PLANT SCIENCE TODAY
Vol 12(4): 1-11
https://doi.org/10.14719/pst.9312

eISSN 2348-1900

RESEARCH ARTICLE

Intron hairpin RNA derived from the HC-Pro gene confers resistance to papaya ringspot virus in transgenic *Nicotiana benthamiana*

Kheta Ram Tak*, Anitha Peter & Deepika Vidyashankar

Department of Plant Biotechnology, University of Agricultural Sciences, Gandhi Krishi Vigyana Kendra, Bangalore 560 065, Karnataka, India

*Correspondence email - khetaram100@gmail.com

Received: 05 May 2025; Accepted: 27 June 2025; Available online: Version 1.0: 29 August 2025; Version 2.0: 01 October 2025

Cite this article: Kheta RT, Anitha P, Deepika V. Intron hairpin RNA derived from the HC-Pro gene confers resistance to papaya ringspot virus in transgenic *Nicotiana benthamiana*. Plant Science Today. 2025; 12(4): 1-11. https://doi.org/10.14719/pst.9312

Abstract

In the present study, cDNA from a papaya leaf sample infected with Papaya Ringspot Virus (PRSV) was used for the amplification of the HC-Pro gene, which yielded an expected amplicon size of ~1350 bp. The partial coding sequence of HC-Pro was used to design a hairpin RNA (hpRNA) construct, which included a cowpea spliceosomal intron (148 bp) inserted between the hpRNA arms (ihpRNA HC-Pro). The potential siRNAs and Virus Induced Gene Silencing (VIGS) sequence predicted by integrated bioinformatics tools (siDirect 2.0 and pssRNAit) identified the best region of 201 bp (557-757 bp). The designed intron hairpin RNA (ihpRNA) HC-Pro was synthesized and cloned into the plant expression vector (pBI121) and the resulting recombinant plasmids (pBI121::CaMV35s::ihpRNA-HC-Pro::NOS) were subsequently transformed into *Agrobacterium tumefaciens* (LBA4404) followed by co-cultivation in *N. benthamiana*, a model plant. Molecular confirmation of the T_0 lines via PCR indicated that three out of four plants were contaminated with *Agrobacterium* in the apoplast. A single line that was positive for ihpRNA HC-Pro amplification and negative for VirG, was forwarded to the T_1 generation. Six out of ten T_1 lines were amplified for ihpRNA HC-Pro. Single copy insertions were confirmed by southern blotting. Bioassay and gene expression tests in T_1 transgenic plants showed lower levels of NbPOD, NbAPX, NbCAT and HcPro, like healthy plants, suggesting that PRSV resistance was successfully achieved through ihpRNA-mediated gene silencing.

Keywords: HC-Pro; ihpRNA; *N. benthamiana*; PRSV; siRNA

Introduction

One of the most destructive viruses that affect cucumber and papaya crops in tropical and subtropical areas is PRSV, which belongs to the Potyvirus family and is of the genus Potyvirus. Two types of PRSV exist: PRSV-P, which can infect cucumbers and other cucurbits and PRSV-W, which can only infect cucumbers and not papaya. Unlike cucumbers and papayas, PRSV does not have a strong impact on cucumbers. The main host plant for PRSV-P is papaya, which exhibits severe symptoms such as mosaic patterns, distorted leaves, greasy streaks on stems and greatly deformed and yield-reducing fruits. Mosaic patterns, mottling of the leaves and a little reduction in fruit quality are often the less severe symptoms displayed by cucumbers infected with PRSV (especially the PRSV-W strain). Aside from its diminutive size (between 760 and 800 nm and 12 nm in diameter), the virus is defined by its filamentous form and monocistronic positive-sense single-stranded RNA. Ten distinct mature proteins are encoded by its 10.8 kb genome. These include P1 protease (59 kb), NIb (63 kb), NIa (48 kb), HC-Pro (52 kb), P3 protease (46 kb), CI (72 kb), Coat Protein (CP) (35 kb) and nuclear inclusion protein a (NIa, 48 kb). The virus is made up of one strand of RNA and has a diameter of 12 nm and a length of 760-800 nm. It is monocistronic and has a positive sense. The approximately 10.8 kb pair viral genome encodes a single large polyprotein, which is auto processed into ten mature proteins. HC-Pro (52k), P1 protease (63k), NIb (59k), P3 protease (46k), CI (72k), NIa (48k) and CP (35k) are essential proteins that span from 5′ to 3′ (1-5). Common papaya symptoms include chlorosis and mosaic on the leaf lamina, petiole and upper trunk; oil streaks that have been absorbed in water and other similar lesions. Rapid transmission of this disease can infect an entire orchard in as little as three to seven months, causing output losses of 100 % (6). It attacks papaya trees at any stage of development and spreads quickly. The vectors that spread PRSV are aphids. Aphids may eat for as little as 15 sec on healthy papaya before they bite into the diseased fruit (7). Management of PRSV and the vector can be achieved using discrete cultural techniques, such as aphid population suppression, virus transmission prevention and systemic roughening of infected plants. However, these treatments have their limitations and are not sustainable in the long run. Two variants, rainbow and sunup have been produced and commercially released and modern methods like genetic engineering are also used for PRSV management, with CP-mediated Pathogen Derived Resistance (PDR) being a common strategy (8). Nevertheless, there have

been reports of papaya's CP-mediated PDR breaking down (9); therefore, new approaches to managing PRSV illness are required.

Another strategy used to manage viral diseases is Posttranscriptional Gene Silencing (PTGS). This method reduces viral gene expression via RNA interference (RNAi) by destroying viral RNA when a small molecule of double-stranded RNA (dsRNA) triggers direct homology-dependent control of gene activity. Another strategy used to control viral diseases is PTGS. Direct homology-dependent control of gene activity causes viral RNA degradation by the action of a tiny dsRNA molecule. A viral gene's expression is decreased by this technique using RNAi. To create highly efficient and specific siRNAs, it is necessary to identify many target regions for potential siRNAs inside viral genes, as the success of RNAi relies on the specific interactions between siRNAs and messenger RNAs (10). Previous research found that ihpRNAs, a specific type of hpRNA that encodes a spliceosomal intron interspersed between hpRNA arms, were able to induce PTGS against RNA viruses with an effectiveness of nearly 100 % (11).

The PRSV HC-Pro gene is part of a family of proteins called cysteine-type proteinases and works as a multifunctional protein that acts on itself. Aphid transmission requires the N-terminal domain, which contains amino acids (aa) 1-100. The central area, which contains regions 101-299 aa, is responsible for RNA silencing suppression and other functions. The C-terminal domain, which contains regions 300-459 aa, contains the proteolytic activity of HC-Pro (12-14). To improve host antiviral silencing activity, it is anticipated that silencing this protein will lead to the downregulation of viral RNAs.

Here, we aimed to develop genetically modified *N. benthamiana* plants that would resist PRSV by using ihpRNA-HC-Pro gene constructs to target the HC-Pro gene.

Materials and Methods

Total RNA extraction, cDNA synthesis and amplification of HC-Pro genes

The papaya leaf samples exhibiting mosaic patterns, leaf distortion and curling, oily streaks and shoe-string leaves symptoms of PRSV collected from the GKVK, Department of Plant Biotechnology, Bangalore, University of Agriculture Sciences were used for total RNA isolation via the lithium chloride (LiCl) method as described earlier (15). The total RNA extracted was used as the template for reverse transcriptase (RT)-PCR. First-strand cDNA was synthesized from total RNA in a 20 µL reaction using a gene-specific reverse primer "(HC-Pro RP: 5'GC AAG CTT GTT GCG CAT ACC CAG GAG AGA GTG 3')". After 5 min at 65 °C, 5 min at 4 °C, 60 min of incubation at 42 °C and lastly 5 min at 85 °C, the reverse transcription process was performed. Hc-Pro gene-specific primers "(RP 5'GC AAG CTT GTT GCG CAT ACC CAG GAG AGA GTG 3')" and "(FP 5'GC GGA TCC ATG TCC AAA AAT GAA GCT GTG GAT 3')" were used for Polymerase Chain Reaction (PCR) amplification of first-strand cDNA generated by reverse transcription. The following conditions were used to conduct the PCR: 5 min of initial denaturation at 94 °C, 30 sec of denaturation at 94 °C with 32 cycles, 45 sec of annealing at 60 °C, 90 sec of extension at 72 °C and finally, 7 min of final extension at 72 °C. A GeneJET Gel Extraction Kit (Cat. No. K0691; Thermo Fisher Scientific) was used to elute the gel after a meticulous extraction of the amplified product.

