Modification of the N-terminal FWKG−αH1 element of potyviral HC-Pro affects its multiple functions and generates effective attenuated mutants for cross-protection

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Abstract
Control of plant viruses by cross-protection is limited by the availability of effective protective strains. Incorporation of an Nla-protease processing site in the extreme N-terminal region of the helper component protease (HC-Pro) of turnip mosaic virus (TuMV) resulted in a mutant virus TuHN3D1 that induced highly attenuated symptoms. Recombination analysis verified that two variations, F7I mutation and amino acid 7-upstream-deletion, in HC-Pro co-determined TuHN3D1 attenuation. TuHN3D1 provided complete protection to Nicotiana benthamiana and Brassica campestris subsp. chinensis plants against infection by the severe parental strain. Aphid transmission tests revealed that TuHN3D1 was not aphid-transmissible. An RNA silencing suppression (RSS) assay by agroinfiltration suggested the RSS-defective nature of the mutant HC-Pro. In the context (amino acids 3–17) encompassing the two variations of HC-Pro, we uncovered an FWKG−α-helix 1 (αH1) element that influenced the functions of aphid transmission and RSS, whose motifs were located far downstream. We further demonstrated that HC-Pro F7 was a critical residue on αH1 for HC-Pro functions and that reinstating αH1 in the RSS-defective HC-Pro of TuHN3D1 restored the protein’s RSS function. Yeast two-hybrid and bimolecular fluorescence complementation assays indicated the FWKG−αH1 element as an integral part of the HC-Pro self-interaction domain. The possibility of regulation of the mechanistically independent functions of RSS and aphid transmission by the FWKG−αH1 element is discussed. Extension of TuMV HC-Pro FWKG−αH1 variations to another potyvirus, zucchini yellow mosaic virus, also generated nonaphid-transmissible cross-protective mutant viruses. Hence, the modification of the FWKG−αH1 element can generate effective attenuated viruses for the control of potyviruses by cross-protection.

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1 | INTRODUCTION

Cross-protection is a natural phenomenon wherein infection by a virus induces resistance in a host plant to exclude subsequent infection by other strains of the virus (McKinney, 1929). Hence, infection by an attenuated viral strain may cross-protect the infected plants against subsequent infection by severe strains of the virus. Cross-protection strains of many plant viruses have been applied on a large scale to control their related severe strains for decades (Gal-On & Shibolet, 2006; Syller & Grupa, 2016; Ziebell & Carr, 2010).

Cross-protection provided by an attenuated virus results from multiple mechanisms of interactive phases. Firstly, the double-stranded RNA replicative intermediates of a systemically established protective virus trigger antiviral silencing through virally derived small interfering (si) RNAs in the host and protect the host against related severe strains in a sequence homology-dependent manner (Hammond et al., 2001). Secondly, the protective virus provides protein-mediated protection by specific mechanisms, including interference with invading viral disassembly (Register & Beachy, 1988) and sequestration of host proteins (Ziebell & Carr, 2010) essential for invading viral functions. Lastly, the protective virus triggers host innate immune responses such as salicylic acid (SA) signalling that also cross-talk with the RNA silencing events of the cross-protection process (Kung et al., 2014).

The cross-protection literature (Fulton, 1986; Kung et al., 2014; Lecoq, 1998; Ziebell & Carr, 2010; Ziebell & MacDiarmid, 2017) collectively postulates several essential criteria for a protective virus: a protective virus must be genetically stable and attenuated. Attenuation of an RNA virus strain is due to its weaker RNA silencing suppression (RSS) ability (Kung et al., 2014; Lin et al., 2007) and minimal interference with host microRNA pathways (Kung et al., 2014; Wu et al., 2010).

Protective strains of plant viruses may be isolated from nature (Kondo et al., 2015; Lecoq et al., 1991) or induced by physical (Kosaka & Fukunishi, 1993; Oshima, 1975) or chemical (Rast, 1972; Yeh & Gonsalves, 1984) means. Empirically, protective strains can be generated by replacing genomic region(s) of severe target strains harbouring major disease determinants with the corresponding region(s) from attenuated strains (Albiach-Martí et al., 2010; Chiang et al., 2007). Moreover, protective strains can be rationally designed from severe strains by manipulating functional motifs of RSS proteins. For instance, the HC-Pro R180I mutation of zucchini yellow mosaic virus (ZYMV) Israel (Gal-On & Raccah, 2000) and Taiwan (Lin et al., 2007) strains, and the HC-Pro R182K mutation of turnip mosaic virus (TuMV; Kung et al., 2014) generate attenuated strains that cross-protect host plants against severe strains of the respective viruses.

Potyviruses are one of the largest groups of plant viruses and cause severe economic loss for many crops (Adams et al., 2012). The multifunctional nature of potyviral HC-Pro (Urcuqui-Inchima et al., 2001) largely depends on its interaction with itself, other viral proteins, and host proteins (Poque et al., 2018; Valli et al., 2018) to form different functional complexes. The spatiotemporal availability of HC-Pro in host cells (Valli et al., 2014) in different complexes appears to govern the intricate coordination of potyviral functions.

In the N-terminal region of HC-Pro, Robaglia et al. (1989) identified a 34 amino acid cysteine (C)-rich domain (amino acids 25–58 in TuMV) and postulated its potential to be a zinc finger possibly involved in HC-Pro self-interaction. The extreme N-terminal region (ENTR) of HC-Pro (amino acids 1–24 in TuMV) has been presumed to be devoid of elements essential for HC-Pro structure, protein–protein interaction, and functions. In several potyviruses, HC-Pro ENTR has been manipulated for in planta expression of exogenous proteins (Chen et al., 2007; Dolja et al., 1992, 1993; German-Retana et al., 2000, 2003; Guo et al., 1998) and for monitoring protein purification with tags (Blanc et al., 1999; Ruiz-Ferrer et al., 2004). However, the pronounced effects of several reported mutations (Blanc et al., 1998; Torres-Barceló et al., 2008; Yamboa et al., 2008), small deletions (Atreya & Pirone, 1993; Guo et al., 1999), and insertions (Varrelmann et al., 2007) in HC-Pro ENTR, and addition of amino acid residues at the HC-Pro N-terminus (Ruiz-Ferrer et al., 2004) have so far been neglected.

In this investigation, a highly attenuated virus TuHN3I expressing an HC-Pro with variations (F7I mutation and amino acid 7-upstream-deletion [aa7-upstream-deletion]) was obtained during incorporation of an Nla-protease processing site in HC-Pro ENTR of TuMV-RC4 (Chen et al., 2003). In inoculated plants, TuHN3I provided complete cross-protection by excluding wild-type (WT) severe virus superinfection. Recombinants of TuHN3I with WT virus indicated that the F7I mutation and aa7-upstream-deletion variations co-determined TuHN3I attenuation. RSS assay showed that the two variations together abolished RSS activity and that either of these variations did not. However, mutant viruses expressing HC-Pro with either one or both of these variations were nonaphid-transmissible. In the HC-Pro ENTR context (amino acids 3–17 in TuMV) encompassing these variations, we unearthed a sequence-structural element, designated the FWKG–α-helix 1 (αH1) element, that showed self-interactive and regulatory properties and influenced symptom expression and the mechanistically independent functions of RSS and aphid transmission. We previously reported the components of the FWKG–αH1 element, that is, the conserved motif FWKG and α-helix 1 (Yeh et al., 2011). Recently, Rodamilans et al. (2018) independently predicted the same α-helix.

Extension of the F7I mutation and aa7-upstream-deletion variations to the ZYMV HC-Pro FWKG–αH1 element also generated attenuated ZYMV mutant viruses with complete cross-protection ability and the nonaphid-transmissibility characteristic. Our findings provide a novel approach for developing nonaphid-transmissible

**KEYWORDS**
cross-protection, FWKG–α-helix 1 element, helper component protease, silencing suppression, symptom attenuation, Turnip mosaic virus, Zucchini yellow mosaic virus
attenuated strains to control potyviral infection of crop plants by cross-protection.

