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

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Summary. Helper component protease (HC-Pro) is a potyvirus-encoded multifunctional protein and a major determinant of symptom expression in a susceptible plant. Here, we show the involvement of clover yellow vein virus (ClYVV) HC-Pro in necrotic symptom expression in broad bean (*Vicia faba* cv. Wase). ClYVV no. 30 induces lethal necrosis. Its spontaneous mutant (MM) that did not lead to lethal necrosis was isolated. Mapping with chimeric viruses between ClYVV no. 30 and MM attributed the symptom attenuation to two mutations from ClYVV no. 30 to MM at positions 27 (threonine to isoleucine) and 193 (aspartic acid to tyrosine) in HC-Pro. Although neither mutant with the single amino acid substitution at position 27 or 193 (ClYVV/T27I or D193Y) successfully induced the lethal necrosis, ClYVV/T27I still retained the ability to induce necrotic symptoms, but ClYVV/D193Y scarcely did. The virus accumulation of ClYVV/D193Y was also less than that of ClYVV no. 30. The mutations are located in a putative zinc finger domain and N-terminal side one of two RNA binding domains, respectively. RNA-silencing suppression (RSS) activity of P1/HC-Pro was weakened by both mutations in *Nicotiana benthamiana*. Our results suggest a correlation between viral virulence and RSS function and the importance of the two domains in HC-Pro.

Introduction

Clover yellow vein virus (ClYVV) is a species of the genus *Potyvirus* within the family *Potyviridae*. Several proteins [2, 3, 5-7, 10, 12, 19, 22, 23, 36], as well as noncoding regions [20], from different members of the genus *Potyvirus* modulate disease symptoms in various plant species. Within a species, more than one gene also appears to condition symptom development when introduced to different hosts [22, 23].

A major symptom determinant in potyvirus infections is the helper component protease (HC-Pro). Point mutations in the HC-Pro of potyviruses alter symptom expression in different hosts. In the tobacco vein-mottling virus (TVMV) system, the substitution of Lys (K) in the KITC domain dramatically attenuated the symptoms in *Nicotiana tabacum* [2, 3]. Klein et al. [10] also found that a mutation in TVMV HC-Pro caused a cyclic symptom pattern from sporadic to severe symptoms on the same host. In another case, HC-Pro was found to cause disease symptoms in zucchini yellow mosaic virus (ZYMV) [6], plum pox virus [23], and Potato virus Y (PVY) [29]. HC-Pro is a multifunctional protein capable of self-interaction, RNA binding, including of short interfering RNA (siRNA), RNA-silencing suppression (RSS), interaction with the 20S proteasome and proteolysis; it also plays a role in various steps of the potyvirus infection cycle [4, 13, 14, 16, 33]. Recently, Kasschau et al. [9] showed that turnip mosaic virus-induced disease was partly explained by HC-Pro RSS activity. However, the manner in which some of the

functions of HC-Pro contribute to symptom expression still remains to be examined.

Several isolates of ClYVV have been differentiated based on host reactions, serological properties, and nucleotide sequences of the coat protein (CP) [24]. These isolates are grouped into two strains based on host reactions to *Chenopodium amaranticolor*, *C. quinoa*, *Nicotiana clevelandii*, *N. benthamiana*, broad bean (*Vicia faba*), and *Trifolium repens* that correspond to two serotypes as determined using enzyme-linked immunosorbent assay (ELISA) and nucleotide sequences of the CP gene [24]. However, lethal necrosis in broad bean [24] is caused by inoculation with either strain.

We isolated a spontaneous ClYVVmutant MM, which is originally isolated from systemically infected leaves of French bean having inoculation for the virus derived from the infectious clone of ClYVV strain no. 30 (ClYVV no. 30). MM causes necrotic symptoms on inoculated and systemic leaves of broad bean without lethal necrosis. Newly emerging leaves develop mosaic symptoms and the plant survives the viral infection. We then characterized chimeric viruses between the RNA genomes of ClYVV no. 30 and its mutant using a reverse genetic system with a full-length cDNA clone of ClYVV no. 30. Two amino acid mutations in the HC-Pro gene dramatically attenuated the virulence of the mutant in broad bean. This report is the first step in understanding the role of HC-Pro as a symptom determinant of ClYVV.

Materials and Methods

Virus sources and plants

Wild-type ClYVV no. 30 was maintained as pClYVV [27]. A mutant that induces mosaic symptoms in broad bean (*V. faba* cv. Wase) was isolated during screening tests for differential reactions on bean varieties by the wild-type virus (Y. Tacahashi, unpublished data). The virus was recovered from the plasmid by inoculation to broad bean. Infected leaves were used to inoculate test plants. A spontaneous mutant was originally isolated from the systemically infected leaves of the French bean (*Phaseolus vulgaris* cv. Hakuti-Burma). Subsequent transfers of the viral culture exhibited mild symptoms on broad bean, typically developing mosaic symptoms under greenhouse conditions. The culture was designated as a mosaic mutant, MM.

Cloning and sequencing the MM genome

Total RNA was isolated from broad bean leaves infected with MM using TRIZOL reagent (Invitrogen Carlsbad, CA, USA). The MM RNA was amplified using an RNA-PCR kit (TaKaRa Otsu, Shiga, Japan), with specific primers, and was directly cloned to plasmid vectors. The MM genome was divided into three major fragments. *Cla* I–*Nhe* I (CN) was amplified using primers 652F (5'ATGGCACAAATCATGATTGG 3') and 657R (5' CATTTGATTTGGATTCCATGACAAACCACTTTG 3') and inserted into pHSG 396

(TaKaRa); *Nhe* I–*Sac* I (NS) was amplified using 677F (5'AGACGAGCTAGGTGCC 3') and 449R (5' GTGATGTAGGATCAGTTTGTAGAGT 3') and inserted into pGEM-Teasy (Promega Madison, WI, USA); *Sac* I–*Sac* I (SS) was amplified using 448F (5' GTTGTTTTAAACAAAATGGCTATAC 3') and 297R (5' CCTTTTTTTTTTCTCGCTCTATAAAGATCAG 3') and inserted into pGEM3zf(+) (Promega). Each of the ClYVV-MM fragments was directly sequenced from the original vector of insertion or the internal fragments were PCR-amplified and recloned to pGEM-Teasy. The cloned inserts were sequenced using a model 373 DNA sequencer (Applied Biosystems).

cDNA modifications and plasmid construction

A chimeric viral construct pClYVV/CN (Fig. 1) was prepared using the cloned fragments of the MM genome. Segments of MM were exchanged at the unique restriction sites in pClYVV-Pst/CP lacking the *Pst* I site in the CP gene without any change of the amino acid sequence [15]. The other two major chimeric constructs (pClYVV/NS and pClYVV/SS; Fig. 1) were prepared in the same manner as pClYVV/CN. Four additional chimeric viruses (Fig. 2B) were created from pClYVV/CN by exchanging MM genome segments at the unique restriction sites *Cla* I (C), *Nde* I (Nd), *Bgl* I (B), *Stu* I (S), and *Nhe* I (N) in the CN region.

