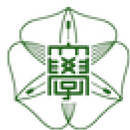


HOKKAIDO UNIVERSITY

Title	Heterologous expression of viral suppressors of RNA silencing complements virulence of the HC-Pro mutant of clover yellow vein virus in pea
Author(s)	Atsumi, Go; Nakahara, Kenji S; Wada, Tomoko Sugikawa; Choi, Sun Hee; Masuta, Chikara; Uyeda, Ichiro
Citation	Archives of Virology, 157(6), 1019-1028 https://doi.org/10.1007/s00705-012-1281-3
Issue Date	2012
Doc URL	http://hdl.handle.net/2115/49369
Type	article (author version)
File Information	TextFigsSupFigs.pdf

Instructions for use

Hokkaido University Collection of Scholarly and Academic Papers : HUSCAP

Heterologous expression of viral suppressors of RNA silencing complements virulence of the HC-Pro mutant of *Clover yellow vein virus* in pea

Go Atsumi[1], Kenji S. Nakahara[1], Tomoko Sugikawa Wada[1], Sun Hee Choi[1,] Chikara Masuta[2], and Ichiro Uyeda[1]

(1) Pathogen-Plant Interactions Group, Graduate School of Agriculture, Hokkaido University, Sapporo 060-8589, Japan

(2) Laboratory of Cell Biology and Manipulation, Graduate School of Agriculture, Hokkaido University, Sapporo 060-8589, Japan

Corresponding author:
Ichiro Uyeda
Tel: +81 11 706 2473
Fax: +81 11 706 2483
E-mail: uyeda@res.agr.hokudai.ac.jp

Abstract

Many plant viruses encode suppressors of RNA silencing, including the helper component-proteinase (HC-Pro) of potyviruses. Our previous studies showed that the D-to-Y mutation at amino acid position 193 in HC-Pro (HC-Pro-D193Y) drastically attenuated the virulence of *Clover yellow vein virus* (ClYVV) in legume plants. Furthermore, RNA-silencing suppression (RSS) activity of HC-Pro-D193Y was significantly reduced in *Nicotiana benthamiana*. Here, we examined the effect of expression of heterologous suppressors of RNA silencing, i.e., *Tomato bushy stunt virus* p19, *Cucumber mosaic virus* 2b, and their mutants, on the virulence of the ClYVV point mutant with D193Y (Cl-D193Y) in pea. P19 and 2b fully and partially complemented the Cl-D193Y multiplication and virulence, including lethal systemic HR in pea, respectively, but the P19 and 2b mutants with defects in their RSS activity did not. Our findings strongly suggest that the D193Y mutation exclusively affects RSS activity of HC-Pro and that RSS activity is necessary for ClYVV multiplication and virulence in pea.

Introduction

RNA silencing acts as an effective antiviral defense in plants according to the following three observations [19]. First, virus-specific small RNAs (sRNAs) accumulate in host cells, suggesting that virus RNAs are subjected to the RNA-silencing machinery of the host [6]. Second, host mutants in major components of the RNA-silencing machinery, including Dicer-like proteins, Argonaute proteins, and RNA-dependent RNA polymerases, exhibited increased virus susceptibility, suggesting that the host utilizes these components for antivirus defense [12, 14, 19, 31]. Finally, many plant viruses express proteins that can suppress RNA silencing (viral suppressor of RNA silencing, VSR) [3]. VSRs are highly diverse in sequence, structure, and target point of RNA silencing, suggesting that each protein has independently gained the ability to suppress RNA silencing [3]. Several reports have suggested that suppression of RNA silencing by viruses leads to symptom expression. *Turnip mosaic virus* (TuMV) encodes P1/HC-Pro, which functions as a VSR. Kasschau et al. [9] found that the developmental defects induced by TuMV were partially expressed, as a result of perturbation of the miRNA-regulated developmental pathway, by P1/HC-Pro in *Arabidopsis thaliana.* Omarov et al. [16] demonstrated that suppression of RNA silencing was important for *Tomato bushy stunt virus* (TBSV) virulence (the ability to induce severe symptoms) in *Nicotiana benthamiana*.

Clover yellow vein virus (ClYVV), a member of the genus *Potyvirus*, causes severe systemic cell death in many cultivars of pea and broad bean [18, 33]. In pea, ClYVV systemically induces cell death in PI 118501, but not in PI 226564 [7, 18]. Genetic analyses indicated that the cell death is controlled by a single incompletely dominant gene, *Cyn*1 [18]. Furthermore, cell death is suggested to be caused by a hypersensitive reaction (HR)-type defense pathway in pea PI 118501 [1, 18]. However, viruses were not restricted to the primary infection area and moved systemically, leading to the systemic induction of an HR-like response (systemic HR: SHR) [1, 18, 33].

Previous studies have demonstrated that an aspartic acid (D) to tyrosine (Y) mutation at amino acid (aa) position 193 of ClYVV HC-Pro substantially attenuates the ability to induce cell death systemically and to activate an HR-like defense pathway in PI 118501 [1]. Furthermore, the same mutation significantly compromised the RNA-silencing suppression (RSS) activity of P1/HC-Pro, suggesting that RSS activity is required for SHR induction by ClYVV in PI 118501 [1, 33]. However, it is possible that

the mutation simultaneously affects several functions other than RSS because HC-Pro is a multi-functional protein [24]. In this study, we examined the effect of expressing heterologous VSRs, i.e., TBSV p19 and *Cucumber mosaic virus* (CMV) 2b, on virulence expression and accumulation of the ClYVV point mutant with D193Y (Cl-D193Y) in pea PI 118501. TBSV p19 and CMV 2b expression restored multiplication and virulence, including SHR of Cl-D193Y in pea, but their RSS-defective mutants did not. Our findings strongly suggest that the D193Y mutation exclusively affects RSS activity of HC-Pro and that RSS activity is necessary for ClYVV multiplication and virulence in pea.

Materials and methods

Plant growth conditions and viral infections

Pea (*Pisum sativum*) was cultivated in a growth chamber at 21°C with a 16-h photoperiod. Viral inocula were prepared as described previously [20, 33]. First, broad bean (*Vicia faba*) was inoculated with infectious cDNA using particle bombardment. The upper leaves were harvested and ground in an inoculation buffer (0.1 M Tris-HCl, pH 7.0, 1% 2-mercaptoethanol). The crude sap was mechanically inoculated onto the third leaves of 2-week-old plants. At the same time, all plants were inoculated with inoculation buffer alone as a negative control (mock inoculation). Plasmid inoculations were conducted by rub-inoculation of plasmid solution (5–10 μg/leaf) onto leaves dusted with carborundum powder.

Construction of plasmids

ClYVV infectious cDNA has been synthesized and developed as a vector in legumes [11, 25, 30]. The construction of pCl-WT/GFP and pCl-D193Y/GFP was described previously [33]. We replaced the GFP sequence in pCl-D193Y/GFP with p19, p19m of TBSV, 2b, and 2bm of CMV isolate Y [16, 23, 26]. In brief, p19, p19m, 2b, and 2bm were inserted into ClYVV-CP-XB-pBS3 and subsequently sub-cloned into pClYVV-D193Y ΔCP to obtain pCl-D193Y/p19, -p19m, -2b, and -2bm [30]. p19m has an A-to-G mutation at nucleotide (nt) positions 223 and 232 (arginine to glycine change at aa positions 75 and 78), whereas 2bm has a C-to-T mutation at nt position 136 (arginine to cysteine change at aa position 46) [5, 16]. Mutations in p19m and 2bm were introduced by PCR using primers that included these mutations [5]. pCl-WT and -D193Y/p19, -p19m, -2b, and -2bm tagged with FLAG at the C-terminal end were constructed in the same way. To create pCl-FINK/GFP and p19, the *Bgl* II-*Bgl* II fragments of pCl-

WT/GFP and pCl-D193Y/p19 were replaced with *Bgl* II-*Bgl* II fragments that had an R-to-I mutation (G-to-T at nt position 542) in HC-Pro (BB/HC-Pro-FINK) [22]. The mutation in BB/HC-Pro-FINK was created by PCR.

pWCl/p19-, p19m-, 2b-, and 2bm-FLAG were constructed as follows: p19, p19m, 2b, and 2bm were fused to the sequence encoding the FLAG peptide at the 3'-end and were introduced into a white clover mosaic virus vector (WClMV vector; pWCl/p19-, p19m-, 2b-, and 2bm-FLAG). In the WClMV vector, the complete cDNA sequence of the WClMV RC strain was flanked by the CaMV 35S promoter, and the multi-cloning site was located between the triple gene block and CP (Fig. 6) [7, 15].

The construction of pBE2113/P1HC-WT, /D193Y, pIG121/GFP, and /IR-GFP has been described previously [33]. P1HC-FINK was created using PCR and replaced with the GUS gene in pBE2113 (pBE2113/P1HC-FINK). P19-, p19m-, 2b-, and 2bm-FLAG were introduced into the pBE2113 vector. pBIC/p19 was provided by T. Okuno [26].

