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From Hypo- to Hypersuppression: Effect of Amino Acid Substitutions on the RNA-Silencing Suppressor Activity of the *Tobacco etch potyvirus* HC-Pro

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ABSTRACT

RNA silencing participates in several important functions: from the regulation of cell metabolism and organism development to sequence-specific antiviral defense. Most plant viruses have evolved proteins that suppress RNA silencing and that in many cases are multifunctional. *Tobacco etch potyvirus* (TEV) HC-Pro protein suppresses RNA silencing and participates in aphid-mediated transmission, polyprotein processing, and genome amplification. In this study, we have generated 28 HC-Pro amino acid substitution mutants and quantified their capacity as suppressors of RNA silencing in a transient expression assay. Most mutations either had no quantitative effect or completely abolished silencing suppression (10 in each class), 3 caused a significant decrease in the activity, and 5 significantly increased it, revealing an unexpected high frequency of mutations conferring hypersuppressor activity. A representative set of the mutant alleles, containing both hypo- and hypersuppressors, was further analyzed for their effect on TEV accumulation and the strength of induced symptoms. Whereas TEV variants with hyposuppressor mutants were far less virulent than wild-type TEV, those with hypersuppressor alleles induced symptoms that were not more severe than those characteristic of the wild-type virus, suggesting that there is not a perfect match between suppression and virulence.

SMALL RNAs, including micro RNAs (miRNAs) and short interfering RNAs (siRNAs), are key components of an evolutionarily conserved RNA-based gene regulation system documented in fungi, plants, and animals that is implicated in various biological processes from development to antiviral defenses (Ratcliff *et al.* 1997; Waterhouse *et al.* 2001; Baulcombe 2002; Voinnet 2002; Ding *et al.* 2004; Chen *et al.* 2005; Wilkins *et al.* 2005; Kim and Nam 2006; Ding and Voinnet 2007). The silencing pathway is triggered by the presence of double-stranded RNAs (dsRNA) or single-stranded RNAs with stem-loop structures that are processed by several Dicer proteins into ~21- to 24-nt short RNAs, including siRNAs, miRNAs, and others, which are incorporated into an RNA-induced silencing complex (RISC) to promote a sequence-specific cleavage or translation arrest of transcripts of complementary sequence (Hammond *et al.* 2001; Waterhouse *et al.* 2001; Voinnet 2002; Carrington and Ambros 2003; Bartel 2004; Baulcombe 2004; Pfeffer *et al.* 2004; Brodersen and Voinnet 2006; Buchon and Vaury 2006; Chapman and Carrington 2007).

RNA silencing is responsible for important endogenous functions, including regulation of cellular transcripts, guiding heterochromatin formation and transcriptional repression of transposon, processing of noncoding RNA precursors that control developmental timing and leaf polarity, and regulation of stress (Hammond *et al.* 2001; Carrington and Ambros 2003; Bartel 2004; Xie *et al.* 2004; Voinnet 2005; Brodersen and Voinnet 2006). RNA silencing also represents a natural defense system against viruses because it is activated by the structured RNAs or the dsRNAs produced during the replication cycles of different classes of viruses and subviral pathogens (Ratcliff *et al.* 1997, 1999; Voinnet 2001; Waterhouse *et al.* 2001; Ding *et al.* 2004; Lecellier and Voinnet 2004; Chen *et al.* 2005; Wilkins *et al.* 2005; Buchon and Vaury 2006; Ding and Voinnet 2007).

Viruses are inducers and targets of RNA silencing, but they have also evolved strategies to counteract this defense mechanism (Kasschau and Carrington 1998; Voinnet *et al.* 1999; Roth *et al.* 2004; Li and Ding 2006). Silencing suppression is a common property of plant viruses and suppressor proteins are considered as pathogenicity determinants, needed for efficient accumulation. Found in most viruses, silencing suppressor proteins show a tremendous structural and sequence diversity that has been explained as an evolutionary convergence toward a common functional necessity (Li and Ding 2006). The *sine qua non* condition for the operation of natural selection is the existence of genetic variation affecting fitness. This condition is amply fulfilled by viral silencing suppressors. For example,

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focusing only on potyviruses, it has been shown that two amino acid replacements in the HC-Pro suppressor of *Clover yellow vein virus* are sufficient to attenuate the symptoms and reduce virus accumulation (Yambao *et al.* 2008), whereas a single mutation in *Plum pox virus* HC-Pro had a similar effect (González-Jara *et al.* 2005). As a final example, mutations in the conserved motifs of *Zucchini yellow mosaic virus* HC-Pro also produced attenuated viruses on the natural host squash and also abolished the ability to elicit hypersensitive responses in other local lesion hosts (Lin *et al.* 2007). All in all, viral suppressor proteins probably undergo strong selective pressures for optimal adaptation to the host since a successful infection will rely on the tight balance between the host silencing response and viral counter-defense mechanisms (Moissiard and Voinnet 2004).

As with many other viruses, *Tobacco etch potyvirus* (TEV) encodes in its genome a suppressor protein named HC-Pro (Kasschau *et al.* 1997; Anandalakshmi *et al.* 1998; Kasschau and Carrington 1998, 2001; Llave *et al.* 2000; Mallory *et al.* 2002). HC-Pro is a multifunctional protein involved in a wealth of functions (reviewed in Urcuqui-Inchima *et al.* 2001): it (i) acts as proteinase during the auto-proteolytic processing of the viral polyprotein, (ii) interacts with the stylet of aphids during transmission, (iii) displays RNA-binding activity and is involved with genome amplification, (iv) is required for entry into and exit from the vascular system, (v) interferes with the 20S proteasome (Ballut *et al.* 2005), (vi) limits methylation of viral-derived small RNAs (Ebhardt *et al.* 2005; Yu *et al.* 2006), and (vii) is essential for symptom development. Therefore, it participates in replication, systemic movement, and vector transmission. Mutagenesis analyses have allowed for defining several functional domains in potyvirus HC-Pro, although some functions are overlapping. Oversimplifying, the N-terminal region is essential for the transmission process but dispensable for infection; in the C-terminal region, the proteinase and movement domains overlap; and the central region is implicated in RNA silencing and genome amplification and overlaps with the movement domain (Kasschau *et al.* 1997; Plisson *et al.* 2003; Varrelmann *et al.* 2007).

