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Molecular characterization of pea seed-borne mosaic virus and the RNA silencing suppression mechanism of HC-Pro

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Abstract

Pea seed-borne mosaic virus (PSbMV) is a significant pathogen that affects the yield and quality of leguminous crops. In recent years, PSbMV has emerged as the dominant viral threat to legume cultivation in Yunnan Province, China. In this study, the complete genome of PSbMV-YN isolate (GenBank accession: PP746839) with a length of 9919 nucleotides excluding the poly(A) tail was obtained by segmented amplification combined with rapid amplification of cDNA ends (RACE). Phylogenomic analysis of full-length polyprotein-encoding nucleotide and predicted amino acid sequences revealed that PSbMV-YN clusters within the P1 pathotype clade. Genetic mapping of local pea varieties demonstrated *elF4E*, a gene tightly linked to the *sbm1* locus, as the susceptibility genotype, revealing the mechanistic basis for the prevalence of the P1 pathotype in Yunnan pea populations. Further analysis of the highly variable region of the *P1* cistron in PSbMV-YN indicates that the P1 protein could be classified as Type A, which generally lacks RNA silencing suppressor activity. Using a GFP suppression assay, we further validated the RNA silencing suppressor activity of HC-Pro in both *Nicotiana benthamiana* and its natural host—*Pisum sativum* L. Additionally, immunoprecipitation sequencing (IP-seq) revealed that HC-Pro binds to small RNAs of variable lengths, thereby effectively inhibiting both local and systemic RNA silencing to promote viral infection. This study reports the first genomic sequence of a Chinese isolate of PSbMV and provides a comprehensive analysis of interactions between HC-Pro with endogenous small RNAs.

Keywords PSbMV, *Pisum sativum* L., *elF4E*, HC-pro, Immunoprecipitation sequencing

Background

Pea (*Pisum sativum* L.), a member of the *Fabaceae* family and *Fabaceae* subfamily, is an annual or biennial cool-season climbing herbaceous plant and one of the earliest crops domesticated by humans. Peas are characterized by a short growing period, strong adaptability, and high genetic diversity, making them an important economic and food crop on a global scale (Smykal et al. 2012; Tayeh et al. 2015). They are rich in nutritional elements such as protein, minerals, and vitamins, and possess medicinal values including anti-inflammatory properties, modulation of gut microbiota, and the ability to lower blood sugar and lipid levels (Ndiaye et al.

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2012; Rungruangmaitree and Jiraungkoorskul 2017). Additionally, due to their cold tolerance and soil adaptability, peas are often used as a cover crop in agricultural practices, thereby playing a significant role in promoting soil improvement, efficient utilization of fertilizers, and sustainable agricultural development (Badaruddin and Meyer 2001; Grimaud et al. 2013; Zhang et al. 2020). However, due to limited disease resistance and changes in cultivation and environment, pathogens including viruses usually cause serious diseases in peas (Swanson and MacFarlane 1999; Fowkes et al. 2021).

Pea mosaic disease caused primarily by PSbMV is a significant threat to the yield and quality of leguminous crops. PSbMV was first reported in Japan in 1967 (Inouye 1967). Since then, the virus has been reported in several regions of the United States, including California, Idaho, Washington, Maryland, New York, Oregon, Vermont, Wisconsin, and North Dakota (Mink et al. 1969; Beck et al. 2018). In the late 1980s, the virus was also detected in Sichuan and Jiangsu Provinces in China. Recent surveys have revealed a high detection rate of PSbMV in legume cultivation areas in Yunnan Province, where it has emerged as the dominant viral pathogen. Notably, there has been an absence of comprehensive report or analysis concerning the full genomic sequences of PSbMV isolates in China, and the pathogenic strain types endemic in China also remains unclear. This lack of clarity has hindered the development of precise, targeted control strategies, leading to recurring outbreaks of PSbMV in specific legume cultivation regions.

PSbMV belongs to the genus *Potyvirus* in the family *Potyviridae*. The virus particles are filamentous, non-enveloped, and exhibit helical symmetry structure. Each virion contains a positive single-stranded RNA approximately 9.9 kilobases (kb) in length (Makkouk et al. 2012). The RNA genome contains a large open reading frame (ORF) spanning 9618 nucleotides, predicted to encode a polyprotein with a molecular weight of approximately 364 kDa (364 K). This ORF is flanked by a 5' untranslated region (UTR) consisting of 143 nucleotides and a 3' UTR comprising 163 nucleotides excluding the poly(A) tail (Johansen et al. 1991). This polyprotein is subsequently cleaved proteolytically into various functional proteins by virally encoded proteases (Makkouk et al. 2012). Additionally, this ORF could be prematurely terminated via transcriptional slippage mechanism, leading to the generation of a smaller ORF. This ORF could encode a polyprotein of P1-HCPro-P3NPIPO (Johansen et al. 1991; Sun et al. 2024). PSbMV is primarily categorized into three pathogenic strains, namely P1, P2, and P4, which correspond to four different resistance genes in peas: *sbm1*, *sbm1¹*, *sbm2*, *sbm3*, and *sbm4*. Among these, *sbm1* and *sbm1¹* confers resistance to the strain P1, *sbm2* and

sbm3 to the strain P2, and *sbm4* to the strain P4 (Provvidenti and Alconero 1988; Gao et al. 2004a, 2004b; Makkouk et al. 2014). In addition, PSbMV also includes the P3 pathogenic strain, which mainly infects broad beans and certain pea varieties (Torok and Randles 2007). To combat different pathogenic strains, the introduction of corresponding resistance genes can be employed to prevent disease and yield loss caused by PSbMV infection in peas. However, the lack of complete genomic assembly for Chinese PSbMV isolates and lack of an analysis of their pathogenic type prevents us from selecting the appropriate resistance genes for disease control.

In plants, RNA silencing is a crucial antiviral mechanism that promotes the degradation of viral RNA, thereby inhibiting viral infection. However, in the ongoing "arms race" with plants, viruses have also evolved multiple strategies to counter plant RNA silencing processes (Ismayil et al. 2018; Yang et al. 2020; Wang et al. 2021; Zhao et al. 2021). For example, viruses encode silencing suppressors that interfere with the RNA silencing pathway, thus facilitating viral infection (Ding 2010; Jiang et al. 2012; Wang et al. 2012; Guo et al. 2019; Zhu et al. 2024). Silencing suppressors in several potyviruses (members of the genus *Potyvirus*) have been identified, such as tobacco etch virus (TEV), turnip mosaic virus (TuMV), and potato virus A (PVA) (Anandalakshmi et al. 1998; Kasschau et al. 2003; De et al. 2020). While the HC-Pro protein is widely recognized as a key silencing suppressor within potyviruses (Shiboleth et al. 2007), other viruses in the family *Potyviridae*, such as Triticum mosaic virus (TriMV) and Sweet potato mild mottle virus (SPMMV), employ a different mechanism. Despite their genomic similarity to potyviruses, these viruses rely on the P1 protein as the primary silencing suppressor instead of HC-Pro (Giner et al. 2010; Tatineni et al. 2012). This highlights the functional diversity of silencing suppressors within the *Potyviridae* family. Although previous studies have demonstrated that these suppressors can bind to small RNAs to suppress RNA silencing (Shiboleth et al. 2007), our current understanding of their interactions with endogenous small RNAs remains incomplete and lacks a comprehensive perspective.