RNA extraction, reverse transcription, and PCR (real-time PCR)

Leaves were homogenized in liquid nitrogen, and total RNA was isolated using Trizol reagent (Invitrogen, CA, USA) according to the manufacturer's instructions [1]. Each RNA sample was treated with RNase-free DNase I (TaKaRa, Shiga, Japan), and 1–2 μg of total RNA was reverse-transcribed using cloned AMV RTase (Invitrogen). The RT reaction mixture (20 μl) contained 1–2 μg of total RNA, 2.5 μM random 9-mers, 1 mM dNTP, 5 mM DTT, 40 U RNase inhibitor (Wako, Osaka, Japan), 4 μl 5× cDNA synthesis buffer, and 15 U of cloned AMV RTase. Samples were first incubated at 25°C for 10 min, then at 45°C for 60 min, and finally at 85°C for 5 min. The PCR primers for the detection of ClYVV and sequencing of D193Y and the R-to-I mutation in the FRNK motif were 5'-GATATGGTAGCCTGACAAATG-3' and 5'-GTTGCAAGTTCTCTCGTACC-3'. Real-time PCR was performed using the DNA Engine Opticon 2 System (Bio-Rad, CA, USA) [1]. The reaction mixture (25 μl) contained 0.625 U ExTaq (Takara), 2.5 μl of 10× ExTaq buffer, 0.2 mM dNTP, 0.2 μM forward and reverse primers, SYBRgreen (×30,000 dilution, Invitrogen), and cDNA obtained by reverse-transcribing 5–10 ng of total RNA. Samples were incubated for 5 min at 95°C, followed by 40 cycles of 95°C for 10 s, 53°C (for *SA-CHI* [accession number, L37876]), 55°C (*HSR203J* [AB026296]), or 58°C (ClYVV) for 30 s, and 72°C for 20 s. Transcript levels were normalized to that of *18S* rRNA (U43011), and means and

1 standard deviations were calculated. The primers used for real-time PCR were as follows: SA-CHI-F, 5'-
2 CGGTTCAACCTCCGAATACT-3'; SA-CHI-R, 5'-TGTTGTTGAAGGTGTCACCG-3'; HSR203J-F,
3 5'-GTCCGGTTGGCTTAGAATCTAC-3'; HSR203J-R, 5'-GTAGTGCTCATGGTCACGTCACG-3';
4 18SrRNA-F, 5'-CGTTCTTAGTTGGTGGAGCGAT-3'; 18SrRNA-R, 5'-
5 CCATAGTCCCTCTAAGAAGCTG-3'; ClYVV-F, 5'-ATTGATCTAACACCCCACAACC-3'; and
6 ClYVV-R, 5'-CTAACCTTGCCTTTCCAGTTTG-3' [1].
7

8 **Western blotting**

9 For ClYVV CP and HC-Pro detection, leaf tissues were ground in PBS-Tween (0.01 M phosphate
10 buffer, pH 7.0, 0.05% Tween 20) after homogenization in liquid nitrogen. The crude extracts were
11 centrifuged for 5 min at 14,000 rpm. The supernatants were mixed with an equal amount of 2×Laemmli
12 sample buffer and used for SDS-PAGE. For detection of RNA-silencing suppressors (p19, p19m, 2b, and
13 2bm), leaf tissues were ground in 1×Laemmli sample buffer, spun down, and the supernatants were used
14 for SDS-PAGE. For Western blotting, mouse monoclonal antibody against ClYVV HC-Pro [32], rabbit
15 polyclonal antibody against ClYVV CP [1], or mouse M2 monoclonal antibody against FLAG (Sigma-
16 Aldrich, MO, USA) was used as primary antibody, and alkaline phosphatase-conjugated goat anti-mouse
17 (Bio-Rad) or anti-rabbit (Invitrogen) IgG was used as secondary antibody.
18

19 **Double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA)**

20 Leaf tissues were homogenized in 40× volume of PBS-Tween and spun down, and the supernatants
21 were used as antigens. DAS-ELISA was conducted using a mouse anti-ClYVV CP IgG as the first
22 antibody and rabbit anti-ClYVV CP as the second antibody. After washing, alkaline phosphatase-
23 conjugated goat anti-rabbit IgG was added, followed by the substrate solution (1 mg disodium ρ-
24 nitrophenyl-phosphate hexahydrate in 1 ml 10% diethanolamine). The intensity of the signal was
25 measured at an optical density (OD) of 405 nm.
26

27 **Northern hybridization**

28 Leaves were homogenized in liquid nitrogen, and total RNA was isolated using Trizol reagent
29 (Invitrogen) according to the manufacturer's instructions [1]. Total RNA (3 μg) was dissolved in RNA

denaturing pre-mix (1×MOPS buffer, 50% formamide, 17.5% formaldehyde), heated at 70°C for 15 min, separated in a 1.2% denaturing gel, and blotted onto a nylon membrane (Hybond N; GE Healthcare, Buckinghamshire, UK). The membrane was hybridized with a digoxigenin (DIG)-labeled, GFP-specific RNA probe in hybridization buffer (DIG Easy Hyb; Roche Diagnostics GmbH, Mannheim, Germany). The chemiluminescence signals were detected with CDP-Star reagent (New England Biolabs, MA, USA) using a LAS-4000-mini camera system (GE Healthcare).

Trypan blue staining

Trypan blue staining was performed as previously described [1, 13]. The lactic acid–phenol–trypan blue solution (2.5 mg/ml trypan blue, 25% [w/v] lactic acid, 23% Tris-EDTA-saturated phenol, 25% glycerol and water) was heated to 70°C and leaves were vacuum-infiltrated with the solution. Leaves were then incubated in boiling water for 2 min and cooled for 1 h. Finally, the lactic acid–phenol–trypan blue solution was replaced with chloral hydrate solution (25 g in 10 ml water) for de-staining.

DAB staining

Leaves were vacuum-infiltrated with DAB solution (1 mg/ml 3,3-diaminobenzidine [DAB]-HCl, pH 3.8) [27] and incubated under high humidity conditions at room temperature until brown precipitates were observed. Finally, leaves were de-stained and fixed in a 3:1:1 ethanol/lactic acid/glycerol solution. As a positive control, pea leaves were infiltrated with H_2O_2 solution using a needleless syringe.

***Agrobacterium*-mediated transient assay**

To assess RNA-silencing suppression activity, we utilized an *Agrobacterium*-mediated transient assay in *N. benthamiana* leaves as previously described [33]. In brief, the *Agrobacterium* KYRT1 strain was transformed with pIG121/GFP and /IR-GFP, pBE2113/P1/HC-Pro, /p19-FLAG, and /2b-FLAG (wild-type and mutant), /GUS (negative control), and pBIC/p19 (positive control) [26]. Agrobacteria carrying each construct were suspended in MES buffer (10 mM MES, 10 mM $MgCl_2$, pH 5.7) and the suspensions were adjusted to OD 600 nm = 1.5 (GFP and test constructs [wild-type or mutant ClYVV P1/HC-Pro, p19 and GUS]) or 0.015 (IR-GFP). Then, acetosyringone was added to the suspensions (final concentration, 200 μM), followed by incubation at room temperature for 4 h. Equal amounts of the

suspensions were mixed, then infiltrated into *N. benthamiana* leaves using needleless syringes. For work described in Supplementary Fig. 6, equal amounts of the suspensions adjusted to OD 600 nm = 1.0 were mixed.

GFP fluorescence was detected using an Illumatool Tunable Lighting System LT-9500 with the excitation light at 470 nm and emission long-pass filter at 515 nm [33].

Results

Expression of heterologous suppressors of RNA silencing enhanced virulence of the attenuated *Clover yellow vein virus* HC-Pro mutant

In a previous study, we showed that the D-to-Y mutation at aa position 193 in HC-Pro of ClYVV abolished the lethal SHR phenotype in broad bean and pea [1, 33]. The RSS activity of HC-Pro-D193Y was drastically decreased by the *Agrobacterium*-mediated transient assay in *N. benthamiana* [33]. However, it is possible that the mutation in HC-Pro simultaneously affects several functions other than RSS [24]. To verify the requirement for RSS activity in virulence expression, we tested whether expression of heterologous VSRs could restore multiplication and virulence of Cl-D193Y in pea PI 118501. We selected the two well-known VSRs: TBSV p19 and CMV 2b [2, 8, 29]. We introduced each VSR into Cl-D193Y (Supplementary Fig. 1) [21, 23].

