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Double-stranded RNA confers resistance to pepper mottle virus in *Nicotiana benthamiana*

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Abstract

RNA interference (RNAi) is a regulatory mechanism of gene expression mediated by small RNAs. By using the RNAi technique, exogenous double-stranded RNA (dsRNA) designed to target mRNA, suppresses target gene expression levels in plants. In this study, we adopted the RNAi mechanism as a tool to protect plants from viruses. We designed and synthesized several dsRNAs targeting the pepper mottle virus (PepMoV) genes HC-Pro and NIb. When used on *Nicotiana benthamiana* plants, these dsRNAs protected the plant against viral infection over a specific period. By optimizing dsRNA and virus injection time, the protection efficiency of dsRNA by targeting virus genes could be maximized. It seems that exogenous dsRNA-derived RNA-induced silencing complex was able to defend the host against viral infection instantly. Furthermore, each dsRNA designed to target different regions within a transcript had varying levels of effects on virus survival in the host plants. When targeting the middle part of both the NIb and HC-Pro genes using the dsRNAs, the highest viral growth inhibitory effect was observed. An RLM-5′ RACE was performed using plant leaves infected with PepMoV after dsRNA treatment and it was observed that most of the mRNA cleavages occurred close to the 3′ part within the dsRNA target position on the mRNA. These results suggest that the dsRNA tool can be used as a plant vaccine platform for crop protection.

Keywords: RNA interference, Gene silencing, Pepper mottle virus, Exogenous dsRNA

Introduction

Pepper mottle virus (PepMoV), a member of the genus *Potyvirus* which is the largest genus of plant RNA virus, infects most *Capsicum* sp. and causes significant economic losses. PepMoV forms a flexuous rod-shaped virion containing a single-stranded plus sense RNA genome of about 10 kb in length and is transmitted by aphids in a non-persistent manner in fields. Viral genomic RNA, which is covalently linked to a viral-encoded protein (VPg) at the 5′ end and contains a polyadenylated tail at the 3′ end, encodes a large polyprotein that is cleaved by three virus-specific proteases to yield 11 mature proteins [1, 2]. thirteen PepMoV isolates were collected from five provinces in Korea [3]. Since viruses of the genus *Potyvirus* are transmitted by aphids, the preferred method of controlling transmission is by using pesticides to get rid of the aphids [4, 5]. However, there are several disadvantages of pesticide use that include the development of pesticide resistance in insects and the elimination of bacteria that are beneficial to crops. Therefore, research on sustainable control methods that target the virus is necessary.

RNA interference (RNAi) is a regulatory mechanism of gene expression induced by small RNA such as small interfering RNA (siRNA) or microRNA (miRNA). Recently, there has been a focus on the use of RNAi induced by double-strand RNA (dsRNA) to control plant viruses [6]. RNA interference is a result of the endogenously induced gene silencing effects of miRNA but can also be triggered by exogenous dsRNA leading to

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the regulation of desired genes. The dsRNA in plants is cleaved by DICER-LIKE proteins to produce siRNAs which subsequently associate with Argonaute (AGO) proteins to form the core of RNA-induced silencing complexes (RISCs). The silencing of desired genes occurs when the siRNA–RISC complex is base-paired with the complementary mRNA triggering the cleavage of mRNA [7]. This silencing effect forms the basis for plant pathogen control using RNAi [8, 9].

Research has shown that topical application of dsRNAs derived from target transcript sequences can control plant viruses including the pepper mild mottle virus (PMMoV), tobacco mosaic virus (TMV), and bean common mosaic virus (BCMV) [10–13]. A topical application could induce down-regulation of desired genes, but the development of efficient dsRNA delivery tools is essential so that RNAi can be applicable under field conditions.

The selection of target genes and securing transcript sequences in viruses and insects are of paramount importance following RNAi induction by dsRNA. Several studies have reported the use of the replicase gene in the control of PMMoV, tobacco etch virus, and alfalfa mosaic virus [10, 14] as well as the coat protein (*CP*) gene for TMV, sugarcane mosaic virus, [15–18]. Specifically, BCMV could be regulated when targeting *CP* and Nuclear Inclusion b (*NIb*) genes by application of dsRNA [11]. On the other hand, CP and Helper component-proteinase (*HC-pro*) genes were selected as target genes to control papaya ringspot virus so that dsRNAs conferred high resistance to plant against viral infection [19].

