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Turnip crinkle virus coat protein mediates suppression of RNA silencing in *Nicotiana benthamiana*

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Abstract

All of the protein products of *Turnip crinkle virus* (TCV; *Tombusviridae*, Carmovirus) were tested for their ability to suppress RNA silencing of a reporter gene after transient expression in *Agrobacterium*-infiltrated *Nicotiana benthamiana* leaves. Only the capsid protein, P38, showed suppression activity, although this was not obvious when P38 was expressed as part of a TCV infection of the same tissues. When P38 was expressed from a PVX vector, symptoms with enhanced severity that correlated with increased PVX RNA accumulation were observed. This contradiction between ectopic expression of P38 and TCV infection could be accounted for if the active determinant of suppressor activity within P38 was sequestered within the capsid protein structure. The N-terminal 25 amino acids were shown to be important for this activity. This region forms part of the unexposed R-domain that interacts with the RNA within the virus particle. This observation throws light on some of the complex biology exhibited by TCV.

Keywords: Turnip crinkle virus; RNA silencing; Suppressor; Coat protein; Capsid; Resistance; Nicotiana; Arabidopsis

Introduction

Systemic infection of host plants by viruses is the consequence of competing interests for virus replication and host defense. It has been shown in recent years that in the absence of specific resistance genes, plants are still able to mount a defense response in the form of RNA silencing. This process, first identified in plants, has also been studied in animals and fungi and may be ubiquitous for all eukaryotes. For RNA viruses, viral double-stranded RNA (dsRNA) (potentially that formed as a viral replicative intermediate) is recognized as foreign and related sequences are degraded in a homology-dependent process mediated by cytoplasmic nucleases. Although RNA silencing can be activated in single cells it is not cell-autonomous and in plants a signal transmits the specificity of the silencing to adjacent cells and systemically to remote tissues. This topic has been comprehensively reviewed in the last two years (Carrington et al., 2001; Vance and Vaucheret, 2001; Voinnet, 2001; Waterhouse et al., 2001; Baulcombe, 2002).

RNA silencing is a very potent protective mechanism, to the extent that when activated through a transgene it can virtually eliminate homologous sequences from the tissues. To counter this defense mechanism, many viruses have developed strategies to suppress RNA silencing. This was first recognized for the potyvirus protein P1-HC-Pro (Anandalakshmi et al., 1998; Brigneti et al., 1998; Kasschau and Carrington, 1998), and later for a wide range of other viruses, leading to the suggestion that this was a property associated with most viruses (Voinnet et al., 1999). To date nearly twenty examples of suppressor activity have been reported, all of which are viral non-structural proteins. None of these proteins show sequence similarity and hence appear to have evolved independently to counter silencing-mediated defense. Accordingly, these proteins do not all target the same stages in the underlying mech-

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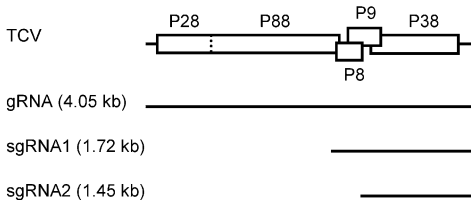

Fig. 1. Schematic representation of TCV genome organization. The TCV open reading frames (ORFs) encoding the five viral proteins (P28, P88, P8, P9, P38) are shown as open boxes within the 5′ and 3′ untranslated regions of the linear genome. The leaky termination site at the end of ORF P28 that allows read-through synthesis of P88 is indicated by a dotted line. The genomic and subgenomic RNA are represented by continuous lines. SgRNA1 expresses P8 and P9; sgRNA2 expresses P38.

anism for RNA silencing (Voinnet, 2001). For example, HC-Pro is able to reverse a previously established transgene-silenced state (Llave et al., 2000) whereas cucumovirus 2b protein (Brigneti et al., 1998) and tomato bushy stunt virus p19 protein can only suppress the intitiation of transgene silencing (Voinnet et al., 1999). Recently the potato virus X (PVX) 25K suppressor protein was shown to block the local spread of the silencing signal (Voinnet et al., 2000), while cucumber mosaic virus 2b prevents systemic translocation of the signal (Guo and Ding, 2002). Most of these suppressors were identified by their ability to interfere with the local and systemic silencing of the green fluorescent protein gene (*GFP*) in *GFP*-transgenic *Nicotiana benthamiana* (Brigneti et al., 1998). However, prior indications as to their identity sometimes came from a correlation with data obtained from mutagenesis to suggest that they were involved in some way with symptom modulation and systemic virus movement, which is a factor that conceivably could provide an indicator as to the nature of the suppressor in other viruses.

