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RNA silencing-suppressor function of *Turnip crinkle virus* coat protein cannot be attributed to its interaction with the *Arabidopsis* protein TIP

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The interaction of the coat protein (CP) of *Turnip crinkle virus* (TCV) with a host protein, TCV-interacting protein (TIP), from *Arabidopsis thaliana* has been reported previously. This interaction correlates with the ability of TCV CP to elicit the resistance response that is mediated by the resistance gene *HRT* in *Arabidopsis* ecotype Di-17. It has also been established that TCV CP is a suppressor of RNA silencing, a process by which the host plant targets viral RNA for degradation. These results have led to the speculation that TIP might be a component of the RNA-silencing pathway and that TCV CP suppresses RNA silencing through its interaction with TIP. In the current report, a number of TCV CP mutants have been investigated for their ability to suppress RNA silencing. These mutants include single amino acid substitution mutants that are known to have lost their ability to interact with TIP, as well as deletion mutants of TCV CP that are of different sizes and from different regions of the protein. Results showed that each of the single amino acid substitution mutants tested retained high levels of RNA silencing-suppressor activity. In addition, a mutant containing a 5 aa deletion in the region that is known to be critical for TIP interaction retained the ability to suppress RNA silencing significantly. Larger deletions in all regions of TCV CP abolished silencing-suppressor activity. It can be concluded from these results that the RNA silencing-suppressor activity of TCV CP cannot be attributed to its ability to interact directly with TIP.

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INTRODUCTION

Viruses constitute a major group of plant pathogens. Plants respond actively to virus invasion by employing several distinct defence mechanisms that recognize and counteract the attack. RNA silencing, one of these active defence mechanisms, targets the invading viral RNA in a sequence-specific manner for subsequent destruction (Baulcombe, 2002). The RNA-silencing pathway uses viral RNA as a template to generate small RNA species of 21–24 nt, termed small interfering RNAs (siRNAs). These siRNAs of viral origin then serve as the guide for further destruction of the invading viral RNA genome as it advances systemically. As the specificity of RNA silencing is determined by the genome of the invading virus, this pathway can target any virus specifically. It is now recognized to be a ubiquitous defence strategy operating against viral pathogens and transposons. RNA silencing is probably an evolutionarily ancient plant defence mechanism, given that the majority of well-studied plant viruses are known to have evolved the ability to evade this defence by encoding suppressors of RNA silencing (Moissiard & Voinnet, 2004; Silhavy & Burgyán, 2004).

A second well-studied active plant defence mechanism is the resistance response, which is mediated by a specific resistance gene (*R* gene) in the plant host. This form of defence is conditioned by the presence of a corresponding avirulence or effector gene in the pathogen. This gene-for-gene interaction triggers a hypersensitive response at the site of invasion that limits the spread of the pathogen (Dangl & Jones, 2001). The specificity of this innate defence system has long been speculated to be due to direct recognition of the effector protein by the host R protein (Gabriel & Rolfe, 1990). However, molecular characterization of several R proteins and corresponding effector proteins, followed by attempts to demonstrate their physical interactions, has verified such direct recognition events in only three systems (Tang *et al.*, 1996; Jia *et al.*, 2000; Deslandes *et al.*, 2003). Instead, several reports have identified other novel host proteins [e.g. TCV-interacting protein (TIP), RIN4 and PBS1] that play an essential role in mediating the *R* gene response (Ren *et al.*, 2000; Mackey *et al.*, 2002, 2003; Shao *et al.*, 2003). In these cases, the pathogen effectors interact with these novel host proteins and modify them biochemically, leading to *R* gene-dependent resistance. These findings lend fresh support to the 'guard hypothesis' (Dangl & Jones, 2001) as an

explanation of how *R* genes function. This hypothesis postulates that R proteins in the nucleotide-binding sites/leucine-rich repeat domains class, to which most of the characterized R proteins belong, function by 'guarding' key components (such as TIP, RIN4 or PBS1) of host basal defence pathways. It is proposed that pathogens would have evolved the ability to target key components of the basal defence system in order to invade successfully. To counteract the pathogen invasion, plants have evolved a surveillance system composed of *R* genes that effectively detect changes in components of the basal defence system that are brought about by pathogen attack. Although the plant basal defence system has not been well-defined, our recent results from studies on *Turnip crinkle virus* (TCV) and its interaction with the host plant *Arabidopsis thaliana* raised the speculation that the RNA-silencing pathway could well represent a basal defence system that is targeted by viruses, components of which could then be surveyed by *R* genes.