Previous studies have induced RNAi by expressing small hairpin RNA through transient expression using *Agrobacterium*-mediated transformation in plants. Recently, it has been observed that the utilization of the nanoparticle, Bioclay as a delivery method for dsRNA extended the protection time for plants by up to one month against PMMoV and BCMV [11, 12]. Topical applications are the promising method of delivering dsRNAs as they reduce the burden of plant transformation and give plants resistance to various viruses by simply loading nanoparticles. Furthermore, various studies on tools that can deliver siRNA and dsRNA into the plant cell have allowed us to break down the barrier, i.e., cell walls that plant cells have [12, 18, 20, 21].

In previous studies, issues to do with the synthesis of dsRNA for targeted transcription have not been given much attention. However, it is of importance that the synthesis of dsRNA is efficient as there exist factors that determine inefficiency including target position, length of dsRNA, and sequence preference of RNAi-processes. For example, shortening dsRNAs has been shown to lower virus suppression efficiency [10]. Additionally, a study with *Paramecium* revealed that the DCLs involved in dsRNA processing (DCL2, DCL3, and DCL5) had a preference for the cleavage sequence [22]. The current study was aimed at identifying the most effective target gene for use in controlling PepMoV via RNAi in *Nicotiana benthamiana*.

Materials and methods

Preparation of plant material and virus inoculum

N. benthamiana was used as an indicator host plant in this study. *N. benthamiana* plants were grown in a growth chamber at 25 °C under a 16 h light and 8 h dark photoperiod. Seedlings were selected for inoculation when the plants were three weeks old. An infectious full-length cDNA clone of PepMoV isolate 134 (pPepMoV:GFP-134) was described, characterized [23], and used. The pPepMoV:GFP-134 was transformed into *Agrobacterium tumefaciens* strain GV3101 and kept at − 80 °C in a freezer for use in agro-infiltration.

Double-stranded RNA design and synthesis

Several dsRNAs were designed for *HC-Pro* and *NIb* genes in PepMoV (Additional file 1: Table S1). For each gene, three dsRNAs were designed to target 5′, central, and 3′ regions in a transcript, and a certain length was overlapped between each dsRNA. This is because we considered all factors in RNAi-involved processes following the dsRNA treatment such as the length of the dsRNA and continuous sequence. Therefore, dsRNAs targeting *HC-Pro* had the same length of 530 bp with 111 bp overlapping between each dsRNA. The *NIb* had a length of 1,557 nt and the dsRNA designed to target it was 555 bp in length. There was a 54 bp overlap between the dsRNAs. The Renilla luciferase gene (*R. luciferase*) was used for control dsRNA and dsRNA targeting *R. luciferase* was designed in a length of 500 bp. To make the desired length of dsRNA, the primer in each DNA template was designed and combined with the T7 promoter sequence 5′-TAATACGACTCACAT ATAAGAGAG-3′ (Additional file 2: Table S2). A total of six pairs of primers was used in the PCR for a binary pSNU1.1 vector and a DNA template was obtained for dsRNA synthesis [23]. A sample of 1 μg of DNA template was used in the dsRNA synthesis. The dsRNA was synthesized using MEGAscript RNAi kit (Thermo Fisher Scientific, Waltham, USA) according to the manufacturer's instructions and the quality was checked by loading it into 1% agarose gel after synthesis.