2 | RESULTS

2.1 | Construction of mutants of TuHN\textsuperscript{NF} with variations in HC-Pro

WT virus TuHN\textsuperscript{NF} was generated in planta from infectious construct pTuHN\textsuperscript{NF}. By incorporating an Nla-protease processing site in the HC-Pro extreme N-terminal region (ENTR), pTuHN\textsuperscript{NF} was modified into pTuHN\textsuperscript{DF}, generating an attenuated virus TuHN\textsuperscript{DF} expressing a mutant HC-Pro, namely HN\textsuperscript{DF}, with aa7-upstream-deletion (Table 1 and Figure 1a). During the construction of pTuHN\textsuperscript{DF}, we also obtained pTuHN\textsuperscript{DI}, which generated a highly attenuated virus TuHN\textsuperscript{DI} expressing a mutant HC-Pro, namely HN\textsuperscript{DI}, with F7I mutation in addition to aa7-upstream-deletion. For uniform nomenclature, the N-terminally generated a mutant virus TuHN \textsuperscript{NI} expressing F7I mutant HC-Pro (Table 1 and Figure 1a).

Moreover, to assess the F7I mutation’s individual contribution to TuHN\textsuperscript{NI} attenuation, pTuHN\textsuperscript{IF} was modified into pTuHN\textsuperscript{DF}, which generated a mutant virus TuHN\textsuperscript{DF} expressing F7I mutant HC-Pro (HN\textsuperscript{DI}). (Table 1 and Figure 1a).

2.2 | Mutant virus TuHN\textsuperscript{DI} induced highly attenuated symptoms with recovery

To assess the effects of HC-Pro F7I mutation and/or aa7-upstream-deletion on viral symptoms, Chenopodium quinoa plants were mechanically inoculated individually with WT pTuHN\textsuperscript{NF} and mutant pTuHN\textsuperscript{NI}, TuHN\textsuperscript{DF}, and pTuHN\textsuperscript{DI} constructs. After 7 days, progeny viruses from C. quinoa were introduced into plants of Nicotiana benthamiana and Brassica campestris subsp. chinensis, and infections were verified by reverse transcription (RT)-PCR and sequencing.

WT virus TuHN\textsuperscript{DF} induced chlorotic lesions in C. quinoa plants, severe mosaic followed by wilting in N. benthamiana plants, and severe mosaic in B. campestris subsp. chinensis plants (Table 1 and Figure 1b). Mutant virus TuHN\textsuperscript{DF} induced diffuse yellow spots in the leaves of C. quinoa plants, mosaic without wilting in N. benthamiana plants, and mosaic (not severe as that of TuHN\textsuperscript{NF}) in B. campestris subsp. chinensis plants. Mutant virus TuHN\textsuperscript{NI} induced significantly milder symptoms of diffuse yellow spots in C. quinoa plants, and mottling in N. benthamiana and B. campestris subsp. chinensis plants. Remarkably, differing from other mutant viruses, TuHN\textsuperscript{DI} induced highly attenuated symptoms of faint diffuse pale-green spots/areas in C. quinoa, and mild mottling in plants of N. benthamiana and B. campestris subsp. chinensis, followed by a systemic recovery from symptoms (Table 1 and Figure 1b). The attenuated symptoms of the mutant viruses TuHN\textsuperscript{DF}, TuHN\textsuperscript{NI}, and TuHN\textsuperscript{DI} were classified as attenuated symptom type (AT)-I, AT-II, and AT-III, respectively (Table 1 and Figure 1b).

2.3 | Genetic recombination verified HC-Pro F7I mutation and aa7-upstream-deletion co-determined the highly attenuated symptoms of TuHN\textsuperscript{DI}

To verify whether HC-Pro variations determined symptom attenuation of TuHN\textsuperscript{NI}, genetic recombination of WT TuHN\textsuperscript{NF} and mutant TuHN\textsuperscript{DI} viruses was performed. The recombinant virus TuHN\textsuperscript{DI}-S with WT HC-Pro (Nco-KpnI region) in the attenuated mutant TuHN\textsuperscript{DI}-skeleton induced WT symptoms in C. quinoa and N. benthamiana plants, similar to WT virus TuHN\textsuperscript{NF}. In contrast, the recombinant virus TuHN\textsuperscript{DF}-Nco-M with HC-Pro of attenuated virus TuHN\textsuperscript{DI} in the WT TuHN\textsuperscript{DF}-skeleton induced attenuated symptoms similar to attenuated virus TuHN\textsuperscript{DI} in these hosts (Figure 1c). Thus, the results verified that HC-Pro F7I mutation and aa7-upstream deletion co-determine the attenuated symptoms of TuHN\textsuperscript{DI}.

2.4 | Accumulation of WT virus and mutant viruses in infected plants

By indirect ELISA with anti-TuMV-RC4 antiserum, accumulation levels of WT TuHN\textsuperscript{NF} and mutant TuHN\textsuperscript{NI}, TuHN\textsuperscript{DF}, and TuHN\textsuperscript{DI} viruses were assessed in C. quinoa plants 4 days postinoculation (dpi) and in N. benthamiana plants 10 dpi. The AT-III symptom-inducing TuHN\textsuperscript{DI} accumulated significantly less than the WT virus, while the AT-I and AT-II symptom-inducing mutant viruses accumulated almost to WT level (Figure S1a,b).

The accumulation dynamics of TuHN\textsuperscript{DI} in N. benthamiana were monitored to 21 dpi. The level of accumulation of TuHN\textsuperscript{DI} increased to 9 dpi and sharply decreased afterwards in a zigzag pattern characteristic of earlier characterized cross-protective strains of potyviruses (Kung et al., 2014; Lin et al., 2007). Until 10 dpi, WT TuHN\textsuperscript{NF} accumulated more than TuHN\textsuperscript{DI}. However, beyond this time point, the systemically wilting host plants did not support TuHN\textsuperscript{NF} accumulation (Figure 2).

2.5 | Genetic stability of attenuated virus TuHN\textsuperscript{DI}

To test the genetic stability of TuHN\textsuperscript{DI}, viral progenies were transferred from infected C. quinoa plants to healthy N. benthamiana and B. campestris subsp. chinensis plants. In these hosts, beyond 10 serial passages (i.e., >100 days), the TuHN\textsuperscript{DI} progenies induced attenuated symptoms like their progenitor (data not shown). Sequencing of the HC-Pro HN\textsuperscript{DI} cDNA amplified from infected plants confirmed the genetic stability of TuHN\textsuperscript{DI} (data not shown).

2.6 | Cross-protection effectiveness of attenuated virus TuHN\textsuperscript{DI}

The cross-protection effectiveness of attenuated virus TuHN\textsuperscript{DI} was tested by challenge-inoculating TuHN\textsuperscript{DI}-protected plants with
| WT/mutant viruses | WT/mutant HC-Pros | HC-Pro aa7 identity and aa7-upstream condition | Symptoms on host | Symptom type |
|-------------------|-------------------|-----------------------------------------------|-----------------|--------------|
|                   |                   |                                               | Chenopodium quinoa | Nicotiana benthamiana | Brassica campestris subsp. chinensis | |
| aa7-upstream-native |                   |                                               |                 |                       |                                      |  
| TuHN⁴F            | HN⁴F              | SAAGAN- F⁷WKG                                 | Chlorotic lesions | Severe mosaic and wilting | Severe mosaic | WT                     |
| TuHN⁴I            | HN⁴I              | SAAGAN- I⁷WKG                                 | Faint diffused yellow spots | Mild mosaic | Mild mosaic | AT-II                  |
| aa7-upstream-deleted |                   |                                               |                 |                       |                                      |  
| TuHN⁵F            | HN⁵F              | ΔΔΔΔΔΔα⁷WKG                                  | Diffused yellow spots | Mosaic | Mosaic | AT-I                   |
| TuHN⁵I            | HN⁵I              | ΔΔΔΔΔΔα⁷WKG                                  | Faint pale-green spots | Mild mottling | Mild mottling | AT-III                    |
| aa7-upstream-modified |                   |                                               |                 |                       |                                      |  
| TuHN⁶F            | HN⁶F              | SAAMASA- F⁷WKG                              | Chlorotic lesions | Severe mosaic and wilting | Severe mosaic | WT                     |
| TuHN⁶I            | HN⁶I              | SAAMASA- F⁷WKG                              | Faint diffused yellow spots | Mild mosaic | Mild mosaic | AT-II                  |