At 13 days post-inoculation (dpi), plants of pea line PI 118501 infected with wild-type ClYVV tagged with GFP (Cl-WT/GFP) were stunted and showed systemic cell death, whereas those infected with Cl-D193Y/GFP were not infected (Fig. 1) [1]. Plants infected with Cl-D193Y carrying p19 (Cl-D193Y/p19) showed cell death in inoculated and upper leaves and were severely stunted, resulting in plant death, which was at a comparable level to Cl-WT/GFP infection (Fig. 1a and bA, B, K, and L). However, Cl-D193Y carrying the RSS-defective mutant p19m, which has an arginine to glycine mutation at aa positions 75 and 78, did not induce symptoms in either inoculated or upper leaves (Fig. 1a and bC and M) [16]. Trypan blue staining of leaves inoculated with each virus indicated that cell death was extensively induced in Cl-WT/GFP and Cl-D193Y/p19 infections but not in Cl-D193Y/GFP and Cl-D193Y/p19m infections (Fig. 1bF–J). We conducted a similar experiment using CMV 2b (Fig. 2). Cl-D193Y/2b induced cell death in inoculated and upper non-inoculated leaves but did not kill the plants (Fig. 2A, F, K, P, and U). Plants infected with Cl-D193Y/2b were not stunted but showed severe mosaic

1 symptoms associated with cell death in the upper leaves (Fig. 2U and P). Plants infected with Cl-D193Y
2 carrying the RSS-defective mutant 2bm, which has an arginine to cysteine mutation at aa position 46, did
3 not show any symptoms (Fig. 2B, G, L, Q, and U) [5].

4 Many reports have indicated that the host RNA-silencing machinery suppresses virus accumulation
5 [3], and we previously reported that the virus accumulation of Cl-D193Y was significantly decreased [1].
6 Thus, we investigated whether p19 or 2b expression could restore virus accumulation of Cl-D193Y. The
7 use of an antibody against ClYVV CP for Western blot analysis of the upper non-inoculated leaves
8 showed that both p19 and 2b expression significantly enhanced ClYVV accumulation, but RSS-defective
9 p19 or 2b mutant expression did not; there was only a weak signal for Cl-D193Y/2bm, but those for Cl-
10 D193Y/p19m and /GFP were not detectable (Fig. 3a). We also compared CP accumulation in the upper
11 non-inoculated leaves using DAS-ELISA (Supplementary Fig. 2). As observed in Western blotting, there
12 were no significant differences in Cl-WT/GFP, Cl-D193Y/p19, and Cl-D193Y/2b. In DAS-ELISA, we
13 could successfully detect CP accumulation of Cl-D193Y/p19m and /GFP in the upper non-inoculated
14 leaves, and found that accumulation was significantly lower than that of Cl-WT/GFP.

15 We compared the virus RNA accumulation in upper non-inoculated leaves using real-time PCR (Fig.
16 3b), and found that Cl-D193Y/p19 accumulation was 180-times and twice as high as Cl-D193Y/GFP and
17 Cl-WT/GFP, respectively. Cl-D193Y/2b accumulation was about 27-times higher than that of Cl-
18 D193Y/GFP, but was about 35% of Cl-WT/GFP. Here, we confirmed by RT-PCR that the Cl-D193Y
19 series maintained the D193Y mutation and the inserted fragment in the upper non-inoculated leaves (data
20 not shown).

21

22 **Differences in protein accumulation of RNA-silencing suppressors**

23 We compared the RSS activities of p19, p19m, 2b, and 2bm used in this study with an
24 *Agrobacterium*-mediated transient assay in *N. benthamiana* leaves. GFP, the inverted repeat sequence of
25 GFP (IR-GFP; trigger of GFP mRNA silencing), and the suppressors of RNA silencing (wild type or
26 mutant, which were tagged with FLAG at the C-terminal end) were expressed and compared for their
27 RSS activities. P19-FLAG- and 2b-FLAG-expressed areas showed strong GFP fluorescence, whereas
28 p19m-FLAG and 2bm-FLAG displayed weak fluorescence (Supplementary Fig. 3a). Northern blot
29 analysis indicated that GFP mRNA correlated with the intensity of GFP fluorescence (Supplementary Fig.

1 3a and b). The accumulations of p19-, p19m-, 2b-, and 2bm-FLAG proteins were confirmed by Western
2 blotting using an antibody against the FLAG peptide. The result showed that p19-FLAG and 2b-FLAG
3 proteins accumulated more efficiently than p19m-FLAG and 2bm-FLAG proteins (Supplementary Fig.
4 3c).

5 We investigated whether the RSS-defective properties of p19 and 2b mutants resulted from
6 decreased protein stabilities. We compared the RSS activity of p19m and 2bm with that of p19 and 2b
7 using serial dilutions of *Agrobacterium* suspensions for infiltration. Although the accumulation level of
8 p19-FLAG protein in the area infiltrated with 1/8 the amount of p19m-FLAG was lower than that of
9 p19m-FLAG, GFP fluorescence in the p19-FLAG-expressed area was significantly stronger than in the
10 p19m-FLAG-expressed area (Supplementary Fig. 3d and f). Northern blot analysis indicated that GFP
11 mRNA level in the p19-expressed (1/8 dilution) area was higher than in the p19m-FLAG-expressed area
12 (Supplementary Fig. 3e). A similar experiment was done for 2b-FLAG and 2bm-FLAG proteins; however,
13 we could not examine the accumulation of 2b and 2bm in a reproducible fashion with the dilution
14 approach (data not shown). It is possible that p19 could protect its own mRNA from RNA-silencing-
15 mediated degradation by RSS activity, which would lead to effective p19 protein accumulation, while
16 p19m was not able to protect its mRNA. To assess this possibility, non-tagged p19 protein was co-
17 expressed with p19- and p19m-FLAG to block RNA-silencing-mediated degradation of the mRNAs. The
18 results showed that p19m-FLAG protein accumulated to a similar level as p19-FLAG protein in the p19-
19 expressed area, but did not in the control GUS-expressed area (Supplementary Fig. 4a). The same result
20 was obtained by ClYVV P1/HC-Pro co-expression (data not shown). We conducted a similar experiment
21 using 2b and 2bm. In contrast to p19, neither p19 nor P1/HC-Pro expression could restore the
22 accumulations of 2bm protein (Supplementary Fig. 4b). Finally, we investigated the stabilities of p19,
23 p19m, 2b, and 2bm proteins in pea. FLAG-tagged p19, p19m, 2b, and 2bm were expressed in the wild-
24 type ClYVV (which has a (wild-type) strong suppressor of RNA silencing), and their relative
25 accumulations to CP were compared, because foreign protein and CP were expressed as one precursor
26 polyprotein. The results showed that there were no significant differences between p19 and p19m
27 (Supplementary Fig. 4c); however, 2bm-FLAG protein accumulated less than 2b-FLAG (Supplementary
28 Fig. 4d).

29

1 **Ability of Cl-D193Y to induce a host HR-like defense pathway was restored by expression of**
2 ***Tomato bushy stunt virus* p19.**

3 Previous reports have shown that Cl-WT can significantly activate a host HR-like defense pathway
4 including expression of marker genes for salicylic acid (SA)-mediated signaling and HR-related pathways,
5 but Cl-D193Y cannot [1]. To examine whether p19 expression can restore the ability to induce an HR-
6 like defense pathway in Cl-D193Y infection, we performed expression analysis of the SA-responsive
7 chitinase gene (*SA-CHI*; accession number L37876) and the HR-related gene homologous to tobacco
8 *HSR203J* (*HSR203J*; AB026296) in Cl-D193Y/p19-inoculated tissue [1, 4, 17]. At 7 dpi, Cl-D193Y/p19
9 significantly induced *SA-CHI* expression (Fig. 4a). Furthermore, it also induced *HSR203J* expression at 5
10 dpi (Fig. 4b); however, Cl-D193Y/p19m did not significantly induce either *SA-CHI* or *HSR203J* (Fig. 4a
11 and b).

12 Reactive oxygen species, such as hydrogen peroxide (H_2O_2), are generated during an HR [28]. We
13 analyzed H_2O_2 generation using 3'-diaminobenzidine (DAB) staining in the ClYVV infection [27], and
14 found that H_2O_2 was generated along the veins in Cl-WT/GFP-inoculated tissue, but not in mock- and Cl-
15 D193Y-inoculated tissues (Fig. 4c). In Cl-D193Y/p19-inoculated tissue, H_2O_2 generation was extensive,
16 as also observed in the Cl-WT/GFP infection (Fig. 4c).

