농학석사학위논문

고추모틀바이러스 방제를 위한 Double-Stranded RNA 활용에 대한 연구

Plant Protection from Viral Infection through the Application of Double-Stranded RNA in *Nicotiana Benthamiana*

2021 년 2 월

서울대학교 대학원

농생명공학부 응용생명화학전공

윤 정 연

A Dissertation for the Master of Science

Plant Protection from Viral Infection through the Application of Double-Stranded RNA in *Nicotiana Benthamiana*

February 2021

Jeongyeon Yoon
Applied Life Chemistry Major
Department of Agricultural Biotechnology
Seoul National University

고추모틀바이러스 방제를 위한
Double-Stranded RNA 활용에 대한 연구

Plant Protection from Viral Infection
through the Application of Double-Stranded RNA
in *Nicotiana Benthamiana*

지도교수 신 찬 석

이 논문을 농학석사학위논문으로 제출함
2021 년 1 월

서울대학교 대학원
농생명공학부 응용생명화학전공

윤 정 연

윤정연의 석사학위논문을 인준함
2021 년 1 월

위 원 장 권 용 훈 (인)

부 위 원 장 신 찬 석 (인)

위 원 이 상 기 (인)

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, helper component-proteinase (HC-Pro) and nuclear inclusion b (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.

Key words: crop protection, exogenous dsRNA, gene silencing, helper component-proteinase, *nicotiana benthamiana*, nuclear inclusion b, *pepper mottle virus*, RNA interference

Student Number: 2019-27043

Table of Contents

List of Figures

List of Tables

List of Abbreviations

AGO	Argonaute
BCMV	Bean common mosaic virus
cDNA	Complementary DNA
dpi	Days post-inoculation
dsRNA	Double-stranded RNA
GFP	Green fluorescent protein
GSP	Gene specific primer
HC-pro	Helper component-proteinase
miRNA	microRNA
NIb	Nuclear inclusion b
PepMoV	Pepper mottle virus
PMMoV	Pepper mild mottle virus
qRT-PCR	Quantitative real-time PCR
RISC	RNA-induced silencing complex
RLM-5′ RACE	RNA Ligase mediated-5′ rapid amplification of cDNA ends
RNAi	RNA interference
siRNA	Small interfering RNA
TMV	Tobacco mosaic virus

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 [Siaw et al., 1985; Riechmann et al., 1990]. thirteen PepMoV isolates were collected from five provinces in Korea (Kim et al., 2009]. 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 (Westwood and Stevens, 2010; Groen et al., 2017). 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 (Mitter et al., 2017). RNA interference is a result of the endogenously induced gene silencing effects of miRNA but can also be triggered by exogenous dsRNA leading to 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 (Baulcombe, 2004). This silencing effect forms the basis for plant pathogen control using RNAi (San Miguel and Scott, 2016; Gogoi et al., 2017).

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) (Tenllado and Díaz-Ruíz, 2001; Konakalla et al., 2016; Mitter et al., 2017; Mitter et al., 2017). 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 (Tenllado and Díaz-Ruíz, 2001; Tenllado et al., 2003) as well as the coat protein (*CP*) gene for TMV, sugarcane mosaic virus (Yin et al., 2009; Gan et al., 2010; Shen et al., 2014; Dubrovina and Kiselev, 2019). Specifically, BCMV could be regulated when targeting *CP* and Nuclear Inclusion b (*NIb*) genes by application of dsRNA (Worrall et al., 2019). 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 (Vadlamudi et al., 2020).

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 (Mitter et al., 2017; Worrall et al., 2019). 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 (Tang et al., 2006; Silva et al., 2010; Mitter et al., 2017; Dubrovina and Kiselev, 2019).

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 (Tenllado and Díaz-Ruíz, 2001). Additionally, a study with *Paramecium* revealed that the DCLs involved in dsRNA processing (DCL2, DCL3, and DCL5) had a preference for the cleavage sequence (Hoehener et al., 2018). 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 (Tran et al., 2019), 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. 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 500bp. To make the desired length of dsRNA, the primer in each DNA template was designed and combined with the T7 promoter sequence 5′-TAATACGACTCACATATAAGAGAG-3′ (Table 1). 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 (Tran et al., 2019). 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 (Tran et al., 2019). *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 100µg) 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 (495nm) 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. ADNase 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 & 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 (Liu et al., 2012) as it is stable even when infected with a virus (Table 2). The expression level of the GFP was normalized by *L23* and analyzed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

