Inhibition of RNA silencing by the coat protein of *Pelargonium* flower break virus: distinctions from closely related suppressors

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Viral-derived double-stranded RNAs (dsRNAs) activate RNA silencing, generating small interfering RNAs (siRNAs) which are incorporated into an RNA-induced silencing complex (RISC) that promotes homology-dependent degradation of cognate RNAs. To counteract this, plant viruses express RNA silencing suppressors. Here, we show that the coat protein (CP) of *Pelargonium* flower break virus (PFBV), a member of the genus *Carmovirus*, is able to efficiently inhibit RNA silencing. Interestingly, PFBV CP blocked both sense RNA- and dsRNA-triggered RNA silencing and did not preclude generation of siRNAs, which is in contrast with the abilities that have been reported for other carmoviral CPs. We have also found that PFBV CP can bind siRNAs and that this ability correlates with silencing suppression activity and enhancement of potato virus X pathogenicity. Collectively, the results indicate that PFBV CP inhibits RNA silencing by sequestering siRNAs and preventing their incorporation into a RISC, thus behaving similarly to unrelated viral suppressors but dissimilarly to orthologous ones.

To counteract RNA silencing-mediated host defences, many plant and some animal viruses have evolved RNA silencing suppressor proteins (Qu & Morris, 2005; Voinnet, 2005). These proteins do not share any obvious sequence or structural similarity across viral groups and might interfere with the RNA silencing pathway at different stages (Roth et al., 2004). So far, the best characterized of the viral suppressors is the p19 protein of tombusviruses. Detailed studies have demonstrated that this binds siRNAs, thus making them unavailable for RISC (Lakatos et al., 2004; Silhavy et al., 2002; Vargason et al., 2003; Ye et al., 2003). Recent results demonstrate that distinct unrelated viral suppressors also inhibit silencing by sequestering siRNAs (Lakatos et al., 2006; Mérai et al., 2006), while others might inactivate silencing by binding dsRNAs without obvious size selection, which could interfere with Dicer activity (Chao et al., 2005; Lu et al., 2005; Mérai et al., 2005, 2006). Viral suppressors that apparently exert their action by different mechanisms have also been described (Baumberger et al., 2007; Trinks et al., 2005; Wang et al., 2005; Zhang et al., 2006).

*Pelargonium* flower break virus (PFBV), genus *Carmovirus*, family *Tombusviridae*, has a single-stranded positive-sense genomic RNA that encodes two proteins involved in replication, p27 and its read-through product p86 (the viral RdRP), two movement proteins, p7 and p12, and the coat protein (CP) p37 (Rico & Hernández, 2004). Studies on turnip crinkle virus (TCV) and hibiscus chlorotic ringspot virus (HCRSV), both carmoviruses, have shown...
that their CPs are strong suppressors of RNA silencing (Meng et al., 2006; Qu et al., 2003; Thomas et al., 2003). In the case of melon necrotic spot virus, the third member of the genus for which RNA silencing has been investigated, CP exhibited an apparently weaker suppressor activity and a weak suppressor function was also reported for the movement protein p7b (Genovés et al., 2006). Whereas TCV CP has been proposed to interfere with the processing of dsRNA mediated by a Dicer enzyme (Deleris et al., 2006; Méri et al., 2006; Qi et al., 2004; Qu et al., 2003), HCRSV CP action was determined to occur at or before the dsRNA generation step (Meng et al., 2006), suggesting that homologous proteins may block silencing by different mechanisms. To explore this issue further here, we have identified and characterized suppressor activity from PFBV. These results highlight a considerable diversification in the molecular basis of the suppressor activity of closely related proteins.

To identify potential RNA silencing suppressors of PFBV, individual PFBV ORFs were amplified from the infectious clone pSP18-IC (Rico & Hernández, 2006) with the Expand High Fidelity PCR system (Roche) and subsequently inserted under the control of the cauliflower mosaic virus (CaMV) 35S promoter into the binary vector pMOG800. Agrobacterium tumefaciens was transformed with the resulting plasmids or with equivalent binary constructs (Supplementary Fig. S1, available in JGV Online) harbouring the green fluorescent protein (GFP) (Herranz et al., 2005) or the tombusviral p19 (Voinnet et al., 2003) gene and these were used to infiltrate leaves from GFP-transgenic Nicotiana benthamiana plants (line 16c) (Ruiz et al., 1998) as described previously (Qu et al., 2003; Voinnet et al., 2000).

