remained at a very low level only in the leaves co-inoculated with the recombinant virus expressing NSs, although in the leaves

co-inoculated with PVX.CP or PVX.CPm, the level was lower than that observed for the empty PVX vector (Figure 5B, bottom panel). These results suggest that CP and CPm ToCV proteins exhibit RNA silencing suppression activity when expressed from the heterologous vector PVX.

ToCV CP, CPm and p22 accentuate PVX infection.

Numerous observations indicate that the expression of silencing suppressors from a heterologous virus enhances symptom severity (Brigneti et al., 1998). Therefore, the effect of ToCV CP, CPm and p22 proteins on the virulence of the unrelated virus PVX was studied. PVX infection of *N. benthamiana* resulted in mild mosaic symptoms at 7 dpi that progressed to no symptoms in some leaves as a result of recovery from viral infection (Figura 6A). In contrast, *N. benthamiana* plants inoculated with PVX recombinant viruses expressing CP, CPm or p22 exhibited more severe symptoms and did not recover from viral infection. Evident systemic symptoms could be observed in PVX.CP and PVX.CPm-infected plants as early as 5 dpi consisting in chlorosis and leaf curling in young non-inoculated leaves that evolved to chlorotic and necrotic mottling and deformation in all the leaves. In contrast, with the recombinant PVX expressing p22, systemic symptoms were slightly delayed in *N. benthamiana* plants, which exhibited evident leaf curling in young non-inoculated leaves at 8 dpi. However, in this case symptoms progressed so rapidly and severely that plants died 2-3 days later (Figure 6A). As shown in Figure 6B, enhanced virus virulence correlated with enhanced accumulation of PVX genomic RNA in young tissues (right panel) that could even be observed in ethidium bromide stained gels of total RNAs prior to northern blot analysis (left panel), in contrast to the non-recombinant PVX (analysis could not be performed

for dying PVX.p22 plants). Retention of CP and CPm sequences in systemically invading recombinant PVX.CP and PVX.CPm was confirmed by using CP and CPm-specific probes (data not shown).

Taken together, these findings demonstrated that expression of ToCV CP, CPm and p22 ORFs enhanced the virulence of the unrelated virus PVX, that (for CP and CPm) correlated with enhanced PVX accumulation probably due to RNA silencing suppression.

Discussion

One of the most common strategies used by plant viruses to counteract the antiviral RNA silencing defense response of plants consists of encoding suppressor proteins. The complexity of the genome of the members in the family *Closteroviridae* could make them strong inducers of RNA silencing (Dolja et al., 2006). To efficiently counteract an RNA silencing defense response some members of this family have been shown to encode multiple RNA silencing suppressor (Lu et al., 2004). Similarly, our data suggest that another member of this family, the crinivirus ToCV, also adopts the strategy of encoding several viral suppressor proteins to counteract the plant RNA silencing defense response.

In this study, by using the classical *A. tumefaciens* co-infiltration assay, we have identified the ToCV RNA-1 encoded p22 as an effective silencing suppressor. Its ability to suppress both sense RNA and dsRNA induced silencing in agroinfiltrated *N. benthamiana* leaves clearly indicates that it acts downstream of the formation of dsRNA. The fact of suppressing dsRNA induced silencing is relevant for the biology of this virus because dsRNAs are produced during ToCV replication. Interestingly, the results of local assays showed that in leaves in wich GFP silencing was triggered by either

sense or dsRNA, expression of p22 sustained GFP fluorescence for at least 30 days, the loss of fluorescence being more related with leaf decay than to degradation of GFP mRNA. To our knowledge, this is the first time that such a lasting local suppressor activity has been reported for a silencing suppressor. Our data reveal that although the presence of p22 reduces drastically siRNA accumulation in wild and transgenic GFP *N. benthamiana* plants, they are not completely eliminated (Figures 2B and 3B). However, although p22 could not counteract the induction of RNA silencing, the low levels of GFP siRNAs found through time indicated that a stand-by state of the local silencing response is accomplished, allowing part of the GFP mRNA to remain intact. Then, p22 clearly interferes with initial stages of RNA silencing. Although very effective as a local suppressor, p22 failed to block cell-to-cell or long-distance spread of the systemic silencing signal (Figure 4). Therefore, the mechanism of action of ToCV p22 seems to differ from that of its ortholog in the genome of the crinivirus *Sweet potato chlorotic stunt virus* (SPCSV), which interferes with cell-to-cell and systemic spread of the silencing signal (Kreuze et al., 2005). Moreover, differences also seem to exist at the local level because while ToCV p22 can maintain a reduced level of siRNAs for a long period, SPCSV p22 is only effective in reducing the levels for a short period of time (Kreuze et al., 2005). In this latter case, only the cooperative effect of the endonuclease activity of RNase3 (not present in the ToCV genome) results in a reduction of siRNAs accumulation, thus enhancing the SPCSV p22 suppressor activity (Kreuze et al., 2005).