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 (Park and Shin, 2014). 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 1μg 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; Table 3). 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 (Table 3). 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 500–600 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). 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, 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 RNA 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* transcripts, 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 (Ho et al., 2010). Furthermore, studies suggest that dsRNA efficiency is determined by guanine-cytosine (GC) content (Birmingham et al., 2007). However, all of the dsRNAs used in the present study had similar GC contents 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,

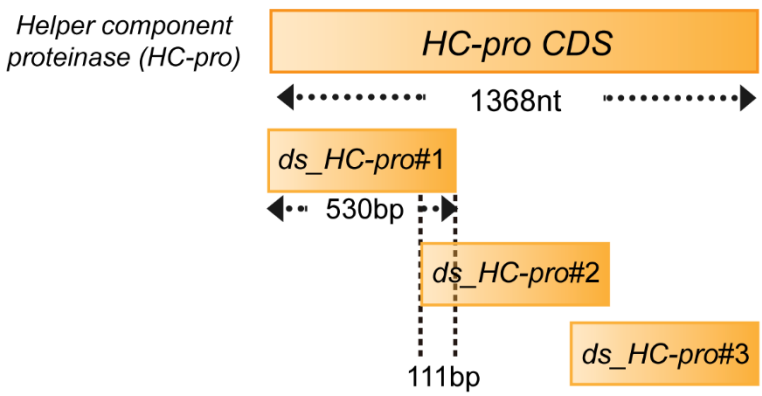
RNAi technology would be a useful alternative for crop protection in future agriculture. We conclude that dsRNA is an important means of suppressing plant viruses and diseases. Future studies in this area should focus on the use and ease of application of RNAi technologies in plant protection (Mitter et al., 2017).

Figures and Tables

Figure 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.

a


Helper component proteinase (HC-pro)
HC-pro CDS
1368nt
ds_HC-pro#1
530bp
ds_HC-pro#2
ds_HC-pro#3
111bp

b

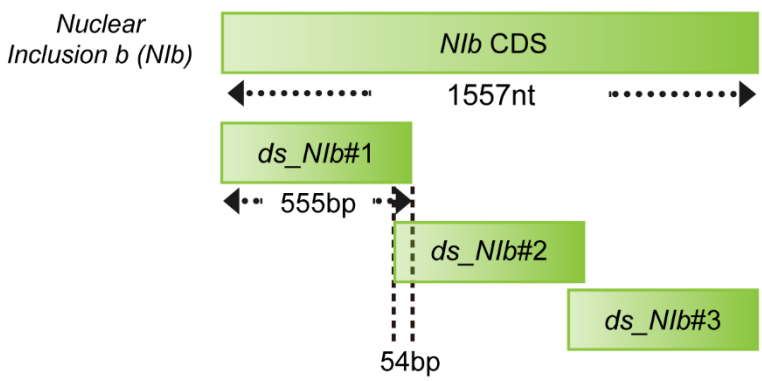

Nuclear Inclusion b (NIb)
NIb CDS
1557nt
ds_NIb#1
555bp
ds_NIb#2
ds_NIb#3
54bp

Figure 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.