In this work, we focused our attention on the RNA-silencing suppressor activity of HC-Pro. The HC-Pro suppression mechanism is complex, interfering with the RNA-silencing pathway at least two stages. First, HC-Pro reduces, although it does not completely eliminate, the processing of dsRNA by Dicer, since siRNAs can still be detected (Mallory *et al.* 2002; Dunoyer *et al.* 2004). Second, despite the presence of siRNAs, degradation of mRNA is prevented, suggesting that HC-Pro also likely interferes with the assembly or activity of RISC (Dunoyer *et al.* 2004; Chapman and Carrington 2007). This second point of action is also supported by the fact that the level of the labile intermediate in the miRNA biogenesis, miRNA*, increases in the presence of the protein (Mallory *et al.* 2002; Kasschau *et al.* 2003; Dunoyer *et al.* 2004; Chapman and Carrington 2007). Alterations of endogenous miRNAs by HC-Pro may have profound morphological effects, contributing to symptom severity (Kasschau *et al.* 2003; Dunoyer *et al.* 2004; Chapman and Carrington 2007).

In this study, a collection of HC-Pro amino acid substitution mutants was created. The suppressor activity of each mutant was quantified by determining the accumulation level of a reporter green fluorescent protein (GFP) mRNA in *Nicotiana benthamiana* leaves co-infiltrated with an *Agrobacterium tumefaciens* strain carrying the GFP reporter gene and HC-Pro (Voinnet *et al.* 2000; Johansen and Carrington 2001). Next, the *in vivo* effect of HC-Pro alleles with altered suppression activity on TEV accumulation and symptom development was explored. TEV infectious clones carrying a subset of the mutant HC-Pro proteins were assessed by inoculating *N. benthamiana* plants with RNA transcripts of each mutant genotype. Overall, mutants carrying hypersuppressor HC-Pro alleles accumulated at higher levels and were more virulent than mutants with hypo-suppressor HC-Pro alleles, although hypersuppressors were not more virulent than wild-type TEV. The relationship between the viral ability to suppress host silencing and its virulence is discussed, including possible trade-offs between suppression and other HC-Pro functions.

MATERIALS AND METHODS

Plasmid constructs and infectious clones: The plasmid pTEV7DA with an infectious TEV clone (GenBank accession DQ986288), kindly provided by James C. Carrington (Oregon State University), was used as a source of wild-type virus and template for site-directed mutagenesis. Mutants in the HC-Pro cistron were generated by site-directed mutagenesis using the Quickchange II XL kit (Stratagene) following the directions of the manufacturer. Eleven mutant genotypes, designated as CLA, corresponded to amino acid substitutions at sites that are conserved among all potyviral HC-Pro proteins so far characterized. The mutants T1 and T2 corresponded to amino acid substitutions at the homologous sites of those described by González-Jara *et al.* (2005) as essential for *Plum pox virus* HC-Pro suppression activity. The 11 PC mutants corresponded to a single random amino acid substitution and have been described before (Carrasco *et al.* 2007a,b). Finally, four previously described alanine-scanning mutants (Kasschau and Carrington 2001) were also generated (labeled as AS). All the mutants were sequenced with an Applied Biosystems PRISM DNA sequencer 3100, and the correctness of mutations was verified.

Vector pBIN61 (Bendahmane *et al.* 2000) and its derivative pBIN61-GFP, containing the mGFP5 cDNA (Haseloff *et al.* 1997), were the gift of David C. Baulcombe (Sainsbury Laboratory, John Innes Centre, Norwich, UK).

DNAs coding for mutant and wild-type HC-Pro proteins were amplified using the forward primer VP-680 [5′-ATGCGGGATCC*ATG*AGCGACAAATCAATCTCTGAGG-3′; contains a *Bam*HI site (underlined) upstream from the initiation codon (italics)] and the reverse primer VP-681 [5′-GATCGCCCCGGG*TTA*TCCAACATTGTAAGTTTTCATTTCG-3′; contains a *Cfr*91

site (underlined) downstream from the termination codon (italics)]. PCR-amplified DNAs were digested with *Bam*HI/*Cfr*91 and subcloned in pBIN61 vector using standard procedures (Sambrook *et al.* 1989).

Transient expression assays and quantification of suppression activity: pBIN61-HC-Pro plasmids were electroporated into *A. tumefaciens* strain C58C1. Culture growth and induction was performed as described by Hamilton *et al.* (2002). Prior to co-infiltration, culture OD_{600} was adjusted to 0.1. Each *N. bentamiana* leaf was co-infiltrated with each of three different 1/1 culture mixes of transformed *A. tumefaciens*: (i) pBIN61-GFP and pBIN61, (ii) pBIN61-GFP and wild-type pBIN61-HC-Pro, and (iii) pBIN61-GFP and mutant pBIN61-HC-Pro (Figure 1). Each mutant was assayed following this scheme in four leaves of different plants for subsequent real-time quantitative RT–PCR (RT–qPCR) assays; independent cultures were infiltrated in 20 leaves from different plants for GFP fluorescence visualization.

Total RNA was extracted from agroinfiltrated areas 6 days post-inoculation (dpi). Up to 100 mg of plant tissue was processed with the RNeasy plant mini kit (Qiagen) according to the manufacturer's instructions. To remove genomic and plasmid DNA, 600 ng of total RNA were treated using the TURBO DNA-free (Ambion) kit in a reaction volume of 20 μl. Each RNA sample was used as template for three independent reverse transcription reactions performed with TaqMan reverse transcription kit (Applied Biosystems). Volumes were set up to 20 μl, containing 120 ng of template DNA-free RNA and oligo$(dT)_{16}$ primer. Primers for RT–qPCR were designed with the aid of Primer Express software (Applied Biosystems) using default parameters. Oligonuclotides GFP-F (5′-CGTGCAGGA GAGGACCATCT-3′) and GFP-R (5′-CGTGTCTTGTAGTT CCCGTCG-3′) amplify a 51-bp fragment from GFP mRNA; primers GADPH-F (5′-GGTGTCAAGCAAGCCTCTCAC-3′) and GAPDH-R (5′-GATGCCAAGGGTGGAGTCAT-3′) yield an equal-length fragment from glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA selected as internal control.