Green fluorescent protein (GFP)-tagged ClYVV (ClYVV-GFP) was prepared as follows. The GFP gene was inserted into pClYVV-Pst/CP between the NIb and CP genes to create pClYVV/deltaCla/CP-GFP following a previously described procedure [37].

Point mutations in the P1 and HC-Pro genes were created using a QuikChange Site-Directed Mutagenesis kit (Stratagene La Jolla, CA, USA) or PCR using primer extension mutagenesis. After successful substitution of the specific amino acid(s), the CN clone with point mutations at amino acid position 182 in P1 (pClYVV/P1E182G-GFP) and amino acid positions 27 (pClYVV/T27I-GFP), 33 (pClYVV/I33V-GFP), 51 (pClYVV/R51I-GFP), and 193 (pClYVV/D193Y-GFP) in HC-Pro was exchanged with that of pClYVV/deltaCla/CP-GFP. A double mutant with both point mutations at positions 27 and 193 (pClYVV/T27I-D193Y) and revertants of pClYVV/T27I-GFP and pClYVV/D193Y-GFP were also created as described above.

To examine the RSS activity of ClYVV P1/HC-Pro, the GUS gene in the binary vector pIG121 was replaced with GFP or an inverted repeat of the full-length GFP open reading frame sandwiched between sense and antisense GFP sequences to create pIG121-GFP and pIG121-IR-GFP. The GUS gene in binary vector pBE2113 was replaced with P1/HC-Pro with/without mutation(s) and HC-Pro to make pBE2113-P1/HC-Pro and pBE2113-HC-Pro, respectively.

Plant inoculation and symptom observations

Inoculation of chimeric clones was done as described previously [26]. The virus was recovered from the plasmid by inoculation to broad bean. Infected leaves were used to inoculate test plants. All broad beans inoculated with the wild, chimeric, or point-mutation clones were maintained in a growth chamber at 23°C with 16 h of light and 8 h of dark.

Enzyme-linked immunosorbent assay (ELISA)

Double antibody sandwich (DAS)-ELISA tests with polyclonal antisera raised against ClYVV isolate no. 30 were used. Crude extracts of five to six inoculated leaves of broad bean were homogenized at 1/5 (wt/vol) in 0.01 M phosphate-buffered saline (PBS) containing 0.05% Tween (PBS-T). A negative control consisting of the extract from noninfected broad bean was added to each plate. The substrate was incubated for 50 min at room temperature and the absorbance of the mixture was measured at 405 nm.

Analysis of RNA-silencing suppression by agroinfiltration

To detect the silencing suppressor activity of ClYVV no. 30 and the mutant P1/HC-Pro, we used the *Agrobacterium tumefaciens* infiltration method [34]. Transgenic *Nicotiana benthamiana* line 16c expressing GFP [21] were infiltrated with an overnight culture of *A.*

tumefaciens KYRT-1 (OD600 = 1) carrying pBE2113-GFP. For coinfiltration, equal volumes of *A. tumefaciens* culture (OD600 = 1) carrying pBE2113-GFP and a culture carrying pBE2113-P1/HC-Pro, pBIC-P19 [28], of which P19 is a tomato busy stunt virus (TBSV) RNA-silencing suppressor, or pBE2113 to express the GUS gene as a control were mixed as described by Silhavy et al. [25]. GFP fluorescence was detected visually using the Illumatool Tunable Lighting System LT-9500 with the excitation light at 470nm, emission long pass filter at 515nm. Plants were photographed using the Canon EOS D30 digital camera. GFP mRNA was detected by northern blotting with a ^{32}P-labeled cDNA probe basically according to manufacture's manual with nylon membrane (Hybond N; GE Healthcare Buckinghamshire, England).

RESULTS

Nucleotide sequencing of the spontaneous ClYVV mutant MM

To facilitate the identification of nucleotide and amino acid changes in the spontaneous ClYVV mutant MM, we cloned and sequenced the genome of MM from the *Cla* I (P1) to the *Sac* I restriction enzyme site (CP; Table 1, Fig. 1B). MM has a very high sequence similarity to wild-type ClYVV of 99% at the nucleotide and amino acid levels. A total of 22 nucleotide substitutions were found in all genes except the 6k1 and NIa-Pro genes. Of these nucleotide substitutions, 15 were responsible for 15 amino acid changes. The remaining seven nucleotide substitutions were found in HC-Pro (two substitutions), P3

(two), 6k2 (one), NIa-VPg (one), and NIb (one). These nucleotide substitutions did not alter the amino acids sequences produced from the genes.

Since mutations in the *Cla* I – *Nhe* I fragment (Fig. 1) should have crucial role in symptom attenuation of MM as mentioned in the next paragraph, we independently cloned and sequenced MM genomic fragments in this region several times. Amino acid mutations, isoleucine (I) to valine (V) at position 33 of HC-Pro (I33V) occurred 4 times from 4 independent cloning and sequencing, aspartic acid (D) to tyrosine (Y) at position 193 of HC-Pro (D193Y) occurred 3 times from 3 independent cloning and sequencing and arginine (R) to I at position 51 of HC-Pro (R51I) occurred twice from 4 independent cloning and sequencing while the other five mutations in this fragment were only found to occur once, suggesting heterogeneity of MM culture.