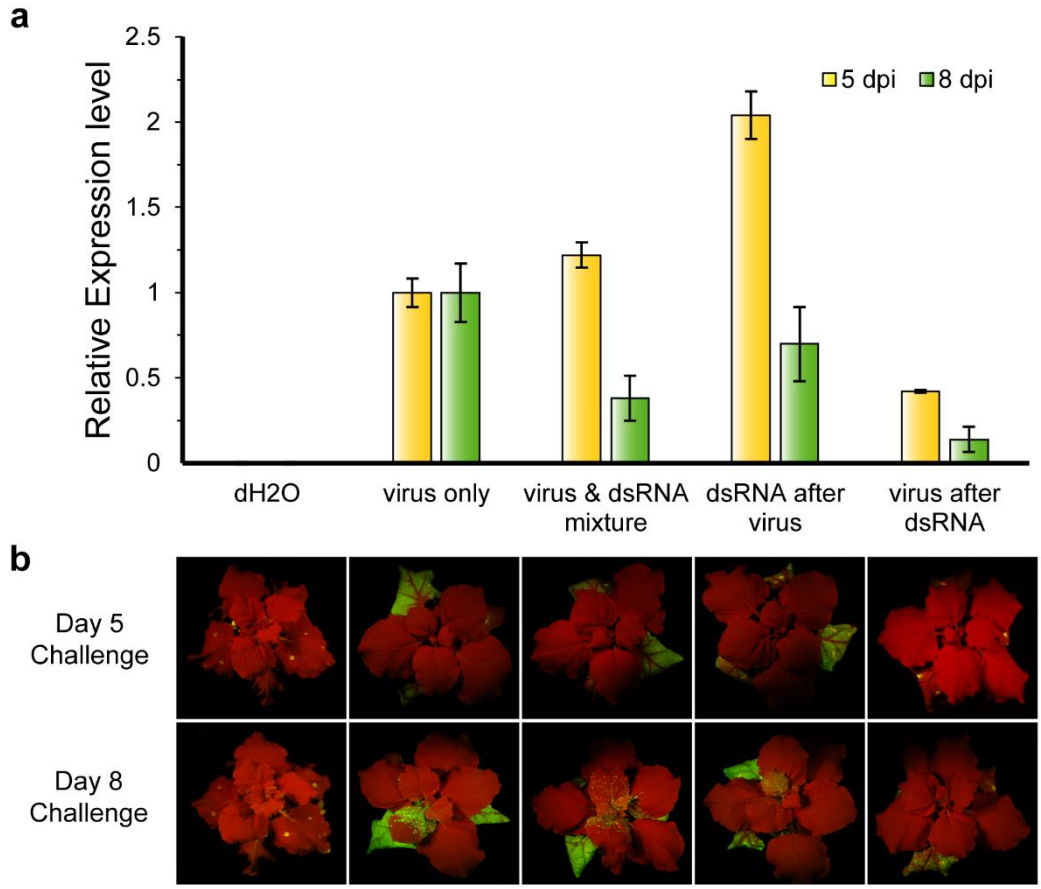

a
Relative Expression level
2.5
2
1.5
1
0.5
0
5 dpi
8 dpi
dH2O
virus only
virus & dsRNA mixture
dsRNA after virus
virus after dsRNA
b
Day 5 Challenge
Day 8 Challenge

Figure 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.

