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BRIEF REPORT

HC-Pro hypo- and hypersuppressor mutants: differences in viral siRNA accumulation in vivo and siRNA binding activity in vitro

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Abstract Viruses have evolved mechanisms to suppress the RNA silencing defense of their hosts, allowing replication and systemic colonization. In a recent study, we found that the effect of mutations in the RNA silencing suppressor of tobacco etch virus (TEV) was variable, ranging from complete abolition of suppressor activity to significantly stronger suppression. Whereas hyposuppressor mutants were less virulent and accumulated fewer viral particles than the wild type, hypersuppressors induced symptoms similar to those of the wild type and accumulated particles to similar levels. Here, we further characterize a set of these mutants in terms of their ability to bind in vitro and induce accumulation in vivo of virus-derived siRNAs. Hyposuppressor alleles are less efficient at binding siRNAs than hypersuppressors, whereas the latter are not different from the wild type. As a consequence of lower viral accumulation, plants infected with virus bearing a hyposuppressor allele also accumulate less virus-derived siRNA.

HC-Pro was the first viral protein identified as a suppressor of RNA silencing [1, 3, 8, 14]. Different experiments have led to propose models for the mechanism of HC-Pro silencing suppression, one of them being the inhibition of RISC assembly through sequestering 21-nt siRNA. Duplex-siRNA binding represents a common mechanism for RNA silencing suppression that has evolved independently in, at least, the families Tombusviridae, Potyviridae and Closteroviridae of positive strand RNA viruses [9].

In a previous study, we described the effect of amino acid substitutions on the RNA silencing suppressor activity of tobacco etch virus (TEV, genus *Potyvirus*, family Potyviridae) HC-Pro [16]. We estimated the relative suppression activity of a collection of HC-Pro alleles by quantifying the amount of GFP mRNA present by real-time quantitative RT-PCR after co-infiltrating *Nicotiana benthamiana* leaves with cDNAs carrying each allele together with a plasmid expressing a GFP reporter [7]. Among these mutants, three caused a significant decrease in activity (hyposuppressors), and five significantly increased it (hypersuppressors). Interestingly, the match between suppression activity and virus accumulation and virulence was imperfect. Despite heterogeneity among HC-Pro alleles, virus carrying hyposuppressor alleles accumulated, on average, less and produced milder infections than the wild-type virus. By contrast, virus carrying hypersuppressor alleles did not differ, on average, from the wild-type virus in either trait [16]. Here, to gain further insights into the relative impact that mutations in HC-Pro exert over the silencing pathway, we examine the effect of some mutants on the metabolism of viral siRNAs, both in vivo and in vitro.

First, we examined whether the different HC-Pro mutants selected differentially affect the accumulation of TEV-derived siRNAs. A radioactively labeled in vitro transcript corresponding to the plus strand of TEV was used as probe in a northern blot analysis. Total RNA was extracted from 0.25 g of tissue from inoculated plants with Trizol (Invitrogen), and the small RNA fraction (<200-nt) was purified using a miRACLE miRNA Isolation Kit (Stratagene). Equal amounts of small RNAs were separated

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by denaturing PAGE in a 15% gel. Figure 1 shows the signal intensities of the siRNA hybridization bands in the northern blot analysis of *N. benthamiana* plants infected with different TEV clones bearing several HC-Pro hypo- and hypersuppressors alleles, as well as the wild type. The sensitivity of the method to detect differences between genotypes was evaluated as the percentage of added variance component over the total observed variance [15]. The maximum likelihood estimator of this variance component was 90.24%, indicating that the method is quite sensitive. A nested ANOVA, in which each allele was nested within its corresponding suppression category, revealed that significant differences exist among suppressor categories in the amount of TEV-derived siRNAs accumulated in infected plants ($F_{3,\ 6} = 72.720$, $P < 0.001$) despite the fact that different alleles within each category were also heterogeneous in their response ($F_{6,\ 18} = 6.763$, $P = 0.001$). A post-hoc Tukey's test [15] shows that plants infected with hyposuppressor HC-Pro alleles produced significantly less TEV-specific siRNAs than wild type. However, this amount did not differ among hypersuppressor and wild type alleles ($P = 0.848$). On average, plants infected with hyposuppressor alleles accumulated five fold less [12.480 ± 3.946 (± SEM)] TEV-specific siRNAs than plants infected with the wild type virus (74.407 ± 26.692) and hypersuppressor mutants (67.979 ± 9.272).