Inoculation of virus and dsRNA to plant

The infectious clone, pPepMoV:GFP-134, was transformed into *A. tumefaciens* strain GV3101, and

agroinfiltration was conducted as described previously [23]. *Agrobacterium* strains to harbor the pPepMoV:GFP-134 clone were individually added to 1 ml of YEB broth containing 50 µg/ml of rifamycin and 100 µg/ml kanamycin. After the broth cultures had grown for 24 h at 28 °C and 200 rpm, 0.1 ml of each was transferred to 4.9 ml of YEP broth containing the same antibiotics and 200 µM acetosyringone. The 5-ml cultures were grown under the same conditions for 16 h before the *Agrobacterium* cells were collected by centrifugation at 3000 × *g* for 10 min. The cells were suspended and diluted in MMA buffer (pH 5.7, 10 mM MES, 10 mM $MgCl_2$, and 200 µM acetosyringone). Finally, 4 ml dsRNA of 6.25 ng/µl concentration (total 100 µg) and the virus inoculum were then infiltrated to fully expanded leaves of *N. benthamiana* by syringe to their backsides.

Determination of green fluorescent protein (GFP) expression

The infiltrated leaves were photographed with a digital camera (Nikon 7200, Tokyo, Japan). Green fluorescent expression was determined under a blue light lamp (Dark Reader Hand Lamp HL32T, Thermo Fisher Scientific, Waltham, USA) using a camera equipped with a long-pass filter (495 nm) combined with a green filter (G(X1), Hoya, Japan).

Total RNA isolation

Since the virus and dsRNA received the same treatment in the selected leaves, any part of the three leaves could achieve the same flow result. Some of the treated leaves were collected and kept frozen. Sampling was carried out a total of three times depending on the appearance of the virus replication. Five days post-inoculation (dpi), active proliferation was assessed. Movement as well as replication of the virus was determined at 8 and 12 dpi in the upper systemic leaves. Total RNA was extracted using RiboEX (GeneAll, Seoul, South Korea) according to the manufacturer's instructions. The RNA quantity and quality were confirmed using Nanodrop and loading 500 ng of RNA onto 1% agarose gel. DNase treatment was carried out to remove any residual DNA in the total RNA using Recombinant DNase I (Takara Bio, Kusatsu, Japan) according to the manufacturer's protocol.

Reverse transcription and quantitative real-time-PCR (qRT-PCR)

Complementary DNA (cDNA) was synthesized using 500 ng of total RNA and oligo dT primer using Primescript reverse transcriptase (Takara Bio, Kusatsu, Japan). Quantitative real-time PCR with an SYBR green detector was carried out using cDNA diluted with an appropriate proportion of gene-specific primer. AccuPower 2X GreenStar qPCR Master Mix (10 µl) (Bioneer, Daejeon, South Korea) was used for amplification. The GFP expressed by PepMoV was targeted with *L23* as the reference gene [24] as it is stable even when infected with a virus (Additional file 2: Table S2). The expression level of the GFP was normalized by *L23* and analyzed using the $2^{-\Delta\Delta CT}$ method [25].

RNA Ligase mediated-5′ rapid amplification of cDNA ends (RLM- 5′RACE)

RLM-5′RACE was performed to identify the cleavage positions within the target mRNA following dsRNA induced RNAi in *N. benthamiana* infected with PepMoV [26]. The GeneRacer kit (Thermo Fisher Scientific, Waltham, USA) was used according to the manufacturer's guidelines with some minor modifications. The cleaved mRNA exposes the phosphate at 5′. Using this principle, when reacting a 5′ RNA adapter and 1 µg of total RNA isolated from tobacco leaves challenged with dsRNA and PepMoV, the adapter only attaches to the cut RNA fragments. After the adapter ligation reaction, complementary DNA (cDNA) synthesis proceeded using a gene-specific primer (GSP; Additional file 2: Table S2). Complementary DNA was used for conducting a touchdown PCR with a 5′ adapter primer and GSP. After the touchdown PCR, the products obtained were used to conduct a nested PCR with 5′ adapter nested primer and nested GSP (Additional file 2: Table S2). To check the PCR product band, amplified DNA fragments were loaded onto 2% agarose gel, and DNA templates of the desired size were obtained. Finally, DNA templates were used in a TOPO cloning reaction and transformed into chemically competent DH5α cells. After obtaining various plasmids from cells and check the sequence of insert in plasmid.