In designing strategies for the controlled modulation of virus replication, we wanted to assess and identify suppressor activity from *Turnip crinkle virus* (TCV). TCV is a member of the family *Tombusviridae*, genus Carmovirus. It has a positive single-strand RNA (4054 nt) encapsidated into an icosahedral capsid of 30 nm diameter. Its RNA encodes five open reading frames (ORFs) (Carrington et al., 1989; Hacker et al., 1992) expressed from the genomic and two subgenomic (sgRNA1 and 2) RNAs (Fig. 1). Both P28 and P88 are translated from the genomic RNA by read-through of the P28 amber termination codon and are involved in virus replication (Hacker et al., 1992). The P8 and P9 proteins, synthesized from two overlapping ORFs of the 1.7 kb sgRNA1, are required for virus cell-to-cell movement and systemic spread (Hacker et al., 1992; Li et al., 1998). The P38 capsid protein is expressed from the 1.45 kb sgRNA2 and also plays a host-dependent role in systemic movement (Heaton et al., 1991; Hacker et al., 1992; Cohen et al., 2000). TCV has a broad host range, which predominantly includes members of the *Cruciferae* but also comprises *N. benthamiana* as an experimental host. We have exploited this latter host to determine the nature of any silencing suppressor activity from TCV. We show that the P38 structural protein from TCV is a very strong suppressor of RNA silencing, and propose that sequestering the N-terminal region of P38 within virus capsids can control the impact of this activity.

Results

Identification of the TCV encoded suppressor of RNA silencing

We started our work with the premise that most viruses encode suppressors of gene silencing that indirectly regulate the level and speed of virus accumulation, frequently with effects on tissue invasion and disease pathology. To identify the TCV suppressor, individual TCV ORFs were cloned into a T-DNA expression cassette driven by the CaMV 35S promoter in a pBIN19 based vector (pBIN61) in *A. tumefaciens* strain C58C1. These clones were infiltrated individually, but in combination with bacteria carrying 35S-*GFP*, into leaf patches of non-transgenic *N. benthamiana*. For comparison, 35S-*GFP* was also co-infiltrated with 35S-TCV, a cDNA clone of the complete TCV RNA that generates a full infection in *N. benthamiana* or *Arabidopsis thaliana* (data not shown). In this assay, *GFP* was expressed transiently (over 2–5 days) in the infiltrated patch and GFP was visualised as green fluorescence under UV light. After six days only weak fluorescence is visible (Fig. 2). This decline in expression has been attributed to RNA silencing targeting the foreign gene expression (Johansen and Carrington, 2001). Hence, in the presence of a suppressor such as the potyvirus HC-Pro, *GFP* expression remained high and was visualized as bright green fluorescence (Fig. 2). Since the silencing of *GFP* was established *de novo*, the assay had the potential to identify suppressors of initiation and/or maintenance of RNA silencing.

For TCV, the P8, P9 and P38 (coat protein; CP) had all been implicated as disease determinants (Heaton et al., 1991; Hacker et al., 1992). Since all other viral suppressors were viral non-structural proteins, we expected that either P8 or P9 would most likely fulfill this role. In fact, neither of these proteins showed suppressor activity (Fig. 2). Similarly, no activity was seen following co-expression of the replicase-related products P28 or P88 with GFP (Fig. 2). In contrast, co-expression of the TCV P38 with GFP produced intense green fluorescence (Fig. 2), which persisted for at least 22 days. Considering the need for TCV P38 to produce capsids in infected cells it was surprising to see, however, that co-infiltration of 35S-*GFP* and 35S-*TCV* showed barely any change in GFP fluorescence, compared to infiltration with 35S-*GFP* alone (Fig. 2). While the relative concentrations of the agrobacterium culture for virus gene and *GFP* expression were not optimized for each combination, cultures of equal density were used in each case. GFP fluores-