This pattern closely matches that observed for the relationship between suppressor activity and viral load reported in our previous work: both the wild type and hypersuppressor mutants produced, on average, equal amounts of infectious viral particles, whereas hyposuppressor mutants produced, on average, fewer infectious viral particles [16]. Indeed, a significant correlation exists between siRNA accumulation and viral load (Spearman's $\rho_S = 0.833$, 6 *df*, $P = 0.010$), which suggests that the former may be a direct consequence of the latter. This is not surprising at all, since it has been shown that viral genomic RNA and virus-derived 21-nt siRNAs both increase during the time course of an infection [9], suggesting that siRNA production is not inhibited in vivo by the suppressor protein. In our study, we have quantified both free and protein-bound (including HC-Pro-sequestered) siRNAs, and therefore the total amount of siRNAs cannot be taken as a direct proof of the suppression strength of each mutant. Moreover, it has been postulated that HC-Pro acts in other steps along the RNA silencing pathway, and the mutations may be affecting these steps, and not necessarily siRNAs sequestration. However, this interpretation is not fully satisfactory when the maintenance/amplification step of plant RNA silencing is incorporated into the picture, since an efficient suppression would clearly lower siRNAs levels [5].

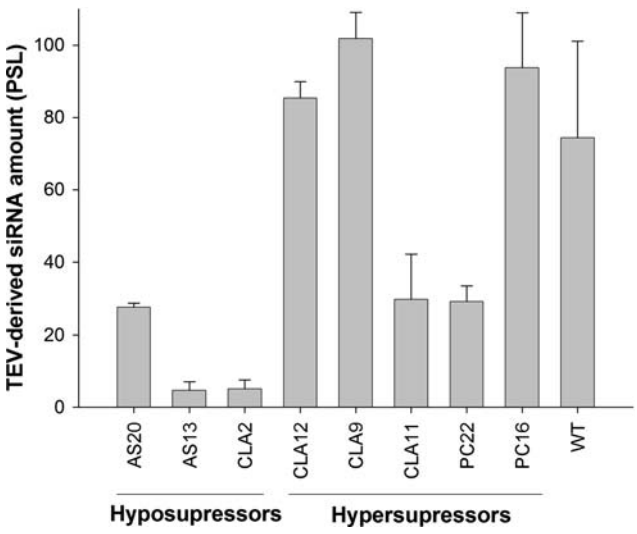

Fig. 1 Accumulation of TEV-derived siRNAs in infected and mock-inoculated plants (three replicates each). Hybridization signals were quantified with a phosphorimager (Fuji). The number of photostimulated luminescence units (PSL) was calculated by subtracting the values of the background and the mock-inoculated controls (error bars correspond to ±1 SEM)

As a consequence of these results, we further analyzed the most interesting HC-Pro alleles with regard to their activity of binding in vitro 21-nt siRNA duplexes containing 3′ 2-nt overhangs in an in vivo/in vitro assay. We transitorily expressed different HC-Pro hyper- and hipo-suppressor alleles in *N. benthamiana* leaves using normalized amounts of *Agrobacterium tumefaciens* cultures. The *A. tumefaciens* clones were transformed with the expression vector pBIN61 containing the different mutant HC-Pro cDNAs as well as the wild-type sequence [12, 16]. Total plant proteins were purified from the agro-infiltrated areas and the concentration normalized to correct for differences during extraction, and an electrophoretic mobility shift assay (EMSA) was performed using a ^{32}P-labelled 21-nt dsRNA with 2-nt 3′ overhangs [11]. Each HC-Pro allele was assayed in triplicate. As before, we also evaluated the sensitivity of this method for detecting differences between genotypes. In this case, the added variance component among HC-Pro alleles is 92.82% of the total (phenotypic) variance, indicating a good sensitivity.