- Viruses in vivo expressed from cDNA constructs.
- Phenylalanine (F)/isoleucine (I).
- For uniform nomenclature, the N-terminally deleted HC-Pros were named as aa7-upstream-deleted HC-Pros, despite the presence of the alanine residue before amino acid 7. Similarly, the N-terminally modified HC-Pros were named as aa7-upstream-modified HC-Pros, although amino acid 7 was shifted to eighth position.
- AT(Attenuated symptom Type)-I, AT-II, and AT-III.
- Replacement of amino acids 4–6 (GAN) by MDASCYHQ/A (engineered Nla-protease site underlined) resulted in an N-terminally deleted HC-Pro on N-terminal processing; a nonnative alanine residue (shown as lower case a) left-over by processing assumed the amino acid 1 position.
- Replacement of amino acids 4–6 (GAN) by MASA reinstated a nonnative α-helix 1 (αH1) (this manipulation was done after understanding the aa7 location on αH1 and disruption of αH1 by N-terminal deletion).

[Correction added on 16 March 2022, after first online publication: Some footnote callouts have been corrected in this version.]
FIGURE 1. Generation of infectious cDNA constructs for wild-type (WT) turnip mosaic virus RC4 (TuMV-RC4) and attenuated mutant viruses, and verification of attenuation determinant(s). (a) pTuHN^N^F (WT TuMV-RC4 cDNA construct) was created as described for TuMV-YC5/p35SYC5 by Chen et al. (2007). pTuHN^D^F (cDNA construct for mutant virus TuHN^D^F, an expression vector) was created from pTuHN^N^F by engineering NcoI and Nhel restriction sites, and Nla-protease cleavage sites in the N-extreme of HC-Pro (site positions/sequence variations shown). During construction of pTuHN^D^F, another mutant construct pTuHN^P^I was also obtained. pTuHN^N^FNco is a severe virus intermediate construct. pTuHN^P^I is a mutant construct created from pTuHN^N^F by HC-Pro F7I point mutation. (b) Symptoms induced by WT TuHN^N^F and mutant TuHN^D^F, TuHN^P^I, and TuHN^D^I viruses in hosts Chenopodium quinoa and Nicotiana benthamiana at 7 days postinoculation (dpi) and in Brassica campestris subsp. chinensis at 14 dpi. (c) The recombinant constructs pTuHN^P^I-S and pTuHN^N^FNco-M (created by exchanging HC-Pro regions of pTuHN^P^I and pTuHN^N^FNco) generated viruses that induced symptoms characteristic of their HC-Pro donors (symptoms/symptom types shown/indicated are described in Table 1).
severe recombinant virus TuMV-RC4-GFP expressing green fluorescent protein (GFP). TuHN\textsuperscript{D1}-protected \textit{N. benthamiana} plants did not develop symptoms until 15 days after TuMV-RC4-GFP challenge inoculation; subsequently, they showed mild mottling and a slight reduction in growth induced by TuHN\textsuperscript{D1}. In contrast, unprotected \textit{N. benthamiana} plants inoculated solely with severe virus TuMV-RC4-GFP developed severe mosaic and wilting as early as 5 dpi (Figure 3a).

In western blotting analysis with anti-GFP antiserum, the unprotected \textit{N. benthamiana} plants inoculated with the GFP-recombinant severe virus TuMV-RC4-GFP produced a GFP signal. Conversely, the TuHN\textsuperscript{D1}-protected and subsequently TuMV-RC4-GFP challenge-inoculated plants did not show a GFP signal (Figure 3b, upper panel). In western blotting with the antiserum to TuMV-RC4, both plants produced a TuMV coat protein (CP) signal (Figure 3b, middle panel). In RT-PCR with CPN-u and CPN-d primers against genomic regions flanking the GFP open reading frame (ORF), the unprotected \textit{N. benthamiana} plants solely inoculated with the GFP-recombinant severe virus TuMV-RC4-GFP yielded 960 bp cDNA encompassing the GFP ORF. However, the TuHN\textsuperscript{D1}-protected and subsequently TuMV-RC4-GFP challenge-inoculated plants did not yield 960 bp cDNA, but yielded 215 bp cDNA, the targeted genomic region of the native virus (Figure 3b, lower panel).

Overall, the host plant phenotypes and nondetection of GFP RNA and protein indicated that TuHN\textsuperscript{D1} provided complete cross-protection to host plants by excluding TuMV-RC4-GFP superinfection. However, although cross-protection against native severe virus TuHN\textsuperscript{NF} was evident from the phenotype (Figure 3a), exclusion of TuHN\textsuperscript{NF} was not confirmed because RT-PCR and western blotting could not discriminate between TuHN\textsuperscript{D1} and TuHN\textsuperscript{NF} (Figure 3b). Similar results were obtained when the cross-protection effectiveness of the attenuated TuHN\textsuperscript{D1} was tested on \textit{B. campestris} subsp. \textit{chinensis} plants (Figure 3c,d).

### 2.7 HC-Pro F7I mutation and/or aa7-upstream-deletion abolishes the aphid transmissibility of TuMV

The aphid transmissibility of the mutant viruses TuHN\textsuperscript{NI}, TuHN\textsuperscript{D1}, and TuHN\textsuperscript{NF} was tested in \textit{B. campestris} subsp. \textit{chinensis} plants. The virus accumulation levels in infected plants were assayed by indirect ELISA with anti-TuMV-RC4 antiserum. At 7 dpi, the mutant viruses TuHN\textsuperscript{NI}, TuHN\textsuperscript{D1}, and TuHN\textsuperscript{NF} accumulated 35%–37% compared to the WT virus, and 56%–60% compared to the nonaphid-transmissible CP DAG-motif-deleted mutant virus TuCP\textsuperscript{DAG} (Figure 4a). In three independent aphid transmission trials, like the negative control TuCP\textsuperscript{DAG}, the mutant viruses TuHN\textsuperscript{NI}, TuHN\textsuperscript{D1}, and TuHN\textsuperscript{NF} were not aphid-transmissible (0%), while the WT virus TuHN\textsuperscript{NF} was highly aphid-transmissible (87%; Figure 4a).

At 10 dpi, while the accumulation of the mutant virus TuHN\textsuperscript{D1} (possessing both variations, F7I mutation and aa7-upstream-deletion) showed only a slight increase, the mutant viruses TuHN\textsuperscript{NI} and TuHN\textsuperscript{D1} (possessing F7I mutation and aa7-upstream-deletion, respectively) accumulated almost to WT level (Figure 4b). In three independent aphid transmission trials, the mutant viruses TuHN\textsuperscript{NI}, TuHN\textsuperscript{D1}, and TuHN\textsuperscript{NF} were not aphid-transmissible (0%), while the WT virus TuHN\textsuperscript{NF} was highly aphid-transmissible (90%; Figure 4b). The above results indicate that either of the variations (F7I mutation or aa7-upstream-deletion) alone or both of the variations together abolished aphid transmission of TuMV.

### 2.8 Mutant HC-Pro HN\textsuperscript{D}I failed to display RSS activity in assay by agroinfiltration

Each pBCo construct expressing WT or mutant HC-Pro was agroinfiltrated into leaves of \textit{N. benthamiana} in combination with GFP-expressing vector pBA-GFP and GFP-silencing inducer pBA-GFI, and
RSS activity was recorded 5 and 9 days post-agroinfiltration (dpa). Both the mutant HC-Pro HN\(^{DI}\) with F7I mutation and HN\(^{DF}\) with aa7-upstream-deletion showed RSS abilities comparable to WT HC-Pro HN\(^{NF}\) (Figure 5a). However, the mutant HC-Pro HN\(^{DI}\) with aa7-upstream-deletion and F7I mutation showed drastically decreased RSS ability (Figure 5a).