Sequencing and bioinformatic analysis

Prior to incubation at 22 °C for 16 hr, the eluted PCR product was ligated to the T/A cloning vector pTZ57R/T using 5 U of T4 DNA ligase and ligation buffer (16). We used the thermal shock method to transform the ligated products into *E. coli* DH5a, maintaining a temperature of 42 °C for precisely 90 sec (17). Blue-white screening and PCR were used to confirm the transformation of consecutive transformants. Chromus Biotech of Bangalore, India, sequenced the recombinant pTZ57R/T carrying the HC-Pro gene and deposited the sequence at NCBI.

After obtaining the nucleotide sequences, they were compared using the BLAST tool, which is based on sequence homology, to other PRSV HcPro sequences that are already in the NCBI database. We used the ClustalW software in BioEdit (v7.2) and Multiple Sequence Alignment (MSA) tools to match sixteen HC-Pro sequences so we could analyze their similarities (18).

Using the maximum likelihood method with 1000 bootstrap repeats, the phylogenetic tree was generated using MEGA-X tools. We chose the Tamura-Nei model of evolution (19) when building the phylogenetic tree.

ihpRNA construction

In silico analysis for the identification of potential siRNAs and selection of VIGS sequences

The PRSV-P HC-Pro gene was studied with siDirect 2.0 and pssRNAit to find possible siRNAs and VIGS targets (20-25). The chosen candidates are displayed in Fig. 1 using bioinformatics tools.

Screening of off target effects of siRNAs in *N. benthamiana*

To find likely off-target sites and very close matches with *N. benthamiana* mRNA sequences in the NCBI database, we used BLAST and pssRNAit. We filtered out siRNAs with substantial off -target effects to boost the validity percentage. We used a minimum off-target effect to select a sequence or fragment of the HC-Pro gene for the construction of the ihpRNA HC-Pro construct.

Development of the ihpRNA HC-Pro construct

A combination of off target analysis and anticipated siRNA technologies allowed for the optimal region selection. The Department of Biochemistry, IARI, New Delhi, generously provided the cloned cowpea intron (300 bp). The intron hairpin loop was designed and constructed using just partial sequences, using the CA-AG eukaryotic splicing rule. We produced the ihpRNA HC-Pro construct by considering and inserting the plant splice site (CA-AG) into the intron sequence.

Generation of transgenic *N. benthamiana* expressing the ihpRNA HC-Pro construct

Subcloning pUC57-ihpRNA-HCPro into pBI121 added it to the plant binary vector. Initially, SacI and BamHI enzymes double digested pUC57 strains with ihpRNA-HCPro and pBI121. We achieved heat inactivation by incubating the reaction mixture at 37 °C for 75 min and 65 °C for 20 min. After separating the

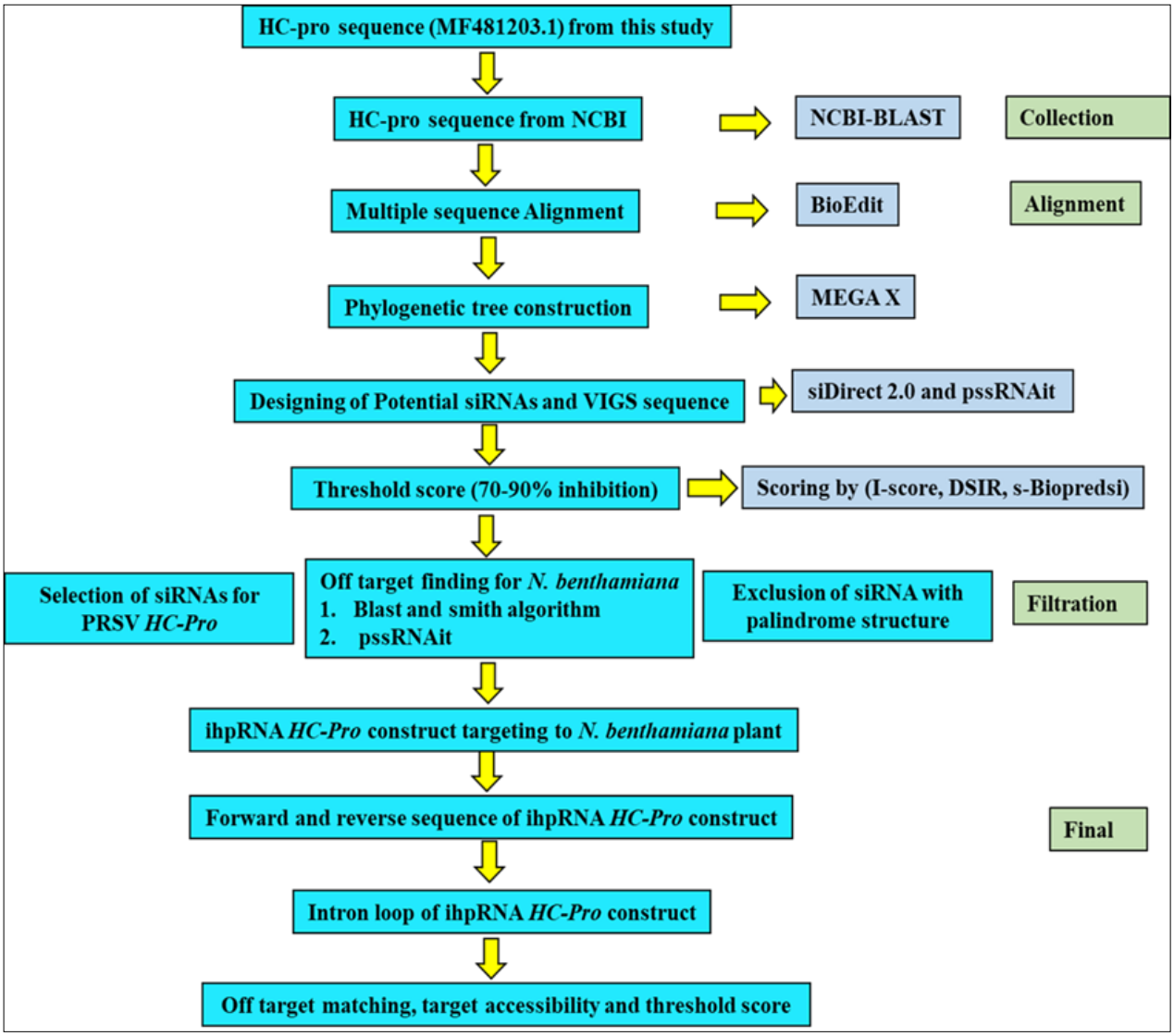

Fig. 1. Schematic overview of the approach adopted in the present study for ihpRNA HC-Pro construction.

DNA on a gel, the ihpRNA-HCPro gene and pBI121 vector were joined together in a 1:3 ratio and introduced into *E. coli* DH5α cells (26).

To confirm that the recombinant *E. coli* DH5α cells contained pBI121::ihpRNA-HC-Pro, we used colony PCR with specific primers.

Forward: "GGATCCCTCTTACATGTGATAATCAATTGGACA" and "Oligo 4 (EcoRI)" Reverse: "GAATTCTTCTGGATA" The clones were moved to *Agrobacterium tumefaciens* strain LBA4404 using a freeze-thaw method and the modified pBI121::ihpRNA-HC-Pro plasmid was found using PCR (27). Co-cultivation was performed with *Agrobacterium tumefaciens* tobacco leaf explants obtained from 3-week-old *in vitro* grown seedlings and used for *Agrobacterium*-mediated transformation (28). Co-cultivation was followed by three subcultures at 15-day intervals. Regenerated shoots were transferred to rooting media for 15 days, then acclimatized in hardening media for another 15 days. T_0 plants were moved to the polyhouse, where they grew vegetatively for 2-3 weeks, flowered in 5-7 weeks and produced seeds in 3-4 weeks. T_1seeds germinated and developed within 3-4 weeks.

Molecular analysis of transgenic plants

PCR amplification

The genomic DNA was extracted from the putative transgenic plants (T_0 and T_1 plants) via the C-TAB method (29). The PCR mixture comprised 50 ng of template DNA, 0.8 µl of 2 mM dNTPs, 0.5 µl of 1 U of Taq DNA Polymerase, 1 µl of 10X PCR buffer and 1 µl of Oligo 1 or Oligo 4, each with a total volume of 10 µl. The amplification was carried out at 94 °C for 30 sec, 94 °C for 4 min, 54 °C for 40 sec, 72 °C for 30 sec and 72 °C for 7 min in a thermocycler. The PCR products were analyzed via 1 % agarose gel electrophoresis and documented.

Southern blot analysis

We used Southern blot hybridization to characterize the integration location and transgene copy quantity in plants that tested positive according to the PCR assays. A specific HC-Pro biotin probe (CTCTTACATGTGATAATCAATTGGACAGGAATG) was used to transfer 30 µg of genomic DNA onto a Biodyne®B membrane after cutting it with HindIII and separating it using 0.8 % agarose gel electrophoresis.