For relative quantitation, each cDNA was amplified in two separate reactions containing 2 μl cDNA in a 25-μl reaction, 1× Power SYBR PCR master mix (Applied Biosystems), and 300 nm GFP or 600 nm GAPDH primers. For standard curve quantitation, a single stock cDNA reaction was prepared as described above from 264 ng treated RNA/20 μl reaction, aliquoted, and stored at −20°. Twofold serial dilutions ranging from 264 to 16.5 ng RNA/20 μl RT reaction were prepared and amplified in triplicate. Amplification and quantification were done using Applied Biosystems Prism 7000 or 7005 sequence detection systems. The suppression activity was expressed as the average quantity of GFP mRNA in the areas co-infiltrated with each mutant HC-Pro relative to wild-type HC-Pro; both values were corrected by subtracting the GFP mRNA quantified in the absence of the suppressor. The expression of the GAPDH endogenous gene was used for mRNA normalization. To account for plant natural variability, each mutant was analyzed in four leaves from different plants, as variation within the plant was negligible (data not shown). To further reduce the effect of outliers, median values will be reported. Confidence intervals for the median were constructed by the Jackknife method.

GFP fluorescence was observed using a hand UV lamp UVGL-58 (UVP). GFP fluorescence images were obtained using a SMZ800 fluorescence stereoscope (Nikon) set with a EX 480/40 DM505 BA510 filter and acquired using the analySIS program (Soft Image System).

To validate the above RT–qPCR method, the relationship between the estimates obtained and the corresponding figures inferred following Kasschau and Carrington (2001) semiquantitative approach has been explored. Kasschau and Carrington (2001) used as a proxy for relative RNA-silencing suppression activity the number of GFP-positive reactions relative to the total number ($n = 20$) of infiltrated spots 6 dpi. Both estimates were highly associated ($R^2 = 0.896$, $F_{1,27} = 231.895$, $P < 0.001$), indicating that the number of infiltration spots showing fluorescence strongly depended on the amount of GFP mRNA present on each spot.

***In vitro* transcription and infectivity assays:** Infectious plasmids were *Bgl*II digested and transcribed to 5′ capped RNAs using SP6 mMESSAGE mMACHINE kit (Ambion). Four-week-old *N. benthamiana* plants were inoculated by abrasion with ~4 μg of transcript inoculum applied to the third true leaf. The whole procedure is detailed in Carrasco *et al.* (2007a).

Virions were partially purified from whole *N. benthamiana* plants as described before (Carrasco *et al.* 2007a). Briefly, 2 ml of 0.5 m borate buffer (pH 8.0) were added per gram of fresh tissue and partially homogenized and later clarified using $CHCl_3/CCl_4$; after centrifugation, virions were precipitated from the upper aqueous phase with PEG8000/NaCl, sedimented at 10,000 × *g* for 15 min, and resuspended in 20 μl/g 0.05 m borate buffer (pH 8.0, 5 mm EDTA).

For titering, 5 μl of partially purified virions and serial dilutions (0.5, 10^{-1}, and 10^{-2}) prepared in borate buffer were inoculated into four fully developed leaves of four *Chenopodium quinoa* (4-week-old) plants, using carborundum as an abrasive. To minimize plant effects, each plant was inoculated with every dilution (Kleczkowski 1949). Concentration of infectious viral particles could then be expressed as the number of lesion-forming units (LFU) per microliter of inoculum. Titer was estimated three times for each viral genotype on three independent full blocks. To control for block effect, wild-type TEV was titered on each block. The advantage of this method over techniques based on quantifying the amount of some viral molecule (*e.g.*, RT–qPCR for detecting RNA molecules) is that it produces a biologically relevant quantity, namely the number of infectious particles produced, rather than properties that may be more or less correlated with infectious particles (*e.g.*, RT–qPCR would count aberrant RNA molecules).

All plants were maintained in the greenhouse at 25° and 16 hr light.

Molecular confirmation of TEV infection: To detect viral RNA in systemically infected leaves, total RNA was extracted from 100 mg of fresh tissue using a standard phenol/chloroform method and resuspended in 40 μl of DEPC-treated water. Primer sequences were HC-ProF (5′-CGGG ATCCGATGCTCGTGCGAAGGTAAC-3′) and CTB2 (5′-GAT CAACATCTCAATTGCACCTTGTG-3′). cDNA synthesis was performed using M-MuLV reverse transcriptase (Fermentas), according to instructions, from up to 200 μg of total RNA. PCR amplification reactions were performed using *Taq* DNA polymerase (Roche). Amplification mixture was prepared according to instructions provided for standard amplification. Cycling parameters were 95° 5 min for template denaturation, 40 cycles (95° 40 sec, 50° 30 sec, 72° 2 min) for DNA amplification, and a final elongation step of 72° 10 min.

To detect viral replication in inoculated leaves, total protein extraction and Western blot analysis were performed by standard protocols (Sambrook *et al.* 1989). Commercial-conjugated anti-TEV coat protein (Agdia) antibody was used for antigen detection and ECL substrate (Amersham) was used for peroxidase luminescent detection.

RESULTS

Distribution of mutational effects in HC-Pro RNA-silencing suppression activity: A collection of 28 TEV