The coat protein (CP) of TCV is a potent effector protein because it is both a suppressor of RNA silencing (Qu *et al.*, 2003) and an elicitor of *R* gene-mediated resistance that is conditioned by the *HRT* gene, identified in ecotype Di-17 of *A. thaliana* (Cooley *et al.*, 2000). We have shown that TCV CP also interacts specifically with the host protein TIP and established that this interaction correlates with the ability of ecotype Di-17 to mount an HRT-mediated resistance response (Ren *et al.*, 2000). These results were cited by Dangl & Jones (2001) as evidence in support of extending the 'guard hypothesis' to include virus pathogens. Since then, we have also established that the TIP protein is a member of the NAC family of transcription factors and that interaction between TCV CP and TIP protein in plant cells prevents the transcription factor from localizing to the nucleus (T. Ren, F. Qu & T. J. Morris, unpublished results). Based on these data, we find it tempting to suggest that RNA silencing might function as a basal resistance pathway that is suppressed by TCV CP through CP–TIP interaction, and that HRT might operate by detecting this interaction as the trigger for mounting the resistance response. This model was supported by Thomas *et al.* (2003) in a paper on the characterization of TCV CP as a suppressor of silencing. In that study, they deleted the N-terminal 25 aa in TCV CP that we had implicated in CP–TIP interaction and found that the resulting deletion mutant had lost the ability to suppress RNA silencing. We felt that it was very important to test our resistance-breaking TCV CP mutants rigorously for their ability to suppress RNA silencing, as these results have important implications with respect to connecting CP–TIP interaction and, hence, the *R* gene-mediated resistance response, to the RNA-silencing pathway. In the current report, we show that TCV CP–TIP interaction is unnecessary for the suppression of RNA silencing by TCV CP. Our data suggest, instead, that the suppressor and resistance-elicitor functions of TCV CP may be distinct, supporting the conclusion that TCV CP interferes with multiple host basal defence pathways.

METHODS

Constructs. The PZP–GFP, PZP–TCVCP, PZP–CPΔ and PZP–TEVHC-Pro constructs were described previously (Qu *et al.*, 2003). Other constructs used in this work are listed in Fig. 1. The corresponding cDNA fragments of TCV CP mutants were PCR-amplified by using appropriate oligodeoxynucleotide primers, with TCV