In this study, legume plants suspected of being infected with PSbMV from Yunnan Province in 2023 were subjected to detection, confirming the presence of PSbMV in the pea cultivation areas. Further, we cloned the full genome of the PSbMV isolate from China (PSbMV-YN) for the first time using a combination of segmented amplification and RACE methods. Subsequent sequence identity and phylogenetic analysis against the full nucleotide and amino acid sequences of the polyprotein of PSbMV isolates from the GenBank database elucidated the pathogenic strain type of the PSbMV-YN isolate.

Furthermore, HC-Pro was identified as the RNA silencing suppressor of PSbMV-YN. HC-Pro randomly binds to small RNAs, effectively inhibiting local and systemic RNA silencing activity. This mechanism enables the virus to counteract the host's antiviral RNA silencing, thereby facilitating viral replication and systemic spread.

Results

Symptoms observation and detection of PSbMV-YN in the pea plants

Pea seeds suspected of being infected with PSbMV were collected from Yunnan. As shown in Fig. 1a, the virus-free healthy seeds are smooth and pale yellow, whereas seeds infected by PSbMV are smaller, wrinkled, and brown. Seedlings developed from the virus-free seeds grow normally (Fig. 1b 1/3), while those from the virus-infected seeds exhibit stunted growth and abnormal morphology (Fig. 1b), indicating that PSbMV-YN is transmitted through seeds. To detect whether theses pea seeds were infected by PSbMV, four pairs of primers (Additional file1: Table S1) were designed based on the complete genome sequences of PSbMV isolates registered in the National Center for Biotechnology Information (NCBI) database. After sowing, the seedlings of

the pea plants were tested. The results showed that the expected size fragments were amplified with all four primer pairs in the diseased plants but not in healthy plants, thus confirming PSbMV infection in diseased plants (Fig. 1c).

After segmental amplification of the genome of the PSbMV-YN isolate, the complete sequence was determined to be 9919 nucleotides (excluding the 3'-polyadenylation sequence) after assembly and correction (Fig. 1d and Additional file1: Table S2). The entire genome contains one large open reading frame (ORF). This ORF is located from nucleotides 144 to 9761 and encodes a poly-protein comprising 3206 amino acid residues (Fig. 1d). Notably, the presence of the "G₁₋₂A₆₋₇" motif within the P3 region suggests that transcriptional slippage may lead to premature termination of this ORF, resulting in the production of a smaller ORF encoding a polyprotein comprising P1-HC-Pro-P3NPIPO (Fig. 1d). The lengths of the 5' and 3' UTRs of PSbMV-YN isolate are 143 nt and 158 nt, respectively (Fig. 1d, Additional file1: Table S2). Analysis of its proteolytic cleavage sites revealed that the PSbMV-YN isolate has nine cleavage sites, i.e. FQIDHY/S, KMYRVG/G, PLFSIQ/A, QEVRCQ/S, ETVRCQ/S, EPVKLE/G, EHV RTE/A, DGVRTQ/S, and

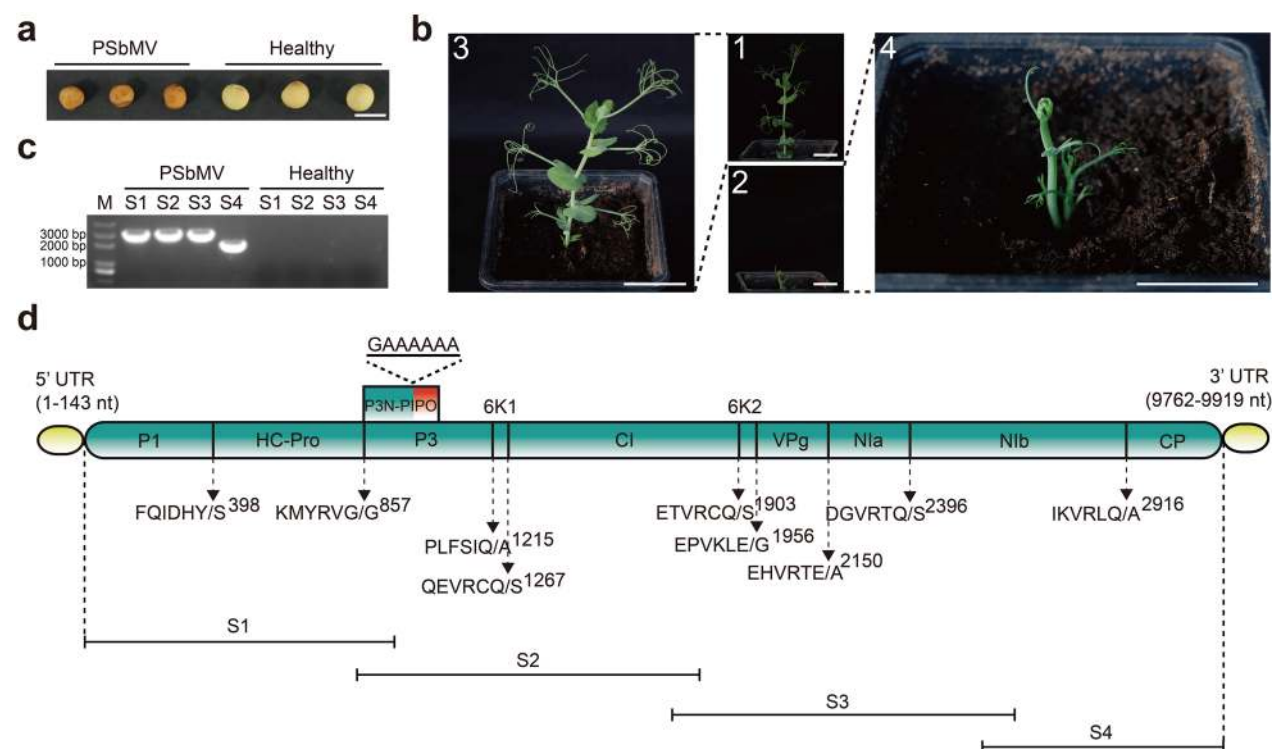


Fig. 1 Detection of PSbMV-Infected seed plants and whole genome acquisition of PSbMV. **a** Phenotypes of virus-infected and health seeds. Scale bars correspond to 1 cm. **b** Plant phenotypes corresponding to virus-infected (1) and health (2) seedlings, with detailed views of plant phenotypes of virus-infected (3) and health (4) seedlings, respectively. Scale bars correspond to 3 cm. **c** RT-PCR detection of PSbMV infected and healthy plants. **d** Assembly of PSbMV-YN genome and diagram illustrating the genome structure of PSbMV-YN

IKVRLQ/A (Fig. 1d). The specific gene locations, and their encoded proteins are detailed in Additional file 1: Table S2.

Pathotype classification and phylogenetic analysis based on full-length genomic sequences

Previous studies relying on partial genomic sequences (e.g., single-gene or CP-based analyses) have faced limitations in resolving viral strain classifications, as truncated datasets may fail to capture critical recombination events or evolutionary signatures (Adams et al. 2005; Gibbs and Ohshima 2010). To address these constraints, we performed sequence identity and phylogenetic analyses using full-length nucleotide and amino acid sequences of the polyprotein and coat protein (CP) of PSbMV-YN (Fig. 2a, b, Additional file 2: Figure S1). Comparative analysis of PSbMV genomes from GenBank revealed divergent sequence identities (80.7–98.8% nucleotide; 88–99% polyprotein amino acid) across isolates (Fig. 2, Additional File 1: Table S3), underscoring substantial genetic diversity and supporting the designation of distinct pathotypes. Notably, the PSbMV-YN isolate demonstrated the highest sequence identity (95.8%–99%) with isolates from the known P1 pathotype isolates (KU870637, MK116871–MK116873, and D10930) at both the nucleotide and amino acid sequence levels (Fig. 2, Additional file 1: Table S3), conclusively classifying it within the P1 pathotype.