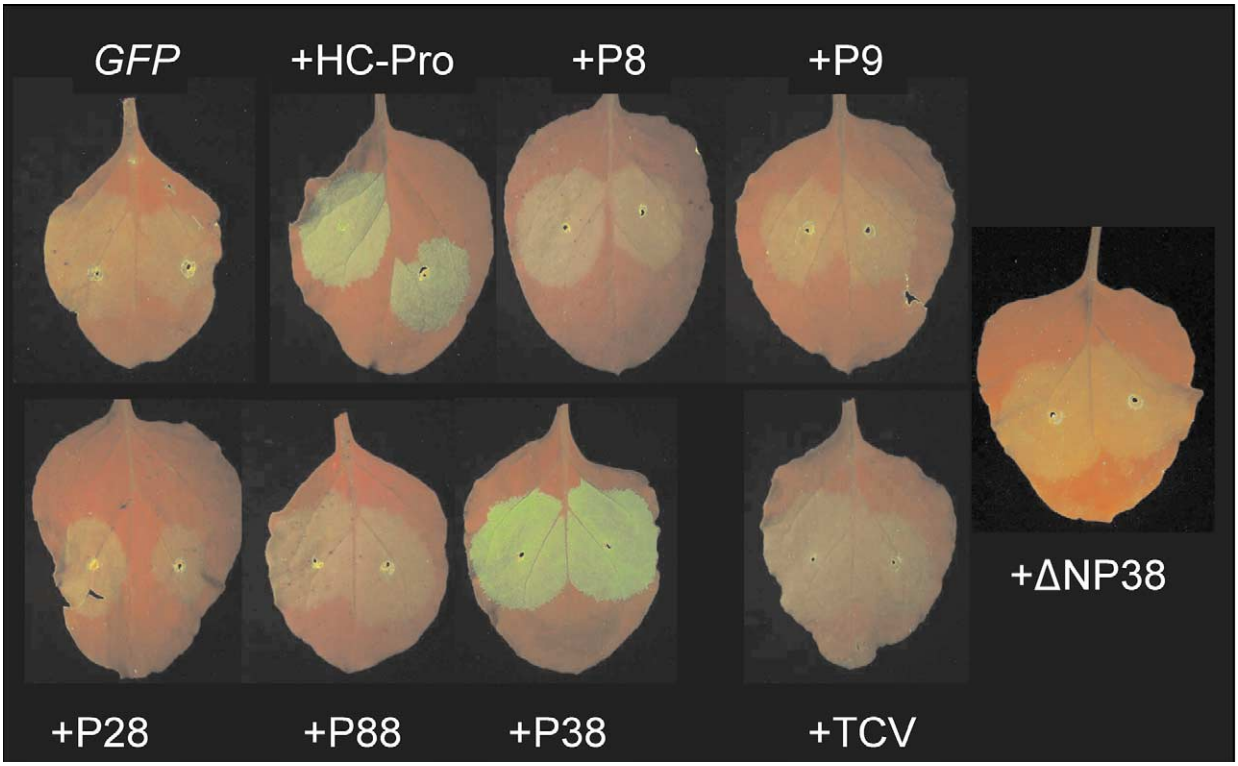

Fig. 2. Effect of potential suppressor activity on 35S-*GFP* expression after agro-infiltration of *N. benthamiana* leaves. Leaves of *N. benthamiana* were agro-infiltrated with 35S-*GFP* (in pBIN61), either in combination with pBIN61 (i.e., empty vector), or with constructs expressing HC-Pro, TCV ORFs P8, P9, P28, P88, P38, ΔNP38 or with 35S-*TCV*. Photographs were taken under UV light to show GFP green fluorescence and the red fluorescence of chlorophyll. Photographs were taken at 6 days post-infiltration when *GFP* expression had declined due to RNA silencing except when a suppressor of silencing was present.

cence in the presence of 35S-*P38* was reproducibly more intense than with the other viral genes, including HC-Pro.

To confirm that the altered fluorescence reflected changes in the steady state levels of *GFP* RNA, northern analysis of RNA extracted from tissues within the infiltrated patch was carried out (Fig. 3). For the expression of *GFP* alone or *GFP* + P8, P9, P28, P88, or TCV, the levels of *GFP* RNA were similar (Fig. 3A). For both *GFP* + HC-Pro and *GFP* + P38 very high levels of *GFP* RNA were detected, with slightly more RNA for the *GFP* + P38 combination. As an indirect measure of P38 accumulation, RNA samples for *GFP* + P38 and *GFP* + TCV were also analyzed for P38-specific RNAs (Fig. 3B). The presence of TCV genomic RNA and sgRNA2 confirmed that the virus was replicating and expressing P38 in the latter tissues.

TCV P38 accentuates PVX infection

Enhanced symptoms resulting from synergistic effects following co-infection of two viruses has been attributed to an enhanced suppression of gene silencing (Pruss et al., 1997). PVX has a relatively weak suppressor of silencing for infections of *N. benthamiana*. To test the impact of P38 on the infection of a heterologous virus, we used a PVX expression vector (pGR107; Jones et al., 1999) for the ectopic expression of P38 and a non-translatable version of P38 (mCP). One leaf on each of four young non-transgenic *N. benthamiana* plants was infiltrated with either PVX (pGR107), PVX-P38 or PVX-mCP. Over 10 days, the leaves infiltrated with PVX or PVX-mCP remained asymptomatic (Fig. 4A), although after six days veinal chlorotic symptoms were increasingly evident on systemically infected leaves (Fig. 4B, shown after 14 days). In contrast, leaves infiltrated with PVX-P38 showed partial and complete necrosis by days six and 10, respectively (Fig. 4A). At six days post-treatment, necrosis was also visible on the systemically infected stem and leaves and the plant shoot had died by day 10-14 (Fig. 4B). Using a TCV-specific probe, we estimated the relative concentration of recombinant PVX RNA in the systemically infected tissues (Fig. 5). PVX-P38 RNA accumulated to a higher level when compared with PVX-mCP, unable to express TCV P38 (Fig. 5). Owing to the early onset of necrosis in infected cells, this was probably an underestimate of the level of replication.