Mapping of viral genes responsible for symptom attenuation

To narrow down the region responsible for the attenuation of viral symptoms, three chimeric clones, pClYVV/CN, pClYVV/NS, and pClYVV/SS, were generated and tested on broad bean (Fig. 1C). Plants inoculated with chimeric virus ClYVV/CN derived from pClYVV/CN, which has the *Cla* I–*Nhe* I fragment from the MM strain, showed very mild chlorotic symptoms 2 weeks post inoculation (Fig. 2B). Symptoms were observed on both inoculated and systemically infected leaves. In contrast, necrosis symptoms typical of the wild type (Fig. 2A) were observed on plants inoculated with viruses derived from

pClYVV/NS and pClYVV/SS. This suggest the involvement of the P1/HC-Pro/P3 domain in symptom attenuation. We prepared four additional chimeric viruses, ClYVV/CN-(NdB+SN), ClYVV/CN-NdB, ClYVV/CN-NdS, and ClYVV/CB (Fig. 2B). The observation of symptoms in broad bean infected with these chimeras revealed that two independent parts in the P1/HC-Pro/P3 region of the MM strain (Fig. 2B, α and β) were involved in symptom attenuation. ClYVV/CB, which contains the α part of the MM genomic cDNA, induced symptoms essentially similar to those of the MM strain. ClYVV/CN-NdB and ClYVV/CN-NdS, which contain the β part of the MM genomic cDNA, induced symptoms similar to those induced by ClYVV/CN, whereas ClYVV/CN-(NdB+SN), which contains neither the α nor the β part of MM genomic cDNA, killed the infected plant. The symptom similarity among ClYVV/CN, ClYVV/CN-Nds, and ClYVV/CN-NdB indicates that the effect of the β part on symptom expression was dominant over that of the α part.

Identification of mutations responsible for symptom attenuation

Compared with ClYVV no. 30, one and four amino acid substitutions occurred in the α and β parts of the MM genome, respectively. To verify which mutations were responsible for the attenuation of viral symptoms, six different point mutants with a single amino acid substitution either in or out of these parts were created, and their infectivity and symptom severity were tested in broad bean (Fig. 3). Because point mutants with either the

substitution of threonine (T) to isoleucine (I) from ClYVV no. 30 to MM at position 27 (ClYVV/T27I-GFP) from ClYVV no. 30 to MM or D193Y (ClYVV/D193Y-GFP) developed attenuated symptoms similar to those developed upon infection by chimeric viruses possessing the α and β parts of the MM genome, these chimeric viruses appear to be represented by ClYVV/T27I-GFP and ClYVV/D193Y-GFP, respectively. Moreover, a double mutant with both substitutions (ClYVV/T27I-D193Y-GFP) developed weaker symptoms similar to those developed upon infection by a chimeric virus possessing both α and β parts of MM genome (ClYVV/CN, Fig. 2). As predicted regarding substitutions outside of the α and β parts, point mutants with amino acid glutamic acid (E) to glycine (G) at position 182 of P1 (ClYVV/P1E182G, data not shown) or I33V (ClYVV/I33V, Fig. 3) led to lethal necrosis, as did ClYVV no. 30. However, another point mutant with a substitution of R51I failed to induce infection in its nine independent clones.

To ensure that accidental mutations, which might also affect viral pathogenicity, were not introduced when creating the point mutants, revertants were recreated from constructs of the point mutants ClYVV/T27I-GFP and D193Y-GFP, and their virulence was examined. Both revertants recovered comparable virulence to that of ClYVV no. 30, seeing that they induced lethal necrosis in broad bean. Thus, the attenuation of symptoms was attributed to these point mutations.

Characterization of point mutants

We next performed a detailed examination of the pathogenicity of the point mutants in broad bean. The cell-to-cell spread of ClYVV no. 30 and the point mutants was first monitored using viruses tagged with GFP. No significant differences occurred among the viruses in their cell-to-cell spread in inoculated leaves (Fig. 3B). However, the GFP signal intensity differed among the viruses. ClYVV/D193Y-GFP developed significantly less GFP signal throughout the observation than did ClYVV no. 30, implying less accumulation of the mutant viruses in infected cells. ELISA with crude sap from GFP foci in the leaves confirmed that the accumulation of ClYVV/D193Y-GFP virus in inoculated leaves was lower than that of ClYVV-GFP, but continued to increase with time (Fig. 4A).

ClYVV/T27I-GFP developed a comparable GFP signal to that of ClYVV/GFP until 5 days after inoculation (dpi), and had a lower GFP signal at 7 dpi. We could not observe the GFP signal on the following days because of severe necrotic reactions in ClYVV/T27I-GFP-inoculated leaves, similar to those in ClYVV/GFP-inoculated leaves (Fig. 3B). This was consistent with the symptom index (Figs. 2B and 3A), demonstrating that whereas ClYVV/T27I retained the ability to produce necrotic symptoms, ClYVV/D193Y scarcely did. Nevertheless, the time-course observations of necrotic symptom expression with ClYVV/T27I-GFP and ClYVV/CB-GFP infection in inoculated and upper leaves revealed that these mutants possess a slight defect in their

ability to induce necrotic symptoms compared to ClYVV-GFP (Fig. 4B and C).

RNA silencing suppression activity of P1/HC-Pro with point mutations

Tomato mosaic virus (ToMV) tagged with GFP forms a halo in the inoculated leaf when it contains a mutation weakening its RSS activity [11], similar to the GFP halo formed with ClYVV/D193Y-GFP (Fig. 3B). Thus, we examined whether the mutations in P1/HC-Pro affected the RSS activity. GFP, inverted repeats of the GFP mRNA sequence, and ClYVV P1/HC-Pro with and without the point mutations were transiently expressed so as to trigger RNA silencing against GFP in transgenic *Nicotiana benthamiana* expressing GFP (16c). The intensity of the GFP fluorescence demonstrated that P1/HC-Pro of the wild-type ClYVV strain no. 30 (WT) possessed RSS activity as did P19, and mutants, T27I, D193Y, and CB, exhibited less RSS activity than did WT (Fig. 5A). Notably, the less RSS activity possessed by P1/HC-Pro, the milder the necrotic symptoms produced by the virus. The P1/HC-Pro were ranked in order of RSS activity (Fig. 5A and B) as follows: WT ≈ P1E182G > T27I ≥ CB > D193Y. The viruses were ranked in order of virulence (Figs. 2B, 3A, 4A and B) as follows: ClYVV ≈ ClYVV/P1E182G > ClYVV/T27I ≥ ClYVV/CB > ClYVV/D193Y. GFP mRNA accumulation was essentially consistent with GFP fluorescence (Fig. 5B). Comparable HC-Pro protein expressed from each of the P1/HC-Pro constructs was accumulated. A band of the expected size for HC-Pro, but not for fused P1/HC-Pro, was detected, even in samples from the infiltrated

zone expressing P1/HC-Pro with the mutation in P1 (P1E182G and CB), indicating that the mutation in the P1 protein did not affect the *cis*-protease activity of the P1 protein. Thus, the defect in RSS activity was not the result of a lack of protease activity (Fig. 5C). We carried out the same experiment without the inverted repeat of GFP mRNA. The RSS activity against sense RNA inducing silencing was similar to the above results in terms of GFP signal intensity.