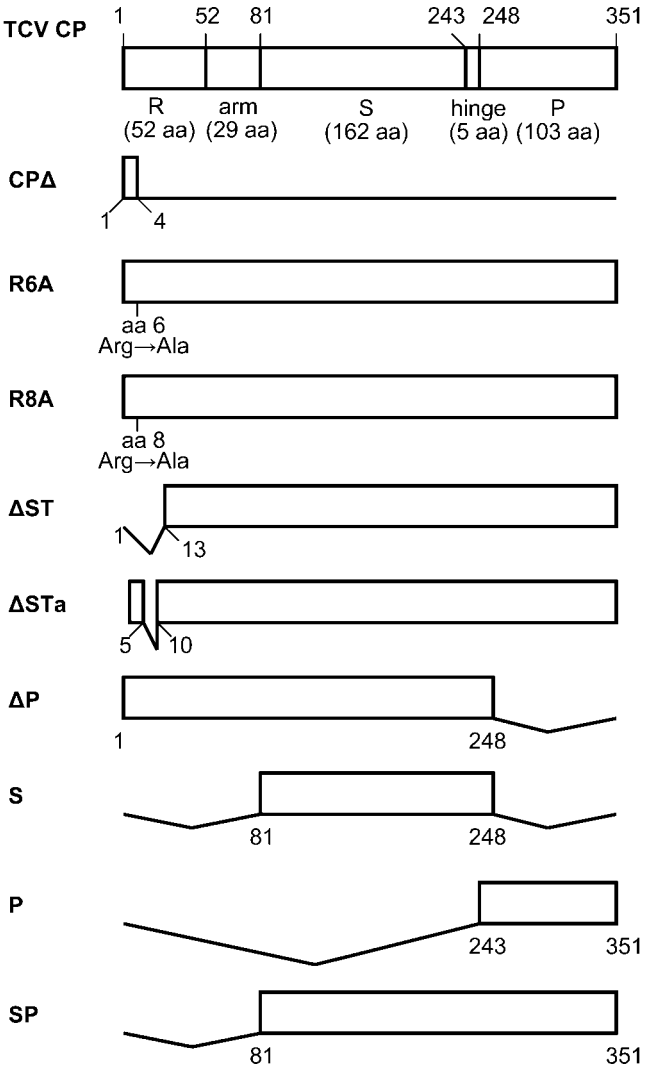

Fig. 1. Diagrams depicting the TCV CP mutants used in this work. The top diagram shows TCV CP, with the different structural domains and their respective sizes indicated. The CPΔ mutant has two consecutive stop codons after the first 4 aa and is not expected to make functional TCV CP in plant cells. Mutants R6A and R8A substitute alanine for arginine residues at positions 6 and 8, respectively. ΔST has aa 2–12 deleted. ΔSTa has aa 6–9 deleted. ΔP has the whole P domain deleted. Mutant S makes a protein that contains the whole S domain plus the 5 aa hinge, whilst mutant P makes a protein containing the hinge and the P domain. Mutant SP has the N-terminal R domain and the arm deleted. Open boxes show the regions of the expressed protein and have an appropriate initiation codon.

cDNA as template. Recognition sites for the restriction enzymes *Nco*I and *Xba*I were incorporated into the 5′ and 3′ primers, respectively, to facilitate subsequent cloning into the plasmid pRTL2 (Carrington & Freed, 1990). The R6A and R8A TCV CP mutants contain arginine to alanine substitutions at aa 6 and 8, respectively. All TCV CP deletion mutants (ΔST, ΔSTa, ΔP, S, P and SP) are in-frame with the TCV CP ORF. The mutant cDNAs were cloned into pRTL2 and the resulting plasmids were sequenced to ascertain that no PCR errors were present. The fragments containing the corresponding mutant cDNAs flanked by the promoter and terminator of *Cauliflower mosaic virus* (CaMV) 35S RNA were released from these plasmids and incorporated into *Pst*I-digested binary vector PZP212.

***Agrobacterium* infiltration.** The constructs described above were introduced into *Agrobacterium* strain C58C1 by electroporation. The detailed agroinfiltration procedure has been described elsewhere (Qu *et al.*, 2003).

Analysis of RNA and proteins extracted from infiltrated leaves. RNA extraction and Northern blot analysis were carried out as reported previously (Qu *et al.*, 2003). Protein extracts were prepared from infiltrated leaves by grinding the leaves in liquid nitrogen and suspending the ground tissue in PBS containing 0·1 % mercaptoethanol. Extracts were then centrifuged at 8000 ***g*** for 10 min and the supernatant fractions were subjected to SDS-PAGE and Western blot analysis following standard procedures (Sambrook *et al.*, 1989).