Phylogenetic reconstruction of polyprotein sequences (nucleotide/amino acid) and CP nucleotide sequences also supported this classification (Fig. 2c, d, Additional File 2: Figure S1d). However, CP amino acid-based phylogeny split P1 isolates into two subclades (Additional File 2: Figure S1c), aligning with prior reports that partial protein sequences may obscure strain discrimination (Adams et al. 2005). All isolates segregated into three clades: P1 (global distribution: Europe, China, India, America), P2 and P4 (geographically restricted to America/Canada) (Fig. 2c, d, Additional file 2: Figure S1c, d). PSbMV-YN clustered within P1, showing closest affinity to the Canadian isolate DSMZ PZ-0301. These results demonstrate the broader dissemination and adaptive potential of the P1 pathotype compared with regionally constrained P2/P4 clades.

Genotyping of *eIF4E* of pea varieties in Yunnan and phylogeographic association analysis

Previous studies established that the *sbm1* locus in *Pisum sativum* L. confers recessive resistance against PSbMV strain P1, with the tightly linked *eIF4E* gene encoding a cap-binding protein essential for viral susceptibility (Gao et al. 2004a, b). Comparative sequence analysis of *eIF4E* alleles between resistant (JI1405, 835) and susceptible

(JI2009, 744) pea lines identified five amino acid polymorphisms correlating with PSbMV P1 resistance: L62W, D73A/D74A, R107G, and K169N. Notably, PSbMV-YN-infected pea accessions from Yunnan Province, China, exhibited identical *eIF4E* sequences to susceptible lines JI2009 and 744 (Additional file 2: Figure S2). These findings collectively demonstrate that Yunnan pea cultivars lack functional *sbm1*-mediated resistance and are genetically susceptible to PSbMV P1 infection, likely due to the absence of resistance-associated *eIF4E* alleles.

To assess geographic structuring among PSbMV isolates, phylogeny-geography association tests were performed. Significant associations ($P \leq 0.05$) were observed for the association index (AI) and parsimony score (PS), indicating a non-random spatial distribution of genetic lineages (Table 1). In contrast, non-significant maximum monophyletic clade (MC) values ($P > 0.05$) suggested weak intra-regional lineage clustering (Table 1). Collectively, these findings imply limited geographic differentiation among PSbMV isolates under the current sampling regime.

Recombination-driven evolution

Recombination is a key driver of viral strain diversity. To investigate the potential role of recombination in the evolution of PSbMV-YN, we conducted a recombination analysis. The results revealed evidence of minor recombination events between the P1 and P2 strains at the terminal regions of the PSbMV-YN genome (Fig. 2e). These findings suggest that, while PSbMV-YN and PSbMV (D10930) are both classified as members of the P1 strain, the evolutionary process of PSbMV-YN may have involved genetic contributions from the P2 strain. Consistent with recombination hotspots, sequence identity, and divergence analyses revealed elevated genetic variability at the 3'-terminal region of the PSbMV genome (encoding CP) (Additional file 2: Figure S1b). This variability likely explains the bifurcation of P1 isolates into two subclades in CP amino acid-based phylogenies (Additional file 2: Figure S1c), further underscoring the limitations of strain classification relying solely on partial sequences.

The protein of PSbMV-YN P1 belongs to type A and lacks silencing suppressor activity

The P1 protein is the first protein encoded by viruses in the *Potyviridae* family and possesses proteolytic activity. It separates from the polyprotein through an autocleavage process (Sun et al. 2024). The C-terminal region contains a conserved protease domain, His-(X8-14)-Asp/Glu-(X31-41)-Ser, and a conserved sequence, Gly-X-Ser-Gly, surrounding the serine residue within this domain (Mao et al. 2022). The conserved protease domain of the