In a time-course analysis for the RSS ability of HN\(^{DI}\), the GFP fluorescence, detectable at 4 dpa, was hardly noticeable at 8 and 12 dpa, suggesting the possible RSS-defective nature of HN\(^{DI}\) (Figure 5b, upper panel). To verify this possibility, RNAs and proteins extracted from agroinfiltrated/GFP-silenced leaf areas were analysed by northern hybridization and western blotting, respectively. The WT HN\(^{NF}\) transcript and protein were detectable at 4, 8, and 12 dpa. In contrast, the mutant HN\(^{DI}\) transcript clearly noticeable at 4 dpa was not detectable at 8 and 12 dpa, indicating that the transcript was targeted by RNA silencing (Figure 5b, lower panel, left side). However, the nondetectability of a distinct HN\(^{DI}\) protein band even at the initial stage of 4 dpa (Figure 5b, lower panel, left side) implied that HN\(^{DI}\) protein was highly unstable.

Co-infiltration of a pBA construct expressing a heterologous RSS protein, that is, watermelon silver mottle virus (WSMoV) NSs protein (Huang et al., 2015) or “nss” (KFTMHNQIF)-tagged (Cheng et al., 2013) cymbidium ringspot virus (CymRSV) P19 protein (Pantaleo & Burgyan, 2008), preserved the mutant HN\(^{DI}\) transcript from host RNA silencing, thereby making HN\(^{DI}\) translation possible. At 8 dpi, the HN\(^{DI}\) transcript accumulated to a level comparable to that of the WT HC-Pro HN\(^{DI}\) transcript (Figure 5b, lower panel, right side). However, the much weaker immunosignal of HN\(^{DI}\) strongly suggests less stability of HN\(^{DI}\) than HN\(^{DF}\), which explains the RSS-defective nature of HN\(^{DI}\).

In western blots (Figure 5b, lower panel), the cross-reactive signal, slightly smaller than the HC-Pro signal, may be confused with N-terminally deleted HN\(^{DI}\) HC-Pro. However, its detection from all the slots, including the empty vector (EV) slot, indicates its origin from the host.
The secondary structural analysis of WT HC-Pro HN\(^{DF}\) by the SYMPRED consensus method (Simossis & Heringa, 2004) predicted the propensity of the context around the F7 residue to assume an \(\alpha\)-helical secondary structure of 15 residues (amino acids 3–17), designated \(\alpha\)-helix 1 (\(\alpha\)H1; Figure S2a). The secondary structural analyses of mutant HC-Pro proteins revealed that in HNNI, the F7I mutation did not affect the integrity of \(\alpha\)H1 (Figure S2b), while in HN\(^{D}\)F and HN\(^{D}\)I, the aa7-upstream-deletion disrupted \(\alpha\)H1 and banished aa7 (F/I) to a random coil context (Figure S2c,d). Moreover, in HN\(^{D}\)I, the F7I mutation and aa7-upstream-deletion together affected the \(\beta\)-strand 1 (\(\beta\)S1; Figure S2a–d).

### 2.10 Reinstatement of \(\alpha\)H1 in RSS-defective HC-Pro HN\(^{D}\)I restores protein RSS function

As shown above (Figure 5a,b), HN\(^{DF}\) (\(\alpha\)H1-disrupted HC-Pro produced by TuHN\(^{DF}\)) was RSS-active, while HN\(^{DF}\) (\(\alpha\)H1-disrupted HC-Pro with F7I mutation produced by TuHN\(^{DF}\)) was RSS-defective. To assess the importance of the \(\alpha\)H1 secondary structure for RSS function, TuHN\(^{DF}\) was modified into TuHN\(^{DF}\) and TuHN\(^{DF}\) by incorporating a nonnative \(\alpha\)H1 (with four mutations and aa7 shifted to position 8; Figure 5c). TuHN\(^{DF}\) induced severe symptoms in tested plants as WT TuHN\(^{DF}\), while TuHN\(^{DF}\) induced attenuated symptoms of type II, similar to TuHN\(^{DF}\) (Table 1), suggesting that their symptoms were determined respectively by F (native) and I (mutant) residues from the reinstated \(\alpha\)-helical structures. Moreover, in HC-Pro HN\(^{DF}\), the structure of \(\beta\)S1 (Figure 5c) was identical not to that in its progenitor HN\(^{D}\)I (Figure S2d), but to that in HN\(^{D}\)I (Figure 5b) expressed by TuHN\(^{D}\), suggesting the influence of the intactness of \(\alpha\)H1 on the folding of \(\beta\)S1.

To test the viability of the reinstated nonnative \(\alpha\)H1 in the RSS function of HC-Pro, two vectors, pBCo-HN\(^{DF}\)I expressing HC-Pro HN\(^{DF}\)I in planta and pBCo-HN\(^{DF}\)F expressing mutant HC-Pro HN\(^{DF}\)F in planta, were constructed. By agroinfiltration, as described above, the RSS abilities of the transiently expressed HN\(^{DF}\)I and HN\(^{DF}\)F were comparable to that of WT HN\(^{DF}\)F (expressed by pBCo-HN\(^{DF}\)F). The results showed that HN\(^{DF}\)F and HN\(^{DF}\)I with reinstated nonnative \(\alpha\)H1 showed RSS abilities equivalent to the WT level (Figure 5d).

### 2.11 Potyviral HC-Pro has a functionally critical conserved FWKG motif inlaid in \(\alpha\)H1

The multiple alignments (Figures S3–S5) of potyviral HC-Pro sequences (107 in total), shown in ClustalW format (Combet et al., 2000), revealed the presence of a highly conserved four amino acid motif F\(_{93}\)-W/F\(_{98}\)-K\(_{42}\)-G\(_{86}\) (subscripts indicate percentage conservation), wherein the F residue (corresponding to TuMV HC-Pro F7) involved in RSS and aphid transmission is the first constituent residue. Considering the predominance of W over F as the second residue and K as the third residue, the motif is called the FWKG motif. The combination of the FWKG motif and \(\alpha\)H1 is called the FWKG–\(\alpha\)H1 element.

As shown in Table 2, minor changes (amino acid substitutions/small insertions/partial deletions) upstream of the first residue of FWKG motif or fusion of a few residues to the HC-Pro N-terminus may not significantly affect potyviral infectivity, viability, symptom expression, RSS, and aphid transmission. However, the first
F residue mutation, F-upstream total deletion (as shown in this study), F-upstream insertion of long polypeptides (such as bacterial β-glucuronidase [GUS] or GFP), and point mutations or small insertions immediately downstream of the F residue can affect HC-Pro functions of symptom expression, RSS, and aphid transmission (references in Table 2).

The FWKG motif’s second aromatic residue (W/F) is also involved in aphid transmission, as shown by the nonaphid transmissibility of tobacco etch virus (TEV) expressing F10L mutant HC-Pro and plum pox virus (PPV)-NAT expressing W8S mutant HC-Pro (Table 2). The importance of the least-conserved third residue in RSS function was indicated by the TEV HC-Pro I11L mutation that rendered the mutant HC-Pro a hypersuppressor of RNA silencing (Table 2). The fourth residue, glycine, may provide functionally important conformational freedom to the motif.

The inlay of the FWKG motif in αH1 and the same-plane occurrence (equal-interval reoccurrence) of three F (or related aromatic/hydrophobic) residues on αH1, that is, FWKG−αH1 element of potyviral HC-Pro (Figures S3–S5). Here, we provide evidence to uncover the mechanisms of previous observations, indicating that changes in this molecular arrangement or steric hindrance to its conformation affect the mechanistically independent functions of RSS and aphid transmission.