Although suppressors of silencing exhibit wide structural diversity and modes of action, counterparts of genes encoding suppressors of RNA silencing can be found in the viral genome among members of the same group of viruses at different locations (reviewed by Li and Ding, 2006). Thus, the members of the genus closterovirus *Beet yellows virus* (BYV), *Beet yellow stunt virus* (BYSV), *Grapevine leafroll-associated*

virus-2 (GLRaV) and *Citrus tristeza virus* (CTV) have been shown to contain in the same part of the genome p21-like proteins with silencing suppression activity (Reed et al., 2003; Lu et al., 2004; Chiba et al., 2006). In the case of the bipartite criniviruses and based on the data for the p22 of SPCSV (Kreuze et al., 2005) and ToCV (shown here), it seems that presence of a protein with RNA protection activity at the end of the RNA-1 molecule could confer some selective advantage. Moreover, the identification of the RNA-1 encoded p34 of the crinivirus *Lettuce infectious yellows virus* (LIYV) as a replication enhancer of LIYV RNA-2 accumulation (Yeh et al., 2000) can also support this hypothesis. However, the existence of members of the genus *Crinivirus* without a similar ORF at the end of RNA1, and the recent detection of SPCSV isolates lacking the p22 ORF (Cuellar et al., 2008) might suggest that presence of viral suppressor genes among members of this genus are evolutionary novel and may represent a recent viral adaptation to the host RNA silencing defense response (Li and Ding, 2006). In fact, the analysis of isolates of SPCSV with or without p22 suggests a recent acquisition of p22 by recombination mediated gene gain, conferring a selective advantage to the p22-encoding isolates (Cuellar et al., 2008).

Although the classical *Agrobacterium* co-infiltration assay has been widely used to identify plant virus suppressors, this assay is unable to identify suppressors that do not exhibit intracellular suppression activity (Lu et al., 2004). Therefore, we also explored ToCV genome for suppressors of silencing by means of a viral vector reasoning also that proteins with a weak intracellular suppression activity could be overlooked. Using this strategy with a PVX vector, we identified two additional ToCV proteins encoded by the RNA-2, CP and CPm, that seemed to exhibit silencing suppression activity. Although it is arguable that the presence of the p25 suppressor of PVX (Voinnet et al., 2000; Bayne et al., 2005) can complicate the interpretation of the

suppression activity of the studied genes, the evidence shown here allows us to conclude that CP and CPm have silencing suppressor activity. The observation that the expression of CP and CPm from the heterologous virus enhances the accumulation and symptom severity of PVX (Figure 6) is consistent with a role for these proteins as suppressors of RNA silencing (Pruss et al., 1997; Anandalakshmi et al., 1998; Brigneti et al., 1998). An extreme example of the enhancement of the viral symptoms was that observed in plants agroinoculated with the PVX recombinant virus expressing the p22 of ToCV which died two or three days after the systemic symptoms were observed. This enhancement of pathogenicity, including death of *N. benthamiana* has also been reported after the expression of several other plant silencing suppressors from a PVX-vector (Brigneti et al., 1998; Chu et al., 2000; Pfeffer et al., 2002; Saénz et al., 2001; Thomas et al., 2003; Delgadillo et al., 2004). A direct association between pathogenicity enhancement and increased PVX accumulation due to suppression of RNA silencing can not be established for p22 because PVX.p22 infected plants rapidly died. One question to solve would be to determine why p22 expressed from a PVX-vector was not capable of suppressing silencing after co-infiltration with 35S-GFP plus PVX.p22. A possible explanation could be that p22 suppresses silencing at an early stage of the RNA silencing process, and the production of p22 from the transient construct could be earlier than from the recombinant virus. Similarly, the suppressor of *Turnip crinkle virus* (TCV), the coat protein (CP), was shown to be very efficient suppressing local GFP silencing when it was expressed from a transient CP construct but not from the infectious TCV genome (Qu et al., 2003; Thomas et al., 2003).