Particle bombardment of detached *Arabidopsis* leaves. The protocol of Després *et al.* (2003) was used with minor modifications. *A. thaliana* (ecotype Col-0) was maintained in a growth chamber at 21 °C (day) and 18 °C (night) with a 10 h photoperiod. Leaves from 4- to 5-week-old plants were placed on plates containing medium with MS salts, micronutrients, B5 vitamins and 1 % sucrose at pH 5·8, and solidified with 0·8 % agar (A-1296; Sigma). The leaves were bombarded at a pressure of 1100 p.s.i. (7500 kPa) and a flight distance of 4 cm by using a Bio-Rad PDS-1000/He biolistic particle delivery system with M10 tungsten particles coated with 1 μg pRTL2–GFP plasmid mixed with 1 μg pRTL2–TCVCP, pRTL2–CPΔ or pRTL2–R6A plasmids. Five leaves were bombarded for each combination of plasmids. The plates containing the bombarded leaves were then kept in the growth chamber for 48 h. The leaves treated with the same plasmids were pooled and total RNA was extracted and subjected to Northern blot analysis.

RESULTS AND DISCUSSION

TCV CP mutants that are unable to interact with TIP retain RNA silencing-suppressor activity

We reasoned that if TIP was a key component of the RNA-silencing pathway and was targeted by TCV CP in order to suppress RNA silencing, then TCV CP mutants that are unable to interact with TIP (Ren *et al.*, 2000) should also lose their ability to suppress RNA silencing. To test this, we assayed several TCV CP mutants for silencing-suppressor activity by using an *Agrobacterium* infiltration assay to demonstrate suppressor activity of the wild-type TCV CP (described previously; Qu *et al.*, 2003). We selected two representative mutants, designated R6A and R8A, that had a single arginine residue replaced with an alanine residue at aa 6 and 8 of TCV CP, respectively (Fig. 1). The R6A mutant was described previously by Ren *et al.* (2000) and a more extensive study of the TIP-binding properties of both mutants is currently in progress (T. Ren, F. Qu & T. J. Morris, unpublished results). Both R6A and R8A mutants lost the ability to interact with TIP in yeast and were unable to trigger HRT-mediated TCV resistance in the resistant *Arabidopsis* ecotype Di-17 (Table 1). The cDNAs of R6A and R8A, flanked by the 35S promoter and transcriptional terminator of CaMV, were incorporated into the binary vector PZP212 prior to introduction into *Agrobacterium* strain C58C1. Leaves of green fluorescent protein (GFP)-transgenic *Nicotiana benthamiana* (GFP 16c) plants were then infiltrated with mixed *Agrobacterium* suspensions containing PZP–GFP and either PZP–R6A or PZP–R8A. The level of expression of GFP mRNA was then monitored by Northern blot analysis 5 days after infiltration (d.a.i.). As expected, GFP mRNA was detected at a lower level in leaves that were infiltrated with PZP–GFP alone than in the mock-inoculated control; this was because of strong induction of silencing by the transiently expressed GFP mRNA (see Fig. 2a; the loading of lane 1 is about twofold lower than that of lane 2 to generate an equivalent GFP mRNA signal). Consistent with our previous results, co-infiltration of PZP–GFP and PZP–TCVCP resulted in a dramatic increase in the level of GFP mRNA accumulation (lane 3), a consequence of the silencing-suppressor activity of TCV CP. Co-infiltration of GFP and the CPΔ construct resulted in a level of GFP mRNA accumulation about equal to that of the buffer control. This served as an additional negative control (Fig. 2a, lane 4). Significantly, both of the mutant TCV CPs (R6A and R8A) that were tested in this assay suppressed RNA silencing as effectively as the wild-type TCV CP. Although the levels of GFP mRNA accumulation that were found in this experiment suggest that the mutant TCV CPs might display a higher suppressor activity than the wild-type TCV CP, additional assays with these mutants failed to confirm this observation consistently. However, based on both visual inspection of leaf fluorescence and GFP mRNA accumulation levels, we can state with confidence that neither mutant displayed any reduction in ability to suppress RNA silencing in *N. benthamiana* leaves, as seen in three independent experiments using this transient assay.

Table 1. Mutants R6A and R8A do not interact with TIP

Interaction with TIP was examined with both yeast two-hybrid assays and *in vitro* binding experiments. R, Resistant; S, susceptible.

TCV CP	Interaction with TIP	*Arabidopsis* response (ecotype)	
		Col-0	Di-17
Wild-type	+	S	R
Mutant R6A*	−	S	S
Mutant R8A†	−	S	S

*Single amino acid change, arginine to alanine at aa 6.
†Single amino acid change, arginine to alanine at aa 8.