Leaf patches expressing GFP alone or together with any of the replication or movement proteins showed high levels of GFP expression at 2 days post-infiltration (p.i.) (data not shown) which was almost completely silenced at 5 days p.i. as assessed by observations of GFP fluorescence (Fig. 1a). However, co-expression of the PFBV CP with GFP resulted in a sustained, very bright green fluorescence which was comparable in intensity and persistency to that visualized in parallel assays with the tombusviral p19, used as a positive control of suppressor activity (Fig. 1a). Northern blot hybridization, performed as previously described (Castano & Hernández, 2005), confirmed that the fluorescence patterns reflected changes in the steady-state levels of GFP mRNA since, at 5 days p.i., GFP mRNA accumulation was very low in infiltration patches expressing GFP alone or in combination with p27, p86, p7 or p12, whereas co-expression of GFP and CP gave rise to GFP mRNA levels equivalent to those observed from expression of GFP plus p19 (Fig. 1b). As indicated above,
RNA silencing is always associated with accumulation of siRNAs. Analysis of siRNAs showed that the levels of GFP siRNAs were strongly diminished in tissues co-expressing GFP and PFBV CP, as reported by Martínez de Alba et al. (2002), which correlated with the high amount of GFP mRNA detected in these tissues (Fig. 1b). Co-expression of GFP and p19 also gave rise to an apparent reduction in siRNA accumulation (Fig. 1b) in agreement with previous observations (Dunoyer et al., 2004; Voinnet et al., 2003). Altogether, the results demonstrated that PFBV CP is able to block sense RNA-induced RNA silencing as efficiently as p19, a potent viral suppressor.

The CP of PFBV can be divided into an internal RNA-binding domain (R), a shell-forming domain (S) and a protruding domain (P) (Lommel et al., 2005). Previous work has shown that adaptation of PFBV to Chenopodium quinoa results in a 5 aa covariation that affects specific positions within the R and P domains (Rico et al., 2006). This C. quinoa-selected CP variant (CPq) was also cloned into pMOG800 and co-expressed with GFP in agroinfiltration assays, to assess whether serial transfer through the local host causes a reduction in the ability of the protein to block RNA silencing, as reported for HCRSV CP (Meng et al., 2006). The GFP fluorescence at 5 days p.i. was indistinguishable from that observed in leaf patches expressing GFP plus CP. As expected, Northern blot analysis showed a high increase in the levels of GFP mRNA which correlated with a low accumulation of GFP-specific siRNAs (Fig. 1). These results indicate that the anti-silencing activity of PFBV CP is not affected by the amino acid substitutions fixed in C. quinoa, and suggested that the local host does not necessarily impose restrictions on viruses that diminish their suppressor function as a strategy to limit the spread of infection. Interestingly, during amplification of the CPq gene, a nucleotide substitution,
which resulted in an amino acid change (P323L) in the P domain, was accidentally introduced into one of the clones. Co-expression of this protein variant, named CP.q.mut with GFP showed that the single amino acid substitution was sufficient to abolish the silencing suppressor function of the PFBV protein (Fig. 1).

Silencing suppressors are commonly involved in enhancement of viral pathogenicity and accumulation of viruses (Voinnet et al., 1999), thus many of them have been shown to accentuate symptoms when expressed by a heterologous virus (Pruss et al., 1997; Valli et al., 2008; Zhou et al., 2006). To investigate this, the PFBV CP gene was cloned in a PVX vector (pPVX202; Sablowski et al., 1995) and the resulting construct (pPVX-CP) was used to mechanically inoculate N. benthamiana and Nicotiana clevelandii plants. Constructs with the CPq (pPVX-CPq) and CPq.mut (pPVX-CPq.mut) genes inserted were also tested. In both hosts, heterologous CP or CPq expression induced stunting and necrosis at 8–10 days post-inoculation, in contrast with the vein clearing and mild chlorotic mosaic elicited by wild-type PVX202 (Fig. 2a). The necrosis of systemic leaves and stems was followed by death of the plants, showing that expression of CP (or CPq) markedly accentuates pathogenicity of the unrelated PVX. Remarkably, symptoms induced by pPVX-CPq.mut were indistinguishable from those observed with pPVX202 (Fig. 2a). Northern blot analysis revealed that CP and CPq, but not CPq.mut, greatly enhanced PVX accumulation, even though the corresponding inserts were retained to similar extents in the recombinant viruses (Fig. 2b). The results showed that increasing pathogenicity and RNA silencing suppression activities of PFBV CP are intimately connected.

To obtain further insight into the mechanism of PFBV CP suppression of RNA silencing, we examined whether PFBV CP was blocking conversion of sense GFP to dsRNA or inhibiting silencing downstream of dsRNA generation. To address this, PFBV CP was co-expressed in N. benthamiana leaves with GFP and double-stranded GFP (dsGFP), the latter was transcribed from a binary construct that harbours both sense and antisense sequences of GFP.