Discussion

To characterize a spontaneous C1YVV mutant (MM) that does not induce lethal necrosis on broad bean, we developed chimeric viruses containing various regions of the mutant genome and assayed their effect on viral symptom expression in the same host (Fig. 1). Because only one of the three major chimeric viruses created, i.e., ClYVV/CN, exhibited attenuated symptoms, the attenuation was attributed to the P1/HC-Pro/P3 region of the MM genome, which was the component in the relevant chimera. Oddly, the mild chlorotic symptoms developed in response to ClYVV/CN were not identical to those associated with MM, which produced necrotic spots on inoculated leaves and systemic mosaic. The following may explain the difference in symptoms between ClYVV/CN and MM. Two independent mutations at positions 27 and 193 in HC-Pro (T27I and D193Y, respectively; Fig. 1, Table 1) were chiefly responsible for the attenuation of viral symptoms (Figs. 2 and 3). Although both point mutants ClYVV/T27I and

ClYVV/D193Y did not induce lethal necrosis in broad bean, ClYVV/T27I produced necrotic symptoms in inoculated and upper leaves, similar to those produced by MM. In contrast, ClYVV/D193Y only induced mild chlorotic symptoms and a double mutant ClYVV/T27I-D193Y induced weaker symptoms, which is similar to those induced by ClYVV/CN. In addition, cDNA clones from the MM genome did not always contain all the mutations. These results suggest that MM is heterogenous and that the population is composed of mutant viruses both with and without D193Y. If true, the mutants lacking D193Y should be dominant over mutants with D193Y in terms of symptom expression. MM would therefore exhibit the symptoms produced by mutants lacking D193Y, that is, the symptoms produced by ClYVV/T27I.

The abovementioned results demonstrate that HC-Pro plays a critical role in the induction of necrotic symptoms. HC-Pro is a multifunctional protein capable of self-interaction, RNA binding, including of a double-stranded form of short interfering RNA, suppression of RNA silencing, interaction with 20S proteasome and proteolysis; it is required for aphid transmission, genome amplification, synergism with some viruses, and systemic movement [4, 13, 14, 16, 33]. Previous deletion and mutation studies, reviewed in Urcuqui-Inchima et al. [33], roughly assigned these functions to domains and motifs in HC-Pro. The T27I is located in a putative zinc finger domain. All cystein residues in this domain are well conserved, and T27I is adjacent to the first cystein residue from the

N-terminal end. It has been proposed that HC-Pro is present as a dimer in infected plants, and an N-terminal region containing a zinc finger domain has been shown to be sufficient for self-interaction [30, 31] though the canonical function of a zinc finger domain is to interact with nucleic acids. On the other hand, two independent RNA-binding domains (Fig. 3A) were identified in PVY HC-Pro in a deletion mutant study [32]. The D193Y occurs in the part corresponding to N-terminal side one (Fig. 3A, a) of the RNA-binding domains. Although the Asp at this position is not conserved among potyviruses, this amino acid is located nine amino acids from the carboxyl end of the conserved motif FRNK and is adjacent to the N-terminal end the conserved motif L×CDNQLD. Thus, these point mutations probably affect HC-Pro functions involving these domains and motifs, resulting in symptom attenuation. Although positions of mutations affecting viral virulence were dispersed over HC-Pro of other potyviruses in previous reports [2, 3, 6, 10, 29], followings were consistent with our results. Substitution of cysteine residues in a putative zinc finger domain of TVMV HC-Pro had profound effects on virulence [3]. Gal-On and Raccah [6] showed that the replacement of a basic amino acid, Arg, with a hydrophobic amino acid, Ile, in the FRNK motif dramatically affected the expression of virus symptoms in cucurbits, without affecting the accumulation of ZYMV.

One of ClYVV HC-Pro functions affected by these point mutations proved to be RSS activity. Moreover, the RSS activity was correlated with the expression of viral necrotic

symptoms, i.e., lower RSS activity of P1/HC-Pro was associated with milder necrotic symptoms (Figs. 2–5). This implies that the RSS activity of P1/HC-Pro is necessary for necrotic symptom development in response to ClYVV. Because RNA silencing is considered part of a key anti-viral defense system in plants [35] and the point mutation D193Y, which weakens the RSS activity, affects the viral accumulation kinetics (Fig. 4A), RSS apparently accelerates viral multiplication in an infected plant and results in more severe virulence. Nevertheless, we independently obtained the following results in pea (*Pisum sativum*): the comparable accumulation of ClYVV/CB and ClYVV/D193Y in an inoculated leaf, the induction of the salicylic acid (SA)-mediated defense reaction in an inoculated leaf showing necrotic symptoms, and the presence of a single semi-dominant allele, *Cyn*1, in a pea line developing tip necrosis by ClYVV infection [8, 18]. This suggests that accelerated virus accumulation is not required to produce necrotic symptoms, but SA-mediated reaction is involved in necrosis symptom expression. Consistently, transgenic expression of tobacco etch virus P1/HC-Pro alters SA-mediated host defense in tobacco [1, 17]. We are now examining whether RSS activity and SA-mediated host reaction are required to produce necrotic symptoms.

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Figure Legend

Fig. 1. Schematic diagram of the full-length clone of ClYVV. (A) Full-length ClYVV cDNA is fused to the CaMV 35S promoter (35S). Open reading frames and untranslated regions are represented by boxes and lines, respectively. P1, protein 1; HC-Pro, helper component protease; P3, protein 3; CI, cylindrical inclusion protein; VPg, genome-linked protein; NIa-Pro, nuclear inclusion protein a protease; NIb, nuclear inclusion protein b; CP, capsid protein. (B) Locations of amino acid differences between ClYVV no. 30 and a spontaneous mutant MM are indicated by black triangles. The amino acid sequence of MM was deduced based on the nucleotide sequencing analysis of the MM genome between the broken lines. (C) Identification of the region involved in the attenuation of symptoms with chimeric viruses between ClYVV no. 30 and MM. Each chimera contained the MM genomic sequence of the region indicated by a gray box. Symptom descriptions: in inoculated leaves / in systemically infected leaves. Symptom abbreviations: CS, chlorotic spots; (CS), weak chlorotic spots; LN, lethal necrosis; M, mottle or mosaic; N, necrosis; NS, necrotic spots.