Results and discussion

The *HC-Pro* and *NIb* genes in PepMoV were selected and various dsRNAs were designed to assess the efficacy of dsRNA in controlling PepMoV in *N. benthamiana*. Three kinds of dsRNA were synthesized for a transcript, and each dsRNA had lengths between 500 and 600 bp. It is noteworthy that the same length was overlapped between each dsRNA to include all the sequence elements within the gene which might affect recognition of RNAi-related machinery. Therefore, for the *HC-Pro* transcript of 1368 nt, 5′, central, and 3′ regions of the transcript are targeted, respectively and each had a length of 530 bp with 111 bp overlapped among these dsRNAs (Fig. 1a). Similarly, for the 1557 nt *NIb* transcript, dsRNAs were designed for a target of 5′, central, and 3′ regions on the *NIb* transcript with 54 bp overlapped among these dsRNAs (Fig. 1b).

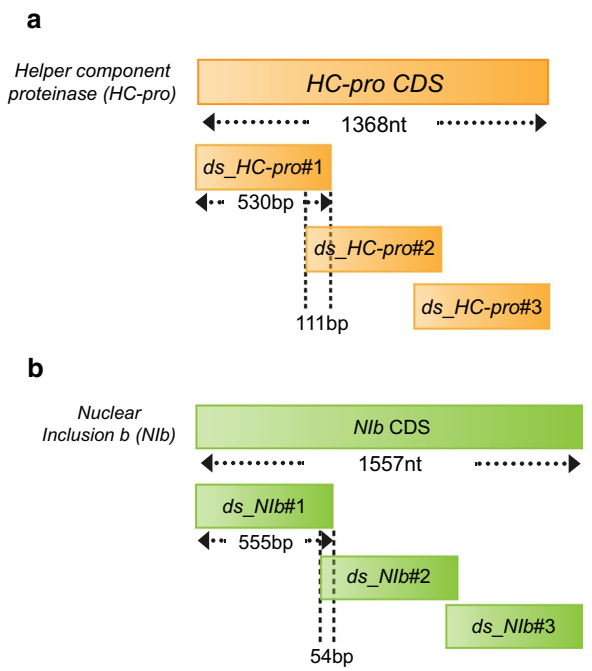


Fig. 1 PepMoV HC-pro (Helper Component-Proteinase) and NIb (Nuclear Inclusion b) gene dsRNA design. A schematic diagram of dsRNA design targeting HC-Pro (**a**) and NIb (**b**) in the pepper motor virus. In the case of HC-Pro dsRNA, it has a constant length of 530 bp and targets the 5′, central, and 3′ regions within the gene. In the case of NIb dsRNA, it is all 555 bp in length and targets 5′, central, and 3′ regions in the gene

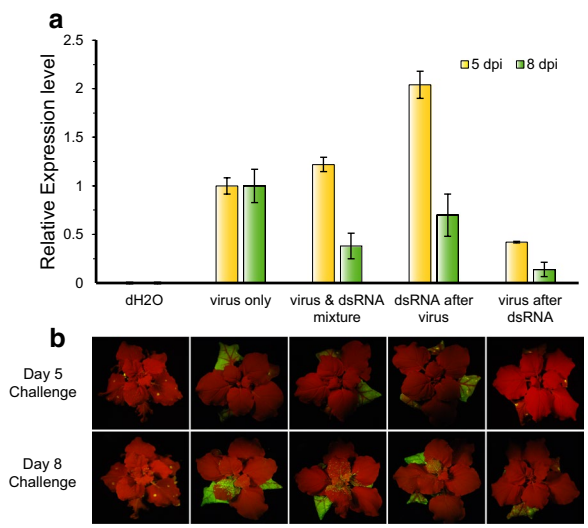


Fig. 2 qRT-PCR results and GFP expression level by adjustment of virus and dsRNA applying time in *Nicotiana Benthamiana*. qRT-PCR results (**a**) and GFP expression levels (**b**) by adjustment of virus and dsRNA application time in *Nicotiana benthamiana*. In qRT-PCR, each bar represents relative expression level of GFP in 5 dpi (yellow) and 8 dpi (green). In the UV treatment, GFP proteins tagged in PepMoV were shown in green light