Bioassay and expression analysis

The PSRV inoculum, which is a pure culture was given to both genetically modified (T_1) and regular *N. benthamiana* plants, ideally those that were one month old and grown in a greenhouse, using the sap inoculation method (6). Researchers regularly monitored PRSV symptoms, including when they first appeared and how severe they became, up until 28 days after inoculation (dpi). The RNA was extracted from the leaf samples

taken at 14- and 28-days post-injection using a Qiagen RNeasy Mini Kit (Cat. No. 74106). Using SYBR Green (Agilent Technologies, Brilliant II SYBR Green qPCR master mix, USA) and a Stratagene mx3005P instrument (Agilent Technologies, USA), the expression levels of the chosen genes, APX, CAT, SOD, POD and ihpRNA HC-Pro, were examined. The amplification conditions included an initial denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 sec and 60 °C for 30 sec. After amplifying the specific primers, the dissociation curve was checked at these temperatures and times: 95 °C for 1 min, 55 °C for 30 sec, increasing the temperature by 0.2 °C each second and finally, 95 °C (fluorescence was continuously recorded from 55 °C to 95 °C). To determine the relative expression level of genes, the mean Ct value of the technical replicates was utilized.

Results

Isolation, identification and sequencing of the HC-Pro gene of PRSV-P

We successfully made copies of a specific gene by creating cDNA from a papaya leaf sample that showed signs of shoestring leaves, using the MMuLV-RT enzyme and HC-Pro reverse primers. The PRSV HC-Pro gene had an amplicon size of 1.3 kb, as shown in Fig. 2a, based on the gel electrophoresis of the amplified products. To sequence the amplified product, it was cloned again and placed into pTZ57R/T (Supplementary Fig. 1a and 1b). The 1.3 kb pair was annotated with the accession number MF481203 and added to the NCBI database. The isolated gene sequence is identified as PRSV HcPro because it closely matches other PRSV HcPro sequences found in the NCBI database (Supplementary Fig. 2a, 2b). Among these sequences, it showed the highest similarity of 97 % with the WB -1 PRSV-P isolate (accession ID: KC149504.1).

The differences among these HcPro gene sequences were analyzed using the ClustalW tool in BioEdit software, which looks at how similar these sequences are to each other. MSA yielded an average of 4.95 % SNPs when aligning the HC-Pro genes (Supplementary Fig. 3). The Indore MP (KC149503) had the highest number of SNPs at 6.05 %, while the Bengaluru -1 (MN103525) gene had the lowest amount at 2.33 % (Supplementary Table 1). Also, as shown in Supplementary Fig. 4, the HC-Pro protein showed an average amino acid variation of 3.3 % when aligned. The HC-Pro gene of Delhi-1 (ATZ76589) showed the lowest amount of variance at 2.33 %, while the genes of Dhaward and Indore MP showed the highest amount of variation at 6.05 % (Supplementary Table 1). Since there were differences in the isolates' SNP and AAP genotypes, it will be difficult to downregulate or delete a specific gene area.

Phylogenetic analysis

Phylogenetic analysis was performed for both the HcPro nucleotide and the deduced amino acid sequences with Tobacco etch virus and the HC-Pro gene as the one outgroup. The phylogenetic analysis of nucleotide sequences revealed two major and distinctly branched clusters: cluster 1 and cluster 2 (Fig. 2b). The present isolate was clustered in subcluster 2 alongside other Bangalore isolates with high bootstrap support. Similar observations were made with the phylogenetic tree of the amino acid sequence, in which two major clusters, cluster 1 and cluster 2 (Fig. 2c), were observed, with the present isolate clustered alongside the Delhi and Bangalore isolates.

In silico design of the intron-containing hairpin RNA (ihpRNA) construct of the HC-Pro gene of PSRV-P

To locate possible siRNA areas, the full 1350 bp HC-Pro gene sequence was utilized. After submitting the sequence, we used the online software pssiRNAit to identify 201 bp long (557-757) siRNA candidates. Using the whole gene sequence and the siDirect (version 2.0) online tool, 49 potential effective siRNAs were found (Supplementary Table 2). A total of 69 VIGS candidates were found by looking at possible siRNA sequences that target the PRSV-P HC-Pro gene, using the whole gene sequence with the pssiRNAit online tool (Supplementary Table 3). Based on the results acquired from these online tools, a 201 bp single nucleotide sequence was chosen from locations 557-757 bp. Through siDirect and pssiRNAit software, 14 efficient siRNA candidates were found (Table 1).

The i-Score designer program identified 180 siRNA sequences from the 201 bp (557-757 bp) HC-Pro gene sequence. Out of all the candidates, ten siRNAs were chosen based on their ranks (1-10) across various scores, including the I -Score, DSIR ands-Biopredsi score (Supplementary Table 4). A range of 96.8- 92.5 % for the DSIR score, a range of 0.874- 0.858 for the s-Biopredsi score and an i score of 80.6- 74.6 % were all used to determine if the HC-Pro gene was inhibited. The 558-base pair sequence of ihpRNA HC-Pro is provided in Appendix 1.

Screening of off target effects of siRNAs in *N. benthamiana*

Off-target prediction in *N. benthamiana* was done using NCBI-BLAST and psRNAit. The results of the BLAST analysis, which did not show any significant similarities between the 201 bp (557-757) individual 14-siRNAs (Supplementary Table 5, Supplementary Fig. 5). Consistent with the pssRNAit web tool, the 14-siRNA sequence had an E value between 0.047 and 0.74 and a query coverage of 76 % to 100 %.

Forward and reverse sequences of the ihpRNA HC-Pro construct

We constructed the ihpRNA HC-Pro construct using the forward and reverse sequences, along with the introns of the HC-Pro gene. The hairpin loop of the ihpRNA construct was made using a 148 bp nucleotide sequence from the cowpea intron. The ihpRNA construct utilized a forward sequence of 201 bp (557-757), with the inverted version serving as the reverse sequence. A simpler version of the ihpRNA HC-Pro gene-building cassette, along with how siRNA is made and expressed is shown in Fig. 3.

Molecular characterization of transformed *N. benthamiana* plants and bioassays

The pBI121::CaMV35s::ihpRNA-HC-Pro::NOS recombinants were transformed via *Agrobacterium*-mediated transformation into *N. benthamiana* to develop PRSV resistant transgenic lines. The ihpRNA-HC-Pro piece was inserted into the pBI121 vector (Fig. 4a, 4b) and put into *N. benthamiana* leaves (Supplementary Fig. 6). PCR analysis of T_0 and T_1 generations confirmed ihpRNA-HC-Pro presence. Genomic DNA from four T_0 plants showed VirG amplification in all but HCP3, which also confirmed construct integration (Fig. 5a). Seeds from HCP3