Fig. 2. Symptom expression in broad bean plants infected with ClYVV no. 30 and chimeric viruses re-constructed from the chimera containing the P1/HC-Pro/P3 region of the MM genome (ClYVV/CN; Fig. 1). (A) ClYVV no. 30 induced necrotic spots on an

inoculated leaf (left), vein necrosis in upper leaves (middle), and finally, resulted in plant death (lethal necrosis, right). (B) Identification of the two parts (α and β) of the chimeric viruses involved in the attenuation of symptoms. Each chimera contained the MM genomic sequence of the region indicated by orange boxes. Symptom descriptions: in inoculated leaves (left panels) / in systemically infected leaves (right panel). Symptom abbreviations: CS, chlorotic spots; (CS), weak chlorotic spots; LN, lethal necrosis; M, mottle or mosaic; N, necrosis; NS, necrotic spots; (NS), weak necrotic spots; VC, vein clearing.

Fig. 3. Pathogenicity of ClYVV point mutants possessing a single amino acid substitution at positions 27 (ClYVV/T27I), 33 (ClYVV/I33V), and 193 (ClYVV/D193Y) in broad bean. (A) Identification of the point mutations involved in the attenuation of symptoms. ClYVV/T27I and ClYVV/D193Y represent mutants containing parts α and β of the MM genome, respectively. ClYVV/T27I-D193Y represents a mutant containing both α and β of the MM genome, ClYVV/CN in Fig. 2. The amino acid positions 27 and 193 fall in a putative zinc finger motif and one (a blue bar) of the RNA-binding domains (a and b blue bars), respectively. (B) Monitoring the infection of broad bean with GFP-tagged ClYVV both with and without point mutations. Amino acid substitutions at either position 27 or 193 weakened the GFP signal intensity, but did not affect spread of GFP signal. Symptom abbreviations: CS, chlorotic spots; (CS), weak chlorotic spots; LN, lethal

necrosis; M, mottle or mosaic; N, necrosis; NS, necrotic spots; (NS), weak necrotic spots; VC, vein clearing.

Fig. 4. Comparison of virulence and accumulation kinetics among ClYVV no. 30 and point mutant viruses. (A) Single infection foci were individually punched out and sampled by monitoring GFP fluorescence. The viral concentration was measured by ELISA at 2, 4, 6, 8, 10, and 12 days post inoculation (dpi) with the ClYVV-GFP (broken line) and ClYVV/D193Y-GFP (line) viruses. The proportion of plants (*V. faba* cv. Uchikoshi-isun) showing necrotic symptoms on (B) inoculated leaves and (C) upper leaves infected with ClYVV-GFP (closed circle with broken line, n = 12), ClYVV/T27I-GFP (open square with broken line, n = 30), and ClYVV/CB-GFP (closed triangle with line, n= 26) was monitored at 1–19 days post inoculation (dpi).

Fig. 5. RNA-silencing suppression (RSS) activity of ClYVV P1/HC-Pro both with and without mutations. GFP, inverted repeats of the GFP mRNA sequence, one of the P1/HC-Pro of strain no. 30 (WT), its mutants at amino acid position 182 in P1 (P1E182G), position 27 in HC-Pro (T27I), or position 193 in HC-Pro (D193Y), and the *Cla* I–*Bgl* I portion (see Fig. 2B) of MM (CB), TBSV P19, or GUS were transiently expressed to trigger RNA silencing against GFP in transgenic *Nicotiana benthamiana* expressing GFP (16c). RSS activity was compared based on (A) GFP fluorescence and (B) accumulation

of GFP mRNA at 3 days after infiltration. (C) Comparable expression of HC-Pro both with and without mutations was confirmed by western blots with monoclonal antibodies against ClYVV HC-Pro [38]. As controls, HC-Pro of the wild type (HC-Pro) and an extract from a ClYVV-infected broad bean leaf (ClYVV) were used. Lane M, protein size marker.