17

18 **p19 expression also restored virulence of the severely attenuated mutant ClYVV with a mutation in**
19 **the highly conserved FRNK motif**

20 Previous studies have found that HC-Pro can bind small RNAs, including siRNA and miRNA, both
21 *in vitro* and *in vivo* and that the highly conserved FRNK motif in HC-Pro is required for sRNA binding
22 [10, 22]. HC-Pro of *Tobacco etch virus* (TEV), *Potato virus Y*, and TuMV with an R-to-I mutation in the
23 FRNK motif (FINK) was not active in suppressing inverted-repeat, sequence-triggered RNA silencing
24 [22]. To examine the requirement of the FRNK motif in ClYVV HC-Pro for RSS activity, we introduced
25 an R-to-I mutation and analyzed RSS activity. GFP, IR-GFP, and ClYVV P1/HC-Pro (wild type or
26 mutants) were expressed transiently using *Agrobacterium tumefaciens* in *N. benthamiana* leaves. As
27 observed in previous studies, at 4 days after *A. tumefaciens* inoculation, strong GFP fluorescence was
28 observed in wild-type P1/HC-Pro and p19-expressed (positive control) areas, whereas weak GFP
29 fluorescence was observed in P1/HC-Pro-D193Y and GUS-expressed (negative control) areas (Fig. 5b)

1 [33]. GFP fluorescence was also very weak in the P1/HC-Pro-FINK-expressed area (Fig. 5b) [22]. The
2 amount of GFP mRNA correlated with the intensity of GFP fluorescence (Fig. 5c). Use of an antibody
3 against ClYVV HC-Pro for Western blotting confirmed that the amount of HC-Pro protein did not differ
4 significantly among the three samples (Fig. 5d) [32].
5 TEV and TuMV with an R-to-I mutation in HC-Pro lost the ability to infect their hosts [22]. We
6 introduced an R-to-I mutation in ClYVV HC-Pro (Cl-FINK) and tested the infectivity. Broad bean was
7 inoculated by particle bombardment using the infectious cDNA, pCl-FINK carrying GFP (pCl-
8 FINK/GFP) [33]. RT-PCR analysis of the upper non-inoculated leaves revealed the presence of virus
9 RNA, although we did not observe any GFP fluorescence in Cl-FINK/GFP-inoculated plants
10 (Supplementary Table 1). Sequence analysis showed that the progeny virus from upper non-inoculated
11 leaves retained the mutation, indicating that Cl-FINK/GFP had infectivity and was able to spread to
12 distant leaves. Cl-FINK/GFP was also inoculated to pea line PI 118501. However, Cl-FINK/GFP
13 accumulation was very low in upper leaves of broad bean to prepare the sap inoculum. Thus, plasmid
14 DNA (pCl-FINK/GFP) was directly inoculated onto pea leaves by rubbing the leaves with DNA solution.
15 However, we could not detect Cl-FINK/GFP infection in upper leaves by RT-PCR, although Cl-WT/GFP
16 could effectively infect and kill the plants (data not shown). To examine whether p19 expression
17 functionally complemented the activity of HC-Pro with an R-to-I mutation, we inoculated Cl-FINK
18 carrying p19 (Cl-FINK/p19) to PI 118501. The results showed that Cl-FINK/p19 could kill the plants
19 completely by 13 dpi (Supplementary Fig. 5).
20
21 **The suppressor of RNA silencing itself did not function as an elicitor**
22 There remains the possibility that strong suppression of RNA silencing itself activates an HR-like
23 response. Therefore, p19 and 2b were expressed in a different context: in the white clover mosaic virus
24 (WCl) vector (Fig. 6a) [7]. WCl carrying GFP induced mild symptoms in pea lines PI 118501 and PI
25 226564 (Fig. 6b and Supplementary Fig. 6). WCl/p19, p19m, 2b, and 2bm tagged with FLAG were
26 inoculated to PI 118501 and PI 226564. WCl/p19 induced partial cell death in inoculated and upper non-
27 inoculated leaves not only in PI 118501 but also in PI 226564 (Fig. 6b). However, WCl/p19 infection did
28 not kill plants of either PI 118501 or PI 226564 (Supplementary Fig. 6). Western blot analysis indicated
29 that p19 protein abundantly accumulated in upper non-inoculated leaves of PI 118501 and PI 226564 (Fig.

6c). 2b expression slightly enhanced symptom development, such as yellowing, but could not significantly induce cell death in PI 118501 and PI 226564 (Fig. 6b). Western blot analysis indicated that 2b protein effectively accumulated in upper non-inoculated leaves in PI 118501 and PI 226564 (Fig. 6c). WCl/p19m and /2bm could not significantly induce cell death, even though p19m and 2bm proteins effectively accumulated in upper non-inoculated leaves (Fig. 6).

Discussion

Our results showed that expression of either TBSV p19 or CMV 2b functionally complemented the virulence of the attenuated ClYVV HC-Pro mutant, or Cl-D193Y, although virulence was fully restored by p19 expression and partially restored by 2b in PI 118501. Either p19 or 2b expression enhanced Cl-D193Y accumulation; p19 expression also restored virulence of the severely attenuated mutant, Cl-FINK, in PI 118501. Furthermore, p19 expression restored the ability of Cl-D193Y to activate an HR-like defense pathway in PI 118501. Because these results strongly suggest the exclusive effect of the D193Y mutation on RSS activity of HC-Pro, our discussion focuses on the critical role of HC-Pro RSS activity in the multiplication and induction of severe disease symptoms, lethal SHR, by ClYVV.

Contribution of RNA-silencing suppression activity of HC-Pro to lethal systemic HR induction by *Clover yellow vein virus* in pea

Previous research demonstrated that infection with ClYVV activated an HR-like defense pathway in PI 118501 [1]. Furthermore, genetic studies suggested that ClYVV-induced cell death in PI 118501 is controlled by a single, incompletely dominant gene, *Cyn*1, which is a proposed resistance gene [18]. These reports put forward that ClYVV-induced cell death is regulated by an R-gene-mediated pathway. However, viruses were not restricted in the primary infection area and spread systemically, resulting in the induction of SHR (Fig. 1a and bA and K) [1]. Analysis using a series of ClYVV P1/HC-Pro mutants indicated that the virulence of ClYVV was correlated with the RSS activity of P1/HC-Pro [1, 33]. D-to-Y mutation at aa position 193 of HC-Pro significantly attenuated the ClYVV virulence and RSS activity, suggesting that reduced RSS activity of mutant HC-Pro results in virulence attenuation (Fig. 1a, bD and N, Fig. 5b) [1, 33]. However, the mutation simultaneously may affect other HC-Pro functions as HC-Pro serves several roles in the viral life cycle (e.g., replication, cell-to-cell and systemic movement) [24]. In

this study, we examined the effect of the expression of heterologous VSRs on virus accumulation and virulence expression of the ClYVV point mutant with D193Y (Cl-D193Y) in pea PI 118501. We selected p19 encoded by TBSV and 2b encoded by CMV as the VSRs. TBSV (family *Tombusviridae*) and CMV (*Bromoviridae*) are viruses that are distinctly different from ClYVV (*Potyviridae*) and whose nucleotide and amino acid sequences are not related. However, P19, 2b, and HC-Pro all appear to interact with small RNAs to suppress RNA silencing [3]. We found that TBSV p19 expression could functionally complement the HC-Pro-D193Y activity for virus accumulation, induction of SHR, and activation of an HR-like defense pathway in the mutant ClYVV infection in PI 118501 (Figs. 1 and 4). CMV 2b could also partially complement the HC-Pro-D193Y function (Fig. 2). The RSS-defective mutants p19m and 2bm could not complement the HC-Pro-D193Y function (Figs. 1 and 2). A previous report demonstrated that the defect in p19m RSS activity derived from its lower affinity to small RNAs [16]. We also confirmed that RSS activity of p19m was significantly decreased, which was not due to lower protein stability than p19 (Supplementary Figs. 3 and 4a). In contrast, the stability of 2bm was lower than that of 2b (Supplementary Fig. 4b). However, a detailed analysis of the sRNA binding properties showed that the R46C mutation affects small RNA binding activity *in vitro* [5]. Taking these findings together, we conclude that the RSS activity of p19 and 2b, but not the sequence, could functionally complement HC-Pro-D193Y activity and the ability for Cl-D193Y to accumulate efficiently and induce cell death. The D193Y mutation is located near the FRNK motif, which is conserved in potyviruses and is required for small RNA binding (Fig. 5a). This suggests that D193Y affects the contact of small RNA duplexes via the FRNK motif. In fact, a mutation in the FRNK motif reduced RSS activity of ClYVV HC-Pro and ClYVV virulence (Supplementary Table. 1). The virulence was restored by TBSV p19, as was also the case with Cl-D193Y (Figs. 1 and 2, Supplementary Fig. 5). Taken together, our findings strongly suggest that the D193Y mutation exclusively affects RSS activity of HC-Pro and that RSS activity is necessary for ClYVV multiplication and virulence in pea.