TCV P38 suppressor activity is associated with the N-terminal 25 amino acids

TCV-CP induces a hypersensitive resistance in *A. thaliana* ecotype Di-0 (Oh et al., 1995; Ren et al., 2000). This

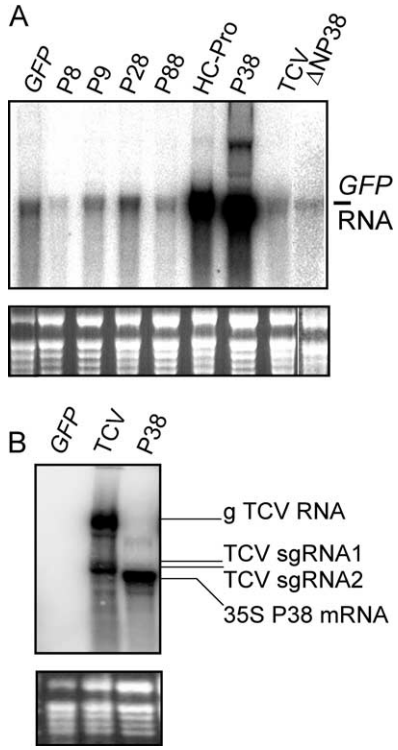

Fig. 3. RNA analysis of the accumulation of *GFP* and TCV-specific RNAs in co-infiltrated tissues. RNA samples from infiltrated leaf patches harvested at 6 days post-infiltration were separated by denaturing gel electrophoresis and the blots analyzed using probes for *GFP* (A) or TCV *p38*(B) sequences. For Panel A, the leaves were agro-infiltrated with 35S-*GFP* (in pBIN61), either in combination with pBIN61 (i.e., empty vector), or with constructs expressing HCPro, TCV ORFs P8, P9, P28, P88, P38, ΔNP38 or with 35S-*TCV*. In Panel B, leaves infiltrated with 35S-*GFP* (in pBIN61) in combination with pBIN61 were compared with those co-infiltrated with constructs carrying 35S-TCV or 35S-P38. The relative loading of the samples is indicated from the ethidium bromide staining of rRNAs (bottom panels).

reaction is strongly associated with the interaction between the N-terminal 25 amino acids of TCV-CP and *A. thaliana* TIP protein (*T*CV-*i*nteracting-*p*rotein; Ren et al., 2000). This domain is essential for virus encapsidation (Qu and Morris, 1997). To determine whether this domain is also correlated with the suppressor activity of TCV in *N. benthamiana*, P38 lacking the first 25 amino acids was cloned downstream of the duplicated CP promoter of pGR107 to give PVX-ΔNP38. *N. benthamiana* plants were also agro-infected with PVX-ΔNP38 (Fig. 4). In contrast, to the extreme necrosis seen with PVX-P38, plants infected with PVX-ΔNP38 were indistinguishable from those infected with PVX-mCP or wild-type PVX, indicating that the N-terminal region of P38 played a role in mediating the extreme pathogenic effects conferred by P38 when expressed from PVX (Fig. 4). To confirm that the role of the N-terminal 25 amino acids was functionally related to gene silencing, ΔNP38 was co-expressed with GFP in non-transgenic *N. benthamiana* plants as described before (Fig. 2). Again ΔNP38 infiltrations showed a level of GFP fluorescence indistinguishable from either 35S-*GFP* alone or from combinations of 35S-*GFP* with other non-suppressor constructs (e.g., 35S-P8). This was reflected in the steady state level of *GFP* RNA (Fig. 3).

The inability of ΔNP38 to suppress silencing could be attributed to instability in the protein. To assess whether the N-terminal deletion influenced protein accumulation, *N. benthamiana* tissues were analysed using a P38-specific antibody (Fig. 6). Tissues systemically-infected with PVX, PVX-ΔNP38 or PVX-P38 were harvested at six days post inoculation, before complete necrosis of PVX-P38 infected tissue. Both ΔNP38 and wild-type P38 were detected by immunoblot analysis although the P38 was more abundant (Fig. 6). This probably reflects the increased replication of PVX in the presence of the P38 suppressor activity. In the same way, tissues infiltrated with 35S-Δ*NP38* or 35S-*P38* were compared. Since in the absence of suppressor activity early expression declines after five to six days due to the impact of silencing (Fig. 2), maximal GFP fluorescence was used to indicate the optimal sampling time for protein analysis. In tissue sampled at two days post-infiltration, both ΔNP38 and P38 could be detected in equal abundance (Fig. 6).