A nested ANOVA of the EMSA data (Fig. 2) highlights significant differences among suppressor categories in their capacity to bind siRNAs ($F_{3,\ 6} = 34.034$, $P < 0.001$) despite the heterogeneity observed between alleles within categories ($F_{6,\ 18} = 3.312$, $P = 0.022$). Once again, a Tukey's test supports the notion that hyposuppressors bind 21-nt siRNAs six fold less efficiently than the other alleles, whereas differences between the wild type and the hypersuppressors are not significant. On the one hand, the binding phenotype is not correlated with the accumulation of specific siRNAs in TEV-infected plants ($\rho_S = 0.517$, 7 *df*,

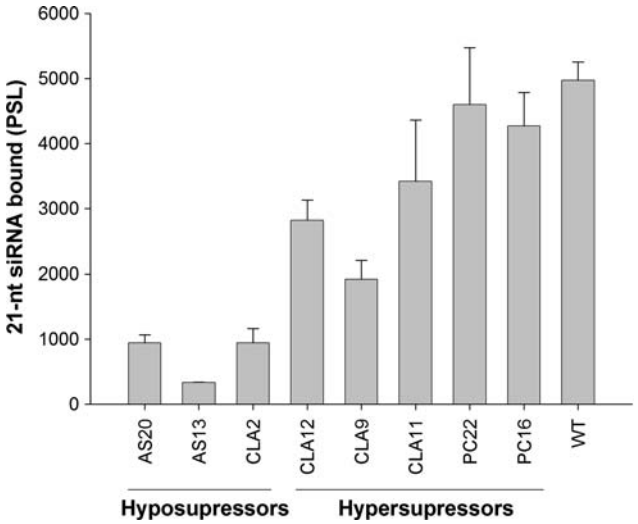

Fig. 2 Electrophoretic mobility shift assay (EMSA) of radioactively-labeled siRNAs by different HC-Pro mutants purified from agroinfiltrated *N. benthamiana* as described in ref. 11 (three replicates each). Mean values of siRNAs binding by HC-Pro mutant alleles quantified by phosphorimaging (error bars correspond to ±1 SEM)

$P = 0.154$) or the number of viral particles ($\rho_S = 0.071$, 6 *df*, $P = 0.867$). On the other hand, the binding phenotype was positively correlated ($\rho_S = 0.717$, 7 *df*, $P = 0.030$) with the silencing suppression activity quantified for the HC-Pro alleles [16].

It has been postulated that two RNA-binding motifs (A and B) are present within the central region of HC-Pro [13]. Interestingly, mutations in the two hyposuppressor alleles showing the weakest binding activity and the lowest TEV-derived siRNA accumulation (Fig. 2; CLA2 and AS13) are located within these domains [16]. The third hyposuppressor mutation (AS20), however, is located at the C-terminus of the molecule, which may interact with the central domain to mediate the suppressor activity [17]. The hypersuppressor mutation PC22 is also located in the RNA-binding motif A. Although its binding phenotype does not differ from that of the wild type (Fig. 2), the amount of TEV-derived siRNA that accumulate in its presence is the lowest among hypersuppressors (Fig. 1), suggesting that it may interfere with siRNA accumulation by a mechanism different from sequestration.

Summarizing the above results, the amount of TEV-derived siRNAs accumulating during the infection of *N. benthamiana* plants has no direct relationship with the activity of HC-Pro sequestering siRNAs. These results suggest that HC-Pro hypersuppression was not achieved by an increase in the ability to bind 21-nt siRNAs. What, then, may be the mechanism for hypersuppression? It has been suggested [2] that HC-Pro interferes with the RNA silencing machinery at other levels, e.g. through activation of an endogenous silencing suppressor of the host. Two recent studies [6, 18] have shown that HC-Pro significantly reduces siRNAs 3′-methylation of siRNAs, making them sensitive to oligouridilation and subsequent degradation [10] and preventing their incorporation into the RISC. Therefore, since we have not found a correlation between HC-Pro hypersuppression and siRNA binding activity, we can postulate that hypersuppression may be associated with alterations in siRNA methylation or with changes in the ability to interact with endogenous suppressors of RNA silencing. Finally, as pointed out in our previous work, it is worth taking into account the hierarchical action of the different Dicer proteins [4] when thinking about HC-Pro interaction with the RNA silencing pathway. An attractive hypothesis would be that wild-type HC-Pro preferentially sequesters 21-nt siRNAs, but some hypersuppressors may bind more efficiently to 22-nt siRNAs.

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