2.12 Variations in the FWKG−αH1 element affect HC-Pro self-interaction

The self-interaction ability of mutant HC-Pros with variations in FWKG−αH1 element was analysed by bimolecular fluorescence
complementation (BiFC). In BiFC, monomers of WT HC-Pro HN\textsuperscript{NF} showed strong self-interaction. In contrast, monomers of mutant HC-Pros HN\textsuperscript{NII}, HN\textsuperscript{DDI}, and ΔN10 self-interacted very weakly, as indicated by relative yellow fluorescent protein (YFP) fluorescence (Figure 6a). Fluorescence (normalized values) estimated from discs of agroinfiltrated \textit{N. benthamiana} leaf areas also confirmed weaker interaction between monomers of each mutant HC-Pro than that between WT HC-Pro monomers (Figure 6b). Figure 6c shows

| Virus name | HC-Pro ENTR condition (native or manipulated) | Manual alignment of native and manipulated HC-Pro ENTRs (manipulations in or upstream of FWKG–αH1 element makes the alignment imperfect towards N-termini) | Phenotype | References |
|------------|---------------------------------------------|---------------------------------------------------------------|-----------|------------|
|            |                                             | Symptom/HC RSS ability | Aphid Trans |
| TaMV       | WT                                          | SAGAAN–FWKGPDRCFLAYRSD–NRE–H\textsuperscript{C} | WTS      | AT         | Present study |
|            | F7I                                          | SAGAAN–FWKGPDRCFLAYRSD–NRE–H\textsuperscript{C} | AS        | AT         | Present study |
|            | F7I + ΔN10                                  | SAGAAN–FWKGPDRCFLAYRSD–NRE–H\textsuperscript{C} | AS        | AT         | Present study |
| ZYMV       | WT                                          | SQQFEVQ–FFQGRGMMFKPR++–SPQIVC | WTS      | AT         | Present study |
|            | F7I                                          | SQQFEVQ–FFQGRGMMFKPR++–SPQIVC | AS        | AT         | Present study |
|            | F7I + ΔN10                                  | SQQFEVQ–FFQGRGMMFKPR++–SPQIVC | AS        | AT         | Present study |
| TEV        | WT                                          | SDKSIEAFFIPYSKKLELRPDG–IS–H\textsuperscript{C} | WTS      | AT         | Torres-Bacelo et al., 2008 |
|            | I1IL                                        | SDKSIEAFFIPYSKKLELRPDG–IS–H\textsuperscript{C} | WTS/HC\textsuperscript{SS} | NK         | Torres-Bacelo et al., 2008 |
|            | F16L                                        | SDKSIEAFFIPYSKKLELRPDG–IS–H\textsuperscript{C} | WTS      | AT         | Blanc et al., 1998, 1999 |
| Yeast-expressed | Yeast-expressed | SDKSIEAFFIPYSKKLELRPDG–IS–H\textsuperscript{C} | NA       | AT         | Ruiz-Ferrer et al., 2004 |
| GUS-tagged | SDKSIEAFFIPYSKKLELRPDG–IS–H\textsuperscript{C} | SDKSIEAFFIPYSKKLELRPDG–IS–H\textsuperscript{C} | AS        | AT         | Dolja et al., 1992 |
| His-tagged | SDKnnnnhasPSDKSIEAFFIPYSKKLELRPDG–IS–H\textsuperscript{C} | SDKnnnnhasPSDKSIEAFFIPYSKKLELRPDG–IS–H\textsuperscript{C} | NA       | AT         | Blan et al., 1999 |
| LMV        | WT                                          | SDVARFNWGYSTCFM1NTPK–DIL–H\textsuperscript{C} | WTS      | AT         | Plisson et al., 2003 |
|            | His-tagged                                  | SDVphhhhhqdvARDNFWGYSTCFM1NTPK–DIL–H\textsuperscript{C} | WTS      | AT         | Plisson et al., 2003 |
|            | Nla Prot-processed                           | SDVpgggggggvARDNFWGYSTCFM1NTPK–DIL–H\textsuperscript{C} | WTS      | AT         | German-Retana et al., 2003 |
|            | GUS-tagged                                  | SDV<GGUS>ARDNFWGYSTCFM1NTPK–DIL–H\textsuperscript{C} | AS        | NK         | German-Retana et al., 2000 |
|            | GFF-tagged                                  | SDV<GGFP>ARDNFWGYSTCFM1NTPK–DIL–H\textsuperscript{C} | AS        | NK         | German-Retana et al., 2000 |
|            | ΔNT1-24 aa                                  | SDVGGGGMGGGSARDF<GGFP>ARDNFWGYSTCFM1NTPK–DIL–H\textsuperscript{C} | AS        | NK         | German-Retana et al., 2003 |
| MVV        | WT [aα8 is Trp]                             | SDGQPQFWDGFTNSNFCKLR–ETD–H\textsuperscript{C} | WTS      | AT         | Guo et al., 1998 |
|            | *GUS-tagged                                 | SDGQPQFWDGFTNSNFCKLR–ETD–H\textsuperscript{C} | Uninfective | NA         | Guo et al., 1998 |
|            | Gsp1 HC-Pro                                 | SDGQPQFWDGFTNSNFCKLR–ETD–H\textsuperscript{C} | Symptomless | NK         | Guo et al., 1998 |
| PPV        | WT [aα8 is Ser]                             | SDGQPQFWDGFTNSNFCKLR–ETD–H\textsuperscript{C} | WTS      | AT         | Varellmann et al., 2007 |
|            | Insertion of pentapeptide egrid            | SDGQPQFWDGFTNSNFCKLR–ETD–H\textsuperscript{C} | HC\textsuperscript{RD} | NAT         | Varellmann et al., 2007 |

Note: All the secondary structural analyses and mechanistic interpretations were done in the present study. The α-helices of FWKG–αH1 elements are highlighted in light brown (HC-Pros carrying larger insertions were not analysed for secondary structures); mutations/natural variations in FWKG motifs shown in red; manipulated regions highlighted olive-green/corresponding residues in lower case; histidine (H) and cysteine (C) residues initiating C-rich domain highlighted grey; deleted residues indicated by Δ; *GUS insertion site not known.

Abbreviations: aa, amino acid; AS, attenuated symptom; AT, aphid-transmissible; HC\textsuperscript{RD}, HC-Pro hypersuppressor; HC\textsuperscript{RD}, HC-Pro RSS-defective; NA, not applicable; NAT, nonaphid-transmissible; NAT\textsuperscript{‡}, PPV\textsuperscript{NAT} FWKG motif W8S natural variation may determine nonaphid-transmissibility; NK, not known; T-I/II/III, Type I/II/III; WTS, wild-type symptom.
FIGURE 6  HC-Pro self-interaction analyses by bimolecular fluorescence complementation (BiFC) and yeast two-hybrid (Y2H) assay. (a) The N-terminal fragment enhanced yellow fluorescence protein (NEYFP)-fused and the C-terminal fragment enhanced yellow fluorescence protein (CEYFP)-fused wild-type (WT) HC-Pro or NEYFP-fused and CEYFP-fused individual mutant HC-Pro (HN^N1, HN^F, HN^N1 or ΔN10) were co-expressed in leaves of *Nicotiana benthamiana* by agroinfiltration. YFP signals were recorded at 60 h post-agroinfiltration (hpa) by confocal fluorescence microscopy. (b) Quantification of YFP fluorescence resulted from BiFC. Using ChemiDoc MP imaging system (Bio-Rad), leaf discs from the agroinfiltrated areas were photographed at 60 hpa (upper panel) and fluorescence quantified and normalized with WT (as 1.0; lower panel). In the mean ± SEM of five independent experiments, the values marked by the same letters are not significantly different (\( p < 0.05 \), one-way analysis of variance). (c) Expression levels of HC-Pro fusion proteins. Total proteins extracted from agroinfiltrated leaf areas 60 hpa were analysed by western blotting with antibody to HC-Pro. (d) Y2H assay of HC-Pro self-interaction. Empty vectors (EV) expressing activation domain (AD) or constructs individually expressing WT (HN^N1F) or mutant (HN^N1, HN^F, HN^N1 or ΔN10) HC-Pro in fusion form with AD or DNA-binding domain (BD) were co-transformed into *Saccharomyces cerevisiae* AH109 and grown on SD medium lacking Leu (L) and Trp (W) or His (H), Leu, and Trp with or without different concentrations of 3-aminotriazole. N-WT, N-terminus of YFP fused at HC-Pro N-terminus; CWT, C-terminus of YFP fused at HC-Pro C-terminus.
immunodetection of comparable expression levels of WT and mutant HC-Pro-fusion proteins.