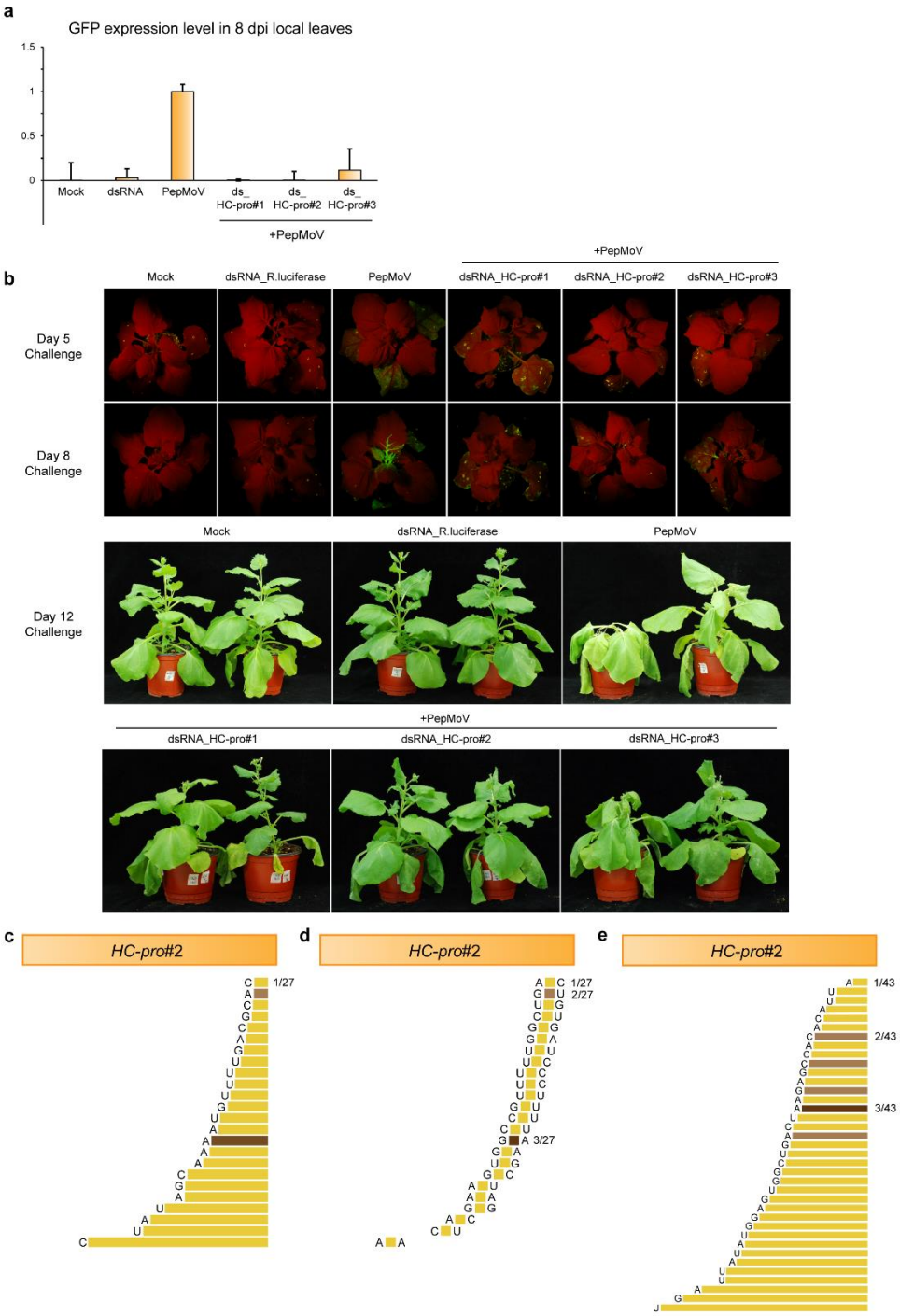

a
GFP expression level in 8 dpi local leaves
1.5
1
0.5
0
Mock
dsRNA
PepMoV
ds_ HC-pro#1
ds_ HC-pro#2
ds_ HC-pro#3
+PepMoV
b
+PepMoV
Mock
dsRNA_R.luciferase
PepMoV
dsRNA_HC-pro#1
dsRNA_HC-pro#2
dsRNA_HC-pro#3
Day 5 Challenge
Day 8 Challenge
Day 12 Challenge
Mock
dsRNA_R.luciferase
PepMoV
+PepMoV
dsRNA_HC-pro#1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

Figure 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.