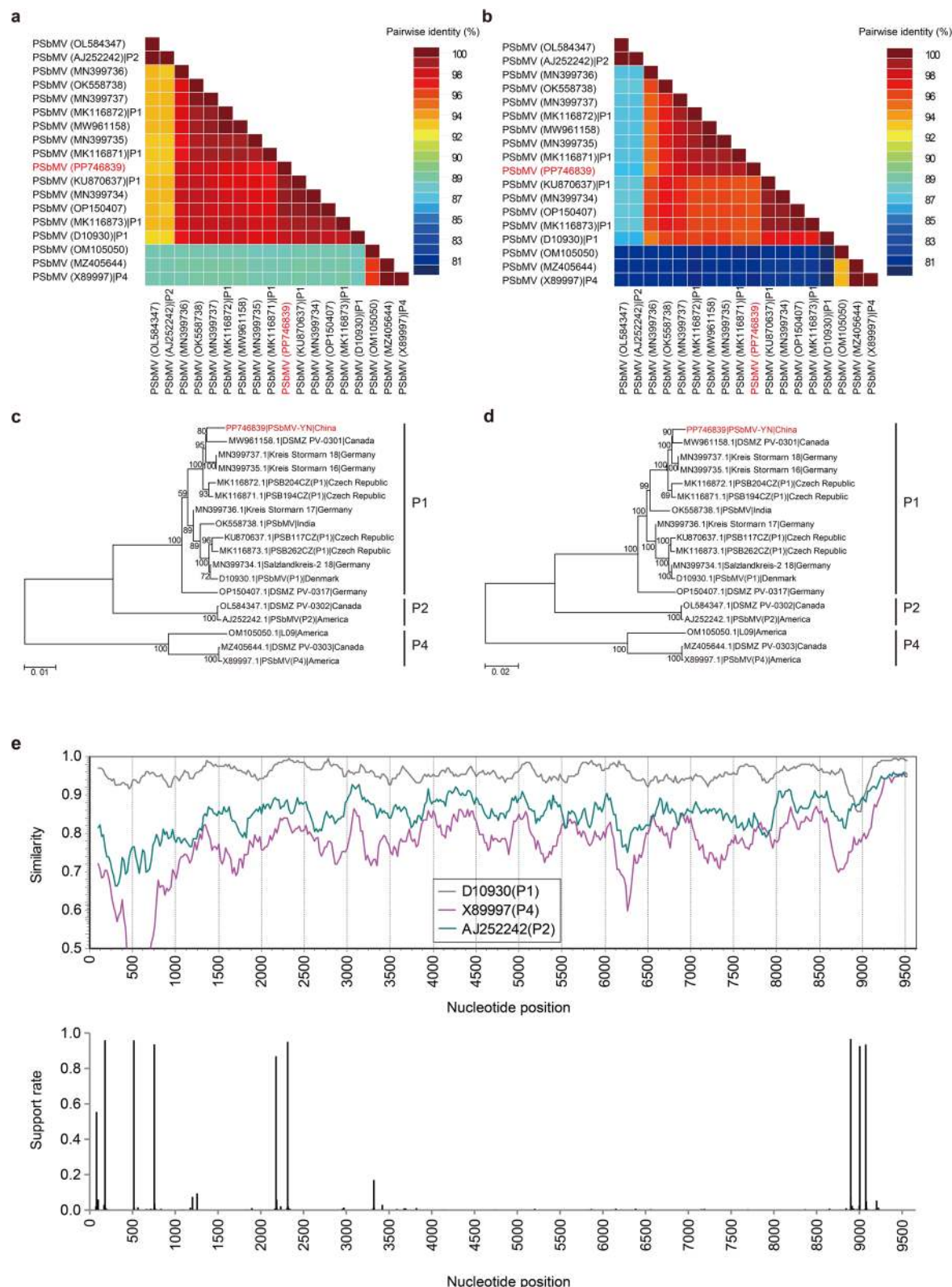


Fig. 2 Pairwise identity matrices, phylogenetic trees, and recombination analysis of the polyprotein. Pairwise identity matrices and phylogenetic trees were constructed based on the amino acids sequence of the polyprotein (**a**, **c**) and the nucleotide sequence of the polyprotein (**b**, **d**). **e** Full-genome similarity with PSbMV (D10930, X89997, and AJ252242) (upper panel) and the corresponding recombination site support rates (lower panel)

Table 1 Tests of geography-phylogeny association for complete genome in PSbMV isolates originated from different countries

Statistic	Isolates	Observed mean (95% HPD)	Null mean (95% HPD)	Significance
AI		1.28 (1.28, 1.30)	1.78 (1.29, 2.16)	0.04*
PS		9.00 (9.00, 9.00)	11.33 (10.00, 13.00)	0.01**
MC (America)	3	1.00 (1.00, 1.00)	1.10 (1.00, 2.00)	1.00 ^{ns}
MC (Others)	3	1.00 (1.00, 1.00)	1.14 (1.00, 2.00)	1.00 ^{ns}
MC (Czech Republic)	4	2.00 (2.00, 2.00)	1.35 (1.00, 2.00)	0.09 ^{ns}
MC (Germany)	5	2.00 (2.00, 2.00)	1.49 (1.00, 2.00)	0.17 ^{ns}
MC (Canada)	3	1.00 (1.00, 1.00)	1.10 (1.00, 2.00)	1.00 ^{ns}

AI, association index; PS, parsimony score; MC, maximum monophyletic clade; HPD, highest probability density interval; "Others" includes the following countries: China, Denmark and India; Significance thresholds: *0.01 < P < 0.05, **0.001 < P < 0.01, ns, not significant

P1 protein from the PSbMV-YN isolate is His-X8-Asp-X31-Ser, and the conserved sequence surrounding serine is Gly-His-Ser-Gly (Fig. 3a). A phylogenetic analysis based on the C-terminal region of P1 protein revealed that P1 proteins from all PSbMV isolates can be classified into two distinct types and the PSbMV-YN P1 protein was grouped into Type A (Fig. 3b). Previous studies have

categorized P1 proteins into Type A and Type B based on their functional diversity. Interestingly, Type A P1 proteins generally lack RNA silencing suppressor activity, while Type B P1 proteins exhibit this function (Mao et al. 2022). These findings suggest that the P1 protein of PSbMV-YN may not possess RNA silencing suppressor activity.

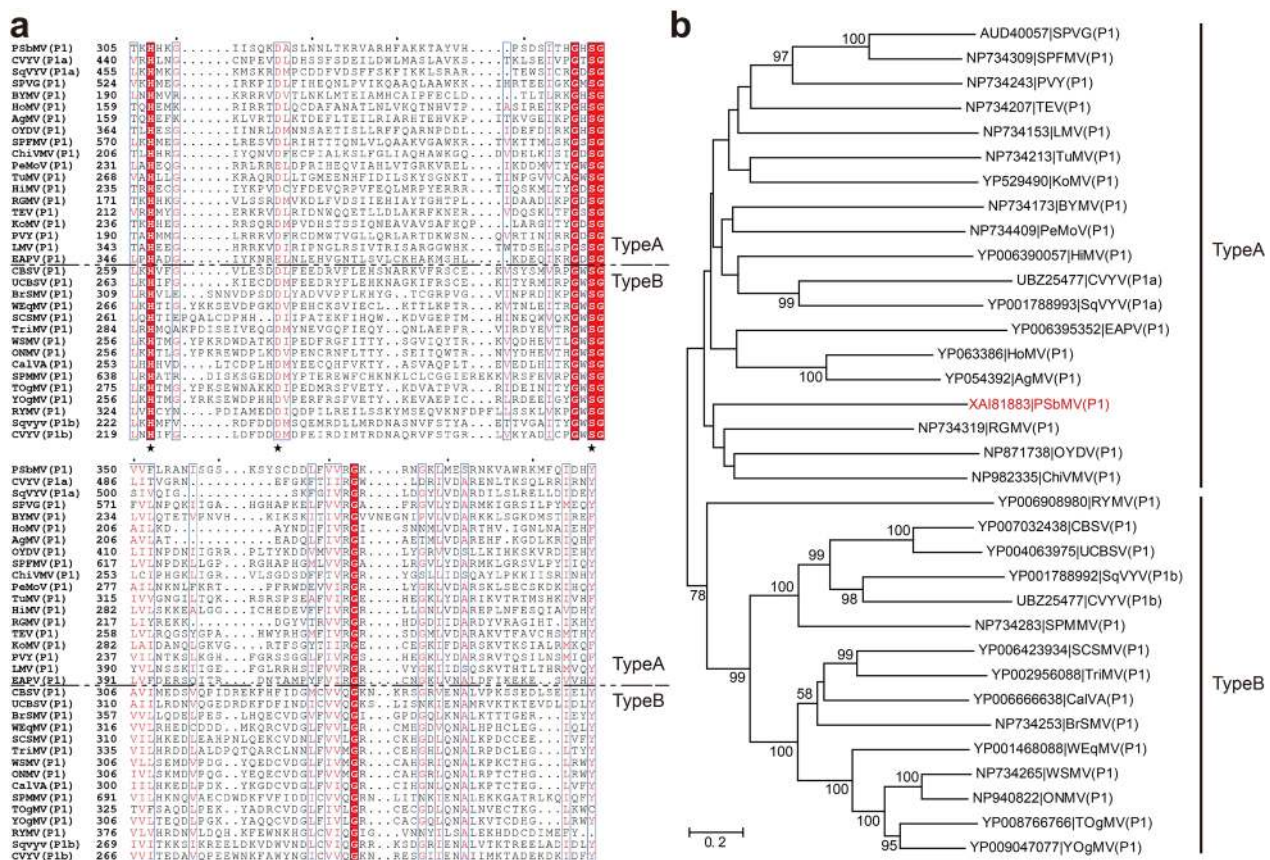


Fig. 3 Alignment and phylogenetic analysis of the conserved domain of P1 in the family *Potyviridae*. **a** Multiple alignment of amino acid sequences of the conserved serine protease domain of P1 proteins. **b** Phylogenetic analysis of amino acid sequences of the conserved serine protease domain of P1 protein