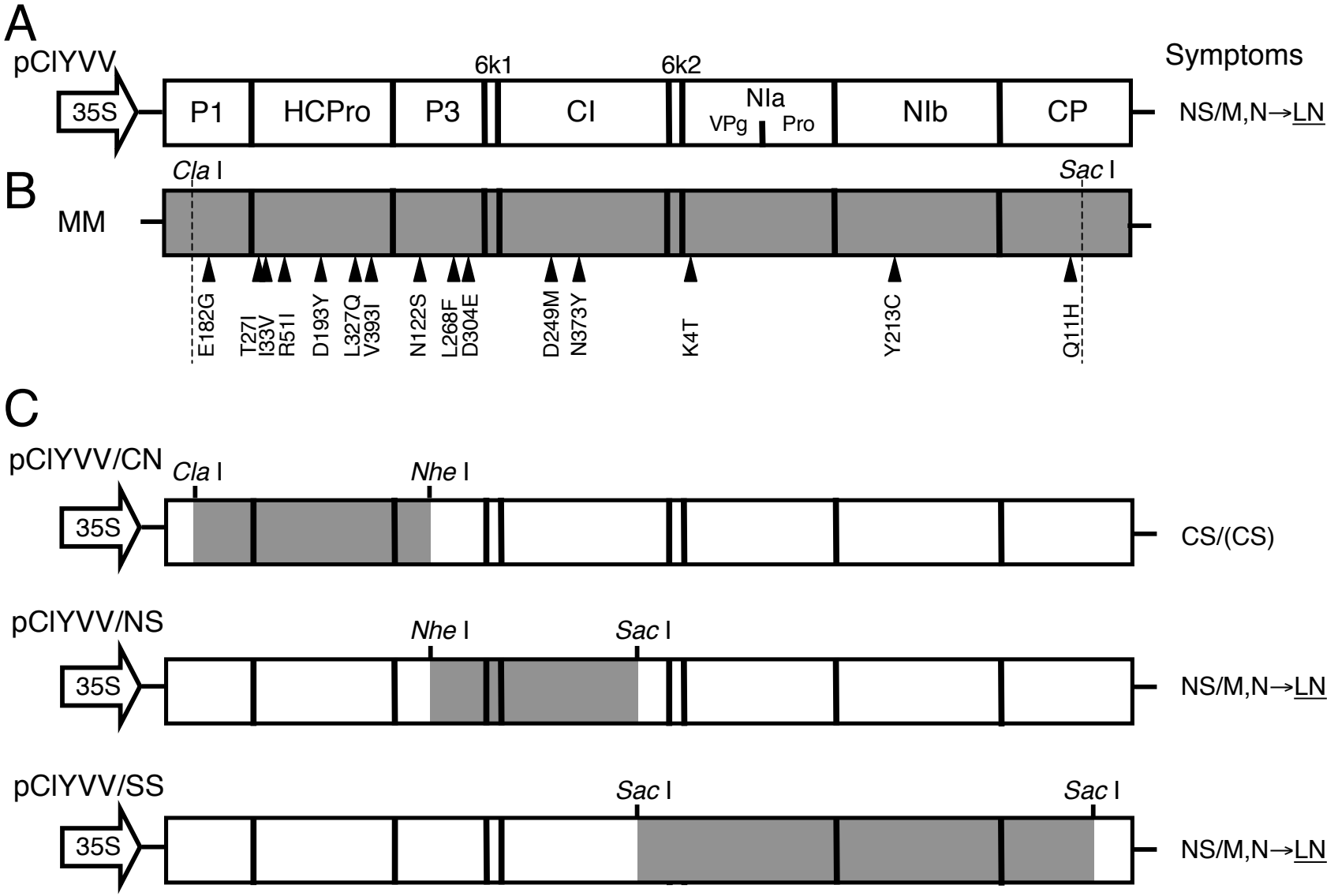
A
pClYVV
35S
P1
HCPro
P3
6k1
Cl
6k2
NIa
VPg
Pro
NIb
CP
Symptoms
NS/M,N→LN
B
MM
Cla I
Sac I
E182G
T27I
I33V
R51I
D193Y
L327Q
V393I
N122S
L268F
D304E
D249M
N373Y
K4T
Y213C
Q11H
C
pClYVV/CN
Cla I
Nhe I
35S
CS/(CS)
pClYVV/NS
Nhe I
Sac I
35S
NS/M,N→LN
pClYVV/SS
Sac I
Sac I
35S
NS/M,N→LN

Fig. 1

A

B

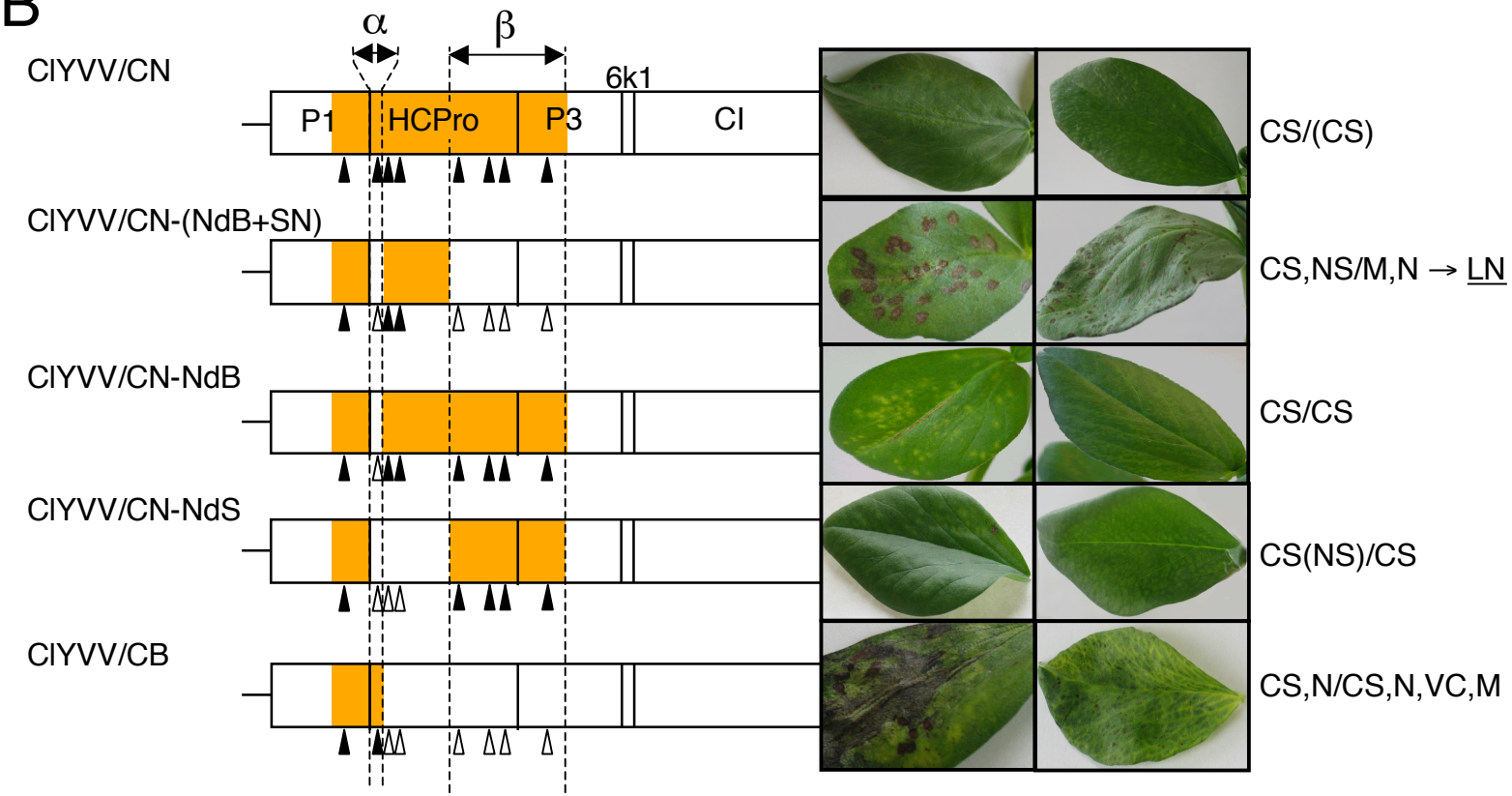
α
β
ClYVV/CN
6k1
P1
HCPro
P3
CI
ClYVV/CN-(NdB+SN)
ClYVV/CN-NdB
ClYVV/CN-NdS
ClYVV/CB
CS/(CS)
CS,NS/M,N → LN
CS/CS
CS(NS)/CS
CS,N/CS,N,VC,M