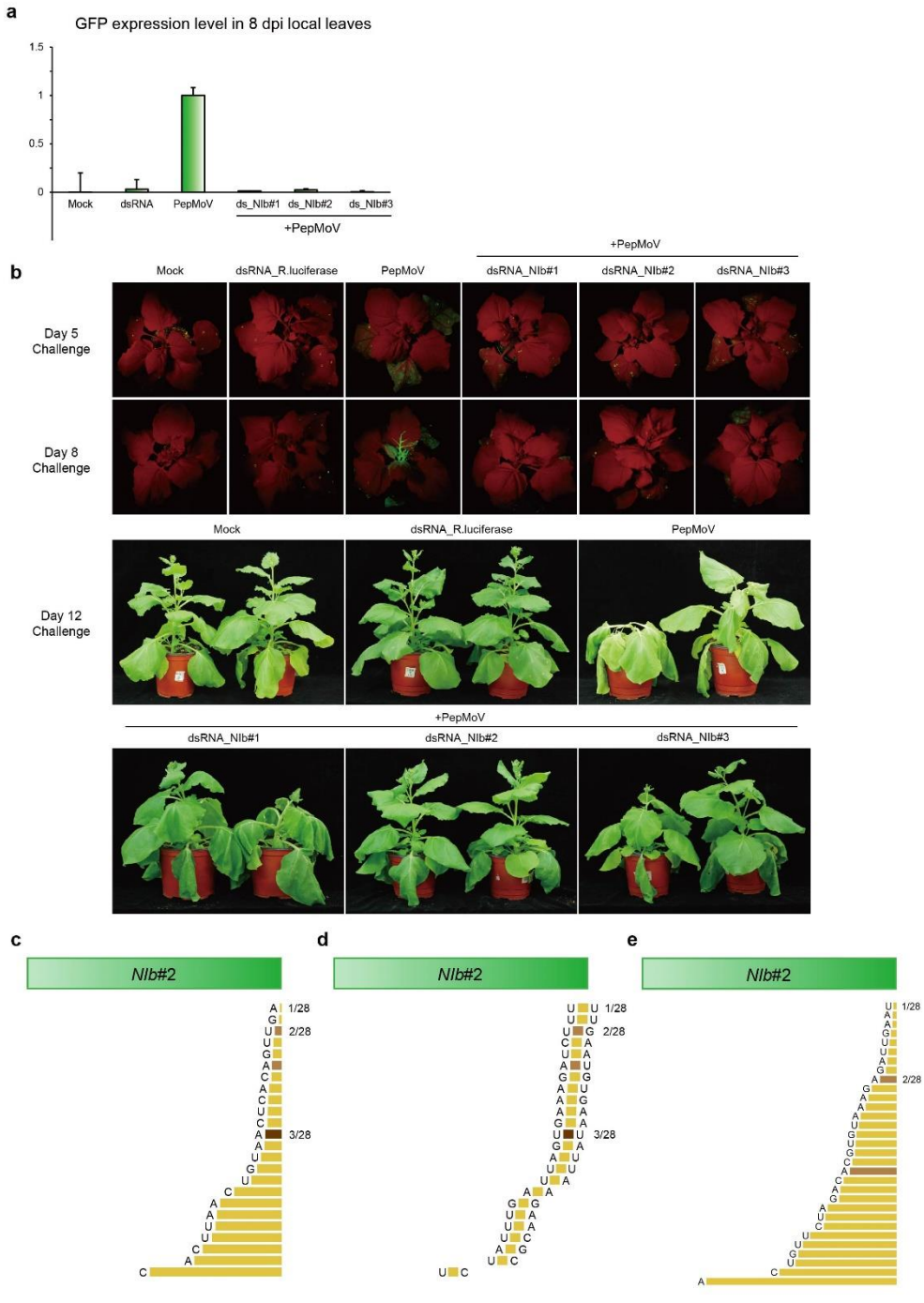

a
GFP expression level in 8 dpi local leaves
1.5
1
0.5
0
Mock
dsRNA
PepMoV
ds_Nlb#1
ds_Nlb#2
ds_Nlb#3
+PepMoV
b
Mock
dsRNA_R.luciferase
PepMoV
+PepMoV
dsRNA_Nlb#1
dsRNA_Nlb#2
dsRNA_Nlb#3
Day 5 Challenge
Day 8 Challenge
Day 12 Challenge
Mock
dsRNA_R.luciferase
PepMoV
+PepMoV
dsRNA_Nlb#1
dsRNA_Nlb#2
dsRNA_Nlb#3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

Table 1. List of primer sequences used in dsRNA synthesis

Name	Sequence (5′ to 3′)
HC-pro#1 For	TAATACGACTCACTATAGGGAGTCAACACCTGAAGCATTTT
HC-pro#1 Rev	TAATACGACTCACTATAGGGAGGCCAAGTCACCTTTCTTT
HC-pro#2 For	TAATACGACTCACTATAGGGAGGAAAGGGAACGAGAATACA
HC-pro#2 Rev	TAATACGACTCACTATAGGGAGTACCAACAACTAAATGTTTCTTAG
HC-pro#3 For	TAATACGACTCACTATAGGGAGGCCAATTGGATGGGAAT
HC-pro#3 Rev	TAATACGACTCACTATAGGGAGACCAACTCTATAGTGCTTTATCT
NIb#1 For	TAATACGACTCACTATAGGGAGGCACACACATCACCTTG
NIb#1 Rev	TAATACGACTCACTATAGGGAGCTTATTAGCCTCTATCTTTTCCTTA
NIb#2 For	TAATACGACTCACTATAGGGAGAATGGATCTCTAAAAGCTGAAC
NIb#2 Rev	TAATACGACTCACTATAGGGAGATCTCCATTAACGAAGAACTTG
NIb#3 For	TAATACGACTCACTATAGGGAGGGTGTGGTGTTTGAAGAAA
NIb#3 Rev	TAATACGACTCACTATAGGGAGCTGATGATGAACTTCATATGTACC
Renilla luciferase For	TAATACGACTCACTATAGGGAGCATCATCCCTGATCTGATCGG
Renilla luciferase Rev	TAATACGACTCACTATAGGGAGAGTTGCGGACAATCTGGA