Discussion

TCV is a very well characterized virus. The atomic structure of its icosahedral capsid has been defined to 3.2 Å (Hogle et al., 1986) and the complete sequence of its genome and its coding potential has been known for 13 years (Carrington et al., 1988). Proteins P28 and P88 have been implicated in viral RNA replication, and proteins P8 and P9 in cell-to-cell movement. P38 encodes the viral capsid protein. It is also required for cell-to-cell movement in *N. benthamiana*, but not in *Arabidopsis*, but is essential for systemic movement in both hosts (Hacker et al., 1992; Cohen et al., 2000). The expression strategy for these proteins, involving in part the use of subgenomic RNAs, has also been well-defined. Despite its apparent lack of organizational complexity, TCV has nevertheless displayed some intriguing biology. It is one of relatively few viruses that are highly virulent on *Arabidopsis*. Despite this, one *Arabidopsis* line derived from ecotype Dijon-0 (Di-0) shows hypersensitive resistance for which the avirulence determinant was shown to be the coat protein P38 (Oh et al., 1995). This resistance appears to be mediated through an *Arabidopsis* protein that interacts with the N-terminus of P38 (Ren et al., 2000). TCV is also frequently associated with both satellite (sat) RNAs and defective-interfering RNAs, which have contrasting impacts on the replication and symptomatology of TCV depending on features of the coat protein (reviewed in Simon, 1999).

We set out to determine the existence and the nature of any suppressor of gene silencing for TCV. As a paradigm for this work, the P19 movement protein from the related

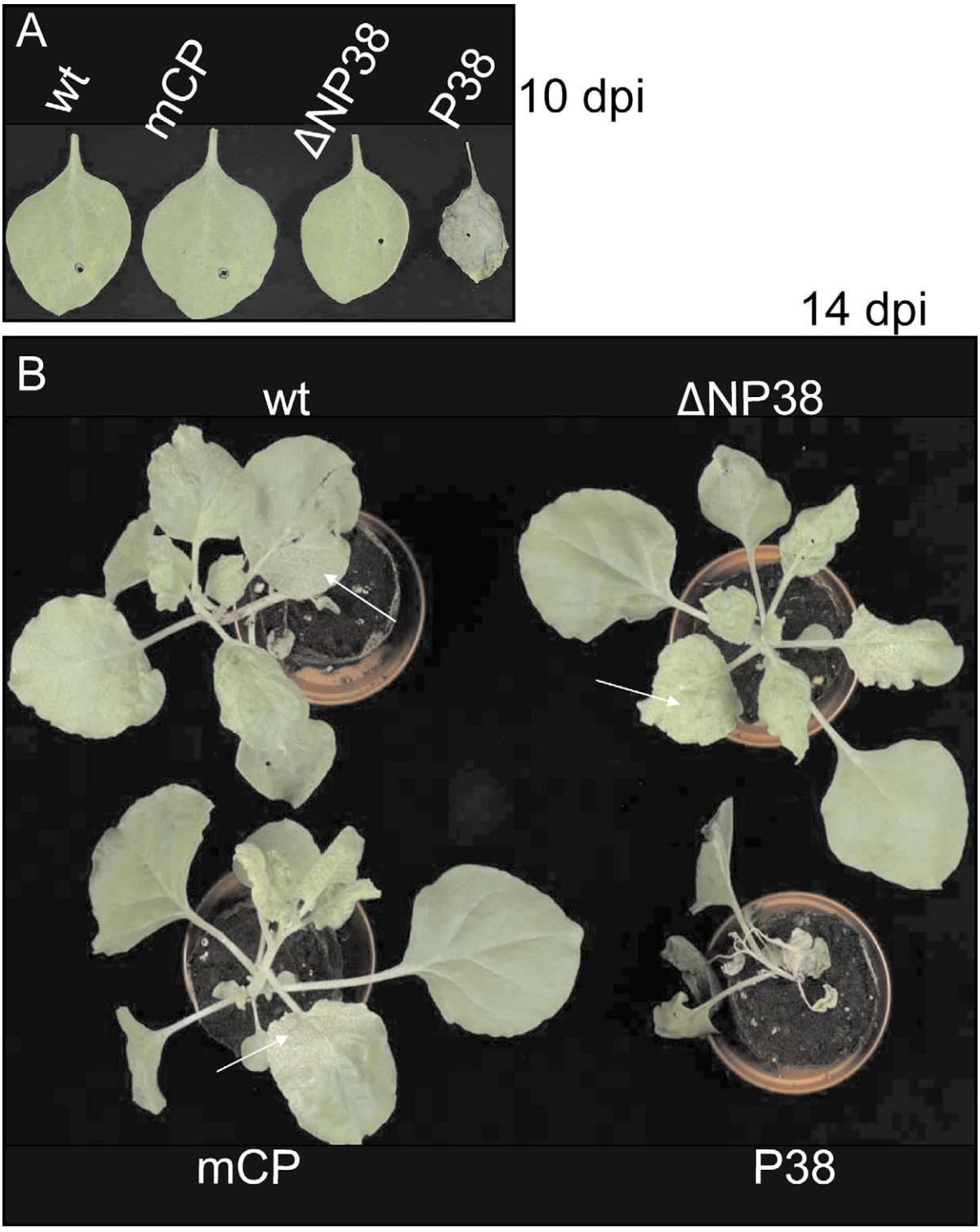

Fig. 4. Phenotypic effects associated with the expression of TCV P38 from the PVX vector. Plants were agro-inoculated with PVX vector constructs containing no insert (wild-type; wt), the TCV P38 cDNA (P38), an untranslatable P38 sequence (mCP), or an N-terminally deleted version of P38 (ΔNP38). After 6 days (10 days shown here) tissues inoculated with PVX-P38 showed severe necrosis while the other infections were asymptomatic. After 10–14 days, plants infected with PVX-wt, PVX-mCP or PVX-ΔNP38 showed the typical chlorotic symptoms of PVX infection (e.g., arrows). Plants infected with PVX-ΔNP38 showed near-complete necrosis.