To test the RNA silencing suppressor activity of PSbMV-YN P1, GFP reporter assays were performed in both *GFP*-expressing (16c) *Nicotiana benthamiana* and *Pisum sativum* L. (natural host of PSbMV). P1, a positive control (T2b) (Li et al. 1999), and negative controls (empty vector, EV; start codon-deleted Δ P1) were co-infiltrated with GFP. In *N. benthamiana*, strong GFP fluorescence was observed at 2 days post-infiltration (dpi) in all groups (Fig. 4a). By 4 dpi, fluorescence intensity sharply declined in P1 and negative controls (Fig. 4b), with complete quenching by 7 dpi (Fig. 4c). Transcript and protein levels mirrored this trend: T2b maintained significantly higher GFP expression, whereas P1 and controls showed no differences (Fig. 4d, e). Parallel experiments in *P. sativum* yielded congruent results. At 2 dpi, robust fluorescence was detected in P1 and controls (Fig. 4f), followed by rapid decline by 6 dpi (Fig. 4g) and complete loss by 11 dpi (Fig. 4h). No significant differences in GFP transcript/protein levels were observed between P1 and controls at 11 dpi (Fig. 4i, j), confirming the absence of silencing suppressor activity in P1. In contrast, the P1/HC-Pro polyprotein exhibited potent suppressor activity in both hosts. GFP fluorescence persisted until 7 dpi in *N. benthamiana* and 11 dpi in *P. sativum* L. (Fig. 4k–t), with transcript/protein levels significantly exceeding controls. These results conclusively attribute silencing suppression to the HC-Pro domain.

PSbMV-YN HC-Pro functions as an RNA silencing suppressor to enhance viral infection

The same method was employed to verify the suppressor activity of HC-Pro. As anticipated, HC-Pro significantly inhibited the silencing of *GFP* (Fig. 5a–j) in *N. benthamiana* line 16c and *P. sativum* L. Both negative controls showed no detectable fluorescence (16c at 7 dpi and *P. sativum* L. at 11 dpi) (Fig. 5c, h), whereas the fluorescence in HC-Pro-infiltrated sites remained bright (Fig. 5c, h), confirming the RNA silencing suppressor activity of HC-Pro.

Viruses encode suppressors to counteract the host's antiviral RNA silencing mechanisms, facilitating further infection and replication, which often results in

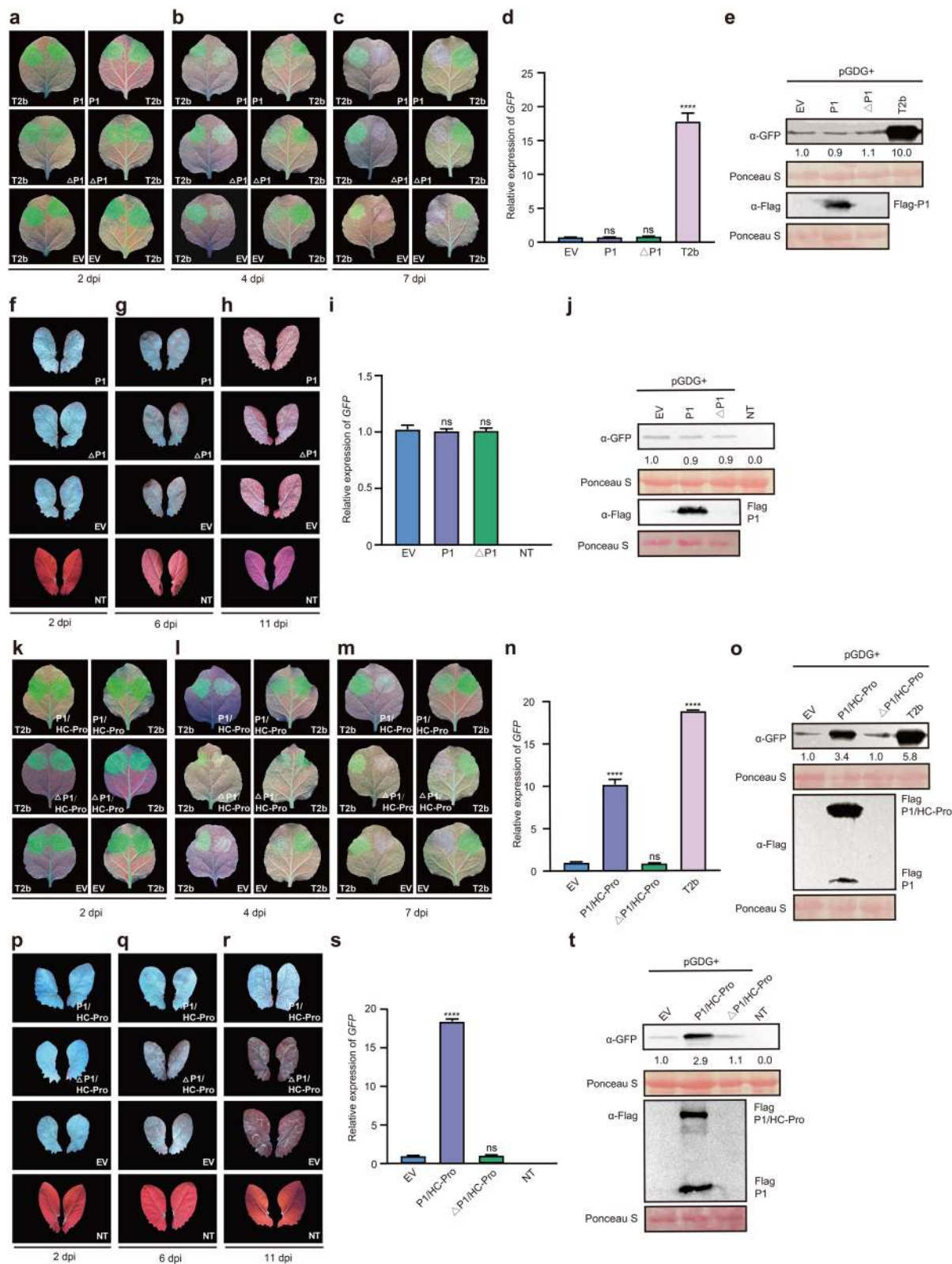
pathogenic phenotypes (Wang et al. 2012). Based on this perspective, we hypothesized that HC-Pro enhances viral infection. To experimentally test this hypothesis, HC-Pro was fused into the PVX genome, and then the HC-Pro-fused PVX construct was inoculated into *N. benthamiana*. At 10 dpi, plants inoculated with wild-type PVX exhibited only mild mosaic symptoms, whereas plants inoculated with HC-Pro-fused PVX developed both mosaic symptoms and prominent necrotic lesions (Fig. 5k). qRT-PCR analysis showed no significant differences in viral *CP* gene between the two treatments (Fig. 5l); however, the accumulation of CP protein was significantly higher in plants infected with HC-Pro-fused PVX (Fig. 5m). This result may be attributed to HC-Pro's ability to suppress small RNA-mediated translational inhibition. At 20 dpi, plants treated with the empty vector PVX still exhibited mosaic symptoms, but the symptoms were slightly improved compared with that at 10 dpi (Fig. 5k). Correspondingly, RT-qPCR and Western blot analyses showed a decrease in viral replication and accumulation (Fig. 5l, m), likely due to the activation of plant RNA silencing mechanisms. In contrast, plants inoculated with PVX-HC-Pro at 20 dpi exhibited more severe mosaic symptoms and persistent necrotic lesions (Fig. 5k). RT-qPCR and Western blot analyses demonstrated significantly higher levels of PVX accumulation compared to 10 dpi (Fig. 5l, m). These findings indicate that HC-Pro functions as a pathogenicity-associated factor, enhancing PVX infection and exacerbating disease symptoms by counteracting host RNA silencing defenses.