Fig. 2

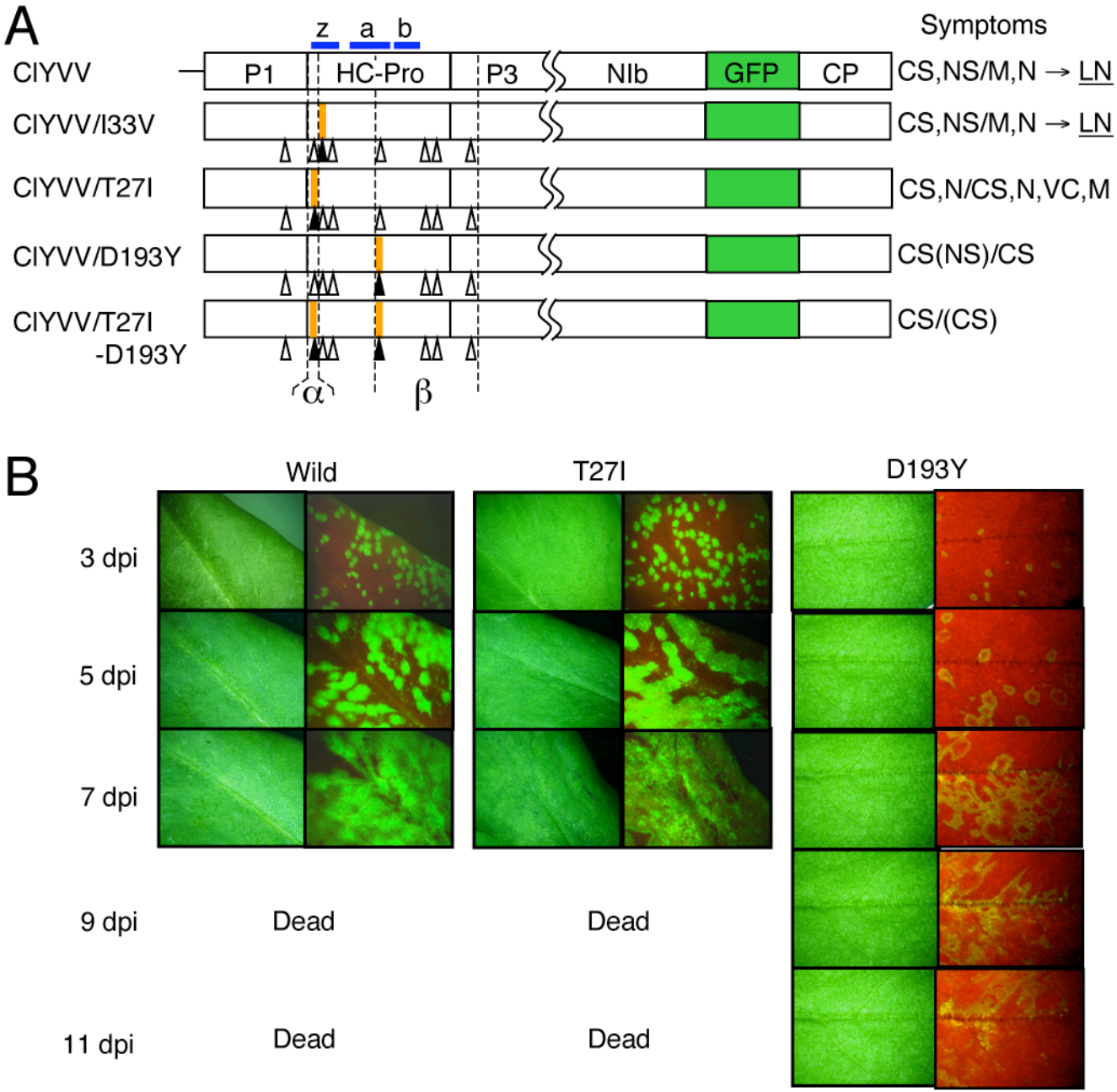
A
z
a
b
Symptoms
ClYVV
P1
HC-Pro
P3
NIb
GFP
CP
CS,NS/M,N → LN
ClYVV/I33V
CS,NS/M,N → LN
ClYVV/T27I
CS,N/CS,N,VC,M
ClYVV/D193Y
CS(NS)/CS
ClYVV/T27I
-D193Y
CS/(CS)
α
β
B
Wild
T27I
D193Y
3 dpi
5 dpi
7 dpi
9 dpi
Dead
Dead
11 dpi
Dead
Dead