TABLE 1

Description of TEV HC-Pro mutants and their phenotypes

Mutant label	Codon(s) changed[a]	Amino acid(s) changed	Domain[b]	Suppressor activity[c]	Symptoms[d]
PC16	1086 AUA → UUA	I11L	Trans	Increased	Etch
PC18	1434 CGC → GGC	R127G	Sup	Null	None
T1	1458 GUA → GCA	V135A	Sup	Null	None
CLA1	1548 AGG → GGG	R165G	Sup	Wild type	Etch
CLA2	1629 GUG → GCG	V192A	Sup-Mov	Reduced	Mild etch
PC20	1632 AAU → UAU	N193Y	Sup-Mov	Wild type	None
PC21	1635 AAC → GAC	N194D	Sup-Mov	Wild type	None
PC22	1654 AAU → AGU	N200S	Sup-Mov	Increased	Etch
CLA3	1755 UAC → CAC	Y234H	Sup-Mov	Wild type	Etch
AS9	1773, 1774 AGG → GCG	R240A	Sup-Mov	Null	None[e]
	1776, 1777 AAA → GCA	K241A			
	1779, 1780 CAU → GCU	H242A			
AS10	1794, 1795 AGA → GCA	R247A	Sup-Mov	Null	None[e]
	1797, 1798 AAG → GCG	K248A			
PC24	1850 CAA → CAU	Q265H	Sup-Mov	Wild type	Etch
T2	1872 GAG → GCG	E273A	Sup-Mov	Null	None
AS13	1951 GAA → GCA	E299A	Sup-Mov-Pro	Reduced	None[e]
	1954 GAU → GCU	D300A			
PC25	1982 AAG → AAU	K309N	Mov-Pro	Wild type	Etch
CLA4	1986 CCA → CUA	P311L	Mov-Pro	Wild type	Etch
CLA5	2085 UAU → UCU	Y344S	Mov-Pro	Wild type	Mild etch
PC26	2118 GUG → UUG	V355L	Mov-Pro	Wild type	Etch
AS20	2134 GAG → GCG	E360A	Mov-Pro	Reduced	Mild etch
	2137 GAU → GCU	D361A			
PC28	2215 GCA → GAA	A387E	Mov-Pro	Null	None
CLA6	2223 UGC → UGG	C390W	Mov-Pro	Null	None
CLA8	2310 GUU → GCU	V419A	Mov-Pro	Wild type	Etch
PC29	2316 GAU → AAU	D421N	Mov-Pro	Null	None
CLA9	2322 UAU → CAU	Y423H	Mov-Pro	Increased	Etch
CLA10	2379 AUU → AUG	I442M	Mov-Pro	Null	None[e]
CLA11	2382 GAA → AAA	E443K	Mov-Pro	Increased	Etch
PC30	2411 GAA → GAU	E452D	Mov-Pro	Wild type	None
CLA12	2415 AAA → ACA	K454T	Mov-Pro	Increased	Etch

[a] Location of mutations on TEV nucleotide sequence (GenBank accession DQ986288) and on the deduced amino acid sequence of HC-Pro protein.

[b] Corresponding functional domains: Trans, transmission; Sup, RNA-silencing suppression; Mov, viral movement; Pro, proteinase.

[c] Relative to wild-type HC-Pro suppressor activity. Classification into functional classes was based on whether the confidence intervals in Figure 2 contained zero and/or one value.

[d] Systemic symptoms displayed by *N. benthamiana* plants inoculated with infectious transcripts.

[e] Asymptomatic infections in which TEV has been detected by RT–PCR in systemic and by Western blot in inoculated leaves.

HC-Pro mutants was generated by site-directed mutagenesis in a pTEV7DA infectious clone. The location of each mutation, its nature, and the phenotype induced are summarized in Table 1. The suppressor activity of each mutant, relative to that of wild-type HC-Pro, was estimated by means of the co-infiltration assay depicted in Figure 1. As previously described, GFP mRNA was silenced after a maximum of 3 days in the absence of a suppressor activity, whereas in the presence of HC-Pro, fluorescence lasted longer (Johansen and Carrington 2001). Samples for quantification of GFP mRNA concentration by RT–qPCR were taken 6 days post-infiltration. The distribution of mutational effects on RNA-silencing suppression activity is shown in Figure 2. Each mutant HC-Pro was classified into one of four functional categories on the basis of the corresponding estimated 95% confidence interval of the median:

i. Null mutants that completely lack suppressor activity (= 0.0). Nine mutants belong to this category (Table 1 and Figure 2).
ii. Mutants with a significant reduction in suppressor activity (>0.0 and <1.0). Three mutants are included in this category (Table 1 and Figure 2). Hereafter, these mutants will be defined as hypo-suppressors since their median activity was 45.85% lower than that of wild type.
iii. Mutants with no effect on the trait and thus performing as wild type (= 1.0). Eleven mutants belong to this category (Table 1 and Figure 2).

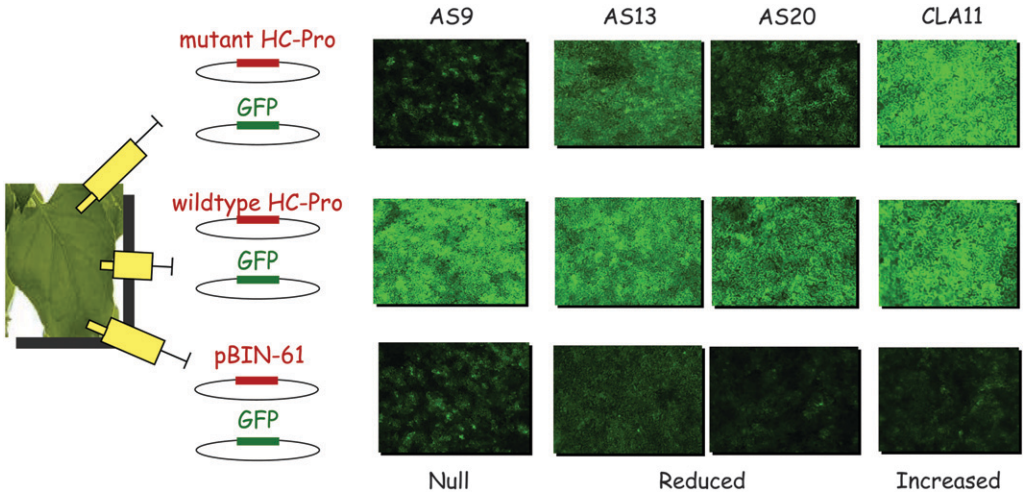

FIGURE 1.—Illustration of the co-infiltration experiment procedure. *N. benthamiana* leaves were infiltrated with three different mixtures: (i) pBIN61-GFP and mutant pBIN61-HC-Pro (first row), (ii) positive control pBIN61-GFP and wild-type pBIN61-HC-Pro (second row), and (iii) negative control pBIN61-GFP and pBIN61 (third row). GFP fluorescence was observed under the stereoscope 6 dpi. Four mutants are shown as examples.

iv. Mutants with increased activity (>1.0). Five mutants belong to this category (Table 1 and Figure 2). The median activity of these mutants was 22.0% larger than that of wild-type HC-Pro. Hereafter, these mutants will be referred to as hypersuppressors.

Roughly speaking, the number of mutations positively affecting suppression activity was half the number of mutations negatively affecting suppression. The differences among these four groups were highly significant (Kruskal–Wallis test: $H = 21.312$, 3 d.f., $P < 0.001$) and, consequently, most of the observed variation (73.1%) was explained by true differences between categories rather than by measurement noise.

No association has been observed between the functional domain in which one mutation was generated and its inclusion in one of the above functional categories ($\chi^2 = 3.400$, 6 d.f., $P = 0.757$), even after collapsing the four categories into neutral and non-neutral ($\chi^2 = 0.127$, 1 d.f., $P = 0.722$). In a continuous trait scale, mutations in different domains do not differ in their effect on suppression activity ($H = 2.829$, 2 d.f., $P = 0.243$). These results are expected for overlapping functional domains.