The results presented here support the hypothesis that a multiple suppressor based strategy might be common in members of the complex family *Closteroviridae.* Thus, as it was reported for *Citrus tristeza virus* (Lu et al., 2004), ToCV also encodes

three suppressors of RNA silencing. This finding highlights the importance of having effective strategies to counter an RNA silencing response for effective infection in plants. To date, ToCV is the first multipartite plant virus that encodes supressors of silencing in more than one genomic RNA molecules. While ToCV RNA-1 encodes an apparently dedicated suppressor protein, p22, the RNA-2 has delegated this suppressor function to the structural proteins CP and CPm. Notably, no other CPm in the *Closteroviridae* has been reported as RNA silencing suppressor, thus increasing the diversity of functions of this type of protein. The different modes of action suggested for the suppressors of RNA silencing encoded by both genomic RNAs of the crinivirus ToCV, clearly shows a greater complexity of silencing suppressor activity for a plant virus.

Materials and methods

Plasmid constructs

Each single ToCV ORF was amplified by reverse transcription-PCR (RT-PCR) with 5´- and 3´-specific primers containing suitable restriction sites from total RNA extracts from ToCV AT80/99 isolate-infected tomato (*Solanum lycopersicum*) plants, and cloned between the *Cauliflower mosaic virus* 35S promoter and the terminator sequence of the *Solanum tuberosum* proteinase inhibitor II gene (PoPit) as previously described (Genovés et al., 2006), but using the binary vector pBin19 (Frisch et al., 1995). To obtain correct expression of papain-like leader proteinase (L-Pro) and RNA-dependent RNA polymerase (RdRp) proteins, a termination and an initiation codon, respectively, were inserted in the constructs. PVX-derivatives containing the ToCV ORFs were constructed in a similar way but cloning the generated fragments created downstream of the duplicated PVX coat protein promoter in pGR107 (Jones et al., 1999). Plasmid 35S-

GFP (Voinnet and Baulcombe, 1997), 35S-GF-IR (Bayne et al., 2005), 35S-HCPro (Hamilton et al., 2002) and pGR107 (Jones et al., 1999) were provided by Dr. David C. Baulcombe (Sainsbury Laboratory, Norwich, United Kingdom). PVX.NSs construct was provided by Dr. Renato O. Resende (Universidade de Brasília, Brasília, Brazil). Each of the constructs was transformed into *A. tumefaciens* strain GV3101 containing the helper plasmid pJIC SA_Rep by electroporation.

Agroinfiltration and GFP imaging

Wild type or transgenic *Nicotiana benthamiana* line 16c constitutively expressing the GFP transgene (provided by Dr. David Baulcombe) were infiltrated with the *A. tumefaciens* GV3101 strain carrying the plasmids indicated above as described by Voinnet et al. (1998). For co-infiltration, equal volumes of individual *A. tumefaciens* cultures (optical density at 600 nm of 1) were mixed prior to infiltration. GFP fluorescence was observed under long-wavelength UV light (Black Ray model B 100AP, UV products, Upland, CA, USA) and photographed using a Coolpix 8700 Nikon digital camera.

RNA analysis

RNA was extracted from leaf tissue using the method described by Noris et al. (1996). For northern blot analysis of GFP and PVX mRNAs, total RNA aliquots (10µg) for each sample were separated on a 1% formaldehyde agarose gels, transferred to nylon membranes and probed with digoxigenin-labelled specific probes for GFP or PVX as described previously (Liu et al., 2004).

For northern blot analysis of GFP siRNAs, low-molecular-weight RNAs were enriched from total RNAs by eliminating high-molecular-weight RNA using 5% polyethylene

glycol (PEG 8000) plus 0.5 M NaCl, separated on a 15% polyacrylamide 7M urea gel, transferred to nylon membranes and probed with a digoxigenin-labelled specific probe for GFP as described previously (Cañizares et al., 2004).

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Figure legends

Figure 1. Schematic representation of the bipartite genome of *Tomato chlorosis virus* showing open reading frames (ORFs) L-Pro, RdRp and p22 encoded by RNA-1 and hsp70, p59, CP, CPm and p27 encoded by RNA-2 screened for silencing suppression activity.