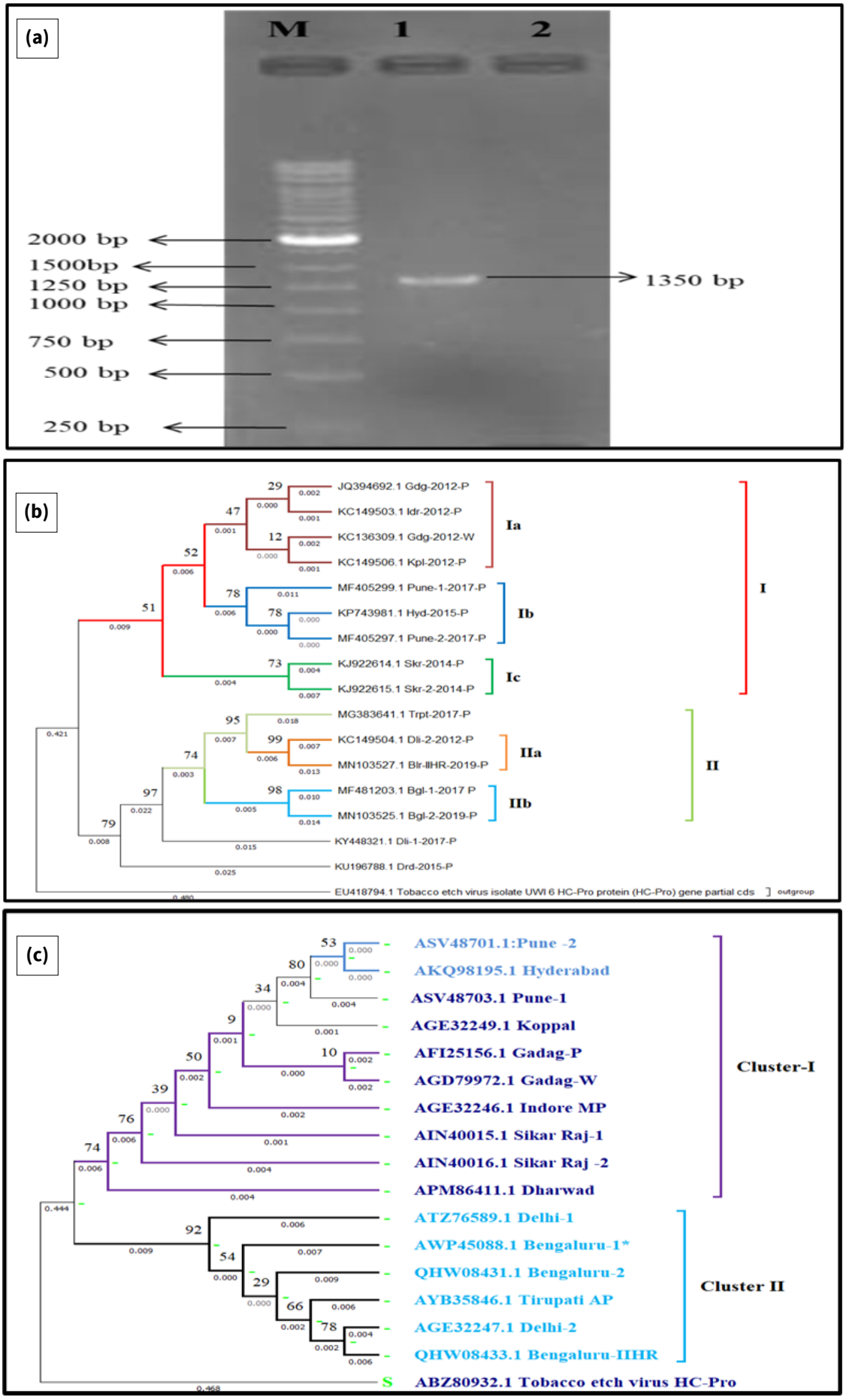

Fig. 2. Molecular and phylogenetic characterization of the Hc-Pro gene of PRSV.

(a) 1 % gel electrophoresis of PCR amplicons Lane 1: PCR amplicon from infected leaf tissue of papaya showing ~1350 bp of the HcPro amplified product. Lane 2: Healthy control; (b) Phylogenetic tree (cladogram) constructed based on the HC-Pronucleotide sequences of PRSV-P isolates; (c) Phylogenetic tree (cladogram) constructed on the basis of the HC-Prodeduced amino acid sequences of PRSV isolates

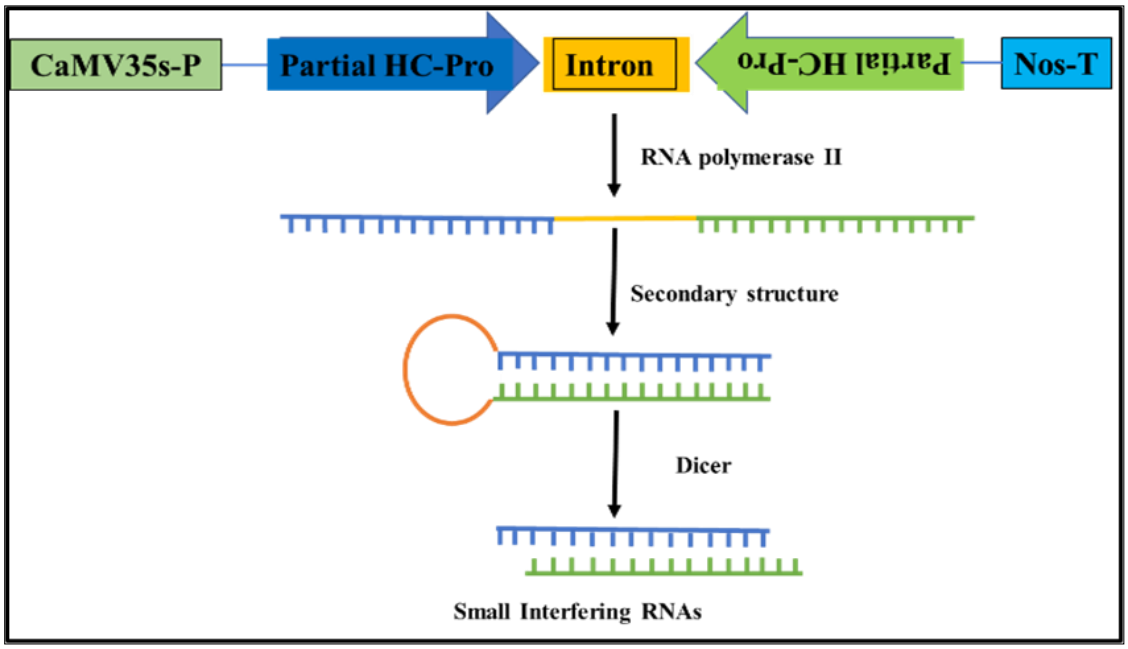

Fig. 3. Diagrammatic representation of the ihpRNA construct, its expression and the formation of siRNAs.

Table 1. List of potential siRNA sequences targeting the PRSV-P HC-Pro gene from the 557-757 bp region

Sr. No.	Target sequence*
1	AUUGAUUAUCACAUGUAAGAG
2	CACCCCAUAAGAAAUUGCCAU
3	UAUUGCCUCUCACCCCAUAAG
4	UUUGGCGUGGUAUUGCCUCUC
5	CAAAGAAUCUUUUGGCGUGGU
6	AAAUAGUUGGCAAAGAAUCUU
7	ACUGUGAUCAAUUUUCUCAAA
8	CAUAACCCUUACUGUGAUCAA
9	CUGUAGUGCUCAUAACCCUUA
10	AUUCUGGUGUUGACUGUAGUG
11	CACCAUUUGGAUUCUGGUGUU
12	UACUUUUCUGACACCAUUUGG
13	AAAUUGCCAAUGGCUACUUUU
14	UCCAAAUUUGUUGAAAAUAUC

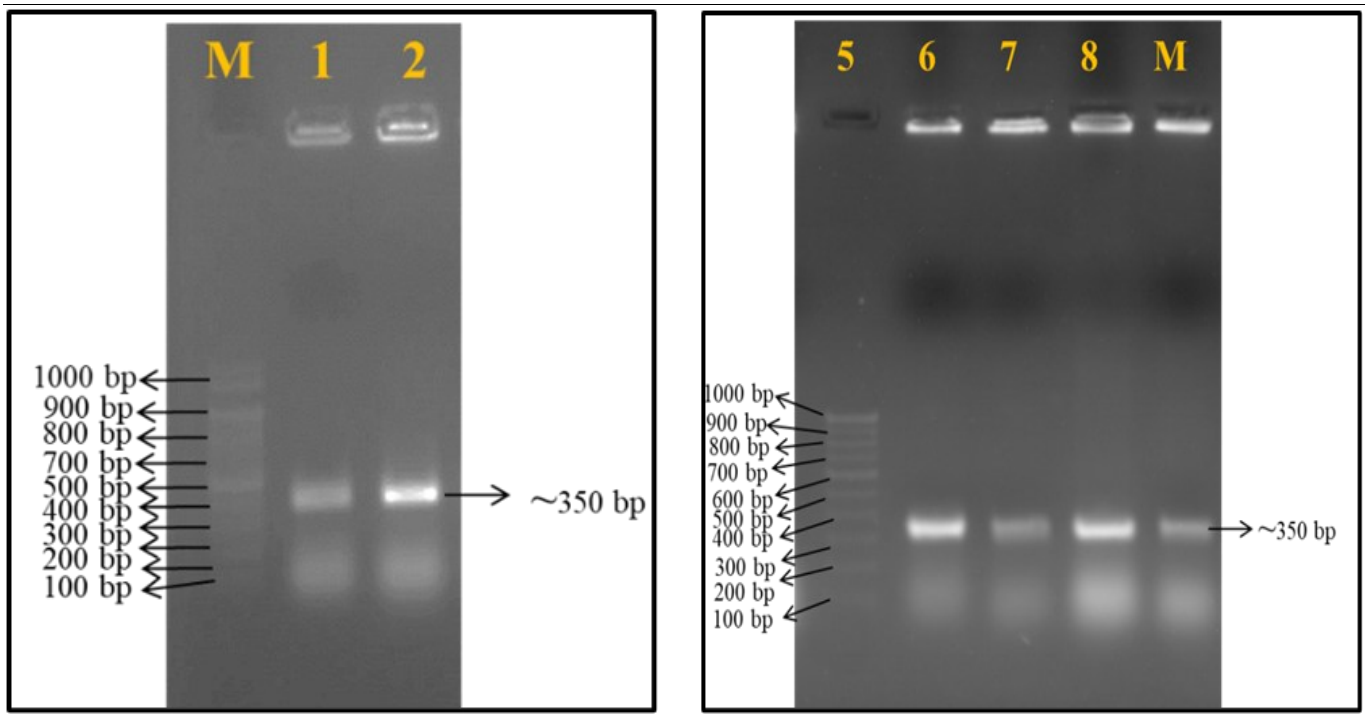

Fig. 4. Cloning of ihpRNA-HCPro into pBI121 and immobilization to *Agrobacterium tumefaciens*.