Relationship between HC-Pro suppression activity and the severity of symptoms: To assess mutant viability, transcripts from reconstituted TEV genomes containing the HC-Pro mutant alleles and wild-type TEV were mechanically inoculated on *N. benthamiana* plants and symptoms were recorded. Asymptomatic plants were analyzed in two different ways: (i) virus amplification and cell-to-cell local movement in inoculated leaves was detected by Western blot using anti-coat protein antibodies and (ii) systemic leaves were analyzed by RT–PCR to detect the presence of TEV (Table 1).

Figure 3 shows the relationship between relative suppressor activity and the symptoms described in Table 1. Activity significantly differed among symptom categories ($H = 13.797$, 2 d.f., $P = 0.001$), with asymptomatic infections associated, on average, with null or weak suppressors and stronger suppressors producing more severe etching. In other words, the stronger the suppressor, the more severe the symptoms that developed in plants (Pearson's correlation coefficient: $r = 0.751$, 27 d.f., $P < 0.001$). Plants infected with null mutants did not showed symptoms 15 dpi. In seven cases, mutant HC-Pro rendered nonviable TEV and the virus was not even detected in the inoculated leaves. However, in three cases (AS9, AS10, and CLA10), mutants were capable of systemic movement and were detected in upper asymptomatic leaves. The three TEV clones bearing HC-Pro mutants classified as hyposuppressors were viable but induced atypically mild etching (CLA2 and AS20) or no symptoms at all (AS13). The five TEV clones carrying HC-Pro mutants classified as hypersuppressors were all viable, inducing a similar etching pattern as wild

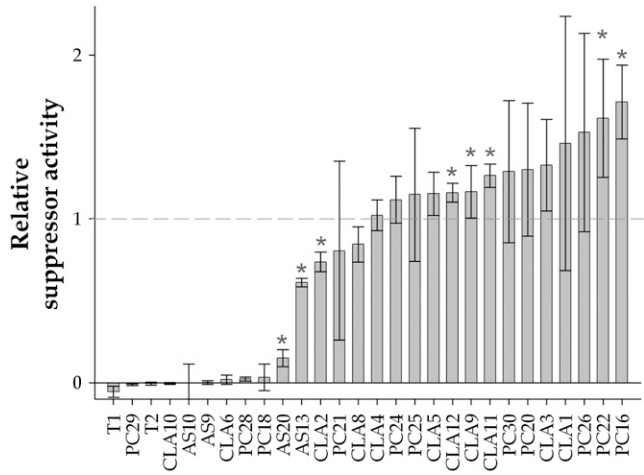

FIGURE 2.—Distribution of mutational effects on RNA-silencing suppressor activity for all HC-Pro mutant genotypes. Values are relative to the wild-type activity; median values are reported. Error bars represent ±1 standard error of the median and were computed by the Jackknife method. Asterisks indicate those cases that significantly differ both from wild type and from the negative control (background measurement).

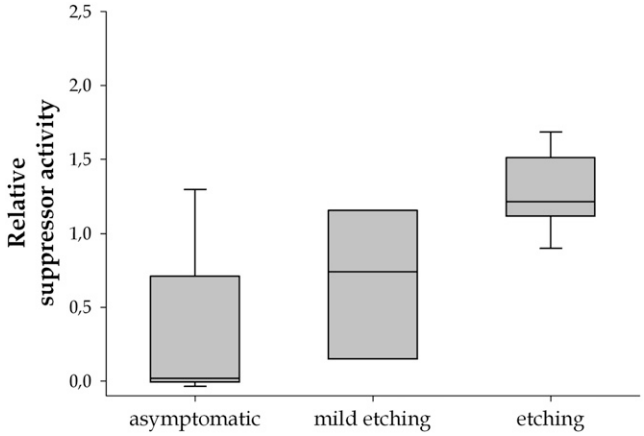

FIGURE 3.—Relationship between symptom severity and relative suppressor activity. Boxes represent the 5 and 95% percentiles and error bars the 95% confidence interval. The horizontal line corresponds to the median activity.

type. In the case of CLA9, plants developed symptoms 1 day sooner than plants infected with wild-type TEV.

In two cases, mutants performing wild-type suppressor activity induced altered symptoms, suggesting that mutations impaired another function. CLA5 produced mild etching in systemic leaves 15 dpi, suggesting that the mutation affected systemic movement, a suggestion compatible with the location of the mutation in the movement-proteinase domain. PC30 was not even detected in inoculated leaves, suggesting that the mutation affected the proteinase activity and thus rendered a nonfunctional protein. Concurrently, the mutation is located in the movement-proteinase domain.

Symptoms described in Table 1 are, somehow, subjective. To avoid this drawback and to gain further insights into the relationship between suppression activity and virulence, the quantitative effect of viral infection on plant growth and vigor was studied for four hypersuppressor (CLA9, CLA11, CLA12, and PC16) and two symptom-producing hyposuppressor (CLA2 and AS20) alleles. Hypersuppressor PC22 was not incorporated in this study because we consistently failed to purify viral particles in large enough amounts to successfully infect new *N. benthamiana* plants. Fresh and dry (after 4 days in a desiccation oven at 100°) weights and the height of the canopy were measured 20 dpi ($n = 7$). Wild-type TEV and mock-inoculated plants served as controls. Figure 4A shows the effect of suppression categories on each morphological trait. Prior to analyses, variables were log transformed to achieve normality of data and homocedasticity of variances. Nested multivariate and univariate model II ANOVA tests were performed. Also, the three variables were collapsed into a first principal component, which explains up to 72.3% of observed variability. This principal component is positively correlated with the three morphological variables measured and its biological meaning is quite straightforward: it is large when plants are heavy and tall and small when plants are light and dwarfed (Figure 4). A nested model II ANOVA was also computed for the principal component values. All tests rendered the same results and thus only those from the analysis of the first principal component are reported. Significant differences exist between suppressor categories (Figure 4B; $F_{4,4} = 170.196$, $P < 0.001$) and among genotypes within categories ($F_{4,48} = 3.818$, $P = 0.009$). More interestingly, a Tukey's post-hoc test indicates that no apparent morphological differences exist between plants inoculated with wild-type TEV and hypersuppressor mutants (Figure 4B; $P < 0.05$) and that plants infected with TEV hyposuppressor mutants are, on average, bigger than plants infected with the wild type, although still significantly smaller than non-infected plants (Figure 4B).