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**Fig. 3.** Exploration of the molecular basis of silencing suppression by PFBV CP. (a) Inhibition of dsRNA-triggered RNA silencing. Leaves of N. benthamiana 16c were agroinfiltrated with construct combinations for expression of GFP plus dsGFP alone (Ø) or together with CP or p19. GFP fluorescence at 5 days p.i. in infiltrated patches is shown on the left and Northern blot detection of GFP mRNA or siRNAs extracted from the same patches is shown on the right. (b) Gel mobility shift assay. $^{32}$P-Labelled double-stranded siRNAs were incubated alone (−) or with crude extracts from mock-agroinfiltrated (Mock) leaves or agroinfiltrated leaves expressing PFBV CP, CPq, CPq.mut or p19. All binding reactions were performed using 22 µl of extract, except p19 for which 5 µl was used. The positions of free siRNA and of protein–siRNA complexes are indicated on the right.
separated by an intron and thus generating GFP dsRNA (Takeda et al., 2002). Combinations of GFP plus dsGFP, either alone or with p19, were also included as controls. The fluorescence in patches expressing GFP plus dsGFP dropped to undetectable levels at 5 days p.i., but patches co-expressing PFBV CP showed considerable fluorescence, which increased in subsequent days, and a strong fluorescence was detected in p19-expressing patches (Fig. 3a). Consistent with the fluorescence patterns, accumulation of GFP mRNA in the PFBV CP-expressing patches was notably higher than in those expressing just GFP and dsGFP, indicating that PFBV CP was able to interfere with dsRNA-triggered RNA silencing (Fig. 3a). GFP mRNA accumulation was greatly enhanced by p19 expression, in agreement with previous reports (Csorba et al., 2007). A large amount of siRNA accumulated in tissues co-expressing GFP and dsGFP alone or together with p19 (Fig. 3a). This was expected, since the tombusviral suppressor specifically binds siRNAs and, therefore, does not inhibit Dicer activity, in contrast with other suppressors that bind to long dsRNAs and compromise their Dicer-mediated processing, as seen with TCV CP (Chao et al., 2005; Lingel et al., 2005; Lu et al., 2005; Mérai et al., 2005, 2006). Remarkably, cleavage of dsGFP to siRNAs was inhibited neither by PFBV CP, which allowed siRNAs to accumulate at levels similar to those observed in the presence of p19, nor in the absence of suppressor proteins (Fig. 3a). These results strongly suggested that PFBV CP does not interfere with Dicer activity and targets the silencing machinery downstream of siRNA generation, thus differing from the CPs of TCV and HCRSV.

The results above indicated that the mechanism for silencing inhibition of PFBV CP could involve sequestering of siRNAs, as reported for different suppressors including p19. To explore this possibility, N. benthamiana leaves were separately agroinfiltrated with constructs for expression of CP, CPq and CPq.mut. In addition, mock-infiltrated leaves and infiltrated leaves expressing p19 were used as negative and positive controls, respectively. Crude extracts were prepared 3 days p.i. and incubated with 32P-labelled positive controls, respectively. Crude extracts were CP, CPq and CPq.mut. In addition, mock-infiltrated leaves separately agroinfiltrated with constructs for expression of siRNAs, as reported for different suppressors including inhibition of PFBV CP could involve sequestering of siRNAs by PFBV CP or CPq, both functional in silencing suppression, also showed siRNA binding activity, giving rise to a complex that migrated more slowly than that generated by p19 extracts. In contrast, those containing CPq.mut, which had no detectable suppressor function, failed to form complexes with siRNAs, even when the amount of extract was increased in the binding mixtures (Fig. 3b and data not shown). Collectively, the results presented here strongly indicate that the silencing inhibition activity of PFBV CP relies on the ability of the protein to sequester siRNAs, as has been shown for phylogenetically unrelated suppressors but not for orthologous ones. In addition, our results might imply that TCV CP and PFBV CP have diverged in their dsRNA-binding properties, with PFBV CP acquiring size-selection separately from the size-independent dsRNA-binding of TCV CP. It has been suggested that selection of siRNAs for binding may reduce host damage, as the corresponding suppressor will not interfere with endogenous long dsRNAs or long structured RNAs (Baulcombe & Molnár, 2004; Vargason et al., 2003). It has also been suggested that the expression of size-independent silencing suppressors might be down-regulated by the virus (Mérai et al., 2006). In the case of TCV CP, this down-regulation is probably accomplished by capsid formation which reduces available suppressor levels (Zhang & Simon, 2003). In this scenario, it is tempting to speculate that capsid formation plus selective binding to siRNAs by PFBV CP might account for the low viral accumulation and the frequent absence of symptoms in PFBV-infected plants.

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References