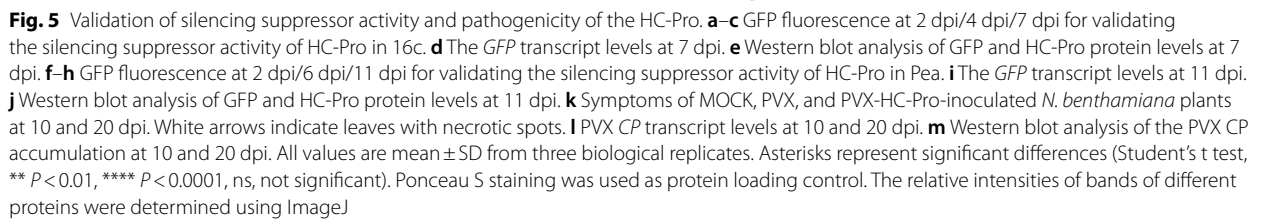
PSbMV-YN HC-Pro suppresses systemic gene silencing by binding to siRNAs

Small interfering RNAs are capable of moving between cells and tissues, facilitating local and systemic gene silencing (Zhao et al. 2021). However, binding by RNA silencing suppressors inhibits small RNA mobility, thereby disrupting systemic gene silencing (Wang et al. 2012). HC-Pro from other potyviruses has been shown to bind small RNAs (Shiboleth et al. 2007). We proposed that PSbMV-YN HC-Pro similarly blocks systemic gene silencing. To analyze its effect on small RNA mobility, we

(See figure on next page.)

Fig. 4 Validation of silencing suppressor activity of P1 and P1/HC-Pro. **a–c** GFP fluorescence at 2 dpi/4 dpi/7 dpi for validating the silencing suppressor activity of P1 in 16c. **d** The *GFP* transcript levels at 7 dpi. **e** Western blot analysis of the GFP and P1 protein levels at 7 dpi. **f–h** GFP fluorescence at 2 dpi/6 dpi/11 dpi for validating the silencing suppressor activity of P1 in *Pea*. **i** The *GFP* transcript levels at 11 dpi. **j** Western blot analysis of the GFP and P1 protein levels at 11 dpi. **k–m** GFP fluorescence at 2 dpi/4 dpi/7 dpi for validating the silencing suppressor activity of P1/HC-Pro in 16c. **n** The *GFP* transcript levels at 7 dpi. **o** Western blot analysis of the GFP and P1/HC-Pro protein levels at 7 dpi. **p–r** GFP fluorescence at 2 dpi/6 dpi/11 dpi for validating the silencing suppressor activity of P1/HC-Pro in *Pea*. **s** The *GFP* transcript levels at 11 dpi. **t** Western blot analysis of the GFP and P1/HC-Pro protein levels at 11 dpi. All values are means $\pm$ SD from three biological replicates. Asterisks represent significant differences (Student's *t* test, **** $P < 0.0001$, ns, not significant). Ponceau S staining was used as protein loading control. The relative intensities of bands of different proteins were determined using ImageJ





employed a method described by Guo and Ding (2002). As illustrated in Fig. 6a, HC-Pro, P19 (a small RNA-binding protein) (Marttinen et al. 2022) or empty vector (EV) were infiltrated into the leaf base, while pGDG (GFP expression construct), which produces mobile GFP-derived small interfering RNAs, was infiltrated into the tip of the same leaf. Ten days post-infiltration, the plants were examined under ultraviolet light to evaluate the silencing effects. The results showed that GFP fluorescence in the upper leaves of plants infiltrated with EV at the leaf base and GFP at the leaf tip began to fade from the veins (Fig. 6a–c), indicating that gene silencing had occurred in these leaves. In contrast, plants infiltrated with the empty vector at the leaf base but without GFP at the leaf tip, as well as those infiltrated with HC-Pro or P19, exhibited normal GFP fluorescence in the upper leaves (Fig. 6a-1/2/4), suggesting that these treatments did not affect the normal expression of GFP in the upper leaves. RT-qPCR and Western blot analyses of the upper leaves revealed similar trends (Fig. 6b, c). These results indicate that small interfering RNAs can systemically spread to the upper leaves, inducing systemic gene silencing, while PSbMV-YN HC-Pro likely inhibits this effect by binding to small RNAs, thereby interfering with their function.

To investigate whether HC-Pro can bind GFP-derived small RNAs, we conducted immunoprecipitation sequencing experiments (Fig. 6d). Leaf regions infiltrated with HC-Pro, as shown in Fig. 6a-4, were collected, and small RNAs were extracted for sequencing. The results demonstrated that HC-Pro binds diverse classes of small RNAs (Fig. 6e), with rRNA- and tRNA-derived sRNAs being the most abundant, consistent with their predominance in the plant endogenous sRNA pool. WebLogo analysis revealed a binding preference for sRNAs enriched in adenine (A) and uracil (U) residues, particularly at their terminal regions (Fig. 6f). Size distribution analysis of HC-Pro-bound GFP-derived siRNAs showed predominant 21-, 22-, and 24-nt species (Fig. 6g), corresponding to canonical products of plant Dicer-like (DCL) family endonucleases. Additionally, GFP-derived siRNAs exhibited no positional bias across the GFP transgene, displaying random distribution patterns (Fig. 6h). Collectively, these data support a model wherein PSbMV HC-Pro suppresses systemic gene silencing by sequestering siRNAs, thereby disrupting their phloem-mediated transport and impeding RISC assembly to evade antiviral RNA silencing.

Discussion

This study utilized segment amplification and RACE methods to obtain the complete genome sequence of the PSbMV-YN isolate. Previous research has indicated

that PSbMV genomic fragments from different strains frequently lead to abnormal growth or complete cessation of growth when they were ligated into vectors and transformed into *Escherichia coli*. In cases where *E. coli* exhibited abnormal growth, the viral fragments in plasmids frequently displayed extensive deletions or random mutations (Johansen 1996). However, in this study, during the segmentation cloning process, the transformed *E. coli* grew normally, and the viral fragments did not show any deletions or nonsense mutations. This allowed for the successful assembly of the complete PSbMV-YN genome (Fig. 1d).

Subsequent phylogenetic analysis classified PSbMV-YN as the P1 pathogenic type. Researchers have used various methods for PSbMV strain identification. For example, the P1 and P4 strains found in Australia were identified through the HC-Pro gene (Torok and Randles 2007); the P1 and P4 strains found in the United States were characterized through host reaction assays and partial nucleotide sequence comparisons (Ali and Randles 1998); and the P2 strain was identified using specific antigens and ELISA experiments (Kasimor et al. 1997). While traditional PSbMV strain differentiation methods have historically served distinct purposes, our genome-wide analysis reveals critical limitations in conventional approaches: (1) targeted gene profiling (e.g., HC-Pro sequencing for Australian P1/P4 strains) offers technical simplicity but risks mis-classification due to localized recombination events; (2) Host differential assays coupled with partial sequencing (U.S. P1/P4 identification) provide phenotypic relevance yet lack molecular resolution; (3) Serological typing (P2 strain via ELISA) enables rapid field detection but fails to discern emerging variants. In this study, both the full-length amino acid and nucleotide sequences of the polyprotein and CP genes were utilized for sequence identity and phylogenetic analyses. The results showed that isolates of the P1 pathotype consistently clustered together in phylogenetic trees constructed using the nucleotide and amino acid sequences of the polyprotein, as well as the nucleotide sequence of the CP gene (Fig. 2c, d, Additional file 2: Figure S1d). However, in the phylogenetic tree based on the CP amino acid sequence, P1 pathotype isolates separated into two distinct clusters (Additional file 2: Figure S1c). This suggests that it is not appropriate to classify PSbMV strains based on the CP amino acid sequence. Instead, the polyprotein amino acid sequence or the complete coding sequence of PSbMV provides a more robust and reliable basis for strain differentiation. These findings also provide valuable insights for the evolutionary analysis of other organisms based on molecular genetic information.