(a) PCR amplification of the recombinant pBI121::ihpRNA-HCPro plasmid isolated from *E. coli* DH5a via ihpRNA-HCPro primers revealed an amplicon size of ~350 bp; (b) PCR amplification of the recombinant pBI121::ihpRNA-HCPro plasmid isolated from *Agrobacterium tumefaciens* via ihpRNA-HCPro primers revealed an expected amplicon size of ~350 bp

produced ten T_1 plants; four showed strong amplification, two showed weak amplification and controls showed none. Southern blot analysis verified single-copy insertions in transgenic lines. Bioassay showed transgenic seedlings resisted PRSV, unlike symptomatic control plants at 28 dpi (Fig. 6).

Gene expression analysis of putative T_1 lines via qRT–PCR

Gene expression study of the NbAPX, NbSOD, NbPOD, NbCAT and PRSV ihpRNA HC-Pro genes was performed on the verified T_1 line at 14 and 28 dpi. Control and transgenic lines with and without PRSV infection were treated at 14 and 28 dpi. The tubulin gene was utilized as a housekeeping gene to normalize gene expression investigations. The housekeeping gene level was used to standardize mRNA expression. The fold change over the control sample was determined from the test sample Ct values. The test samples have elevated HC-Pro, NbPOD, NbAPX and NbCAT genes compared to transgenic T1P1-28 dpi samples (Supplementary Fig. 7). CI1-28 dpi infected samples had 2.83-fold higher HC-Pro gene expression than T1P1-28 dpi samples (Fig. 7). NbSOD gene expression levels in test samples CP1 and TIP1-28 dpi did not differ significantly in fold change.

Discussion

In this study, we used the ihpRNA method to create transgenic

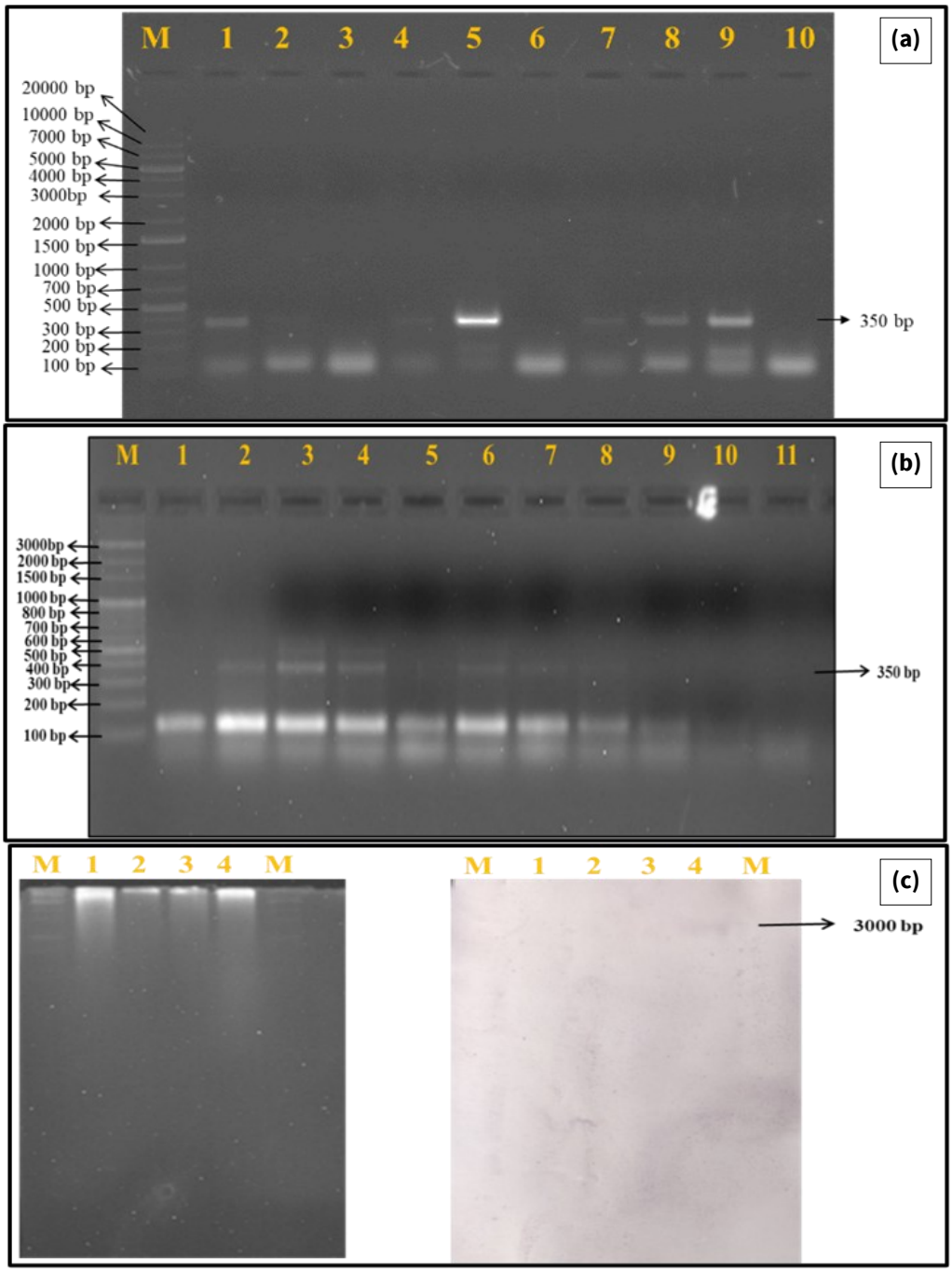

Fig. 5. PCR analysis and confirmation of the integration of ihp-HCPro in transgenic plants.

(a) PCR analysis of the putative T_0 transgenic line via the construction of specific primers. Lane M: 1 kb plus DNA ladder, Lanes 1-4: Transgenic plant (HCP1 to HCP4 with VirG primers), Lane 5: Control *Agrobacterium*, Lanes 6-9: Transgenic plant (HCP1 to HCP4 with specific primers), Lane 10: Control plant (non transgenic); (b) PCR analysis of the putative T_1 transgenic line (HCP3 line progenies) via the construction of specific primers. Lane M: 100 bp plus DNA ladder, Lanes 1-10: Transgenic plants (HCP3.1 -3.10), Lane 11: Control plant (non transgenic); (c) Southern blot hybridization analysis of the ihpRNA HC-Pro copy number in the transgenic plant lines. M: 1 kb plus DNA ladder, Lanes 1-4: Transgenic plant (HCP3.2, 3.3, 3.4 and 3.6)