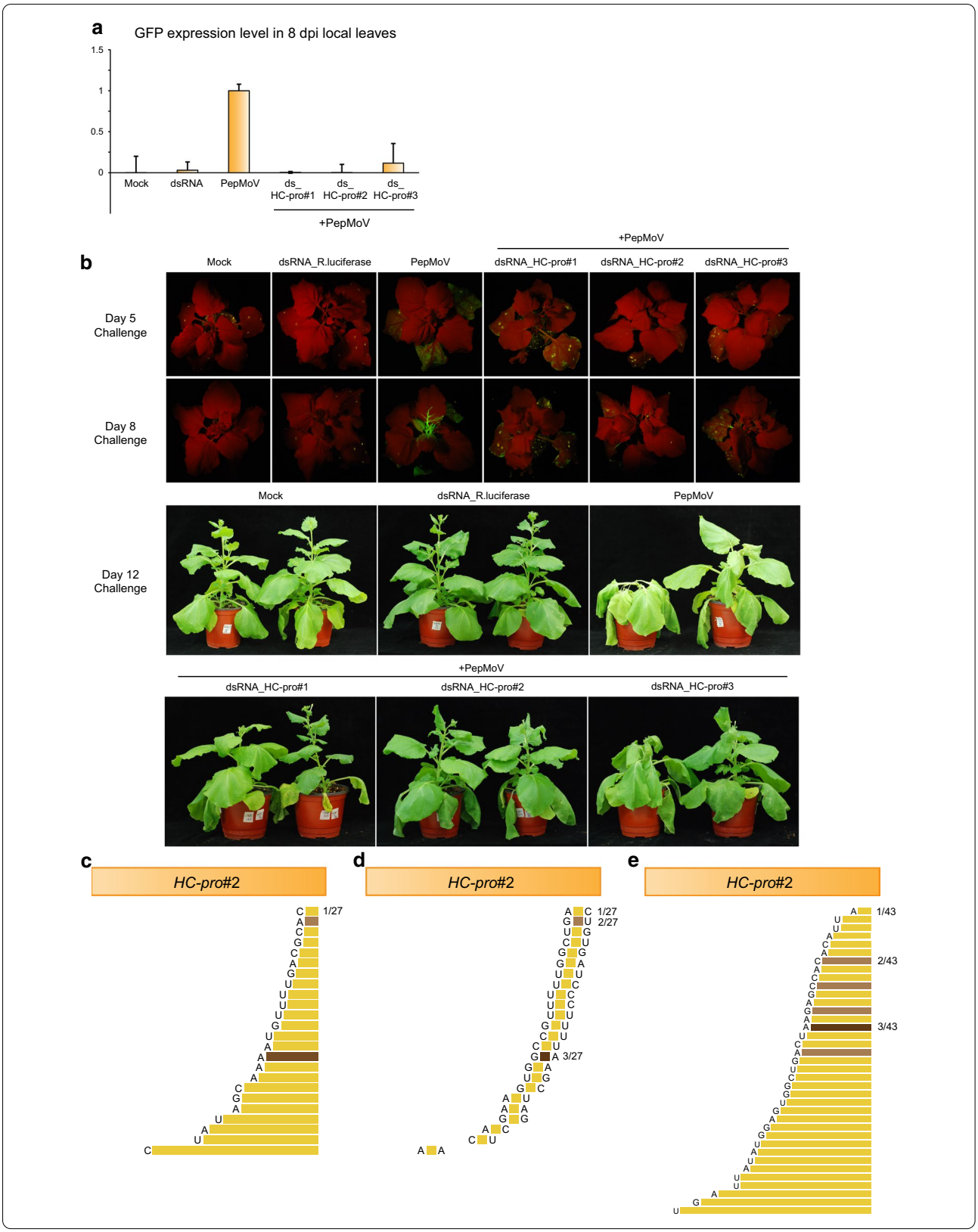
Overally, a total of six dsRNAs were synthesized to assess their effects on PepMoV replication.