For additional supporting data, a yeast two-hybrid (Y2H) assay was also conducted. Conforming to its in planta self-interaction behaviour, WT HC-Pro HNNF showed strong self-interaction also in yeast, while mutant HC-Pros HNNI, HND, and ΔN10 did not show self-interaction (Figure 6d). In contrast, differing from its in planta self-interaction behaviour, HND mutant showed self-interaction in yeast (Figure 6d). This discrepancy could be attributed to differences in protein folding, protein processing, or cellular factors between yeast and plant.

Overall, the results of BiFC and Y2H assays indicate that HC-Pro F7I mutation and/or aa7-upstream deletion drastically weaken HC-Pro self-interaction.

2.13 | Effects of HC-Pro F7I mutation and F7-upstream deletion verified in ZYMV

WT ZYMV, identified in this study as ZHN4F, caused severe yellowing and mosaic symptoms in squash (Cucurbita pepo var. zucchini; Figure 7a). HC-Pro F7I mutation and/or aa7-upstream-deletion in ZHN4F created mutant viruses ZHN4I, ZHN5F, and ZHN5I. While ZHN4I- and ZHN5I-infected C. quinoa plants showed inconspicuous symptoms (Figure 7a), ZHN5F-infected plants exhibited almost normal appearance.

The attenuated ZHN4I and ZHN5I viruses provided complete cross-protection in squash plants, which did not develop any severe symptoms beyond 1 month after WT ZHN4F challenge inoculation (Figure 7b).

3 | DISCUSSION

Of the three TuMV HC-Pro FWKG−αH1 element mutant viruses (Table 1), the HN5I HC-Pro-expressing TuHN5I was selected and tested for its cross-protection effectiveness because of its accumulation pattern’s similarity to those of our earlier reported cross-protective attenuated strains GAC of ZYMV (Lin et al., 2007) and Tu-GK of TuMV (Kung et al., 2014), and its genetic stability. The establishment of TuHN5I in the protected host, striking a balance with host RNA silencing machinery, was evident from its pattern of accumulation in the host: the zigzag pattern of accumulation is a feature of attenuated viruses that provide RNA-mediated cross-protection (Kung et al., 2014; Lin et al., 2007).

Intriguingly, while the HC-Pro HND was RSS-defective, HN4F and HN5I showed RSS abilities comparable to WT HC-Pro (Figure 5a,b). As shown previously by Torres-Barcelo et al. (2008), HC-Pro of a potyvirus (TEV) asymptomatic (N193L, N194D, or E452D) or mildly symptomatic (Y344S) mutant may have WT-comparable RSS ability. Moreover, mutations in HC-Pro may discriminate between miRNA and siRNA pathways (Wu et al., 2010). Hence, the differences in degrees of symptom attenuation and RSS ability of HC-Pros HN4F, HN5I, and HN5I may also be interpreted as the discriminatory effects of their variations on siRNA and miRNA pathways.

The survival of TuHN5I despite its RSS-defective or highly unstable HC-Pro may be intriguing. However, the RSS ability of a transiently expressed HC-Pro cannot be likened to that of a virally expressed HC-Pro because the latter exists not only in mature form, but also in incompletely processed intermediate form(s) and in complexes

Figure 7: Symptoms and cross-protection effectiveness of attenuated variants of zucchini yellow mosaic virus. (a) Symptoms induced by ZHN4F (wild-type [WT] virus expressed from pZHN4F) and attenuated mutant viruses ZHN4I (expressing F7I mutant HC-Pro) and ZHN5I (expressing HC-Pro with F7I mutation and aa7-upstream-deletion) in Chenopodium quinoa plants 5 days postinoculation (dpi) and squash (Cucurbita pepo var. zucchini) plants 15 dpi. (b) Cross-protection effectiveness of the above two attenuated viruses in squash plants. Plants preinfected with attenuated viruses and subsequently challenge-inoculated with severe ZHN4F were protected entirely, as recorded 15 days after challenge inoculation.
with other viral proteins. Moreover, in potyviruses, the viral genome-linked protein (VPg) plays an auxiliary role in RSS (Rajamäki & Valkonen, 2009) by interacting with the host RNA silencing-related protein SG3 (Rajamäki et al., 2014; Cheng & Wang, 2017).

The genetic stability of TuHN I may be because of the complexity of variation, that is, the combined presence of a point mutation and a deletion in the FWKG–αH1 element. Our experiments with ZYMV in this study and other ongoing experiments with papaya ringspot virus (PRSV) and East Asian Passiflora virus (EAPV; data not shown) also corroborate this idea. The mutant HC-Pro HN F, HN I, and HN I, which were equally weaker in self-interaction than the WT HC-Pro, did not support aphid transmission of mutant viruses. The additional properties of genetic stability and nonaphid transmissibility characterize the mutant virus TuHN I as a safe and reliable cross-protective virus.

Although RNA silencing is the principal mechanism of cross-protection provided by TuHN I, the extremely weak self-interaction of HC-Pro HN I may underlie a protein-mediated mechanism. Findings suggesting the involvement of the N-terminal region of HC-Pro in self-interaction (Urcuqui-Inchima et al., 1999a, 1999b) and dissecting interpretations negating its role in self-interaction (Guo et al., 1999; Plisson et al., 2003; Ruiz-Ferrer et al., 2005; Zheng et al., 2011) led to an implicit belief in species-specific differences in HC-Pro self-interaction domains. However, obvious differences in the context and extent of deletions inflicted on HC-Pro, and several other important differences in experimental conditions were not given serious consideration. Hence, it is logical to assume that the observed species-specific differences in the HC-Pro self-interaction domain might be largely due to possible exclusion, by larger deletions, of certain important elements from the biologically relevant HC-Pro self-interaction surface(s) and/or exposure of certain structure-maintaining hidden binding potentials.

In the present study, a point mutation and a small deletion, which respectively caused a sequence and a structural variation in the FWKG–αH1 element, drastically decreased the self-interaction ability of TuMV HC-Pro (Figure 6). Notably, the observed involvement of the FWKG–αH1 element in HC-Pro self-interaction does not appear to be species-specific. The N-terminal amino acids 1-22 (STGDVFWRGFLNRTFLENKPINL) deletion from potato virus A (PVA) HC-Pro, which rendered the deletion mutant protein (HCΔBX) significantly weaker in self-interaction (Guo et al., 1999), strongly corroborates our finding. The lack of infectivity of tobacco vein mottle virus (TVMV) with HC-Pro N-terminal amino acids 5-19 deletion (Atreya & Pirone, 1993) and attenuation of lettuce mosaic virus (LMV) due to HC-Pro N-terminal amino acids 1-24 deletion (German-Retana et al., 2000) may also be because of the abolition of HC-Pro self-interaction, because these regions contain the respective FWKG–αH1 elements (Table 2).

It is now understood that the earlier reported symptom attenuation and abolition of the aphid transmissibility of potyviruses due to the insertion of GUS/GFP (details/citations in Table 2) upstream of the FWKG–αH1 element are because of a possible steric hindrance to the conformational freedom of the FWKG–αH1 element. Interestingly, the earlier reported abolition of the RSS function of PPV-NAT HC-Pro by the pentapeptide CGRRD insertion (Varrelmann et al., 2007) that destroyed αH1 and split the FWKG motif (Table 2) now provides evidence for the crucial role of the FWKG–αH1 element in RSS function. Moreover, F101 mutation in TEV HC-Pro, which rendered the mutant virus nonaphid-transmissible (Blanc et al., 1998), and I111L mutation in TEV HC-Pro, which enhanced the RSS ability of the protein (Torres-Barcelo et al., 2008), further reflect the roles of the FWKG–αH1 element in these mechanistically independent functions.