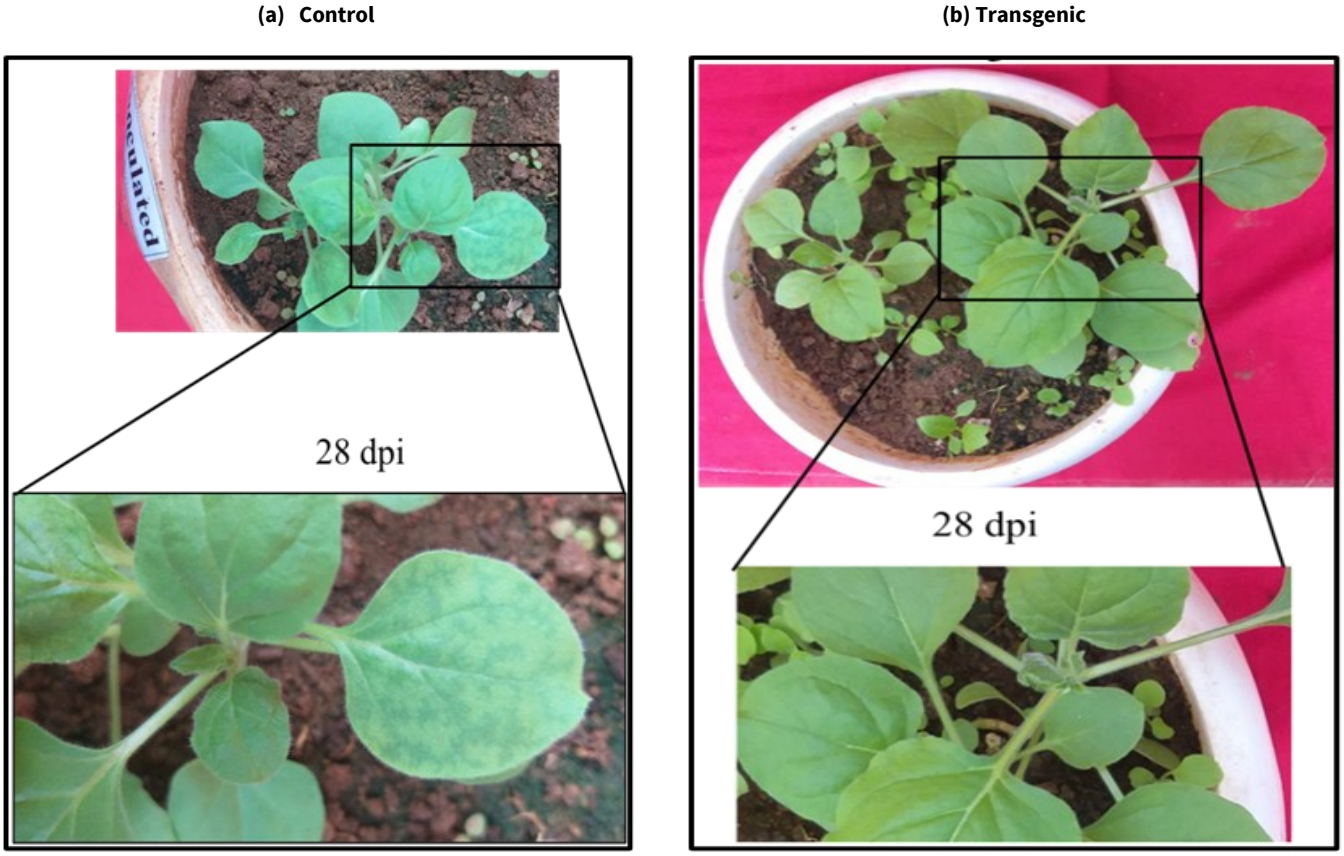

Fig. 6. Bioassay of PRSV in transgenic *N. benthamiana* plants through mechanical sap inoculation (image after 28 dpi).
(a) Symptoms developed in control plants -Non transgenic; (b) No symptoms developed in HCP3.3 transgenic line T_1

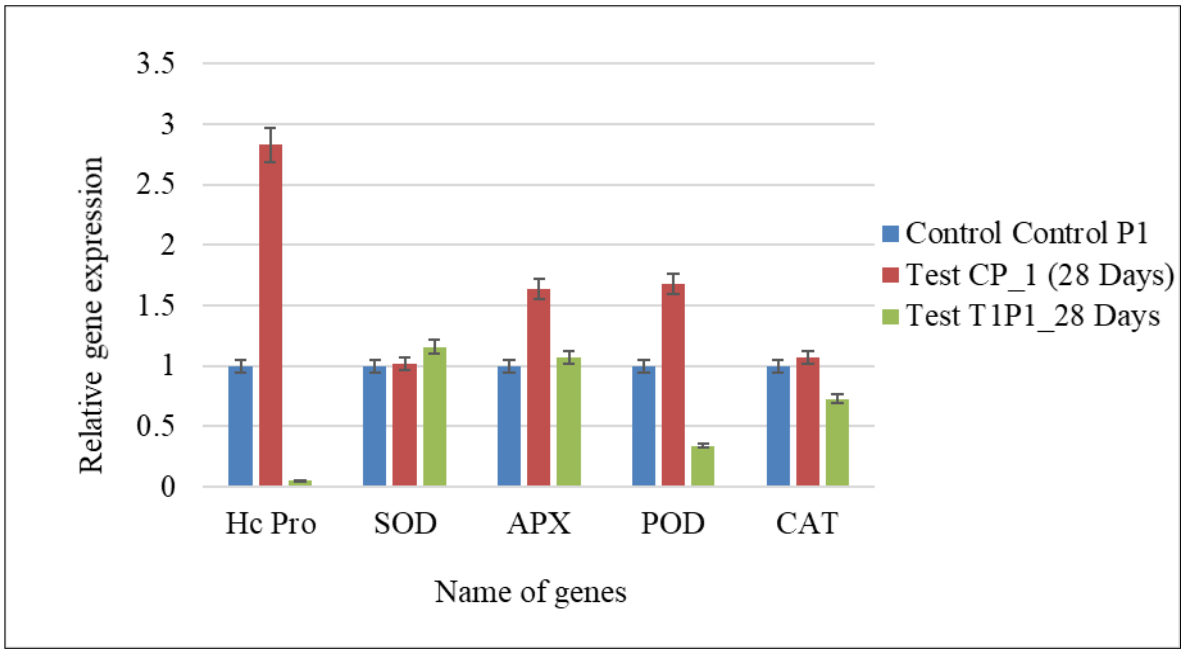

Fig. 7. Relative gene expression data measured via qRT-PCR to examine the effects of PRSV infection on control, nontransgenic infected and transgenic infected plants.

N. benthamiana plants that express a gene construct that is resistant to PRSV via intron-hairpin RNA. When viruses invade plants, they take over the host cell's transcription and translation systems (30). Transgenic plants attach to viral polycistronic genomic RNA at the complementary region whenever they meet it in cells. These siRNAs are created by the transgenic plants themselves. To stop the virus from moving and its proteinase activity from happening, Dicer cleaves the dsRNA that forms due to complementarity. This further limit the formation of HcPro, the helper component proteinase. Through the PTGS pathway, this siRNA imparts PRSV-P viral resistance (31).

While CP-Mediated Resistance (CP-MR) was successful in achieving PDR in papaya to PRSV in the 1990s, its resistance has since broken down, making other PDR techniques crucial. The current study started by isolating the viral HC-Pro gene (~1350 kb) by PCR amplification after total RNA extraction.

Gene lengths ranging from 1.35 kb to 1.37 kb were reported in different global studies on the isolation and cloning of the potyvirus HC-Pro gene (1,32,33). A large chunk of the viral genome went unnoticed in earlier potyvirus biodiversity studies conducted in India, which focused solely on the CP gene (34). Based on the HC-Pro gene sequences of all sixteen Indian isolates (plus one outgroup sequence), a phylogenetic tree was constructed for the present study. Phylogenetic examination of several PRSV genes, such as the P1, Hc-Pro and NIb-CP sequences of several South Indian isolates, showed variety with numerous groups in a previous (35).

Research has shown that ihpRNA, a type of dsRNA, is more effective than single-stranded sense or antisense RNA fragments or hpRNAs in plants at causing PTGS. Intron removal from the spliceosome may facilitate duplex formation by bringing the complementary hairpin arms into alignment, as was seen with the spacer/intron fragment stabilizing the inverted repeat sequences (36,37). The present study validated the possible gene-silencing mechanism of an intron-mediated hairpin construct of HC-pro in *N. benthamiana*. We utilized the siDirect and pssiRNAit tools to identify the possible siRNA regions in HC-Pro. Then, we used scores like the I-Score, DSIR and s-Biopredsi to determine the effective target sites. The I score, DSIR and s-Biopredsi were among the metrics used to determine the threshold score, which indicates an inhibition level of 70-90 % (38).

Two studies found that hpRNA downregulated genes more effectively than other types of RNA. Multiple studies have demonstrated that a 20-200 bp intron is superior for efficient siRNA synthesis. The optimal size of the hairpin was found to be 100-300 bp, that it effectively downregulated the gene PVY-resistant tobacco plants were produced by encoding a spliceable intron-induced PTGS with 96 % efficiency using the transgenic hpRNA of PVY-Nia protease (36-37). Subsequently, hairpin constructions via PPV-P1 and PPV-Hc-Pro gene sequences revealed, over 90 % of transgenic *N. benthamiana* lines exhibited virus resistance (39). Also, transgenic tomato plants have developed full resistance to TMV and CMV, plum pox virus in *N. benthamiana* and *Prunus domestica*, *Citrus tristeza virus* in Mexican lime and tomato yellow leaf curl virus in transgenic tobacco plants (40-42). The expression of an intron hairpin SMV NIb RNA gave substantial RNAi-mediated resistance to various potyvirids. The present findings also agreed with earlier research showing that the ihpRNA HC-Pro gene construct conferred a significant degree of resistance to PRSV in *N. benthamiana* (15).

Due to the absence of symptoms in transgenic *N. benthamiana* plants, which were infected with PRSV-P, high levels of PRSV-P inhibition were found. In contrast, mosaic symptoms were observed in wild-type plants infected with PRSV-P. The qPCR results also showed that the HC-Pro gene was not highly expressed in the transgenic plants that were infected. The current study's findings of elevated POD gene expression in infected *N. benthamiana* (CP1-28 dpi) are consistent. Plants infected with PRSV show an increased defense response, which includes an upregulation of the genes encoding NbAPX, NbPOD and NbCAT. This upregulation could cause an increase in Reactive Oxygen Species (ROS), which in turn could cause hypersensitivity in the infected area(s), PCD and other symptoms of the disease. Infected transgenic plants showed lower levels of NbAPX, NbPOD and NbCAT compared to inoculated control plants. This study suggests that suppressing the HC-Pro gene confers resistance to PRSV infection in *N. benthamiana* through RNA-mediated defense, like what is seen in noninfected control plants and resistant varieties (43,44).