Suppression of RNA silencing in the context of viral disease expression

Developmental defects were observed in *A. thaliana* infected with TuMV; Kasschau et al. [9] suggested that such symptoms were the result of a perturbation of an endogenous miRNA-regulated developmental program by the TuMV-encoded VSR, P1/HC-Pro. This occurs where RSS activity of HC-

1 Pro directly affects virus virulence or induction of symptom expression. In our previous studies, we
2 showed that viral virulence (the degree of SHR induction) is correlated with RSS activity of P1/HC-Pro
3 [1, 33], and accumulation of mutant viruses is also correlated with RSS activity of PI/HC-Pro [1, 33]. In
4 this study, p19 expression restored the accumulation of Cl-D193Y and the ability to induce an HR-like
5 defense pathway and lethal SHR (Fig. 3). Furthermore, 2b expression also restored Cl-D193Y
6 accumulation, but the virus accumulation was lower than for Cl-WT, where 2b could not completely
7 complement the HC-Pro-D193Y function (Fig. 3). These results suggest that suppression of host RNA-
8 silencing machinery is necessary for effective virus accumulation and lethal SHR induction. However, the
9 RSS activity of HC-Pro does not appear to directly affect SHR induction.
10

11 **Suppression of RNA silencing and activation of HR-like pathways**

12 From our studies, we hypothesized that ClYVV had an Avr factor whose effective accumulation
13 was supported by the RSS activity of HC-Pro, resulting in lethal SHR induction in pea. However, it is
14 possible that suppression of RNA silencing itself activates an HR-like pathway. To examine this
15 possibility, we expressed the p19 or 2b gene via the viral vector WClMV , which does not cause
16 significant cell death (Fig. 6). p19 expression from WClMV induced cell death in both inoculated and
17 non-inoculated leaves in PI 118501 and PI 226564 (Fig. 6b). Our previous reports suggested that SHR
18 induction was controlled by a single incompletely dominant gene, resistance *Cyn*1, in PI 118501 [18];
19 however, PI 226564 does not have this allele. Thus, it is likely that p19-induced cell death is not
20 controlled by *Cyn*1-regulated pathways, indicating that p19, or strong suppression of RNA silencing itself,
21 does not induce HR-like pathways in PI 118501. The results suggest that strong suppression of RNA
22 silencing is required for ClYVV to accumulate sufficient Avr factors to activate HR-like pathways and
23 induce SHR in pea. Elucidation of the mechanism of lethal SHR induction by ClYVV in pea will require
24 determination of the Avr factors.
25

26 **Acknowledgments**

27 We are grateful to T. Okuno (Kyoto University) for providing the pBIC-P19. We also thank K. Goto
28 (Hokkaido University) for helpful discussions. This study was supported in part by Grants-in-Aid for
29 Scientific Research (S) and Young Scientists (A) from the Ministry of Science, Education, Culture, and

1 Technology of Japan.

2

3 **References**

4 1. Atsumi G, Kagaya U, Kitazawa H, Nakahara K, Uyeda I (2009) Activation of the salicylic acid
5 signaling pathway enhances Clover yellow vein virus virulence in susceptible pea cultivars. Mol
6 Plant Microbe Interact 22:166-175
7 2. Brigneti G, Voinnet O, Li W, Ji L, Ding S, Baulcombe D (1998) Viral pathogenicity
8 determinants are suppressors of transgene silencing in *Nicotiana benthamiana*. EMBO J
9 17:6739-6746
10 3. Burgyan J, Havelda Z (2011) Viral suppressors of RNA silencing. Trends Plant Sci 16:265-272
11 4. Chang M, Horovitz D, Culley D, Hadwiger L (1995) Molecular cloning and characterization of a
12 pea chitinase gene expressed in response to wounding, fungal infection and the elicitor chitosan.
13 Plant Mol Biol 28:105-111
14 5. Goto K, Kobori T, Kosaka Y, Natsuaki T, Masuta C (2007) Characterization of silencing
15 suppressor 2b of cucumber mosaic virus based on examination of its small RNA-binding
16 abilities. Plant Cell Physiol 48:1050-1060
17 6. Hamilton A, Baulcombe D (1999) A species of small antisense RNA in posttranscriptional gene
18 silencing in plants. Science 286:950-952
19 7. Ido Y, Nakahara KS, Uyeda I (2012) *White clover mosaic virus*-induced gene silencing in pea. J
20 Gen Plant Pathol DOI: 10.1007/s10327-012-0360-3
21 8. Kasschau K, Carrington J (1998) A counterdefensive strategy of plant viruses: suppression of
22 posttranscriptional gene silencing. Cell 95:461-470
23 9. Kasschau K, Xie Z, Allen E, Llave C, Chapman E, Krizan K, Carrington J (2003) P1/HC-Pro, a
24 viral suppressor of RNA silencing, interferes with Arabidopsis development and miRNA
25 function. Dev Cell 4:205-217
26 10. Lakatos L, Csorba T, Pantaleo V, Chapman E, Carrington J, Liu Y, Dolja V, Calvino L, López-
27 Moya J, Burgyán J (2006) Small RNA binding is a common strategy to suppress RNA silencing
28 by several viral suppressors. EMBO J 25:2768-2780
29 11. Masuta C, Yamana T, Tacahashi Y, Uyeda I, Sato M, Ueda S, Matsumura T (2000)

1 Development of clover yellow vein virus as an efficient, stable gene-expression system for
2 legume species. Plant J 23:539-546
3 12. Morel J, Godon C, Mourrain P, Béclin C, Boutet S, Feuerbach F, Proux F, Vaucheret H (2002)
4 Fertile hypomorphic ARGONAUTE (ago1) mutants impaired in post-transcriptional gene
5 silencing and virus resistance. Plant Cell 14:629-639
6 13. Morita-Yamamuro C, Tsutsui T, Sato M, Yoshioka H, Tamaoki M, Ogawa D, Matsuura H,
7 Yoshihara T, Ikeda A, Uyeda I, Yamaguchi J (2005) The Arabidopsis gene CAD1 controls
8 programmed cell death in the plant immune system and encodes a protein containing a MACPF
9 domain. Plant Cell Physiol 46:902-912
10 14. Mourrain P, Béclin C, Elmayan T, Feuerbach F, Godon C, Morel J, Jouette D, Lacombe A,
11 Nikic S, Picault N, Rémoué K, Sanial M, Vo T, Vaucheret H (2000) Arabidopsis SGS2 and
12 SGS3 genes are required for posttranscriptional gene silencing and natural virus resistance. Cell
13 101:533-542
14 15. Nakabayashi H, Yamaji Y, Kagiwada S, Ugaki M, Namba S (2002) The complete nucleotide
15 sequence of a Japanese isolate of white clover mosaic virus strain RC. J Gen Plant Pathol
16 68:173-176
17 16. Omarov R, Sparks K, Smith L, Zindovic J, Scholthof H (2006) Biological relevance of a stable
18 biochemical interaction between the tombusvirus-encoded P19 and short interfering RNAs. J
19 Virol 80:3000-3008
20 17. Pontier D, Godiard L, Marco Y, Roby D (1994) hsr203J, a tobacco gene whose activation is
21 rapid, highly localized and specific for incompatible plant/pathogen interactions. Plant J 5:507-
22 521
23 18. Ravelo G, Kagaya U, Inukai T, Sato M, Uyeda I (2007) Genetic analysis of lethal tip necrosis
24 induced by Clover yellow vein virus infection in pea. J Gen Plant Pathol 73:59-65
25 19. Ruiz-Ferrer V, Voinnet O (2009) Roles of plant small RNAs in biotic stress responses. Annu
26 Rev Plant Biol 60:485-510
27 20. Sato M, Masuta C, Uyeda I (2003) Natural resistance to Clover yellow vein virus in beans
28 controlled by a single recessive locus. Mol Plant Microbe Interact 16:994-1002
29 21. Scholthof H, Scholthof K, Jackson A (1996) Plant virus gene vectors for transient expression of

1 foreign proteins in plants. Annu Rev Phytopathol 34:299-323
2 22. Shiboleth Y, Haronsky E, Leibman D, Arazi T, Wassenegger M, Whitham S, Gaba V, Gal-On A
3 (2007) The conserved FRNK box in HC-Pro, a plant viral suppressor of gene silencing, is
4 required for small RNA binding and mediates symptom development. J Virol 81:13135-13148
5 23. Suzuki M, Kuwata S, Kataoka J, Masuta C, Nitta N, Takanami Y (1991) Functional analysis of
6 deletion mutants of cucumber mosaic virus RNA3 using an in vitro transcription system.
7 Virology 183:106-113
8 24. Syller J (2005) The roles and mechanisms of helper component proteins encoded by potyviruses
9 and caulimoviruses. Physiol Mol Plant P 67:119-130
10 25. Takahashi Y, Takahashi T, Uyeda I (1997) A cDNA clone to clover yellow vein potyvirus
11 genome is highly infectious. Virus Genes 14:235-243
12 26. Takeda A, Sugiyama K, Nagano H, Mori M, Kaido M, Mise K, Tsuda S, Okuno T (2002)
13 Identification of a novel RNA silencing suppressor, NSs protein of Tomato spotted wilt virus.
14 FEBS Lett 532:75-79
15 27. Torres M, Dangl J, Jones J (2002) Arabidopsis gp91phox homologues AtrbohD and AtrbohF are
16 required for accumulation of reactive oxygen intermediates in the plant defense response. Proc
17 Natl Acad Sci U S A 99:517-522
18 28. Torres MA (2010) ROS in biotic interactions. Physiol Plant 138:414-429
19 29. Voinnet O, Pinto Y, Baulcombe D (1999) Suppression of gene silencing: a general strategy used
20 by diverse DNA and RNA viruses of plants. Proc Natl Acad Sci U S A 96:14147-14152
21 30. Wang Z, Ueda S, Uyeda I, Yagihashi H, Sekiguchi H, Tacahashi Y, Sato M, Ohya K, Sugimoto
22 C, Matsumura T (2003) Positional effect of gene insertion on genetic stability of a clover yellow
23 vein virus-based expression vector. J Gen Plant Pathol 69:327-334
24 31. Xie Z, Johansen L, Gustafson A, Kasschau K, Lellis A, Zilberman D, Jacobsen S, Carrington J
25 (2004) Genetic and functional diversification of small RNA pathways in plants. PLoS Biol
26 2:E104
27 32. Yambao M, Masuta C, Nakahara K, Uyeda I (2003) The central and C-terminal domains of VPg
28 of Clover yellow vein virus are important for VPg-HCPro and VPg-VPg interactions. J Gen
29 Virol 84:2861-2869