Tomato bushy stunt virus (TBSV) had been identified as a suppressor of the initiation of gene silencing (Voinnet et al., 1999). (Note: During the progress of this work Qu and Morris (2002) provided unpublished evidence that the TBSV P19 protein could be complemented in its suppressor activity by the TCV coat protein.) Using co-infiltration and

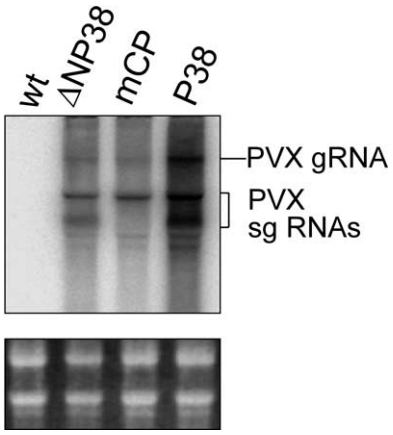

Fig. 5. Recombinant PVX RNA accumulation in systemically infected [b]*N. benthamiana* leaves. RNA samples from leaves systemically infected with recombinant PVX without an insert (wt) or PVX containing the TCV P38 cDNA (P38), an untranslatable P38 sequence (mCP), or an N-terminally deleted version of P38 (ΔNP38), were separated by gel electrophoresis and analysed by hybridisation using a TCV-*P38*-specific probe. The relative migration of PVX genomic (g) or sub-genomic RNAs are indicated. The relative RNA loadings are indicated from the ethidium bromide staining of rRNAs (bottom panel).

expression of *GFP* and viral genes in the model host, *N. benthamiana*, we were surprised to find, however, that none of the TCV non-structural proteins showed suppressor activity. Rather, the coat protein P38 showed strong activity when expressed ectopically, apparently stronger than the equivalent potyvirus protein, HC-Pro. This activity was observed as enhanced GFP fluorescence and *GFP* RNA levels. To test whether this activity was relevant in the context of a virus infection, we expressed P38 and an untranslatable mutant of P38 (mP38) from a PVX vector. P38 had a dramatic effect on the infection phenotype leading to rapid tissue necrosis and plant death. Similar enhanced disease phenotypes are typical of synergistic reactions following co-infections with two viruses and have been associated with an increased suppression of gene silencing (Pruss et al., 1997). This is normally associated with increased accumulation of one or both viruses. We also saw a larger accumulation of PVX in the presence of P38, although this was probably underestimated due to the severity of the tissue necrosis. We could discount the possibility that P38 itself caused tissue necrosis since we saw no such phenotype in the co-infiltration assay where P38 was expressed in the absence of virus infection. Similarly, no phenotype was reported for transgenic *Arabidopsis* expressing TCV P38 (Cohen et al., 2000).

Since the viral coat protein is required in abundance for encapsidation, it was surprising that the co-infiltration assay did not demonstrate an equivalent activity when a complete infectious TCV genome was present. We did not measure the precise timing of the ectopic and virus-driven expression of P38, or measure P38 protein levels after 35S-*TCV* infiltration, although the accumulation of the TCV sgRNA2 during TCV infection indicated that P38 was being expressed. Nevertheless, we know from independent experiments that the peak of TCV replication occurs after the peak of 35S-mediated gene expression (unpublished data). It is possible that our inability to see suppressor activity as increased GFP fluorescence with TCV infiltration may reflect the sensitivity of the assay. Notwithstanding this, others (F. Qu and J. Morris, personal communication) have shown that P38 operates as a suppressor in the context of a TCV infection. In our experiments, it would seem likely that coat protein committed to capsid production could be functionally inactive with respect to silencing suppression. A similar discrepancy between TCV infection and ectopically expressed P38 was observed recently with respect to the complementation of TCV GFPΔCP I, a virus capable of expressing GFP after its insertion into the N-terminal region of P38 (Cohen et al., 2000). This virus is cell-to-cell and systemically restricted in *N. benthamiana* and *Arabidopsis*, respectively, but is complemented for long distance movement in transgenic *Arabidopsis* expressing P38. In contrast, co-infection of this virus with wild-type TCV as helper resulted in the accumulation of the helper but not TCV GFPΔCP I in upper leaves.