The role of ROS in stress-induced PCD regulation is crucial. Both the cell's antioxidant capacity and the transcription of genes involved in stress response are enhanced during an oxidative burst. Enzymes such as Glutathione Reductase (GR), Ascorbate Peroxidase (APX), Superoxide Dismutase (SOD), Catalase (CAT) and Peroxidase (POD) metabolize ROS and generate stress signals that activate defensive systems. While ROS, particularly hydrogen peroxide (H_2O_2), are famous for their involvement in plants' immune responses to bacterial and fungal infections, their function in interactions between plants and viruses has also been proven. Interestingly, ROS appear to have a dual function: first, they provoke the targeted death or limiting of pathogens and host plant cells. Secondly, they serve as a signal that can be diffused and induce genes associated with antioxidants and pathogenicity in neighboring plant tissues (43-45).

Prolonged infection of peach leaves with Potyviridae was associated with elevated APX activity and reduced SOD levels in soluble fractions (46-47). The current investigation also found that APX mRNA levels were higher and SOD levels were lower (46). Nevertheless, the researchers found that APX, CAT and POD activities were reduced in PPV-infected soluble fractions of apricot leaves (47). Researchers studied how antioxidant enzymes respond early in PPV infection, when H_2O_2 levels in chloroplasts go up and the enzymes that help get rid of it (APX and POD activity) go down (48). But here we showed that APX and POD mRNA levels were up in this study. When *Arabidopsis thaliana* plants were infected with the Turnip mosaic virus, POD activity increased (49). In contrast, elevated SOD and CAT activity were observed in Sunflower chlorotic mottle virus (SuCMoV) only before symptom onset, the current study found no change in SOD and CAT mRNA levels at 28 days post-infection (50). The transgenic plants are able to block the synthesis of HC-Pro by the generation of siRNAs. These siRNAs attach to complementary sequences on the viral RNA, causing Dicer to degrade it. Results like these point to incompatibility or reduced viral load in transgenic plants as a result of HC-Pro gene silencing.

Conclusion

This study concluded that the model crop *N. benthamiana* can be used to produce transgenic lines with reduced or no PRSV symptoms upon infection through the PTGS mechanism. The transgenic lines were created using an ihpRNA construct of the HC-Pro gene. Along with CP-MR, another PDR method may help plants fight viral infections. Other crops that are severely affected by potyviruses could potentially benefit from this same approach. To create transgenic lines that are resistant to PRSV infection, researchers may use multigene constructions to suppress multiple viral genes in the future.

Acknowledgements

The authors gratefully acknowledge all the members of thesis advisory committee R L Ravikumar, Nagesha N, Nataraja Karaba and Nagaraju N and they express their deepest gratitude to the Professor and Head of Department Dr. Shyamalamma S and to other staff members, Dr. Veena S Anil and Dr. K M Harinikumar for boosting moral throughout in the course of research work and stay in the department. The first author gratefully acknowledges the financial support provided by the Department of Biotechnology (DBT), Government of India, under the DBT-Junior Research Fellowship (DBT-JRF) program.

Authors' contributions

The authors contributed equally to the paper. All the authors reviewed the results and approved the final version of the manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interest to declare.

Ethical issues: None

References

1. Yeh SD, Gonsalves D. Translation of papaya ringspot virus RNA *in vitro*: detection of a possible polyprotein that is processed for capsid protein, cylindrical inclusion protein and amorphous inclusion protein. Virology. 1985;143(1):260-71. https://doi.org/10.1016/0042-6822(85)90113-8
2. Herold F, Weibel J. Electron microscopic demonstration of papaya ringspot virus. Virology. 1962;18(2):302-11. https://doi.org/10.1016/0042-6822(62)90017-X
3. Purcifull DE. Papaya ringspot virus. CMI/AAB Descr Plant Viruses. 1984:292.
4. Murphy FA. Virus taxonomy: 6th report of the International Committee on Taxonomy of Viruses. Arch Virol Suppl. 1995;10:1-586.
5. Bateson MF, Henderson J, Chaleeprom W, Gibbs AJ, Dale JL. Papaya ringspot potyvirus: isolate variability and the origin of PRSV type P (Australia). J Gen Virol. 1994;75(12):3547-53. https://doi.org/10.1099/0022-1317-75-12-3547
6. Sharma SK, Tripathi S. Horticultural characterization and papaya ringspot virus reaction of papaya Pune Selections. Indian J Hortic. 2019;76(1):32-7. https://doi.org/10.5958/0974-0112.2019.00005.7
7. Gonsalves D, Tripathi S, Carr JB, Suzuki JY. Papaya ringspot virus. Plant Health Instr. 2010;10:1094. https://doi.org/10.1094/PHII2010100401
8. Cheng YH, Yang JS, Yeh SD. Efficient transformation of papaya by coat protein encoding gene of papaya ringspot virus mediated by *Agrobacterium* following liquid phase wounding of embryogenic tissues with carborundum. Plant Cell Rep. 1996;16:127-32. https://doi.org/10.1007/BF01890852
9. Kung YJ, You BJ, Raja JA, Chen KC, Huang CH, Bau HJ, et al. Nucleotide sequence homology independent breakdown of transgenic resistance by more virulent virus strains and a potential solution. Sci Rep. 2015;5:9804. https://doi.org/10.1038/srep09804
10. Mahanthappa N. Translating RNA interference into therapies for human disease. Mol Diagn Ther. 2005;6(8):879. https://doi.org/10.2217/14622416.6.8.879
11. Pandolfini T, Molesini B, Avesani L, Spena A, Polverari A. Expression of self complementary hairpin RNA under the control of the rolC promoter confers systemic disease resistance to plum pox virus without preventing local infection. BMC Biotechnol. 2003;3:7. https://doi.org/10.1186/1472-6750-3-7
12. Hasiów Jaroszewska B, Fares MA, Elena SF. Molecular evolution of viral multifunctional proteins: the case of *potyvirus* HC-Pro. J Mol Evol. 2014;78:75-86. https://doi.org/10.1007/s00239-013-9601-0
13. Atreya CD, Pirone TP. Mutational analysis of the helper component proteinase gene of a potyvirus: effects of amino acid substitutions, deletions and gene replacement on virulence and aphid transmissibility. Proc Natl Acad Sci U S A. 1993;90(24):11919-23. https://doi.org/10.1073/pnas.90.24.11919
14. Atreya CD, Atreya PL, Thornbury DW, Pirone TP. Site directed mutations in the potyvirus HC-Pro gene affect helper component activity, virus accumulation and symptom expression in infected tobacco plants. Virology. 1992;191(1):106-11. https://doi.org/10.1016/0042-6822(92)90171-K
15. Yang X, Niu L, Zhang W, He H, Yang J, Xing G, et al. Robust RNAi mediated resistance to infection of seven potyvirids in soybean expressing an intron hairpin NIb RNA. Transgenic Res. 2017;26:665-76. https://doi.org/10.1007/s11248-017-0041-2
16. Zhou MY, Gomez Sanchez CE. Universal TA cloning. Curr Issues Mol Biol. 2000;2(1):1-7.
17. Hanahan D. Studies on transformation of *Escherichia coli* with plasmids. J Mol Biol. 1983;166(4):557-80. https://doi.org/10.1016/S0022-2836(83)80284-8
18. Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. Nucleic Acids Res. 1994;22:4673-80. https://doi.org/10.1093/nar/22.22.4673
19. Tamura K, Nei M. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol Biol Evol. 1993;10(3):512-26. https://doi.org/10.1093/oxfordjournals.molbev.a040023
20. Naito Y, Yoshimura J, Morishita S, Ui Tei K. siDirect 2.0: updated software for designing functional siRNA with reduced seed dependent off target effect. BMC Bioinformatics. 2009;10:392. https://doi.org/10.1186/1471-2105-10-392
21. ElHefnawi M, Kim T, Kamar MA, Min S, Hassan NM, El Ahwany A, et al. *In silico* design and experimental validation of siRNAs targeting conserved regions of multiple hepatitis C virus genotypes. PLoS One. 2016;11(7):e0159211. https://doi.org/10.1371/journal.pone.0159211
22. Fakhr E, Zare F, Teimoori Toolabi L. Precise and efficient siRNA design: a key point in competent gene silencing. Cancer Gene Ther. 2016;23(4):73-82. https://doi.org/10.1038/cgt.2016.4
23. Nur SM, Hasan MA, Amin MA, Hossain M, Sharmin T. Design of potential RNAi (miRNA and siRNA) molecules for Middle East respiratory syndrome coronavirus (MERS CoV) gene silencing by computational method. Interdiscip Sci Comput Life Sci. 2015;7:257-65. https://doi.org/10.1007/s12539-015-0266-9
24. Sohrab SS, El Kafrawy SA, Mirza Z, Kamal MA, Azhar EI. Design and delivery of therapeutic siRNAs: application to MERS coronavirus. Curr Pharm Des. 2018;24(1):62-77. https://doi.org/10.2174/1381612823666171109112307
25. Shiboleth YM, Haronsky E, Leibman D, Arazi T, Wassenegger M, Whitham SA, et al. The conserved FRNK box in HC-Pro, a plant viral suppressor of gene silencing, is required for small RNA binding and mediates symptom development. J Virol. 2007;81(23):13135-48. https://doi.org/10.1128/jvi.01031-07
26. Sambrook J, Russell DW. Molecular cloning: a laboratory manual. 3rd ed. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press; 2001

27. Jyothishwaran G, Kotresha D, Selvaraj T, Srideshikan SM, Rajvanshi PK, Jayabaskaran C. A modified freeze–thaw method for efficient transformation of *Agrobacterium tumefaciens*. Curr Sci. 2007;93(6):770-2.