Table 2. List of primer sequences used in qRT-PCR

Name	Sequence (5′ to 3′)
eGFP qRT For	GGACGACGGCAACTACAAGA
eGFP qRT Rev	TTGTACTCCAGCTTGTGCCC
NbL23 qRT For	AAGGATGCCGTGAAGAAGATGT
NbL23 qRT Rev	GCATCGTAGTCAGGAGTCAACC

Table 3. List of primer sequences used in RLM 5′ RACE

Name	Sequence (5′ to 3′)
HC-pro GSP1	GAAGTCCTTGGCCTCTTCCTCGCTTA
HC-pro GSP2	AGTCGGTGCGTAAACAGCTGATCGAA
NIb GSP1	CTCCGCTCGGTCCCATTGGAGAA
NIb GSP2	TTCACGTTCTGGGTTCACGGCTAT

References

Baulcombe D (2004) RNA silencing in plants. *Nature* 431, 356-363.

Dubrovina AS and Kiselev KV (2019) Exogenous RNAs for gene regulation and plant resistance. *Int J Mol Sci* 20, 2282.

Livak KJ and Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25, 402-408.

Park JH and Shin C (2014) MicroRNA-directed cleavage of targets: mechanism and experimental approaches. *BMB Rep* 47, 417-423.

San Miguel K and Scott JG (2016) The next generation of insecticides: dsRNA is stable as a foliar-applied insecticide. *Pest Manag Sci* 72, 801-809.

Tenllado F and Díaz-Ruíz JR (2001) Double-stranded RNA-mediated interference with plant virus infection. *J Virol* 75, 12288-12297.

Westwood JH, Stevens M (2010) Resistance to aphid vectors of virus disease. *Adv Virus Res* 76, 179-210.

Hoehener C, Hug I, and Nowacki M (2018) Dicer-like enzymes with sequence cleavage preferences. *Cell* 173, 234-247.e7.

Riechmann JL, Laín S, and García JA (1990) Infectious in vitro transcripts from a plum pox Potyvirus cDNA clone. *Virology* 177, 710-716.

Groen SC, Wamonje FO, Murphy AM, and Carr JP (2017) Engineering resistance to virus transmission. *Curr Opin Virol* 26, 20-27.

Tenllado F, Martínez-García B, Vargas M, and Díaz-Ruíz JR (2003) Crude extracts of bacterially expressed dsRNA can be used to protect plants against virus infections. *BMC Biotechnol* 3, 3.

Tran PT, Fang M, Widyasari K, and Kim KH (2019) A plant intron enhances the performance of an infectious clone in planta. *J Virol Methods* 265, 26-34.

Gogoi A, Sarmah N, Kaldis A, Perdikis D, and Voloudakis A (2017) Plant insects and mites uptake double-stranded RNA upon its exogenous application on tomato leaves. *Planta* 246, 1233-1241.

Kim YJ, Jonson MG, Choi HS, Ko SJ, and Kim KH (2009) Molecular characterization of Korean pepper mottle virus isolates and its relationship to symptom variations. *Virus Res* 144, 83-88.

Konakalla NC, Kaldis A, Berbati M, Masarapu H, and Voloudakis AE (2016) Exogenous application of double-stranded RNA molecules from TMV p126 and CP genes confers resistance against TMV in tobacco. *Planta* 244, 961-969.

Mitter N, Worrall EA, Robinson KE, Xu ZP, and Carroll BJ (2017) Induction of virus resistance by exogenous application of double-stranded RNA. *Curr Opin Virol* 26, 49-55.

Siaw MF, Shahabuddin M, Ballard S, Shaw JG, and Rhoads RE (1985) Identification of a protein covalently linked to the 5′ terminus of tobacco vein mottling virus RNA. *Virology* 142, 134-143.

Silva AT, Nguyen A, Ye C, Verchot J, and Moon JH (2010) Conjugated polymer nanoparticles for effective siRNA delivery to tobacco BY-2 protoplasts. *BMC Plant Biol* 10, 291.

Tang W, Weidner DA, Hu BY, Newton RJ, and Hu XH (2006) Efficient delivery of small interfering RNA to plant cells by a nanosecond pulsed laser-induced stress wave for posttranscriptional gene silencing. *Plant Sci* 171, 375-381.

Gan D, Zhang J, Jiang H, Jiang T, Zhu S, and Cheng B (2010) Bacterially expressed dsRNA protects maize against SCMV infection. *Plant Cell Rep* 29, 1261-1268.