As an additional test of virulence, the effect of infection on plant survival was studied for wild-type TEV, three hyposuppressors (AS20, AS13, and CLA2), and four hypersupressors (CLA12, CLA9, CLA11, and PC16). Batches of *N. benthamiana* plants were infected with each genotype (median number of infected plants, 25; range 14–28). Infected plants were maintained in the greenhouse until systemic necrosis reached all leaves and roots or up to 134 dpi. Survival data were analyzed using a Kaplan–Meir regression in which genotypes were nested within their corresponding suppressor category. Figure 5 shows the average survival curves for each suppressor category; the mean survival time for each TEV genotype is reported in Table 2. Significant differences exist among suppressor categories (Figure 5; Mantel–Cox test: $\chi^2 = 106.721$, 2 d.f., $P < 0.001$). On average, a plant infected with wild-type TEV survived 41 dpi (95% C.I.: 40.416–41.738). Among hyposuppressors, AS20 and AS13 have no effect on plant survivorship and all plants survived until the end of the experiment even though they developed mild symptoms. By contrast, plants infected with CLA2 showed a significant reduction in survival time (Table 2). On average, plants infected with hyposuppressor mutants survived 108 dpi (95% CI: 98.518–117.449), 2.6 times longer than plants infected with wild-type TEV; this difference is statistically significant (non-overlapping 95% C.I.'s). The effect of hypersuppressor mutants was intermediated among wild-type TEV and hyposuppressors. Results ranged from CLA12, which shows the smallest impact on plant survival, and CLA9, which exerts the same impact as wild-type TEV on plant viability (Table 2). On average, a plant infected with a hypersuppressor mutant survived 1.5 times more than plants infected with wild-type TEV (61 dpi; 95% C.I.: 55.249–66.893), but 1.8 times less than plants infected with hyposuppressor mutants. As expected, symptom severity (Figure 4B) and plant survival time were negatively correlated ($r = -0.819$, 5 d.f., $P = 0.024$); that is, the severer the symptoms induced by the virus HC-Pro mutant, the shorter the plants that survived.

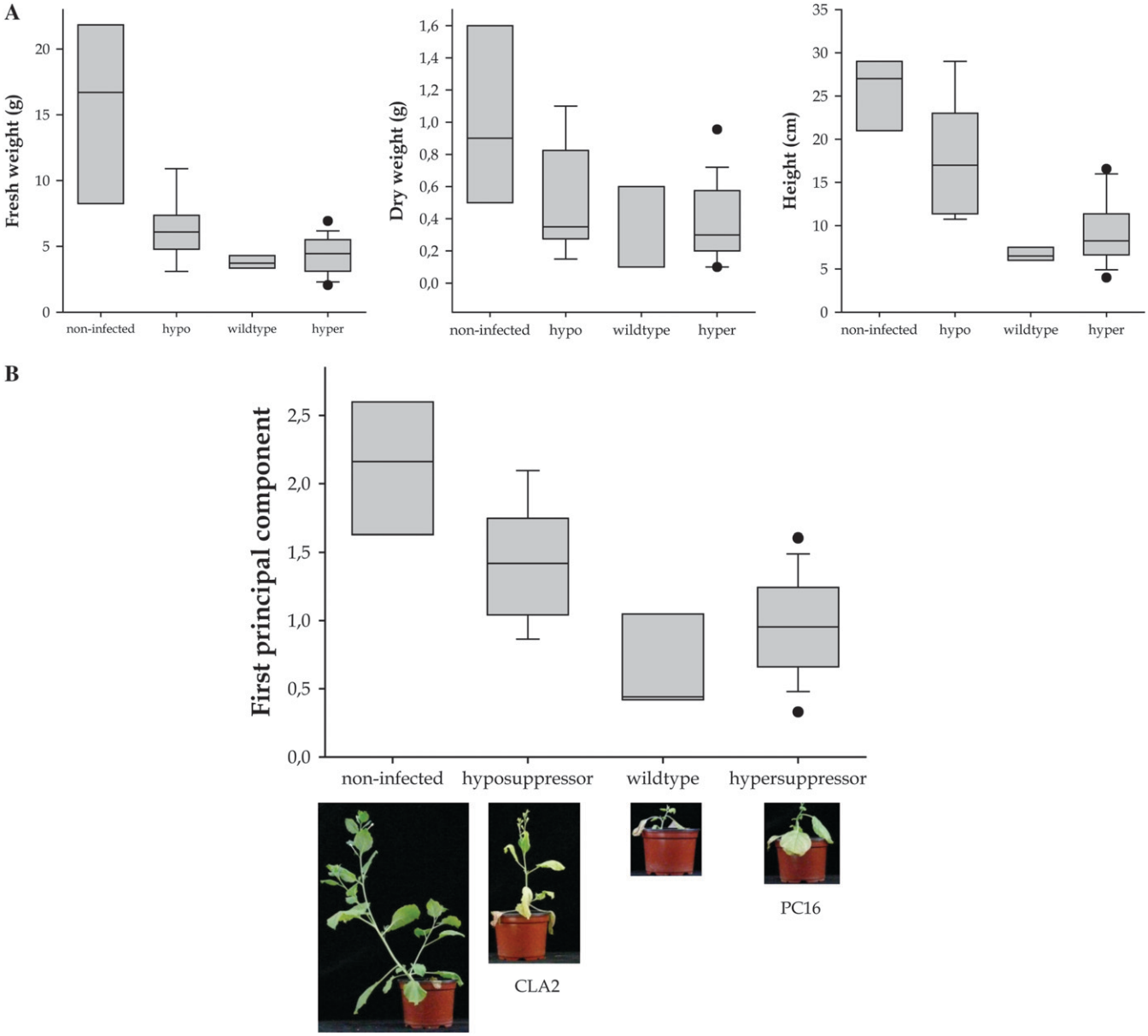

FIGURE 4.—(A) Morphological traits measured in mock-inoculated plants and plants infected with wild-type TEV and hyper- and hyposuppressor mutants. (B) First principal component computed from the three morphological traits: 0.927 × (log fresh weight) + 0.840 × (log height) + 0.779 × (log dry weight). Boxes represent the 5 and 95% percentiles and error bars the 95% confidence interval. The horizontal line corresponds to the median value. Circles represent outliers. Photos show representiative symptoms for each suppressor category.