33. Yambao M, Yagihashi H, Sekiguchi H, Sekiguchi T, Sasaki T, Sato M, Atsumi G, Tacahashi Y, Nakahara K, Uyeda I (2008) Point mutations in helper component protease of clover yellow vein virus are associated with the attenuation of RNA-silencing suppression activity and symptom expression in broad bean. Arch Virol 153:105-115

Figure captions

Fig. 1 Expression of the heterologous suppressor of RNA silencing p19 encoded by TBSV via the Cl-D193Y genome in PI 118501. (a) Viruses were mechanically inoculated, and photographs were taken at 13 dpi. (b) The photographs show symptoms in inoculated (A–E) and upper leaves (K–O) at 13 dpi. Inoculated leaves were stained with trypan blue to visualize dead cells (F–J). Scale bar = 0.5 cm. dpi = days post-inoculation.

Fig. 2 Expression of the heterologous suppressor of RNA silencing 2b encoded by CMV via the Cl-D193Y genome in PI 118501. Viruses were mechanically inoculated, and photographs were taken at 8 dpi (inoculated leaves, A–E) and 13 dpi (upper non-inoculated leaves, F–T; whole plants, U). Photographs in F–J, K–O, and P–T show the second, third, and fourth leaves of inoculated plants, respectively. Scale bar = 0.5 cm. dpi = days post-inoculation.

Fig. 3 ClYVV accumulation in upper non-inoculated leaves. (a) Virus CP accumulation at 8 dpi in upper non-inoculated leaves was compared by Western blotting using an antibody against ClYVV CP. (b) Virus RNA accumulation at 8 dpi in upper non-inoculated leaves was compared by real-time PCR. Right panel is a close-up of data (Cl-D193Y/p19m, 2bm, GFP) in the left panel. Relative levels of virus RNA accumulation to Cl-WT/GFP are indicated. dpi = days post-inoculation.

Fig. 4 Hypersensitive reaction-like defense response in PI 118501 inoculated with Cl-D193Y carrying TBSV p19. (a) *SA-CHI* expression at 7 dpi. Total RNA was isolated from the inoculated leaves and used for real-time PCR. (b) *HSR203J* expression at 5 dpi. All mRNA expression (a and b) was normalized to *18S* rRNA. Error bars indicate the standard deviation of means for three replicates. Dunnett's test was applied to the data. Data from the mock control were used as a control for statistical analysis. ** $P < 0.01$

1 (c) Detection of peroxidase activity. Leaves were stained with 3,3'-diaminobenzidine (DAB) at 8 dpi. As
2 a positive control, pea leaves were infiltrated with H_2O_2 solution using needleless syringes and stained
3 with DAB after incubation at room temperature for 10 min. The black arrow indicates the infiltrated area.
4 Scale bar = 0.5 cm. dpi = days post-inoculation.

5

6 **Fig. 5** Effect of mutation in the highly conserved FRNK motif on RNA-silencing suppression activity of
7 ClYVV HC-Pro. (a) Site of mutation in ClYVV HC-Pro mutants. (b) GFP, an inverted repeat sequence of
8 GFP (IR-GFP), and ClYVV P1/HC-Pro (wild type or mutant) were expressed using an *Agrobacterium*-
9 mediated transient assay in an *N. benthamiana* leaf. GFP fluorescence was detected 4 days after
10 infiltration. (c) Total RNA was extracted from the infiltrated area indicated in (b) and GFP mRNA was
11 detected by Northern hybridization. (d) Total proteins were extracted from the infiltrated area indicated in
12 (b) and analysed by Western blotting using antibodies against HC-Pro.

13

14 **Fig. 6** Expression of either TBSV p19 or CMV 2b using the white clover mosaic virus (pWCl) vector. (a)
15 Schematic representation of the pWCl vector [7]. A multi-cloning site (gray box) was introduced between
16 the triple gene block and CP. (b) pWCl carrying p19, p19m, 2b, 2bm (all tagged with FLAG), or GFP
17 plasmids was rub-inoculated onto leaves of pea lines, PI 118501 and PI 226564. Photographs in upper
18 non-inoculated leaves were taken at 14 dpi. (c) p19, p19m, 2b, and 2bm protein expression was confirmed
19 by Western blotting using an antibody against FLAG.

20

21 **Supplementary Fig. 1** Schematic representation of the ClYVV infectious cDNA used in this study. Full-
22 length cDNA of ClYVV No. 30 isolate was fused to the *Cauliflower mosaic virus* 35S promoter [11, 25,
23 30]. Cl-D193Y encodes a point mutation, aspartic acid to tyrosine, at aa position 193 in HC-Pro [33].
24 Either the TBSV p19 or the CMV 2b coding sequence was introduced into the Cl-D193Y genome [16, 23,
25 26]. p19m has point mutations at the aa positions 75 (arginine to glycine) and 78 (arginine to glycine)
26 [16]; 2bm has an arginine-to-cysteine mutation at aa position 46 [5].

27

28 **Supplementary Fig. 2** ClYVV accumulation in upper non-inoculated leaves. Virus CP accumulation at 8
29 dpi was compared using DAS-ELISA. The signal value of healthy plants was zero.

1

2 **Supplementary Fig. 3** Comparison of RNA silencing suppression activity of FLAG-tagged TBSV p19
3 and CMV 2b (wild type or mutant). (a) GFP, an inverted repeat sequence of GFP (IR-GFP), and
4 suppressor of RNA silencing (p19, p19m, 2b, or 2bm tagged with FLAG) were expressed using an
5 *Agrobacterium*-mediated transient assay in *N. benthamiana* leaves. GFP fluorescence was detected at 4
6 days after infiltration. (b) Total RNA was extracted from the infiltrated area indicated in (a), and mRNA
7 levels of GFP were compared by Northern hybridization. (c) Total proteins were extracted from the
8 infiltrated area indicated in (a), and levels of suppressor proteins were compared by Western blotting
9 using an antibody against FLAG. (d) Comparison of RNA-silencing suppression activity between wild-
10 type and mutant p19 proteins. The amount of *Agrobacterium* carrying p19 was diluted to 1/4 or 1/8 of the
11 amount of *Agrobacterium* carrying p19m. GFP fluorescence was detected at 4 days after infiltration. (e)
12 Total RNA was extracted from the infiltrated area indicated in (d), and mRNA levels of GFP were
13 compared by Northern hybridization. (f) Total proteins were extracted from the infiltrated area indicated
14 in (d), and levels of suppressor proteins were compared by Western blotting using an antibody against
15 FLAG.

16

17 **Supplementary Fig. 4** Protein stabilities of P19 and 2b mutants. (a) Mutant p19 protein was expressed
18 with wild-type p19 or GUS (negative control) using an *Agrobacterium*-mediated transient assay in *N.*
19 *benthamiana* leaves. Total proteins were extracted from the infiltrated area, and the level of p19 mutant
20 protein was compared with that of wild-type p19 by Western blotting using an antibody against FLAG.
21 (b) Mutant 2b protein was expressed with p19, ClYVV P1/HC-Pro, or GUS (negative control), and the
22 level of mutant 2b protein was compared with that of wild-type 2b as in (a). (c) p19 wild-type or mutant
23 protein was expressed via Cl-WT, and the level of proteins compared by Western blotting using an
24 antibody against FLAG. The level of ClYVV CP protein accumulation was used as a control. ClYVV CP
25 was detected by Western blotting using an antibody against ClYVV CP. (d) The level of 2b wild-type or
26 mutant protein was compared as in (c).