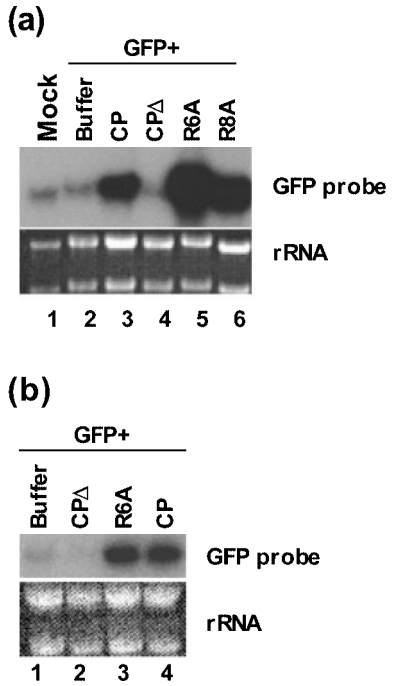

Fig. 2. Mutants with single amino acid substitutions are still capable of RNA-silencing suppression. (a) The TCV CP constructs shown were infiltrated into GFP 16c plant leaves together with the GFP construct. Total RNA was extracted from the infiltrated leaves at 5 d.a.i. and GFP mRNA accumulation was detected by Northern blot analysis using a GFP-specific probe. (b) Each of the TCV CP constructs shown above the photograph, together with the GFP construct, was bombarded into five detached *A. thaliana* (ecotype Col-0) leaves. Two days after bombardment, the leaves were pooled and RNA was analysed as for (a).

We felt that it was also important to confirm the ability of the mutants to suppress RNA silencing in *Arabidopsis* plants, as the presence of a TIP homologue has not been demonstrated in the heterologous *N. benthamiana* system. To do this, particle bombardment was used to deliver plasmids containing the respective expression cassettes into leaf cells of *Arabidopsis* plants (ecotype Col-0). To ensure efficient delivery of the constructs, each plasmid combination was bombarded into five leaves that were then pooled for RNA extraction. As shown in Fig. 2(b), the mutant R6A proved to be as strong a suppressor of RNA silencing as wild-type TCV CP (compare lanes 3 and 4). This result establishes firmly that the inability of TCV CP mutants to interact with TIP had no effect on their ability to suppress RNA silencing. These results confirm the observation in *N. benthamiana* that the R6A mutant retained strong silencing-suppressor activity, effectively unlinking the phenomena of TIP–CP interaction and RNA-silencing suppression by TCV CP. Our result is also supported by the recent report of Zhang & Simon (2003), in which the authors showed that adding two additional amino acid residues to the N-terminus of TCV CP did not affect its silencing-suppression ability.

Suppression of RNA silencing by TCV CP is compromised by in-frame deletions within all structural domains of TCV CP