Urcuqui-Inchima et al. (1999a) demonstrated that the N-terminal 83 amino acid region was sufficient for potato virus Y (PVY) HC-Pro self-interaction and that the highly conserved C25, C53, and H23 within the C-rich putative metal-binding domain (originally proposed by Robaglia et al., 1989) were involved in HC-Pro self-interaction. The present multiple alignments suggest the presence of a HXXC27/CX2C zinc finger in HC-Pro of most potyvirus species (Figures S3 and S4), corroborating an earlier suggestion based on mutational analysis of TVMV HC-Pro (Atreya & Pirone, 1993), and also modified zinc fingers in a few species (Figure S5).

The presence of j51 (whose folding may be affected by the integrity of αH1; Figure S2) at the start of the C-rich domain (Figures S3-S5) appears to define the FWKG–αH1 element as a distinct plane of the HC-Pro self-interaction domain that functions in concert with the downstream C-rich domain in a cooperative manner. Loss of function of either of these sites appears to abolish HC-Pro self-interaction. This unified mechanism of HC-Pro self-interaction, involving the FWKG–αH1 element, which most likely applies to all potyvirus species, should be tested with other potyviruses.

In this study, we deduced an intriguing association between attenuated symptoms/declined RSS and nonaphid transmissibility (Table 2 and Figure 8 references). Scrutiny of the HC-Pro N-terminal region’s C-rich domain reveals a seemingly undissectable interspersion or unity of residues involved in HC-Pro self-interaction and those involved in aphid transmission, RSS, and symptom expression; this situation is far more intricate in the FWKG–αH1 element (Figure 8). In the C-rich domain, the residues H23/24, C25/26, C53/54, and C56/57 (numbering, PVY/TVMV HC-Pro) suggested (Urcuqui-Inchima et al., 1999a) or deduced (Atreya & Pirone, 1993) to be involved in Zn2+ coordination may not be definitively studied for any viral trait because their mutations render the virus extremely inviable, nondetectable, and symptomless (Atreya & Pirone, 1993), possibly because of extreme weakening of RSS. Moreover, almost all the transmission-affecting residues (Atreya & Pirone, 1993; Llave et al., 2002), present exclusively in the C-rich domain, may be involved in folding-assisted positioning of the aforementioned H and C residues to assume a conformation favourable to the formation of the HCCC zinc finger. These facts and the RSS function’s lack of dependence on HC-Pro self-interaction imply that the FWKG–αH1 element and C-rich domain of a HC-Pro monomer involved in transmission-determining interaction with a second HC-Pro monomer may also interact with a host protein, in a mutually exclusive manner, to perform the RSS function.

HC-Pro’s different transition paths to bind various partner proteins may initiate from pathophysiologically induced distinct
conformational states of the FWKG−αH1 element and spread differentially to downstream sites of HC-Pro functions. The variants of the FWKG−αH1 element shown in Table 2 may simulate different pathophysiological induced conformational states of the WT FWKG−αH1 element favourable for different HC-Pro functions. The FWKG−αH1 element may have functional similarity to the primate tau protein's N-terminal 11-residue-motif regulating tau protein differential protein–protein interaction (Stefanoska et al., 2018). The FWKG−αH1 element’s influence on the spatiotemporally separated, mechanistically independent, functions of aphid transmission and RSS probably relies on its possible functioning as a conformational switch regulating differential protein–protein interactions of HC-Pro. That the conserved H residue (H25 in TuMV) in or preceding βS1 (Figures S3–S5) of potyviral HC-Pro is possibly a partly buried protonation site mediating such a conformational switch (Narayan & Naganathan, 2018; Schönichen et al., 2013) is yet to be verified.

TuMV HC-Pro intermonomeric disulphide bond formation (oxidation) during transmission activation (Berthelot et al., 2019a, 2019b) may be under possible H25 protonation-mediated pH-sensitive regulation.

4 EXPERIMENTAL PROCEDURES

4.1 Mutants of WT virus TuMV-RC4: construction, nomenclature, and symptom analysis

WT TuMV-RC4 (Chen et al., 2003) is identified in this study as TuHN²F for nomenclature uniformity with mutants (Table 1). Full-length cDNA of TuHN²F in pTuHN²F (constructed as described for TuMV-YCS/p35SYC5; Chen et al., 2007) was driven by a CaMV 35S promoter to generate WT TuHN²F in planta (Figure 1a). To generate pTuHN²F (an exogenous protein-expression vector),
pTuHN^{D\text{F}} was modified in the HC-Pro extreme N-terminal region (ENTR) by incorporating Ncol and NheI sites, and a context encoding an Nla-protease recognition site. The construct pTuHN^{D\text{F}} generated in planta a mild mutant virus TuHN^{D\text{F}} that expressed an aa7-upstream-deleted HC-Pro. During pTuHN^{D\text{F}} construction, we also obtained pTuHN^{P\text{I}}, which generated a highly attenuated virus TuHN^{P\text{I}} expressing an aa7-upstream-deleted HC-Pro with F7I mutation.

To assess the F7I mutation’s individual contribution to TuHN^{P\text{I}} attenuation, we constructed pTuHN^{P\text{I}}, which generated in planta the mutant virus TuHN^{P\text{I}} expressing an F7I mutant HC-Pro (Figure 1a).

For symptom analyses, pTuHN^{D\text{F}}, pTuHN^{P\text{I}}, pTuHN^{P\text{F}}, or pTuHN^{P\text{I}} constructs were mechanically inoculated into C. quinoa plants. The viral progenies from C. quinoa plants were used for infecting N. benthamiana and B. campestris subsp. chinesis plants.

### 4.2 | Genetic recombination analysis for determinants of TuHN^{P\text{I}} attenuation

To ascertain whether HC-Pro F7I mutation and aa7-upstream-deletion determined TuHN^{P\text{I}} attenuation, the Ncol-KpnI fragments of HC-Pro coding regions of pTuHN^{P\text{I}} and pTuHN^{D\text{F}N\text{co}} (intermediate construct carrying WT virus cDNA with an engineered Ncol site) were exchanged to obtain the recombinants pTuHN^{P\text{I}}-S and pTuHN-N\text{co}-M. The recombinants were analysed for symptoms.

### 4.3 | Genetic stability of attenuated virus TuHN^{P\text{I}}

N. benthamiana plants were inoculated with sap from a single lesion of TuHN^{P\text{I}}-infected C. quinoa leaf. Then serial passaging of viruses from infected plants to fresh uninfected plants was performed at 10-day intervals. After 10 serial passages, the stability of TuHN^{P\text{I}} was checked by RT-PCR and sequencing.

### 4.4 | Analysis of cross-protection effectiveness

The cross-protection ability of attenuated TuHN^{P\text{I}} was analysed on N. benthamiana and B. campestris subsp. chinesis by three independent experiments. The upper two fully expanded leaves of plants at the three to four fully expanded leaf stage were mechanically inoculated with sap from lesions of TuHN^{P\text{I}}-inoculated C. quinoa plants. After 10 days, TuHN^{P\text{I}}-inoculated or buffer-inoculated plants were challenge-inoculated with WT severe virus TuHN^{N\text{F}} or its recombinant TuMV-RC4-GFP on three upper fully expanded leaves and kept in a temperature-controlled greenhouse (23–28°C). Fourteen days after challenge-inoculation, plants were analysed by western blotting for GFP or CP with 1000-fold diluted anti-GFP (Hsu et al., 2004) or anti-TuMV-RC4 (Chen et al., 2003) antiserum. RT-PCR was done with CPN-u (5‘-TAAAGAACCACTGACCTCAAGA-3‘) and CPN-d (5‘-TGCCCTTTCTTGAGCGCCA-3‘) primers corresponding to viral genomic regions flanking the GFP ORF.