In common for several families of small icosahedral viruses, including the *Tombusviridae*, the coat protein can be divided into three structural domains: the C-terminal P domain that extends outwards beyond the virion surface, the central S domain that comprises the virion shell and the N-terminal R domain that interacts with the RNA within the interior of the capsid (Qu and Morris, 1999). One mechanism of regulating the silencing suppression activity could therefore be through the sequestration of active P38 in an inactive capsid form, a potential example of post-translational conformational control. Relatively little is known of the structure and biological activity of non-capsid P38 in vivo. However, to investigate TCV P38 as an avirulence determinant for the *HRT* locus in *Arabidopsis* ecotype Di-0,

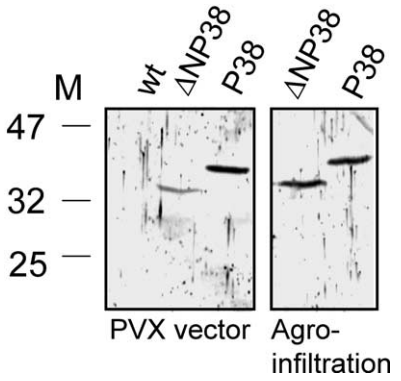

Fig. 6. Immunoblot analysis of P38 and ΔNP38. Protein samples from tissue infected with recombinant PVX without an insert (wt), PVX with a N-terminally deleted version of P38 (ΔNP38) or PVX with TCV P38 cDNA (P38) and tissues agro-infiltrated with 35S-Δ*NP38* or 35S-*P38* were subjected to SDS–PAGE and the P38-specific polypeptides identified on immunoblots using anti-P38 serum. The relative migration of molecular weight markers (M; kDa) is indicated.

Ren et al. (2000) used the yeast two-hybrid system to demonstrate the interaction between the N-terminal 25 amino acids of the P38 R domain and a potential transcriptional activator (NAC domain) protein. We tested a P38 mutant deleted for these 25 amino acids (ΔNP38) for its ability to suppress gene silencing. Neither in the co-infiltration *GFP* assay nor as a synergistic factor for PVX infection was suppression activity detected even though we were able to verify that the protein accumulated in treated tissues. While this indicates that part of the R domain is necessary for suppressor activity it does not show that it is sufficient. Nevertheless, it does provide an explanation for the apparent lack of activity for capsid-associated P38 since the N-terminal 25 amino acids would be inaccessible. Whether or not the suppressor activity is functionally related to the resistance observed in *Arabidopsis* has not been formally tested, although indirect evidence (below) from work with TCV sat RNA-C suggests that this might not be so. It is relevant, however, that preliminary data indicate P38 as a suppressor of silencing in *Arabidopsis* (O.Voinnet, personal communication).

The effects on TCV of either sat RNA C or defective interfering (DI) RNA reveal a complex picture of molecular interactions centered on the coat protein (P38) (Simon, 1999). When the coat protein of the related *Cardamine chlorotic fleck virus* (CCFV) was used to replace TCV P38, sat C or DI RNA accumulation increased over 20-fold relative to that seen with the wild-type TCV, and the normal intensification of symptoms associated with the presence of either sat or DI RNAs was reversed and symptoms were attenuated (Kong et al., 1997a). One interpretation of these data is that the suppressor function of P38 is dependent upon the active concentration of the exposed N-terminal R domain, which is modulated in some way by the presence of the sat or DI RNAs. The CCFV protein may not substitute for this suppressor function. In subsequent experiments (Kong et al., 1997b; Wang and Simon, 1999), a reduced level of P38 accumulation and encapsidation occurred when subtle changes to the P38 initiation codon were made. These changes also overcame resistance in *Arabidopsis* Di-0. A further local compensatory mutant, which restored P38 accumulation and encapsidation to wild-type levels, also restored virulence on susceptible (Col-0) plants, still overcame resistance on ecotype Di-0, and eliminated any effect of sat-RNA C. The fact that the compensatory mutant restored virulence but still overcame resistance suggests that the suppressor and avirulence determinant functions of P38 can be genetically distinguished.

Since virus replication and encapsidation are usually very tightly linked processes and progeny virus particles accumulate to very high concentrations, the use of the coat protein as a suppressor of RNA silencing presents particular challenges for the virus with respect to regulating any impact on the host. Our indirect evidence suggests that, for TCV, these competing demands could be dealt with by placing the determinant of suppressor activity in a location that is not exposed when it exists within the virus particle.

Materials and methods

Plants and viruses

N. benthamiana plants were grown for silencing studies as previously described (Voinnet et al., 1998). A cDNA clone of TCV isolate 'M' in a derivative of pT7E19 (Oh et al., 1995) was obtained from A. Simon. PVX, as an expression vector (pGR107) was obtained from D. Baulcombe.