In view of our results, we felt that it was important to examine more directly the possible connection between the TIP-binding function of the TCV CP R domain and its potential silencing-suppression activity, which was inferred in the report of Thomas *et al.* (2003). These authors showed that removal of the first 25 aa abolished the silencing-suppression activity of TCV CP and suggested that this might be related to the inability of TCV CP to bind to TIP. Our data showed that point mutations in the R domain that abolish TIP interaction do not abolish suppressor activity, indicating that the two phenomena are not connected. However, their results raise the need to examine whether suppression of RNA silencing might require several different domains or an intact CP. To test this possibility, we made a series of deletion mutants by removing specific regions of TCV CP and examined each mutant for its ability to suppress RNA silencing. The structural domains of TCV CP are shown in Fig. 1 (Qu & Morris, 1999). The R domain is located at the N-terminus and is composed of the first 52 aa that interacts with the viral RNA inside the virus shell. The R domain is connected by a 29 aa arm to the surface (S) domain, which makes up the shell of the virion. The S domain connects through a 5 aa hinge to the protruding (P) domain, which forms the 'spike' on the virion surface. The ability of each major structural domain to suppress RNA silencing was tested by transiently expressing each of the six deletion mutants that are shown in Fig. 1 in *N. benthamiana* leaves. The two R-domain mutants contained smaller deletions than the mutant that was tested by Thomas *et al.* (2003). ΔST had 11 aa deleted (aa 2–12) and ΔSTa had only 4 aa deleted (aa 6–9). The entire P domain was removed in ΔP; mutants S and P each contained the entire respective domain, together with the 5 aa hinge. Mutant SP lacked the entire R domain and arm. *Agrobacterium* cultures harbouring binary vectors that were engineered to express each of the mutant proteins were infiltrated into GFP 16c plant leaves, together with the GFP-expressing *Agrobacterium* suspension. GFP mRNA accumulation was monitored by Northern blot analysis as described above (Fig. 3, top panel). It is clear that none of the deletion mutants was able to suppress the silencing of GFP mRNA to the level of full-length TCV CP. Only the ΔSTa mutant, with a deletion of 4 aa in the region of the R domain that encompassed both the R6A and R8A mutations, retained some silencing-suppression activity. It is also noteworthy that the ΔP mutant with an intact N-terminal R domain was completely ineffective at suppressing RNA silencing. When the blot was reprobed with a TCV CP-specific probe (middle panel), only ΔSTa mRNA was detected in appreciable quantities, confirming that this was the only mutant to retain some suppressor activity.

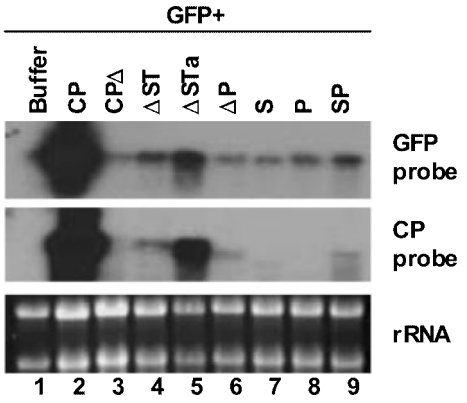

Fig. 3. TCV CP deletion and domain mutants lost most of their silencing-suppressor activity. Northern blot analysis of GFP RNA and TCV CP RNA shows that each of the TCV CP deletion mutants lost most of its RNA silencing-suppressor activity. Each of the constructs shown above the panels was delivered, together with the GFP construct, into GFP 16c plant leaves by agroinfiltration. Total RNA was extracted at 5 d.a.i. and identified by using both GFP-specific (upper panel) and TCV CP-specific (middle panel) probes.

These experiments support the conclusion that retention of CP–TIP interaction ability is not necessary for silencing suppression. Moreover, these results show that large deletions in any part of the protein debilitate the silencing-suppression function, suggesting that the activity probably requires intact TCV CP.

Reduced accumulation of mutant TCV CP does not account for loss of RNA silencing-suppressor activity

The inability to detect suppressor activity for the mutant proteins tested above could be a consequence of the decreased stability of the mutant proteins after translation, or a combination of reduced stability of the proteins and the mutant mRNAs, due to the loss of suppressor activity by the mutant protein. To address the former concern, we extracted proteins from the infiltrated leaves and monitored the titres of each by Western blot analysis using TCV CP antisera. Fig. 4 shows the levels of TCV CP accumulation at 2 d.a.i. Mutant proteins of the expected sizes were detected for most of the mutants, although several were visible at barely detectable levels in the gel (note that CPΔ was not expected to make any protein). Only the wild-type TCV CP showed measurable protein accumulation at 5 d.a.i., suggesting that loss of suppressor activity by each of the other mutant proteins contributed significantly to their lower levels of accumulation (data not shown). To confirm this, each mutant construct was co-infiltrated into *N. benthamiana* leaves together with *Tobacco etch virus* (TEV) HC-Pro, another suppressor of RNA silencing. Fig. 5 shows the result for samples taken at 5 d.a.i., the interval at which maximal protein accumulation was