Liu D, Shi L, Han C, Yu J, Li D, and Zhang Y (2012) Validation of reference genes for gene expression studies in virus-infected Nicotiana benthamiana using quantitative real-time PCR. *PLoS One* 7, e46451.

Birmingham A, Anderson E, Sullivan K, Reynolds A, Boese Q, Leake D et al. (2007) A protocol for designing siRNAs with high functionality and specificity. *Nat Protoc* 2, 2068-2078.

Ho T, Wang L, Huang L, Li Z, Pallett DW, Dalmay T et al. (2010) Nucleotide bias of DCL and AGO in plant anti-virus gene silencing. *Protein Cell* 1, 847-858.

Mitter N, Worrall EA, Robinson KE, Li P, Jain RG, Taochy C et al. (2017) Clay nanosheets for topical delivery of RNAi for sustained protection against plant viruses. Nat Plants 3, 16207.

Shen W, Yang G, Chen Y, Yan P, Tuo D, Li X et al. (2014) Resistance of non-transgenic papaya plants to papaya ringspot virus (PRSV) mediated by intron-containing hairpin dsRNAs expressed in bacteria. *Acta Virol* 58, 261-266.

Vadlamudi T, Patil BL, Kaldis A, Gopal DVRS, Mishra R, Berbati M et al. (2020) DsRNA-mediated protection against two isolates of Papaya ringspot virus through topical application of dsRNA in papaya. *J Virol Methods* 275, 113750.

Worrall EA, Bravo-Cazar A, Nilon AT, Fletcher SJ, Robinson KE, Carr JP et al. (2019) Exogenous application of RNAi-inducing double-stranded RNA inhibits aphid-mediated transmission of a plant virus. *Front Plant Sci* 10, 265.

Yin G, Sun Z, Liu N, Zhang L, Song Y, Zhu C et al. (2009) Production of double-stranded RNA for interference with TMV infection utilizing a bacterial prokaryotic expression system. *Appl Microbiol Biotechnol* 84, 323-333.

초 록

RNA 간섭은 small RNA 에 의해 유전자의 발현이 조절되는 현상이다. mRNA 를 표적하는 double-stranded RNA (dsRNA) 의 처리로 RNA 간섭 현상을 일으킬 수 있으므로, 외래합성 dsRNA 는 식물체 내 표적 유전자의 발현 수준을 억제하기 위해 사용된다. 본 연구에서는 바이러스로부터 식물을 보호하기 위해 RNA 간섭 현상을 이용한다. Pepper mottle virus (PepMoV)의 helper-component-proteinase (HC-pro) 유전자와 nuclear inclusion b (NIb) 유전자를 대상으로, 이를 표적하는 여러 dsRNA 를 디자인하고 합성했다. Nicotiana Benthamiana 에 PepMoV 와 PepMoV 표적 dsRNA 를 함께 처리했을 때, dsRNA 가 특정 기간 동안 바이러스의 감염으로부터 식물체를 보호했다. dsRNA 와 바이러스 처리 기간을 최적화함으로써 dsRNA 의 식물 보호 효율을 극대화하였다. 식물체에 선처리된 외래합성 dsRNA 로부터 비롯된 RNA induced silencing complex 의 생성이 바이러스의 감염으로부터 식물체를 즉각적으로 방어할 수 있었던 것으로 보인다. 또한, 동일 유전자 내의 서로 다른 구간을 대상으로 디자인된 각 dsRNA 는, 동일 유전자를 표적하더라도 바이러스 증식에 미치는 영향의 수준이 서로 달랐다. HC-pro 와 NIb 유전자의 중간 구간을 표적하는 dsRNA 의 처리 시, 가장 높은 바이러스 증식 억제 효과가 관찰되었다. dsRNA 선처리 후 PepMoV 를 감염시킨 식물 잎을 사용하여 RLM-5′ RACE 를 수행한 결과, mRNA 의 3′ 부근에서 대부분의

절단이 발생한 것으로 보인다. 본 연구 결과는 RNA 간섭 현상을 일으키는 dsRNA 가 농작물 보호를 위한 식물 백신 플랫폼으로써 사용될 수 있음을 시사한다.

주요어: 유전자 침묵 현상, 외래합성 dsRNA, 작물 보호, helper component-proteinase, *nicotiana benthamiana*, nuclear inclusion b, *pepper mottle virus*, RNA 간섭

학번: 2019-27043