Given that processing of exogenous dsRNAs into siRNAs is carried out by several RNAi-related mechanisms when dsRNAs are applied within a cell, it is necessary to adjust the application time of the virus and dsRNA to maximize the ability of dsRNA to suppress PepMoV multiplication. In the current study, the virus, and dsRNA were injected in *N. benthamiana* separately or simultaneously to determine the optimum treatment scenario. It was observed that the most significant inhibition of PepMoV replication occurred when plants were treated with dsRNA two days before viral inoculation showing 58 and 86% inhibition at 5 and 8 dpi, respectively (Fig. 2a, b). Plants inoculated either with a mixture of PepMoV and dsRNA or dsRNA following viral inoculation also inhibited viral replication. It seems that the earlier the dsRNA is introduced, the more effective the treatment. Prior treatment of dsRNA might allow the generation of siRNAs from dsRNA and form RISC in advance so that it can respond immediately upon virus infection. Therefore, a certain period is required for dsRNA processing in cells to see the RNAi effect of the treatment of exogenous dsRNA.

When the three dsRNAs targeting the *HC-Pro* gene were injected into *N. benthamiana* leaves before virus inoculation, all three dsRNAs appeared to have virus proliferation inhibiting effects based on their low level of GFP expression compared to leaves treated only with the virus. The qRT-PCR analyses showed that when the 5′ and central regions of the *HC-Pro* transcript were targeted, the virus proliferation inhibition was greater than when the 3′ region was targeted (Fig. 3a). Additionally,

(See figure on next page.)

Fig. 3 Effects of HC-pro dsRNA on suppression of virus replication and HC-pro mRNA cleavage pattern from dsRNA treatment targeting HC-pro transcript. In qRT-PCR results (**a**), bars indicate GFP expression level in inoculated leaves at 8 dpi. In the 5 dpi and 8 dpi (**b**), the level of virus replication was confirmed by observing GFP expression under UV light. At 12 dpi, the growth differences between plants resulting from virus infection are evident. RLM-5′ RACE PCR results on *N. benthamiana* leaves treated with dsRNA corresponding to the central region of the HC-Pro along with PepMoV inoculation. Each bar shows the arrangement of HC-Pro mRNA fragments in leaves applied with virus and dsRNA (**c**), predicted locations of small RNA pools generated from dsRNA treatment (**d**) and the virus only (**e**), respectively. The darkness of bar color indicates the number of repetitions of small RNAs


a
GFP expression level in 8 dpi local leaves
1.5
1
0.5
0
Mock
dsRNA
PepMoV
ds_ HC-pro#1
ds_ HC-pro#2
ds_ HC-pro#3
+PepMoV
b
Mock
dsRNA_R.luciferase
PepMoV
+PepMoV
dsRNA_HC-pro#1
dsRNA_HC-pro#2
dsRNA_HC-pro#3
Day 5 Challenge
Day 8 Challenge
Day 12 Challenge
c
HC-pro#2
1/27
d
HC-pro#2
1/27
2/27
3/27
e
HC-pro#2
1/43
2/43
3/43

(See figure on next page.)