### 4.5 | Accumulation of mutant viruses

Accumulation of mutant viruses in host plants was determined by indirect ELISA, following a standard protocol (Lin, 2015). Leaf disks of 6 mm diameter from three upper fully expanded leaves of infected test plants were collected on different sampling days. Leaf disks were collected from four plants on each sampling day. In indirect ELISA with extracts from 0.1 g leaf disks, the primary antiserum to TuMV-RC4 (Chen et al., 2003) in 1:5000 dilution and the secondary antibody, that is, alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin antibody (Gene Tex, Inc.), in 1:5000 dilution was used. ELISA readings were recorded by measuring the absorbance at 405 nm with a microplate reader (Multiskan FC; Thermo) after 30–60 min of colour development. WT virus-inoculated and buffer-inoculated plants were used as positive and mock controls, respectively.

### 4.6 | Aphid transmission tests

The aphid transmissibility of WT and mutant viruses was tested. Nonaphid-transmissible CP DAG-motif-deleted virus TuCP^{ΔDAG} (constructed by site-directed mutagenesis) was used as a negative control. Each virus (maintained in N. benthamiana) was mechanically introduced into B. campestris subsp. chinesis plants. At 7 and 10 dpi, infected plants were identified by ELISA with TuMV-RC4 antiserum and the levels of accumulation of viruses were recorded. For transmission tests, apterous aphids (Myzus persicae, raised on healthy N. benthamiana plants) were starved for 2 h and transferred onto leaves of infected B. campestris plants. After a 2-min acquisition access period, 10 aphids were transferred to each healthy B. campestris seedlings at the 2–true-leaf stage. Following overnight feeding, aphids were killed and plants were maintained in a greenhouse. On the 10th day, plants were tested by ELISA with anti-TuMV-RC4 antiserum.

### 4.7 | Analysis for RSS activity of WT and mutant HC-Pros

WT (HN^{N\text{F}}) and mutant (HN^{P\text{I}}, HN^{P\text{F}}, and HN^{P\text{D}}) HC-Pro cDNAs were amplified from appropriate templates with specific primers (Table S1). After AscI-BamHI digestion, cDNAs were cloned into pBCo to construct pBCo-HN^{N\text{F}}, pBCo-HN^{P\text{I}}, pBCo-HN^{P\text{F}}, and pBCo-HN^{P\text{D}}.

Each construct was agroinfiltrated into leaves of N. benthamiana in combination with GFP-expressing pBA-GFP and GFP-silencing pBA-GFI constructs (Huang et al., 2015). In the negative control reaction, the pBCo construct carrying HC-Pro cDNA was replaced by pBCo devoid of HC-Pro cDNA (empty vector). In the positive control
reaction, additionally, a pBA construct carrying a cDNA expressing a heterologous viral RSS protein, that is, WSMoV NSs protein (Huang et al., 2015) or “ns” (KFTMHNQIF)-tagged (Cheng et al., 2013) CymRSV P19 protein (Pantaleo & Burgýán, 2008), was included. At different time points, RSS activities were recorded. From RNAs and proteins extracted from areas of GFP fluorescence, HC-Pro RNA and proteins were detected by northern hybridization with a [α-32P]-labelled HC-Pro probe and western blotting with antibody to HC-Pro, respectively (Chen et al., 2003). NSs protein and nss-tagged P19 protein were detected by NSscon monoclonal antibody (Chen et al., 2006).

### 4.8 Secondary structural analysis

Secondary structural analyses of HC-Pros were performed by the SYMPRED consensus method (Simossis & Heringa, 2004): for α-helix predictions, combined results from PSIPred, YASPIN, SSPro2.01 and PROFsec methods were considered; for β-strand predictions, results from YASPIN method were considered (Lin et al., 2005).

### 4.9 Reinstatement of nonnative αH1 in HC-Pro

Following identification of HC-Pro αH1 and its disruption by aa7-upstream-deletion, pTuHN5F and pTuHN6I constructs were created from pTuHN3I by site-directed mutagenesis (primer details in Table S2). These constructs generated TuHN5F and TuHN6I viruses expressing HN5F and HN6I HC-Pros, respectively, with non-native αH1. To verify functioning of nonnative α-helices, HN5F and HN6I cDNAs were amplified (template/primer details, Table S1) and cloned into pBCo to generate pBCo-HN5F and pBCo-HN6I, respectively, and RSS activities were analysed as described above.

### 4.10 Sequence analysis of HC-Pros

Multiple sequence alignment of HC-Pro sequences was done using the ClustalW alignment tool (Combet et al., 2000). The sequence logo display of the alignment was made using the sequence logo generator WebLogo (Crooks et al., 2004).

### 4.11 Analysis of TuMV HC-Pro self-interaction by BiFC assay

The TuMV HC-Pro cDNA region encoding the WT or each of the different mutant HC-Pro proteins was amplified using specific primers from respective full-length infectious cDNA constructs of WT or mutant TuMV. The amplified products were cloned into the pENTR/D vector (Invitrogen) to generate pENTR/THC-Pro, and then transferred to pBA-DC-NEYFP and pBA-DC-CEYFP (Huang et al., 2020) by LR clonase (Invitrogen) to generate the binary vectors pBA-THC-Pro-NEYFP and pBA-THC-Pro-CEYFP expressing HC-Pro-NEYFP (N-terminal fragment enhanced YFP) fusion protein and HC-Pro-CEYFP (C-terminal fragment enhanced YFP) fusion protein, respectively. Overnight culture of A. tumefaciens ABI harbouring pBA-THC-Pro-NEYFP or pBA-THC-Pro-CEYFP was diluted 50-fold in Luria-Bertani (LB) medium with 10 mM 4-morpholineethanesulfonic acid (MES) and 0.5 mM acetylseringone, and grown at 28°C up to an optical density of 1.0 at 600 nm. The cells were resuspended in 10 mM MgCl2 with 1 mM acetylseringone and incubated at room temperature for 3 h before agroinfiltration. At 2 dpa, the agroinfiltrated leaf tissues were examined for regenerated EYFP fluorescent signals by Fluoview FV1000 confocal laser scanning microscopy (Olympus). Using the BIO-RAD ChemiDoc MP Imaging System, leaf discs from three independent agroinfiltrated leaves were analysed, and fluorescence was quantified and normalized to WT. Five independent experiments were conducted.

### 4.12 Analysis of TuMV HC-Pro self-interaction by Y2H assay

Y2H assay was performed as described by Huang et al. (2015). The WT and different mutant HC-Pro coding sequences were amplified from appropriate cDNA constructs and inserted into the yeast GAL4-binding domain vector pGBK7T7 and the GAL4-activation domain vector pGADT7 (Clontech Laboratories Inc.). The required pairs of plasmids were used for cotransforming the yeast reporter strain AH109 following the LiAc/SS-DNA/PEG transformation method. The transformants were grown for 3 days at 30°C on synthetic dropout (SD) medium lacking Leu and Trp, and subsequently transferred to SD medium lacking His, Leu, and Trp and containing 5 mM 3-aminotriazole (3-AT) to check binding activity.

### 4.13 Generation cDNA constructs for ZYMV mutant viruses

The cDNA constructs for ZYMV mutant viruses expressing F7I mutated and/or aa7-upstream-deleted HC-Pros were generated by modifying pZHN6F (synonym p35SZYMV2-26) carrying full-length cDNA of a ZYMV severe strain under the control of a CaMV 35S promoter (Hsu et al., 2004) by site-directed mutagenesis, as described above.

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CONFLICT OF INTERESTS
The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS
J.A.J.R. and S.D.Y. conceived the idea, designed the experiments, and interpreted the results. S.D.Y. guided and supervised the project. J.A.J.R. performed sequence analyses and interpreted the data. C.H.H., C.C.C., W.C.H., H.W.C., P.G., C.H.C., and Y.R.T. performed the experiments. J.A.J.R. and S.D.Y. wrote the manuscript.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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