Figure 2. Identification of p22 as a suppressor of RNA silencing by the *Agrobacterium tumefacies* co-infiltration assay. (A) Photographs taken under UV light of *N. benthamiana* leaves 7 days postinfiltration (dpi) with *A. tumefaciens* harbouring 35S GFP either in combination with the pBIN19 empty vector (vector) or with constructs expressing p22 and HC Pro. (B) and (C) Northern blot analysis of GFP mRNA and siRNA extracted from the zones infiltrated with *A. tumefaciens* harbouring the constructs indicated above each lane, at 3, 7 and 30 days postinfiltration (dpi). Ethidium bromide staining of rRNA and tRNA was used as loading control for mRNA and siRNA, respectively.

Figure 3. Suppression by ToCV p22 of RNA silencing triggered by GFP dsRNA. (A) Photographs taken under UV light of *Nicotiana benthamiana* leaves 7 days postinfiltration (dpi) with *A. tumefaciens* harbouring 35S GFP, 35S GF-IR, either in combination with the pBIN19 empty vector (vector), or with constructs expressing p22 or HCPro. (B) Northern blot analysis of GFP mRNA and siRNA extracted from the zones infiltrated with *Agrobacterium tumefaciens* harbouring the constructs indicated above each lane, at 3 and 7 days postinfiltration (dpi). Ethidium bromide staining of rRNA and tRNA was used as loading control for mRNA and siRNA, respectively.

Figure 4. Effect of ToCV p22 on the spread of systemic silencing of GFP in *Nicotiana benthamiana* line 16c. (A) Photographs taken under UV light of GFP transgenic *N. benthamiana* leaves 10 days postinfiltration (dpi) with *Agrobacterium tumefaciens* harbouring 35S GFP, either in combination with the pBIN19 empty vector (vector), or with constructs expressing p22 and HCPro. (B) GFP fluorescence photographs taken under a UV lamp of the same plant agroinfiltrated with 35S GFP plus 35S p22 at 15, 23 and 30 dpi, respectively.

Figure 5. Suppression by ToCV proteins CP or CPm expressed from the heterologous vector PVX of RNA silencing triggered by GFP mRNA. (A) Photographs taken under UV light of *Nicotiana benthamiana* leaves 6 dpi with *Agrobacterium tumefaciens* harbouring 35S GFP either in combination with PVX (i.e., empty vector), or with the recombinant constructs PVX.CP, PVX.CPm and PVX.NSs. (B) Northern blot analysis of GFP mRNA, genomic PVX RNA and siRNA extracted from the infiltrated zones with *A. tumefaciens* harbouring the construct indicated above each lane, 3 and 6 days postinfiltration (dpi). Ethidium bromide staining of rRNA and tRNA was used as loading control for mRNA and siRNA, respectively.

Figure 6. Enhancement of the pathogenicity of PVX by ToCV proteins. (A) Phenotypic effect observed at 15 dpi in plants agroinoculated with PVX vector containing no insert (PVX), or containing the ToCV proteins CP (PVX.CP), CPm (PVX.CPm) or p22 (PVX.p22). (B) Ethidium bromide-stained RNA gel electrophoresis and northern blot analysis by using a PVX probe of total RNA extracts obtained from young leaves of mock inoculated (mock) plants or plants systemically infected with PVX without an

insert (PVX), or containing the ToCV CP cDNA (PVX.CP), the ToCV CPm cDNA (PVX.CPm).