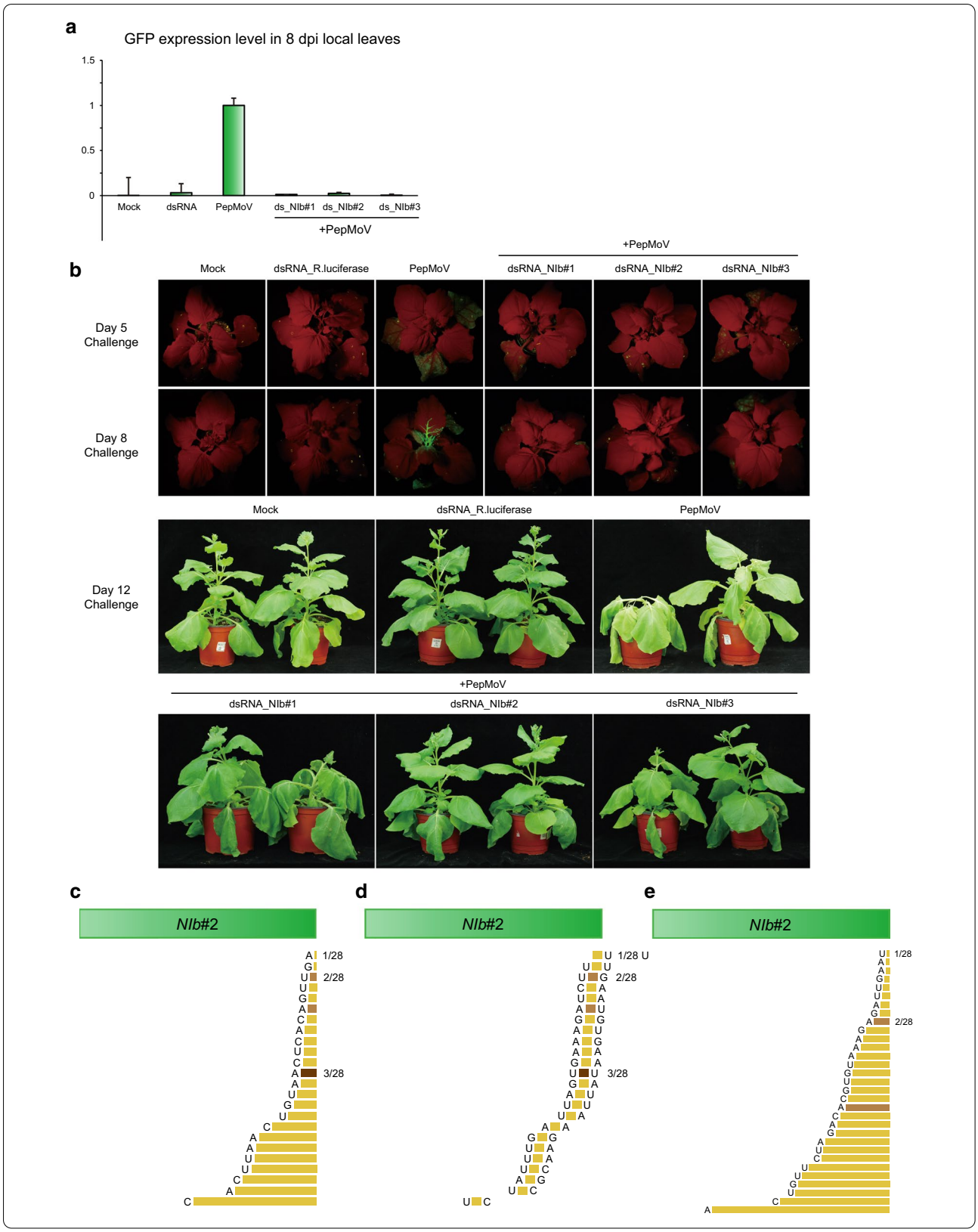
Fig. 4 Effects of NIb dsRNA on suppression of virus replication and NIb mRNA cleavage pattern from dsRNA treatment targeting NIb transcript. In qRT-PCR results (**a**), bars indicate GFP expression level in inoculated leaves at 8 dpi. In the 5 dpi and 8 dpi (**b**), the level of virus replication was confirmed by observing GFP expression under UV light. At 12 dpi, the growth differences between plants resulting from virus infection are evident. RLM-5′ RACE PCR results on *N. benthamiana* leaves treated with dsRNA corresponding to the central region of the NIb along with PepMoV inoculation. Each bar indicates the arrangement of NIb mRNA fragments in leaves challenged with virus and dsRNA (**c**), predicted locations of small RNA pools generated from dsRNA treatment (**d**) and virus only (**e**), respectively. The darkness of bar color indicates the number of repetitions of small RNAs

under UV light the level of GFP expression as a result of virus proliferation was low when dsRNA was targeted at the central region of *HC-Pro*. Considering the phenotype of the plant at 12 dpi when treated with dsRNA targeting the central region of the *HC-Pro* transcript, the plant was at its healthiest compared to the other dsRNA treated plants (Fig. 3b). Even if the three dsRNAs targeted a single viral gene transcript, they might result in differences in their effect on suppressing viral replication among dsRNAs. RLM (RNA Ligase Mediated)-5′ RACE PCR on plant leaves treated with dsRNA targeting the central region of *HC-Pro* showed that most of the cleavages occurred at the 3′ end within the position targeted by dsRNA (Fig. 3c). This pattern suggests that the small RNA derived from the treatment of dsRNA is mainly formed at the 3′ end of the transcript targeted by dsRNA (Fig. 3d). On the other hand, randomly degraded mRNA fragments were found within the *HC-Pro* transcript on plant leaves when only the PepMoV inoculation was carried out (Fig. 3e).