At face value, all these results indicate that, on average, hyposuppressor mutants are also less virulent than wild type but that viruses carrying hypersuppressor mutations are not more virulent than wild-type TEV. Phrased differently, hyposuppressors are hypovirulent whereas hypersuppressors are not hypervirulent. Therefore, other factors in addition to suppressor activity must be contributing to set the upper virulence value.

None of the mutants assayed *in planta* had reverted to the wild-type allele (C. TORRES-BARCELÓ and S. F. ELENA, unpublished results).

Effect of HC-Pro mutations on virus accumulation: To further investigate the consequences of suppression on viral accumulation, the number of infectious viral particles produced by wild-type TEV, two of three hyposuppressors (CLA2 and AS20), and five hypersuppressors (CLA9, CLA11, CLA12, PC22, and PC16) were estimated by counting the number of local necrotic lesions produced in *C. quinoa* leaves inoculated with serial dilutions of each genotype. AS13 hyposuppressor was not included in the analysis because it consistently failed to produce visible lesions in *C. quinoa* leaves. Table 2 shows the average titer for each of these genotypes. Under the culture's environmental conditions, the wild-type TEV produced visible lesions in *C. quinoa* leaves 10–11 dpi. The two hyposuppressor mutants developed

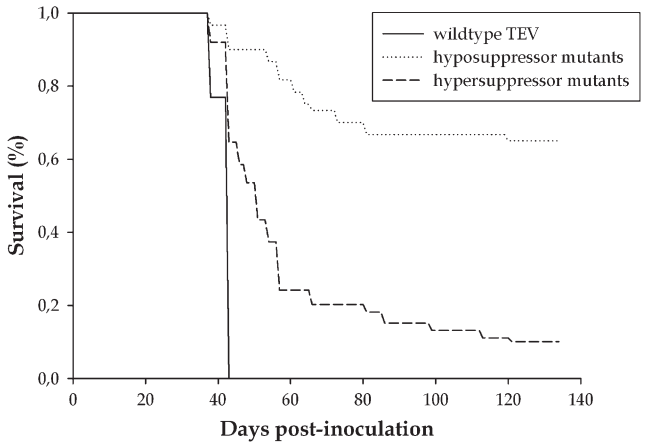

FIGURE 5.—Survival curves of plants infected with wild-type TEV and hyper- and hyposuppressor mutants.

lesions more slowly, being visible only after 15–18 dpi. Furthermore, on average, hyposuppressor mutations significantly reduced 67.37% of the number of infectious particles produced (Table 2; *t*-test: $P = 0.018$). Among the five hypersuppressors, CLA11 and CLA12 delayed the development of local lesions on *C. quinoa* leaves by 3 days (13–15 dpi), but the other three hypersuppressor genotypes did not differ from wild type. On average, hypersuppressor mutations have no significant effect on the accumulation of infectious viral particles (Table 2; *t*-test: $P = 0.958$).

Virus accumulation does not show a correlation with the intensity of symptoms induced in *N. benthamiana* plants. No significant differences in virus accumulation have been found between genotypes producing mild etching (AS20 and CLA2; average titer = 16.461 ± 5.465 LFU/μl) or wild-type-like etching (wild-type TEV, CLA9, CLA12, CLA11, PC16, and PC22; average titer = 44.464 ± 10.253 LFU/μl; Mann–Whitney *U*-test: $P = 0.286$).

DISCUSSION

Here, we have described the effect that amino acid substitutions exert on the suppressor activity of the multifunctional protein HC-Pro encoded by TEV. Mutational effects on suppressor activity, measured in a transient expression assay, ranged from no effect at all (neutral mutations) to complete elimination of activity (lethal mutations). Lying between these two extremes, three mutations induced a significant reduction in activity (from 1.4- to 7-fold reduction). We have qualified these HC-Pro mutants as hyposuppressor alleles. Interestingly, five mutations showed a significant increase in suppression activity (from 1.1- to 2.4-fold increase). We have qualified these HC-Pro mutants as hypersuppressor alleles. A set of HC-Pro alleles was reconstituted into the viral infectious cDNA and their effect on virus accumulation and symptom expression was explored. Suppression activity levels positively correlated with the intensity of symptoms induced, with hyposuppressors inducing milder symptoms and accumulating to lower levels than the wild-type TEV virus. By contrast, the hypersuppressor mutants induced symptoms and accumulated to levels that were not distinguishable from those characteristic of the wild-type virus. These results suggest an asymmetrical response of the virus to mutations affecting their suppressor activity; whereas hyposuppressor mutants clearly have impaired replication (lower accumulation) and mild symptoms, hypersuppressor alleles do not translate their stronger suppression to any quantitative effect on virus accumulation and symptom expression, at least to the resolution of our experimental procedures. Two cases are particularly interesting in this regard. The CLA12 hypersuppressor shows the second largest accumulation of infectious viral particles (Table 2) but affects the survival of plants in a way similar to hyposuppressors. By contrast, the CLA2 hyposuppressor affects plant survival almost similar to hypersuppressors,

TABLE 2

Survival time and virus accumulation in plants infected by TEV with HC-Pro mutants

Suppression category	Mutant	Survival time (dpi)	Average survival time	Infectious titer[a] (LFU/μl)	Average titer
Hyposuppressor	AS20	134.000 ± 0.000	107.983 ± 4.829	10.996 ± 5.153	16.461 ± 5.353
	AS13	134.000 ± 0.00		ND	
	CLA2	56.650 ± 2.954		21.927 ± 9.320	
Wild type		41.077 ± 0.337		50.446 ± 11.701	
Hypersuppressor	CLA12	99.250 ± 5.905	61.071 ± 2.970	60.406 ± 29.375	49.059 ± 12.930
	CLA9	41.167 ± 0.339		76.470 ± 33.703	
	CLA11	47.429 ± 1.323		29.454 ± 18.599	
	PC22	ND		4.306 ± 0.601	
	PC16	49.346 ± 0.994		45.701 ± 13.127	

ND, not determined.

[a] Average number of local necrotic lesions per inoculum (LFU/μl) produced in *C. quinoa* leaves. In all cases, errors represent the standard error of the mean.

despite producing the second lowest amount of infectious particles (Table 2).