Figure 1

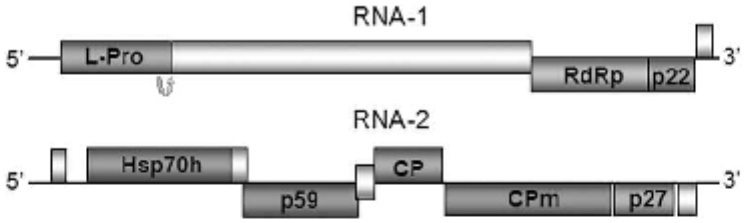
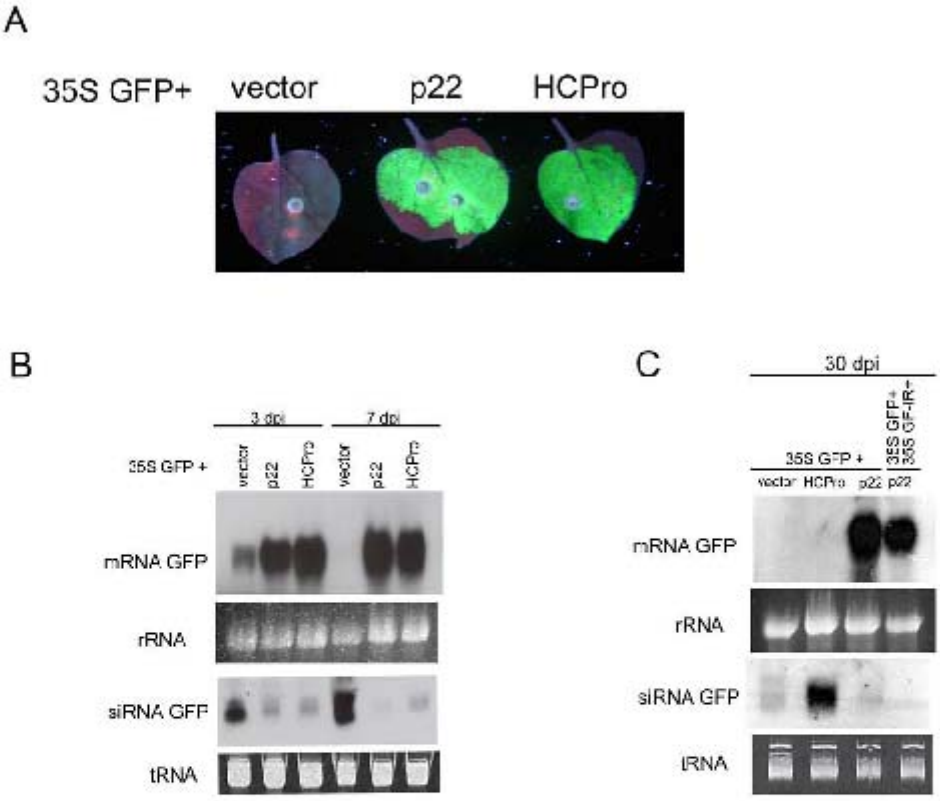


Figure 2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rRNA
siRNA GFP
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Figure 3

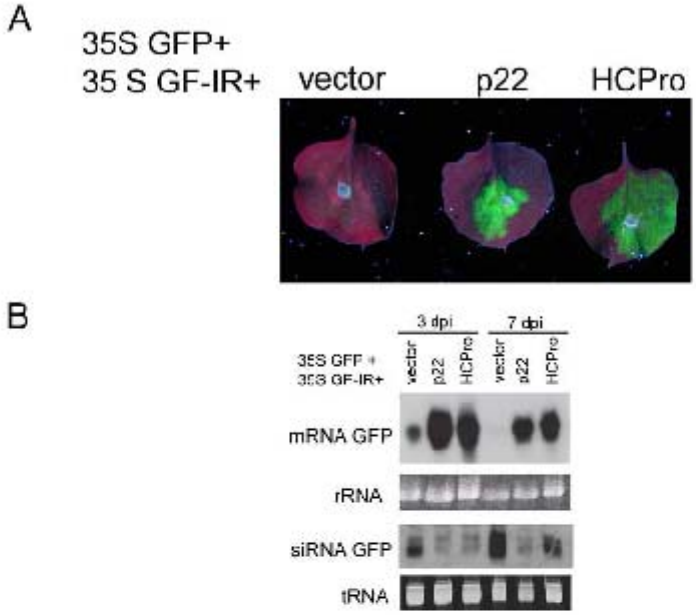
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Figure 4