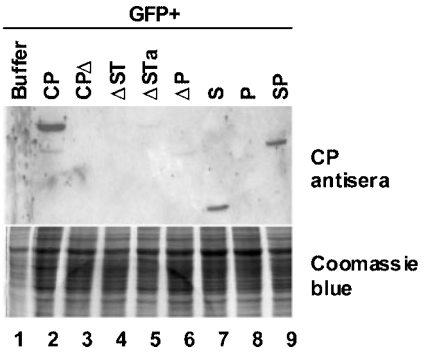

Fig. 4. Accumulation of TCV CP deletion and domain mutants is reduced compared to wild-type TCV CP. Western blot analysis (top panel) showed levels of accumulation of TCV CP in leaves that were infiltrated with each of the mutant constructs, as described in Fig. 3. Leaves were harvested at 2 d.a.i. and the mutant TCV CPs were detected by using TCV CP antisera.

evident. It is clear that the protection provided by TEV HC-Pro permitted substantially higher levels of mutant mRNA accumulation (compare the top panel of Fig. 5 with the middle panel of Fig. 3). Accordingly, mutant proteins could be detected readily for each of the deletion mutants, including mutant P. It is also interesting to note that the ΔSTa mutant, which retained the highest level of suppression activity among the mutants tested, actually accumulated mutant TCV CP to a lower level than most of the other mutants. These results demonstrate that loss of silencing suppression by the TCV CP deletion mutants was probably not only a direct consequence of decreased stability of the mutant proteins.

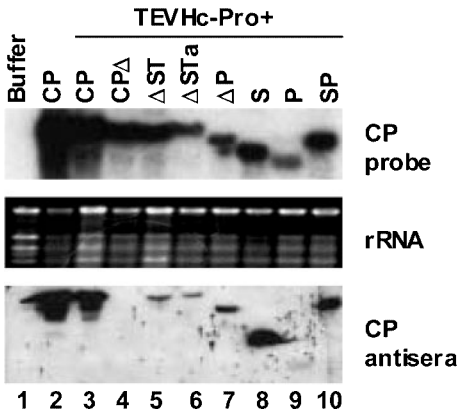

Fig. 5. An exogenous suppressor of silencing enhances mutant TCV CP mRNA expression and protein accumulation. Northern and Western blot analyses show that each of the deletion mutants produced detectable levels of TCV CP-specific mRNA and most produced detectable levels of mutant proteins when expressed in the presence of the TEV HC-Pro silencing suppressor. Protein and RNA samples were collected at 5 d.a.i.

Our inability to map suppressor activity to a specific region or domain of TCV CP suggests that the suppression function may require the intact subunit. This may not be so unexpected, as it is well-established that TCV CP dimerizes before the initiation of TCV assembly on RNA. It has also been suggested that the dimerized state is the stable form of the protein in the cell (Sorger *et al.*, 1986). Interestingly, dimerization has been shown to be essential for silencing-suppression function of the p19 protein of *Tomato bushy stunt virus* (Vargason *et al.*, 2003).

Our inability to connect the silencing-suppressor activity of TCV CP directly with its ability to bind to the TIP factor, which is associated with the *R* gene-mediated resistance response in *Arabidopsis*, is disappointing. It is clear, however, that the hypothesis emerging from studies on bacterial plant pathogens is that the signalling cascade leading to the resistance response initiates from a multi-protein complex of R proteins, effectors and as yet unidentified components of the basal resistance system (Ellis & Dodds, 2003). Our most recent results have confirmed the interaction of TCV CP with the TIP transcription factor *in vivo*. We have also shown that wild-type TCP CP prevents accumulation of TIP in the nucleus, suggesting that this interaction regulates host gene expression. The fact that TCV CP accumulates to such high concentrations in infected plants may explain why it has been selected for multiple functions associated with host–pathogen interactions. Although we have not yet been able to link the two resistance pathways directly, our results do not detract from a role for TCV CP as the effector protein consistent with the 'guard hypothesis', but rather suggest that TCV CP may interfere with multiple basal defence pathways.

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