Although previous studies have established that HC-Pro binds to small RNAs (Shiboleth et al. 2007), its



Fig. 6 HC-Pro inhibits systemic gene silencing through binding to siRNAs. **a** Diagram of *Agrobacterium*-mediated infiltration and GFP fluorescence at 15 dpi. 1, 2, 3, and 4 represent EV, P19 + GFP, EV + GFP, and HC-Pro + GFP groups, respectively. **b** qRT-PCR analysis of *GFP* transcript levels in systemic leaves at 15 dpi. **c** Western blot analysis of GFP protein accumulation in systemic leaves at 15 dpi. **d** Western blot analysis of α-Flag immunoprecipitation for Flag-HC-Pro. **e** Characterization of HC-Pro-binding endogenous small RNAs. SRP RNA: signal recognition particle RNA, rRNA: ribosomal RNA, snRNA: small nuclear RNA, snoRNA: small nucleolar RNA, tRNA: transfer RNA. **f** Binding preference analysis of HC-Pro-associated small RNAs by WebLogo. **g** Length distribution of GFP-derived siRNAs from α-Flag IP (18–30 nt). **h** Distribution of GFP-derived siRNAs in the GFP genome (21–22 nt and 24 nt). All values are mean ± SD from three biological replicates. Asterisks represent significant differences (Student's *t* test, *****P* < 0.0001). Ponceau S staining was used as protein loading control. The relative intensities of bands of different proteins were determined using ImageJ.

interactions with endogenous small RNAs are not yet fully understood and lack a comprehensive analysis. Through IP-seq analysis, we demonstrated that HC-Pro binds to endogenous small RNAs. The amount of endogenous small RNAs bound by HC-Pro correlates closely with their abundance, suggesting that HC-Pro binding may not exhibit length specificity. Small interfering RNAs can move between cells and tissues, facilitating local and systemic gene silencing (Wang et al. 2012). The direct binding of small RNAs by HC-Pro impedes their loading and movement, thereby suppressing both local and systemic gene silencing.

Conclusions

In conclusion, we identified and characterized a PSbMV isolate from infected pea plants in Yunnan Province, China. The complete genome of the PSbMV-YN isolate was successfully sequenced and assembled. Sequence identity and phylogenetic analyses classified PSbMV-YN as belonging to the P1 pathogenic strain. Furthermore, we identified HC-Pro as the RNA silencing suppressor and elucidated its inhibitory mechanism (Fig. 7): PSbMV HC-Pro interacts with siRNAs generated by

Dicer, effectively preventing their loading into Ago proteins. This disruption inhibits the formation of the RISC, thereby suppressing local gene silencing. Furthermore, HC-Pro binding to siRNAs interferes with their movement to systemic tissues, blocking the transport required for initiating systemic gene silencing. This dual mechanism highlights HC-Pro's role as a key viral suppressor of RNA silencing, targeting both local and systemic pathways to enhance viral infection and pathogenicity. By binding siRNAs, HC-Pro not only disrupts their functional activity but also compromises the plant's ability to mount an effective and systemic antiviral defense, providing insights into the molecular strategies employed by PSbMV to counteract host immunity.

Methods

Sample collection

During the summer of 2023, pea plants displaying typical viral symptoms such as leaf deformation, shortened internodes, and shriveled seeds were collected from pea cultivation areas in Yunnan Province. Plant samples were flash-frozen in liquid nitrogen and stored at -80°C ,

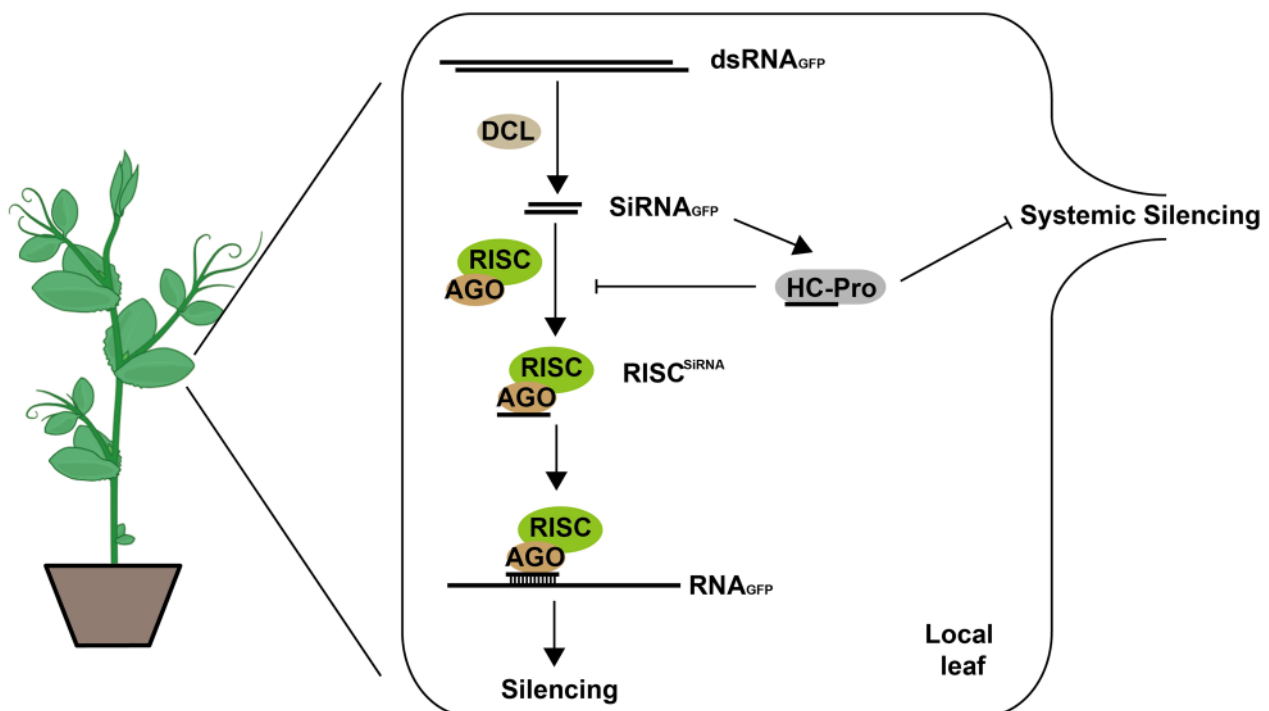


Fig. 7 A working model for PSbMV HC-Pro inhibiting gene silencing. Under normal conditions, after gene transcription, dsRNA is produced through RdRp activity and subsequently cleaved by the Dicer enzyme to form siRNAs. These siRNAs are incorporated into the RISC via Ago proteins, ultimately leading to target RNA cleavage or translation inhibition. Upon entering the cell, PSbMV HC-Pro interacts with siRNAs generated by Dicer, effectively preventing their loading into Ago proteins. This disruption inhibits the formation of the RISC, thereby suppressing local gene silencing. Furthermore, HC-Pro binding to siRNAs interferes with their movement to systemic tissues, blocking the transport required for initiating systemic gene silencing

while seed samples were dried and preserved in a dry incubator.