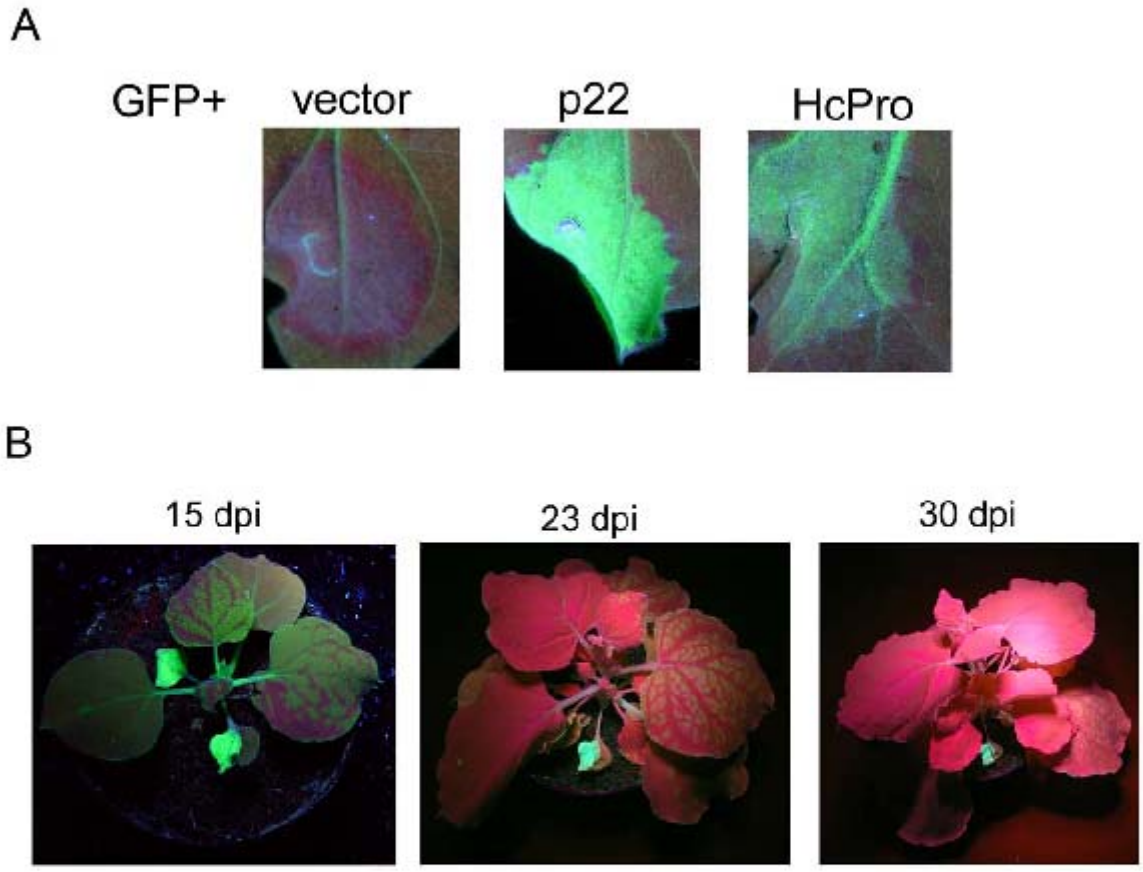
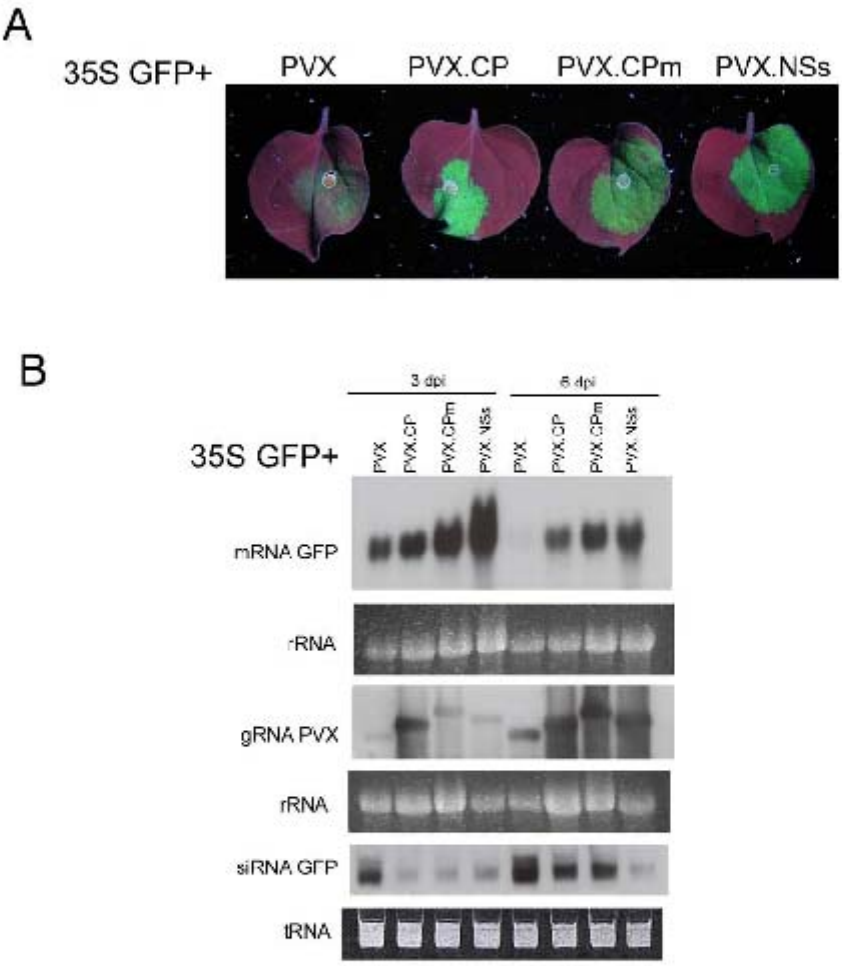