The inhibitory effects on virus growth of three dsRNAs targeting the *NIb* gene of PepMoV were also investigated. In the qRT-PCR results, the level of GFP mRNA expression was significantly lower in which dsRNAs were used to target 5′, central, and 3′ regions of the *NIb* compared to plants that had the virus treatment only (Fig. 4a). GFP expression was lowest in leaves treated with dsRNA targeting the central region of *NIb* (Fig. 4b). Also, when the plant was observed at 12 dpi, no symptoms were observed in the plant treated with dsRNA targeting the central region of *NIb*. Efficiency differences exist between multiple dsRNAs targeting the same *NIb* transcript, and it seems that targeting the central region of *NIb* could most effectively undermine the virus. Results of the RLM 5′ RACE PCR indicate that leaves treated with dsRNA targeting the central region of *NIb* have the *NIb* mRNA being cleaved mainly at the 3′ of the dsRNA target site within the transcript (Fig. 4c). Depending on the cleavage position, it could be expected that the small RNA pool following the application of dsRNA would also be formed on the 3′ side of the central region of *NIb* mRNA (Fig. 4d). However, since the random degradation of viral RNA occurred in leaves treated with the virus only, the cleavage pattern appeared to be evenly distributed within the *NIb* transcript (Fig. 4e).

Double-strand RNAs were cut into siRNA by DCL in plant cells and loaded onto AGO to form RISC, the key component of RNAi. The type of DCL is determined by the origin of dsRNA, and the sequence characteristics of siRNA change the AGO to which siRNA is loaded. In summary, DCL is an important factor in determining dsRNA efficiency. DCL2, DCL3, and DCL4 are known to be involved in the processing of exogenous dsRNA, but their cleavage pattern is yet to be understood. It has been reported that DCLs have a preference for nucleotide composition [27]. Furthermore, studies suggest that dsRNA efficiency is determined by guanine-cytosine (GC) content [28]. However, all of the dsRNAs used in the present study had similar GC content and showed no significant difference. The RACE trial showed that most cleavages occurred on the 3′ side of the target site.

This study provides a viable alternative for controlling the pepper virus by utilizing dsRNA. Given the environmental and biological problems that chemical pesticides have, RNAi technology would be a useful alternative for crop protection in future agriculture. We conclude that dsRNA is an important means of suppressing plant viruses and diseases. Future studies in this area should focus on the use and ease of application of RNAi technologies in plant protection [12].


a
GFP expression level in 8 dpi local leaves
1.5
1
0.5
0
Mock
dsRNA
PepMoV
ds_NIb#1
ds_NIb#2
ds_NIb#3
+PepMoV
b
+PepMoV
Mock
dsRNA_R.luciferase
PepMoV
dsRNA_NIb#1
dsRNA_NIb#2
dsRNA_NIb#3
Day 5 Challenge
Day 8 Challenge
Mock
dsRNA_R.luciferase
PepMoV
Day 12 Challenge
+PepMoV
dsRNA_NIb#1
dsRNA_NIb#2
dsRNA_NIb#3
c
NIb#2
1/28
2/28
3/28
d
NIb#2
1/28
2/28
3/28
e
NIb#2
1/28
2/28

Supplementary Information

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Additional file1: **Table S1**: dsRNA sequence targeting HC-pro and NIb gene.

Additional file 2: **Table S2**: Primer sequence.

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Author's contributions
CS, K-HK conceived the project. JY, MF, DL, and MP performed experiments. CS, K-HK, JY, and MF wrote the manuscript. All authors read and approved the final manuscript.

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Competing interests
None declared.

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