Fig. 3

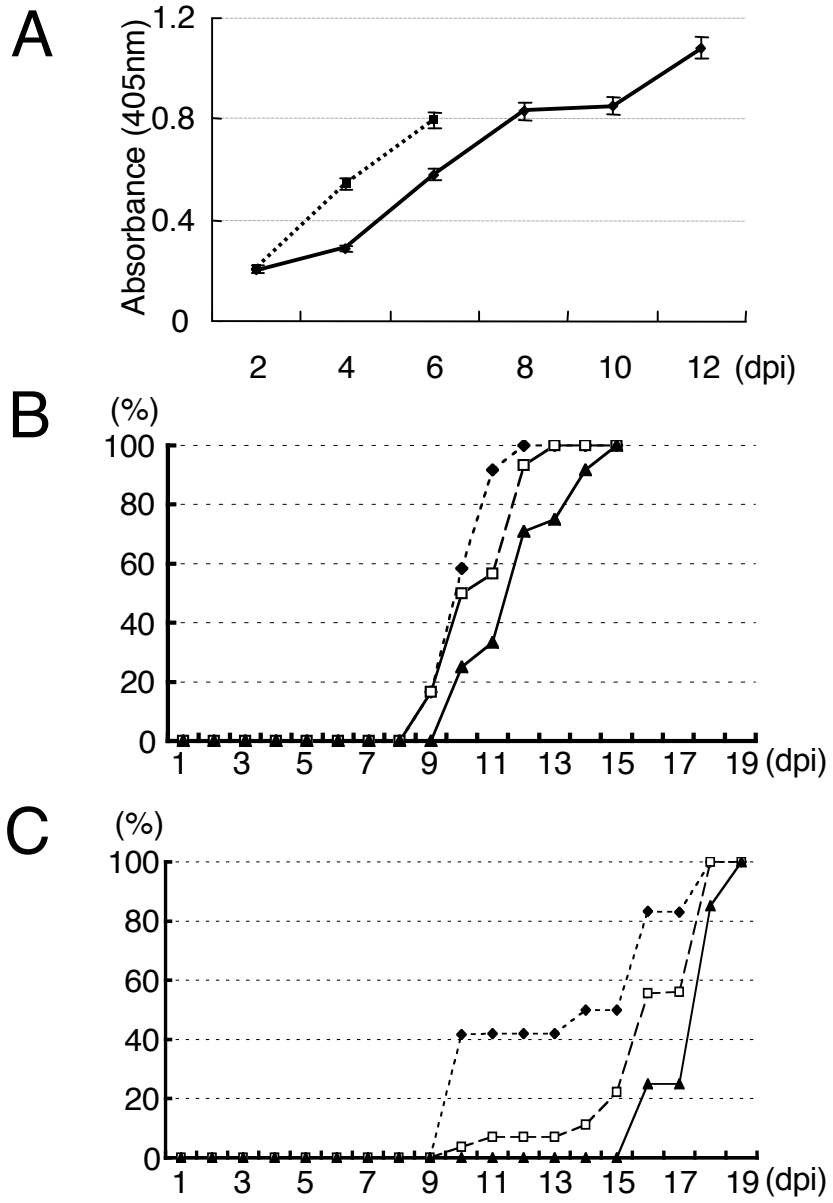
A
Absorbance (405nm)
1.2
0.8
0.4
0
2
4
6
8
10
12
(dpi)
B
(%)
100
80
60
40
20
0
1
3
5
7
9
11
13
15
17
19
(dpi)
C
(%)
100
80
60
40
20
0
1
3
5
7
9
11
13
15
17
19
(dpi)

Fig. 4

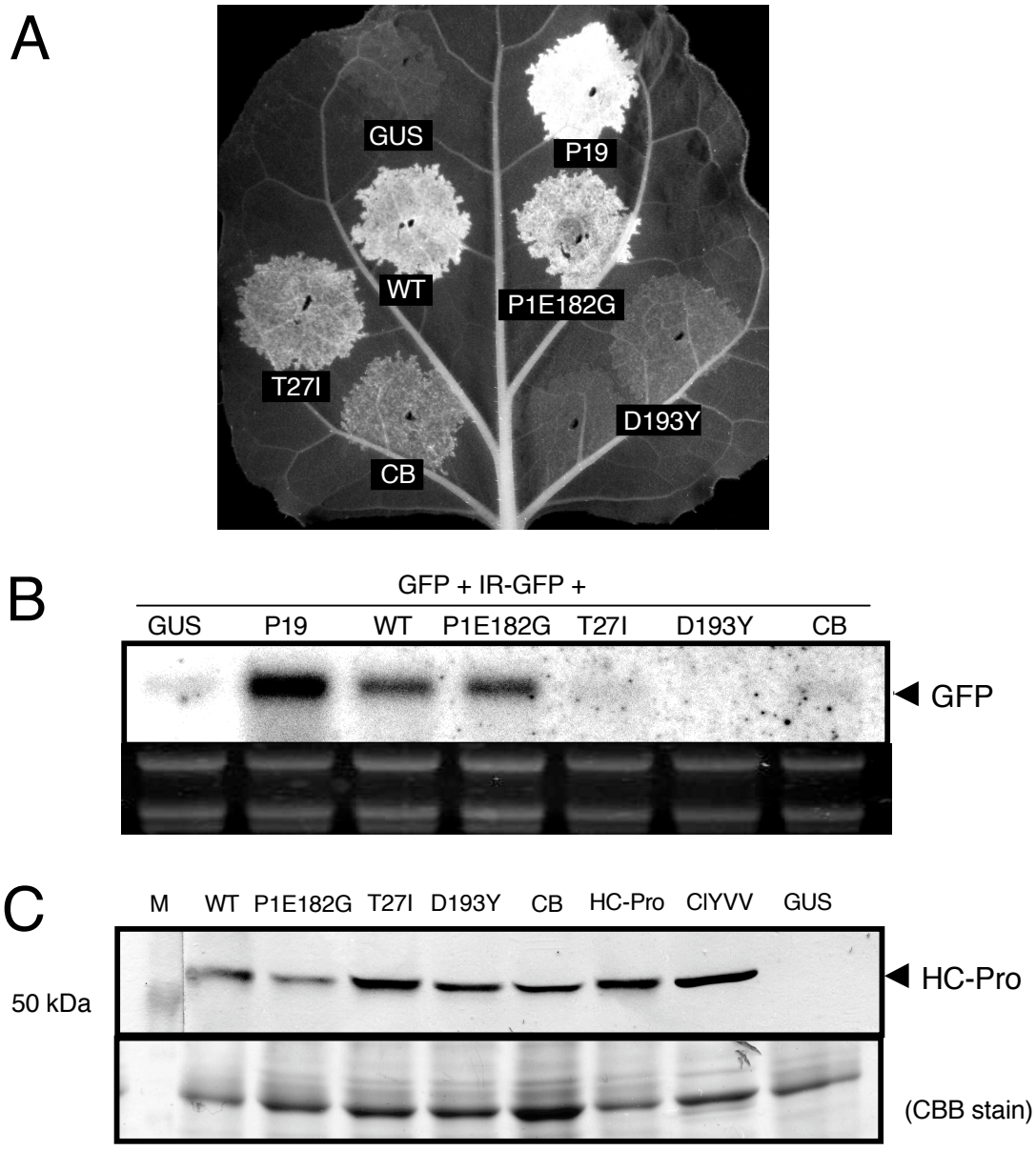
A
GUS
P19
WT
P1E182G
T27I
D193Y
CB
B
GFP + IR-GFP +
GUS
P19
WT
P1E182G
T27I
D193Y
CB
GFP
C
M
WT
P1E182G
T27I
D193Y
CB
HC-Pro
ClYVV
GUS
50 kDa
HC-Pro
(CBB stain)

Fig. 5