In a similar study, Stenger *et al.* (2006) created a collection of single-nucleotide substitution mutants of *Wheat streak mosaic virus* (WSMV) HC-Pro. The collection included both synonymous and nonsynonymous substitutions. Each mutant was evaluated for its ability to systemically move and the severity of the symptoms induced, although no quantitative information about the suppressor activity was provided. Briefly, they found that synonymous substitution did not have an effect on the virus's systemic movement nor on the strength of symptoms. Among the nonsynonymous mutants, most had no effect (57.69%), 15.38% resulted in attenuated systemic infection, and 26.92% abolished systemic infection. Interestingly, the latter mutants were not deficient in proteinase activity, thus suggesting that the mutation was affecting the silencing-suppression activity. These results are in excellent agreement with ours and support the conclusion that most mutations affecting HC-Pro negatively affect potyvirus systemic movement and accumulation.

These results provide further support to the well-established notion that HC-Pro is essential for symptom development and virus accumulation (Kasschau and Carrington 2001; Urcuqui-Inchima *et al.* 2001). In an extreme case of null mutants with no production of HC-Pro protein, it has been described that the potyvirus WSMV was still viable but infectivity was low and infection concurred with the development of very mild symptoms (Stenger *et al.* 2005). Experimental evolution of this deletion mutant by serial passages resulted in the recovery of infectivity and symptoms similar to wild type (Stenger *et al.* 2005), suggesting that hyposuppressor mutations do not represent an evolutionarily stable situation. Furthermore, it was also hypothesized that WSMV may encode a protein other than HC-Pro with RNA-silencing suppressor activity and thus HC-Pro may be functionally redundant and dispensable (Stenger *et al.* 2006). Supporting this possibility, Valli *et al.* (2006) found that a second copy of the P1 serine protease of *Cucumber vein yellowing virus* (a member of the genus Ipomovirus within the Potyviridae family) has RNA-silencing suppressor activity. Whether TEV may encode for a second suppressor protein is a tantalizing possibility that would explain why some suppression-defective mutants were still capable of producing a systemic infection.

Hypersuppressor alleles have not been described before, perhaps because only semiquantitative approaches have been applied to the characterization of HC-Pro mutants (*e.g.*, Kasschau and Carrington 2001). The sensitivity of our RT–qPCR method for quantifying suppressor activity has allowed us to describe five HC-Pro alleles with increased suppression activity, representing 17.86% of the sample size analyzed here. This high frequency opens the question of whether hypersuppressor mutants may be common in nature or, alternatively, impose a fitness burden that precludes their spread in natural viral populations. In other words, has the intermediate suppression activity characteristic of wild type been optimized by natural selection? Although answering this question requires additional experiments, our observation that hypersuppressor mutants reach similar accumulation levels as wild type suggests that the benefits or costs of RNA-silencing hypersuppression, if any, are not associated with virus accumulation.

HC-Pro is a determinant of host range (Sáenz *et al.* 2002; Stenger and French 2004) and thus it may be argued that our estimates of virus accumulation may be affected by the fact that estimates were done by inoculating the mutants on a different host. However, we consider this possibility highly unlikely for the following reason: Stenger and French (2004) performed heterologous cistron replacement experiments in which WSMV HC-Pro was systematically replaced by homologous proteins from other members of the Potyviridae family. When the protein was replaced by that of a different isolate of WSMV (*i.e.*, sequence identity $\geq$86%), host range was not altered. By contrast, when the replacing protein come from a virus belonging to a different genus (*i.e.*, sequence identity $\leq$17%), then host range was largely affected (Stenger and French 2004). These results suggest that changes in host range are not easily achieved by a few mutations in HC-Pro, as they are in our case.

Here we have shown that the degree of RNA-silencing suppression shows a positive association both with virus accumulation and the strength of symptoms. However, we have failed to find such positive association between the severity of symptoms and the level of virus accumulation. That is, severer symptoms are not explained by a larger accumulation of viral particles. This lack of correlation is not an unexpected result since there are examples of uncorrelated changes in plant virus accumulation and virulence. For example, we have recently observed a lack of correlation between viral fitness and virulence for a collection of TEV random single-nucleotide substitution mutants (Carrasco *et al.* 2007b). Similarly, it was shown that when *Barley stripe mosaic virus* was evolved by serial horizontal transfers, its virulence increased with no concomitant increase in viral load (Stewart *et al.* 2005). As a final example, necrogenic and non-necrogenic variants of *Cucumber mosaic virus* did not differ in their accumulation in tomato plants (Escriu *et al.* 2000). Most theoretical models seeking to explain the evolution of virulence assume that it is a side effect of virus replication and accumulation (Ebert 1998; Brown *et al.* 2006). However, virulence would not depend on within-host replication if the extent of damage was not proportional to the amount of viral particles, as is the case with hypersensitive responses (Morel and Dangl 1997), or if expressing the systemic acquired resistance pathway is costly for the plant (Heidel *et al.* 2004), or if allocating resources to defense detracted plants from vegetative growth or reproductive effort (Heil 2001).

Expression of both Dicer proteins DCL4 and DCL2 is necessary for conferring antiviral immunity in *Arabidopsis thaliana* against RNA viruses (Deleris *et al.* 2006). DCL4 acts as the primary sensor and produces 21-nt siRNAs that guide RISC. In a second step, DCL2 forms 22-nt siRNAs with antiviral activity, but these siRNAs are manifested only when DCL4 is inactivated (Deleris *et al.* 2006). Plants deficient in DCL2 and DCL4 presented higher accumulation of *Tobacco rattle virus* RNA and stronger symptoms than wild-type plants, thus proving that virus accumulation depends on the strength of RNA silencing. This is in good agreement with our observation that the stronger the RNA-silencing suppression activity may be, the more viruses accumulate and the more severe the symptoms are. Indeed, this study also suggests a possible explanation for why TEV hypersuppressors do not significantly differ from wild type either in accumulation or in symptoms. If TEV HC-Pro kidnaps mainly the 21-nt siRNAs produced by DCL4 but has no significant activity in the 22-nt siRNAs produced by DCL2, a hypersuppressor mutant will more efficiently sequester 21-nt siRNA, but will be controlled by 22-nt siRNAs to the same extent as the wild-type virus.

In experimental evolution work currently in progress, we are exploring the pathways of compensatory evolution in restoring the wild-type level of suppressor activity and the molecular basis of such compensations.

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