28. Khanna H, Daggard G. *Agrobacterium tumefaciens* mediated transformation of wheat using a superbinary vector and a polyamine supplemented regeneration medium. Plant Cell Rep. 2003;21:429-36. https://doi.org/10.1007/s00299-002-0529-x

29. Doyle J. DNA protocols for plants. In: Hewitt GM, Johnston A, editors. Molecular techniques in taxonomy. Berlin, Heidelberg: Springer.1991:283-93. https://doi.org/10.1007/978-3-642-83962-7_18

30. Komarova AV, Haenni AL, Ramírez BC. Virus versus host cell translation: love and hate stories. Adv Virus Res. 2009;73:99-170. https://doi.org/10.1016/S0065-3527(09)73003-9

31. Ruanjan P, Kertbundit S, Juříček M. Posttranscriptional gene silencing is involved in resistance of transgenic papayas to papaya ringspot virus. Biol Plant. 2007;51:517-20. https://doi.org/10.1007/s10535-007-0110-0

32. Castillo XO, Fermin G, Tabima J, Rojas Y, Tennant PF, Fuchs M, et al. Phylogeography and molecular epidemiology of Papaya ringspot virus. Virus Res. 2011;159(2):132-40. https://doi.org/10.1016/j.virusres.2011.04.011

33. Yeh SD, Jan FJ, Chiang CH, Doong TJ, Chen MC, Chung PH, et al. Complete nucleotide sequence and genetic organization of papaya ringspot virus RNA. J Gen Virol. 1992;73(10):2531-41. https://doi.org/10.1099/0022-1317-73-10-2531

34. Wei JY, Liu DB, Li XY, Zhou P. Variation in the coat protein encoding gene of Papaya ringspot virus isolates from multiple locations of China. J Integr Plant Biol. 2007;49(7):1062-9. https://doi.org/10.1111/j.1672-9072.2007.00477.x

35. Mishra R, Patil S, Patil A, Patil BL. Sequence diversity studies of papaya ringspot virus isolates in South India reveal higher variability and recombination in the 5′ terminal gene sequences. Virus Dis. 2019;30:261-8. https://doi.org/10.1007/s13337-019-00512-x

36. Wesley SV, Helliwell CA, Smith NA, Wang M, Rouse DT, Liu Q, et al. Construct design for efficient, effective and high throughput gene silencing in plants. Plant J. 2001;27(6):581-90. https://doi.org/10.1046/j.1365-313X.2001.01105.x

37. Soler N, Plomer M, Fagoaga C, Moreno P, Navarro L, Flores R, et al. Transformation of Mexican lime with an intron hairpin construct expressing untranslatable versions of the genes coding for the three silencing suppressors of *Citrus tristeza virus* confers complete resistance to the virus. Plant Biotechnol J. 2012;10(5):597-608. https://doi.org/10.1111/j.1467-7652.2012.00691.x

38. Ahmed F, Senthil Kumar M, Dai X, Ramu VS, Lee S, Mysore KS, et al. pssRNAit: a web server for designing effective and specific plant siRNAs with genome wide off target assessment. Plant Physiol. 2020;184(1):65-81. https://doi.org/10.1104/pp.20.00293

39. Nicola Negri ED, Brunetti A, Tavazza M, Ilardi V. Hairpin RNA mediated silencing of *plum pox virus* P1 and HC-Pro genes for efficient and predictable resistance to the virus. Transgenic Res. 2005;14:989-94. https://doi.org/10.1007/s11248-005-1773-y

40. Hu Q, Niu Y, Zhang K, Liu Y, Zhou X. Virus derived transgenes expressing hairpin RNA give immunity to *Tobacco mosaic virus* and *Cucumber mosaic virus*. Virol J. 2011;8:41. https://doi.org/10.1186/1743-422X-8-41

41. Hily JM, Ravelonandro M, Damsteegt V, Bassett C, Petri C, Liu Z, Scorza R. Plum pox virus coat protein encoding gene intron hairpin RNA (ihpRNA) constructs provide resistance to plum pox virus in *Nicotiana benthamiana* and *Prunus domestica*. J Am Soc Hortic Sci. 2007;132(6):850-8. https://doi.org/10.21273/JASHS.132.6.850

42. Fuentes A, Ramos PL, Fiallo E, Callard D, Sánchez Y, Peral R, et al. Intron-hairpin RNA derived from replication associated protein *C1* gene confers immunity to Tomato yellow leaf curl virus infection in transgenic tomato plants. Transgenic Res. 2006;15:291-304. https://doi.org/10.1007/s11248-005-5238-0

43. Bahar T, Qureshi AM, Qurashi F, Abid M, Zahra MB, Haider MS. Changes in phytochemical status upon viral infections in plant: a critical review. Phyton. 2021;90(1):75. https://doi.org/10.32604/phyton.2020.010597

44. Radwan DEM, Fayez KA, Mahmoud SY, Lu G. Modifications of antioxidant activity and protein composition of bean leaf due to Bean yellow mosaic virus infection and salicylic acid treatments. Acta Physiol Plant. 2010;32:891-904. https://doi.org/10.1007/s11738-010-0477-y

45. Overmyer K, Brosché M, Kangasjärvi J. Reactive oxygen species and hormonal control of cell death. Trends Plant Sci. 2003;8(7):335-42. https://doi.org/10.1016/S1360-1385(03)00135-3

46. Hernández JA, Rubio M, Olmos E, Ros Barceló A, Martínez Gómez P. Oxidative stress induced by long term plum pox virus infection in peach (*Prunus persica*). Physiol Plantarum. 2004;122(4):486-95. https://doi.org/10.1111/j.1399-3054.2004.00431.x

47. Hernández JA, Díaz Vivancos P, Rubio M, Olmos E, Ros Barceló A, Martínez Gómez P. Long term plum pox virus infection produces an oxidative stress in a susceptible apricot, *Prunus armeniaca*, cultivar but not in a resistant cultivar. Physiol Plant. 2006;126(1):140-52. https://doi.org/10.1111/j.1399-3054.2005.00581.x

48. Díaz Vivancos P, Clemente Moreno MJ, Rubio M, Olmos E, García JA, Martínez Gómez P, et al. Alteration in the chloroplastic metabolism leads to ROS accumulation in pea plants in response to plum pox virus. J Exp Bot. 2008;59(8):2147-60. https://doi.org/10.1093/jxb/ern082

49. Manacorda CA, Mansilla C, Debat HJ, Zavallo D, Sánchez F, Ponz F, et al. Salicylic acid determines differential senescence produced by two *Turnip mosaic virus* strains involving reactive oxygen species and early transcriptomic changes. Mol Plant Microbe Interact. 2013;26(12):1486-98. https://doi.org/10.1094/MPMI-07-13-0190-R

50. Arias MC, Luna C, Rodríguez M, Lenardon S, Taleisnik E. *Sunflower chlorotic mottle virus* in compatible interactions with sunflower: ROS generation and antioxidant response. Eur J Plant Pathol. 2005;113:223-32. https://doi.org/10.1007/s10658-005-7559-5

Additional information

Peer review: Publisher thanks Sectional Editor and the other anonymous reviewers for their contribution to the peer review of this work.

Reprints & permissions information is available at https://horizonepublishing.com/journals/index.php/PST/open_access_policy

Publisher's Note: Horizon e-Publishing Group remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Indexing: Plant Science Today, published by Horizon e-Publishing Group, is covered by Scopus, Web of Science, BIOSIS Previews, Clarivate Analytics, NAAS, UGC Care, etc
See https://horizonepublishing.com/journals/index.php/PST/indexing_abstracting

Publisher information: Plant Science Today is published by HORIZON e-Publishing Group with support from Empirion Publishers Private Limited, Thiruvananthapuram, India.