Vector construction

Total RNA was extracted from the plants using the AG RNAex Pro RNA extraction kit (Aike Rui Bio-Engineering, Hunan, China). The cDNA was synthesized using a reverse transcription kit (Vazyme Biotech, Nanjing, China). Fragments were amplified using primers for PSbMV whole and the silencing suppressor genes (Additional file1: Table S1). The PCR products were purified and recovered using a DNA gel purification kit. The whole-genome amplification fragments were ligated with the pEASY-Blunt Zero vector, and the silencing suppressor gene fragments were cloned using the Cloning Express Ultra One Step Cloning kit (Vazyme Biotech, Nanjing, China) with the pCambia2300-35S-Flag vector. The ligation products were transformed into Trans1, and after verification by colony PCR, three clones were selected from each and sent to Qingke Biotechnology Co., Ltd. for sequencing.

Amplification of genomic terminal sequences

The 5' terminal fragment of PSbMV was obtained using the SMARTer RACE 5'/3' Kit (TaKaRa, Japan), with the following steps: Reverse transcription was performed using the SMARTScribe RT enzyme with the primer PSbMV5'-1R, and the SMARTer II A Oligonucleotide was included in the reaction system. Using this cDNA as a template, the 5' terminal sequence of PSbMV was amplified with the long primer and the primer PSbMV5'-2R. Due to the poly(A) tail structure of PSbMV, the 3' terminal sequence was directly amplified using the specific primers and Oligo(dT)₂₁. The recovery, ligation, and transformation for PCR products were identical to those described previously, and ten clones for each fragment were selected and sent to Beijing Qingke Biotechnology Co., Ltd. for sequencing.

Sequence assembly, identity analysis, phylogenetic analysis, and recombination analysis

The full-length viral genome was separated into four fragments to amplify. Four pairs of primers (gPSbMV1F/1R, gPSbMV2F/2R, gPSbMV3F/3R, and gPSbMV4F/4R Additional file1: Table S1) were designed based on the complete genome sequences of PSbMV isolates registered in the NCBI database. The segmented contigs of PSbMV were assembled using DNAMAN 6.0 software and subjected to whole-genome alignment analysis using SnapGene 5.2 software. The sequence encoding the silencing suppressor was also aligned and analyzed using SnapGene 5.2 software. Sequence identity analysis of different PSbMV isolates was conducted using DNAMAN

6.0 software. Phylogenetic analysis was performed using MEGA 7.0 software using neighbor-joining with 1000 bootstrap replicates after aligning sequences with the software Muscle (Qiao et al. 2023). Recombination analysis and reliability validation were performed using SimPlot3.5 and the website (<http://www.datamonkey.org>).

Phylogeny-geography association analysis

The effect of geographic origin on PSbMV populations was evaluated by phylogeny-trait association analysis, and BaTS 2.0 was used to compute an association index (AI), parsimony score (PS), and maximum monophyletic clade (MC). The significance of the association between isolates and regions is primarily determined based on their respective *P*-values. If all *P*-values are ≤ 0.05 , it is concluded that there is a significant association between the isolates and regions.

Agrobacterium-mediated transient expression

Agrobacterium tumefaciens transformation was carried out using the freeze-thaw method, and positive clones were screened by colony PCR. Positive colonies were picked and grown overnight in 3 mL of liquid Lennox Broth (LB) medium containing at 28 °C. The following day, the culture was diluted 1:100 into fresh liquid LB medium containing kanamycin and rifampicin and grown at 28 °C until the OD₆₀₀ reached approximately 0.8. The bacteria were harvested by centrifugation at 4000 *g* for 5 min at room temperature and resuspended in infiltration solution (10 mM MES, 10 mM MgCl₂, and 200 μ M acetosyringone) to an OD₆₀₀ of 1.0. After incubation at room temperature for 2–3 h, the suspension was infiltrated into 16c *N. benthamiana* leaf discs and Pea leaf discs. The infiltrated plants were then placed in a greenhouse for cultivation.

RT-qPCR and Western blot analysis

Total RNA was extracted from *N. benthamiana* leaves using a phenol-based method. cDNA was synthesized using 500 ng of total RNA through reverse transcription. Real-time quantitative PCR was performed with *NtEF-1 α* or *PsTubulin* as the reference gene. Data analysis was conducted using the 2^{− $\Delta\Delta$ Ct} method, and graphs were generated using GraphPad Prism 8 software.

Total protein was extracted from *N. benthamiana* leaves at 7 dpi and subjected to SDS-PAGE gel electrophoresis. After electrophoresis, proteins were transferred to a nitrocellulose membrane using a semi-dry transfer method and then blocked in 5% non-fat milk for 1 h. Immunoblotting was performed with primary antibodies against GFP, Flag, and PVX CP, and secondary antibodies were HRP-conjugated Goat Anti-Mouse IgG (TransGen Biotech, Beijing, China). Protein

visualization was achieved using the SuperKine™ West Femto Maximum Sensitivity Substrate (Abbkine Scientific, Georgia, USA). Rubisco was used as a loading control.

IP-Northern and small RNA sequencing

N. benthamiana leaves infiltrated with Flag-HC-Pro for 10 dpi were collected, ground in liquid nitrogen, and lysed with IP buffer (20 mM Tris-HCl pH 7.5, 100 mM NaCl, 2 mM MgCl₂, 1 mM DTT). The lysate was centrifuged to collect the supernatant (Input), which was incubated with Anti-DYKDDDDK Agarose (Abmart, Shanghai, China) at 4 °C for 2–3 h. The beads were washed thoroughly with IP buffer to obtain the IP product. Small RNAs were extracted from the IP product using RNAiso for Small RNA (TaKaRa, Japan) and sequenced by BGI Genomics (Shenzhen, China).

Abbreviations

CP	Coat protein
dpi	Days post-infiltration
kb	Kilobases
ORFs	Open reading frames
PVA	Potato virus A
PSbMV	Pea seed-borne mosaic virus
RACE	Rapid amplification of cDNA ends
SPMMV	Sweet potato mild mottle virus
TEV	Tobacco etch virus
TriMV	Triticum mosaic virus
TuMV	Turnip mosaic virus
UTRs	Untranslated regions

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s42483-025-00373-x>.

Additional file 1: Table S1. Primers used for detection and amplification of PSbMV-YN. **Table S2.** Genomic information of PSbMV-YN. **Table S3.** Sequence identities between PSbMV-YN and PSbMV isolates.

Additional file 2: Figure S1. Pairwise identity matrices and phylogenetic trees using amino acid and genomic sequences of PSbMV CP gene. **Figure S2.** Alignment of protein sequences for Pea-YN eIF4E and eIF4E from resistant (JI1405 and 835) and susceptible (JI2009 and 744) pea lines.

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Author contributions

JM and MW designed the project and conducted the experiments. All authors analyzed the data and reviewed the manuscript. JW, SZ, MW, and JM wrote the paper. All authors read and approved the final manuscript.

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Data availability

The datasets used and analyzed during the current study are available from the corresponding author at a reasonable request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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