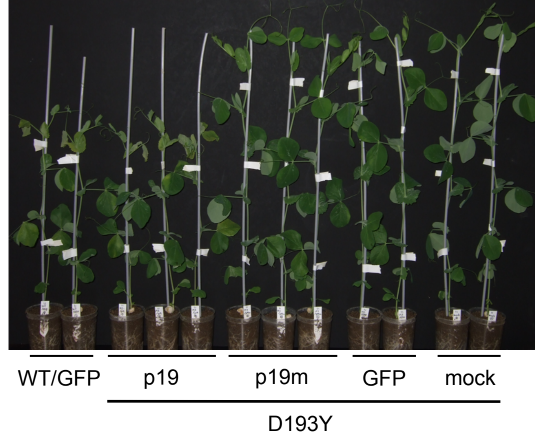
27

Supplementary Fig. 5 Effect of TBSV p19 expression from ClYVV, which has an R-to-I mutation in the HC-Pro FRNK motif, on symptom expression in PI 118501. Symptoms were monitored, and photographs were taken at 11 dpi.

Supplementary Fig. 6 Expression of either TBSV p19 or CMV 2b using the white clover mosaic virus vector. Photographs in upper non-inoculated leaves were taken at 14 dpi.

Fig. 1

(a)



(b)

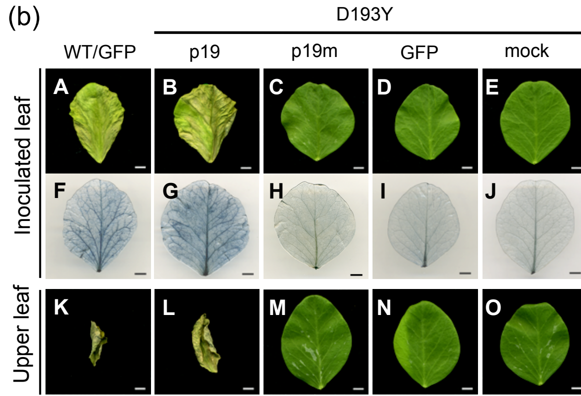


Fig. 2

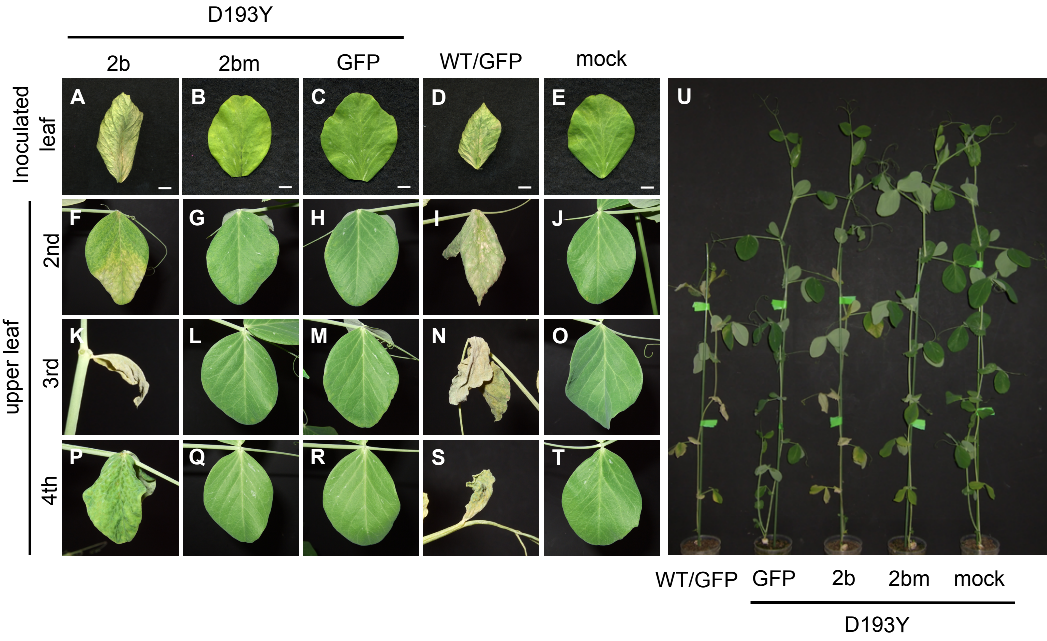
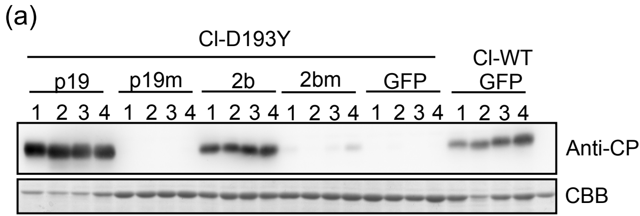


Fig. 3

(a)



(b)

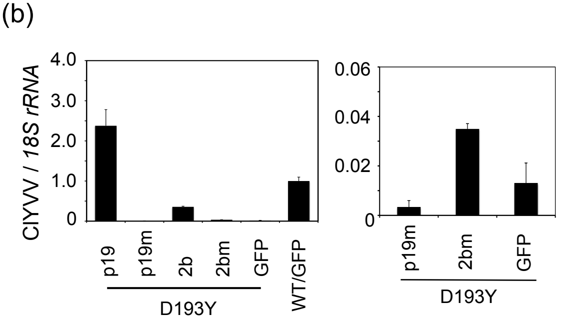


Fig. 4

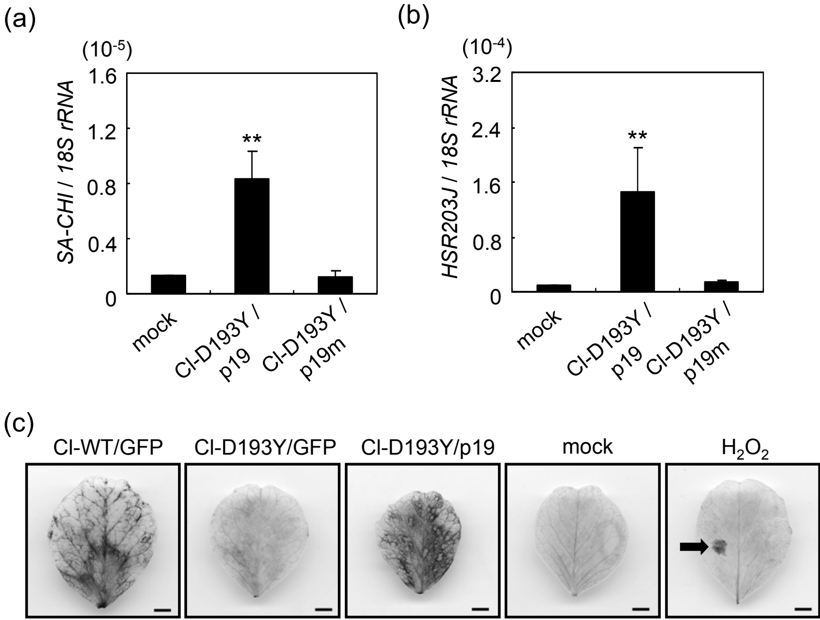


Fig. 5

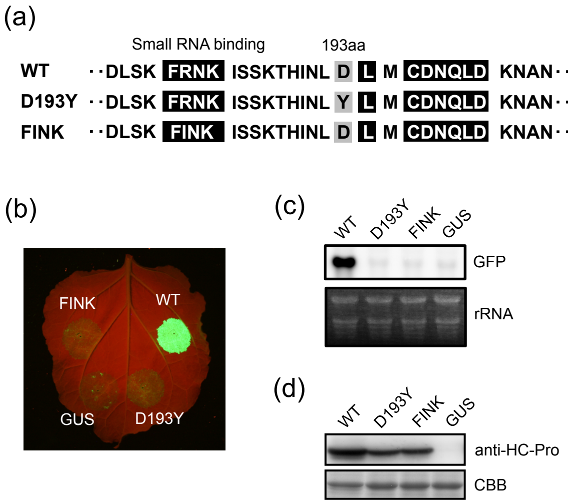


Fig.6

(a)

35S
replicase
TGB1
TGB3
TGB2
MCS
CP

(b)

p19 -FLAG
p19m -FLAG
2b -FLAG
2bm -FLAG
GFP

PI 118501
PI 226564

(c)