Constructs

35S-*GFP*, containing the GFP ORF cloned into the binary expression vector pBIN61, has been described before (Voinnet and Baulcombe, 1997). TCV was PCR amplified from the cDNA of TCV-M (Oh et al., 1995) using primers (TCVS1 and TCVA1; Table 1) and *Pfu* polymerase (Stratagene). To generate 35S-TCV, the product with adapted ends was digested with *Xba*I and *Xma*I for directional cloning into *Xba*I/*Xma*I digested pBIN61, using standard molecular biology techniques. For individual TCV cistrons, specific primer pairs (see Table 1) for P28 (TCV p28/88 5′ and TCV p28 3′), P88 (TCV p28/88 5′ and TCV p88 3′), P8 (TCV p8 5′ and TCV p8 3′), P9 (TCV p9 5′ and TCV p9 3′) and P38 (TCV p38 5′ and TCV p38 3′) were similarly used to create constructs 35S-P28, 35S-P88, 35S-P8, 35S-P9 and 35S-P38, respectively. PVX derivatives PVX-P38, PVX-mP38 and PVX-ΔNP38 were constructed in a similar way using primer pairs TCV p38 5′Cla and TCV p38 3′, TCV p38 m5′Cla and TCV p38 3′, and TCV p38 ΔN25 and TCV p38 3′. The fragments created by PCR amplification were directionally cloned into the *Cla*I and *Sma*I sites downstream of the duplicated coat protein promoter in pGR107 (Jones et al., 1999). Sequencing showed that mutated P38 differed from wild-type P38 only at the site of mutation. 35S-HC-Pro (ex. *Potato virus Y*) was obtained from D. Baulcombe (Hamilton et al., 2002).

Virus infection and agro-infiltration

The agrobacterium infiltration method used has been described previously (Voinnet et al., 1998). Clones of different pBIN61 constructs in *Agrobacterium tumefaciens* strain C58C1 were grown to $OD_{600} = 1$ and were mixed in equal volumes prior to co-infiltration. Derivatives of PVX were propagated in *A. tumefaciens* strain GV3101, grown to $OD_{600} = 0.5$, and infiltrated into non-transgenic *N. benthamiana* plants. GFP expression was observed and photographed under UV light as described previously (Voinnet et al., 1998).

Table 1
PCR primers used in the construction of TCV expression cassettes

TCV primer	Sequence
TCV S1	GCACATCTAGAGGTAATCTGCAAATCCCTGG
TCV A1	GACTGCCCGGGCAGGCCCCCCCC
TCV p28/88 5′	GGGTTCTAG**ATG**CCTCTTCTACACACACTC
TCV p88 3′	TCATCTAGAACCCGGGCGTCGCTGTCGC**TTA**GAGA
TCV p28 3′	AGAACCCGGG**TTA**CTAGCGGACAAAAGAGATCG
TCV p8 5′	GGGTTCTAG**ATG**GATCCTGAACGAATTCC
TCV p8 3′	AGAACCCGGG**TTA**GAAGTTGAAGTTGATTGAGAC
TCV p9 5′	CGCCCGGGTTCTAG**ATG**AAGGTTCTGCTAGTCACG
TCV p9 3′	AGAACCCGGG**TTA**TCATTTTCCATTTCCAGTGTT
TCV p38 5′	GGGTTCTAG**ATG**GAAAATGATCCTAGAG
TCV p38 3′	AGAACCCGGG**TTA**CTAAATTCTGAGTGCTTGC
TCV p38 5′Cla	GGATCGAT**ATG**GAAAATGATCCTAGAG
TCV p38 m5′Cla	GGATCGAT**TAG**GAAAATGATCCTAGAG
TCV p38 N25Δ	GGATCG**ATG**TGGTCAACCCTAACCAGCAGAC

Note. Restriction sites are underlined. Start and Stop codons are in bold.

Northern analysis

RNA was extracted from plant tissue using Tri-reagent (Sigma) following the manufacturer's protocol. RNA electrophoresis and gel blot analyses were performed as described previously (Jones et al., 1998). Equal amounts of total RNA (2.5 æg) were separated by agarose gel electrophoresis under denaturing conditions and transferred to Hybond-NX membranes. Membranes were hybridized with ^{32}P-labeled probes specific for *GFP*, or TCV-P38 using standard techniques. After washing, the blots were exposed using phospho-imager plates.

Immunoblot analysis

N. benthamiana tissues, systemically infected with PVX constructs, were harvested at 6 d post-inoculation, rapidly frozen and homogenised in 1× Laemmli sample buffer. Leaf tissue patches harvested two d post-infiltration were processed similarly. Equalized loadings of approximately 20 mg tissue equivalent were electrophoresed on a 10% SDS-polyacrylamide gel and blotted to nitrocellulose. The immunoblot was incubated with anti-TCV-P38 serum (T. J. Morris, personal communication) at a dilution of 1/500, washed, and the specific reactivity visualized using alkaline phosphatase-conjugated goat anti-rabbit serum and nitroblue tetrazolium as a colorimetric substrate, following standard techniques.

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