Figure 5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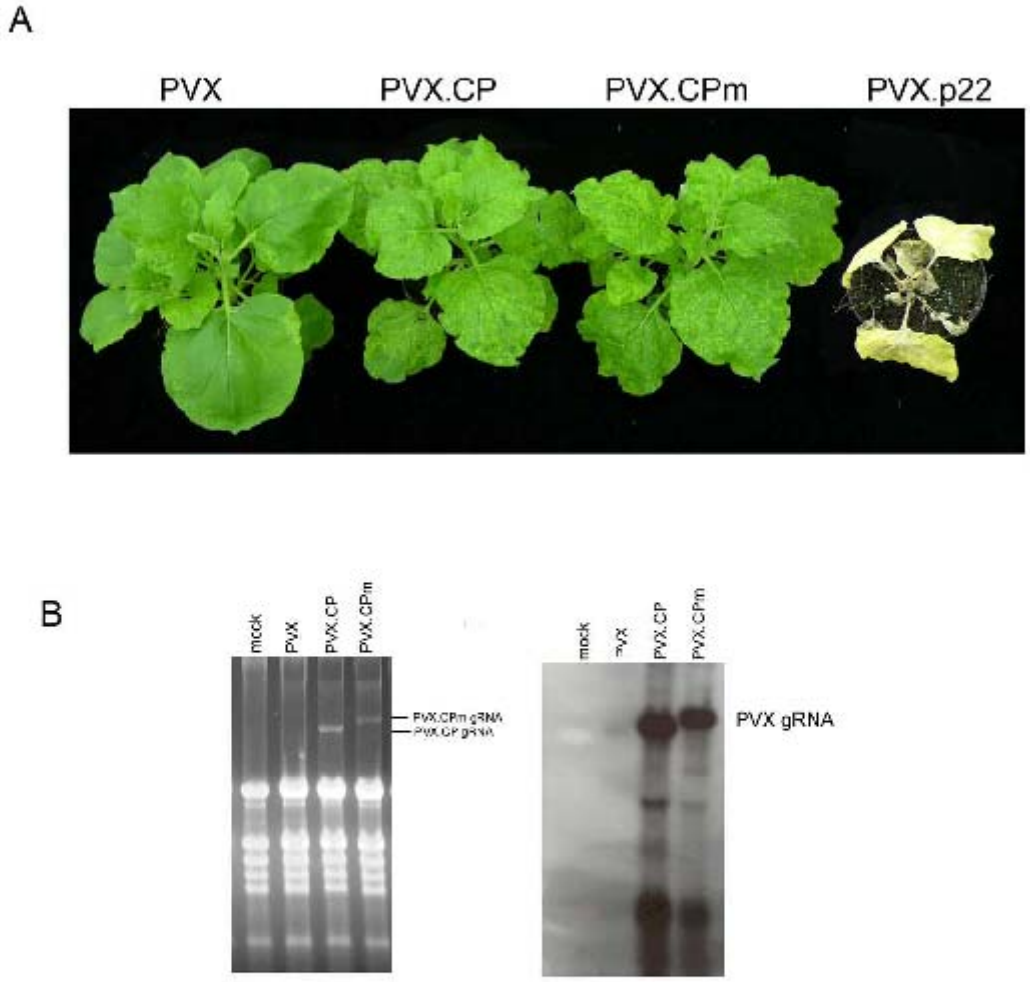
siRNA GFP
tRNA

Figure 6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mock
PVX
PVX.CP
PVX.CPm
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