PI 118501
PI226564

p19-FLAG
p19m-FLAG
healthy
2b-FLAG
2bm-FLAG
healthy
p19-FLAG
p19m-FLAG
healthy
2b-FLAG
2bm-FLAG
healthy

anti-FLAG
CBB

Fig. S1

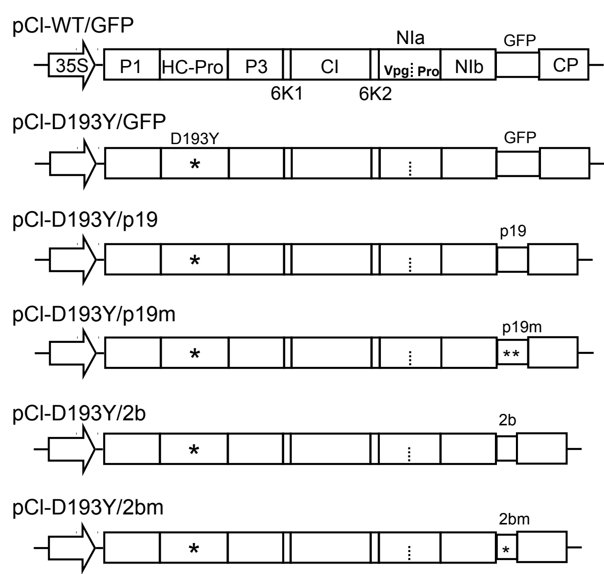


Fig. S2

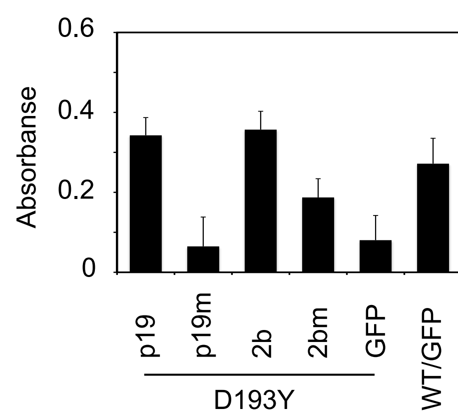


Fig. T1

Supplementary Table 1 Effect of R-to-I mutation in FRNK motif of HC-Pro on the ability for ClYVV to infect broad bean.

	pCl-FINK/GFP	pCl-WT/GFP
Exp.1[a]	2/3[b, c]	2/2
Exp.2	2/4	2/2

[a] Infectious cDNA, pCl-FINK/GFP and pCl-WT/GFP were inoculated to broad bean by particle bombardment

[b] Infected / total plants

[c] Infection in upper uninoculated leaf was confirmed by RT-PCR

Fig. S3

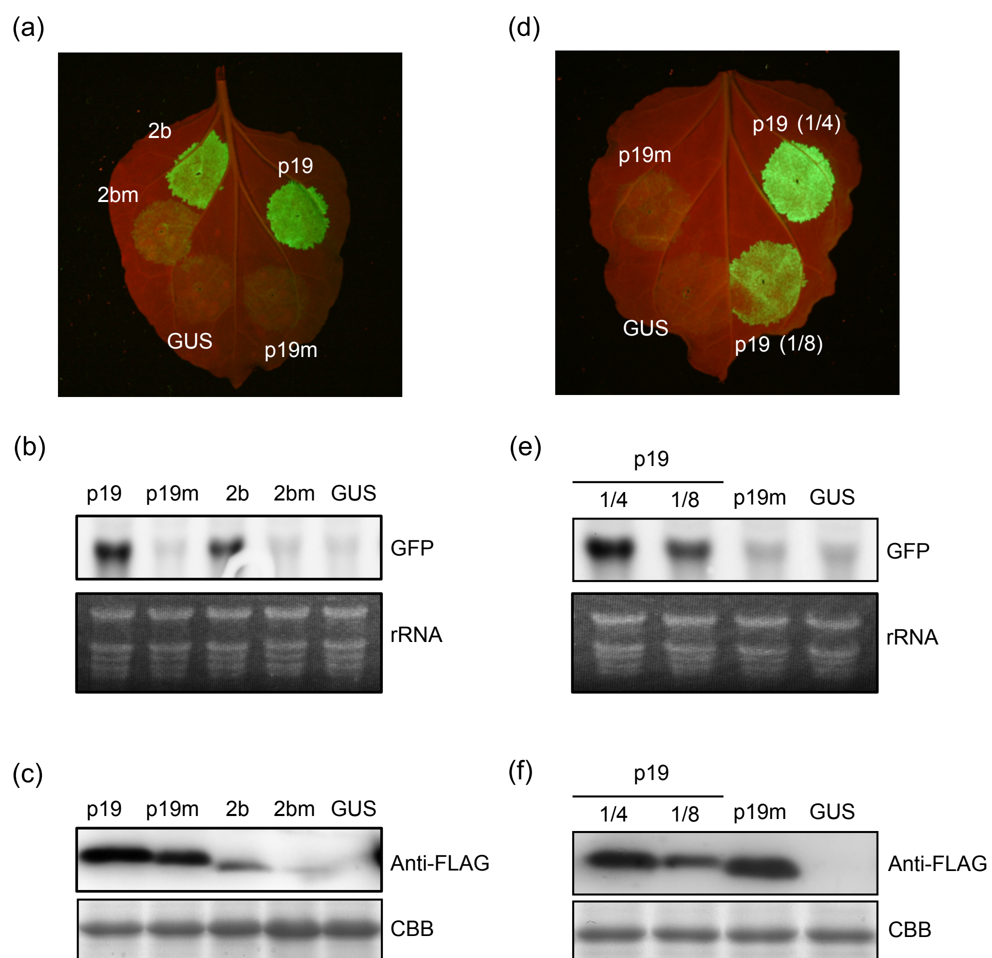


Fig. S4

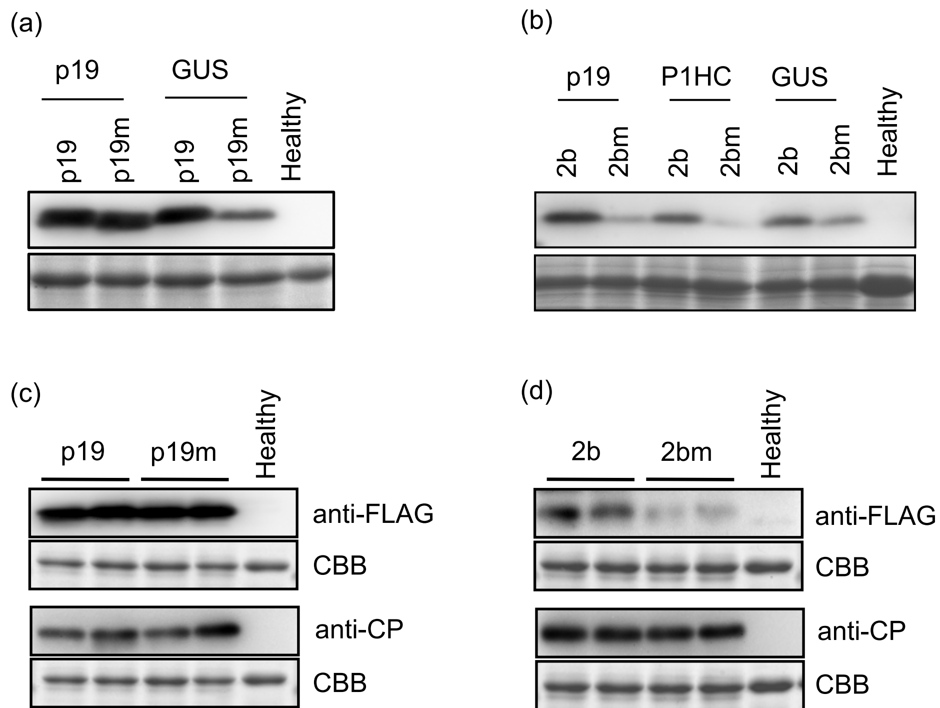


Fig. S5

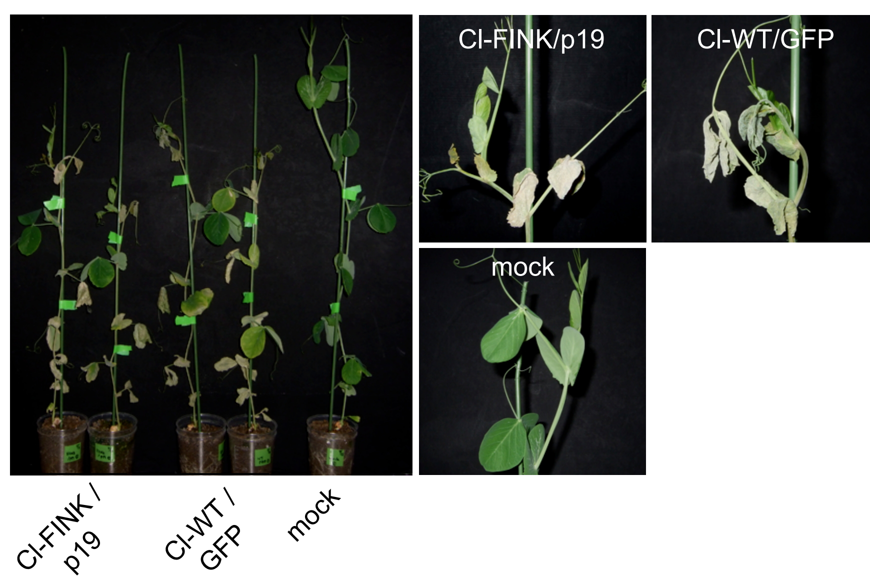


Fig. S6