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Suppression of *Phytophthora capsici* using double-stranded RNAs targeting *NLP* effector genes in *Nicotiana benthamiana*

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Abstract

RNA interference (RNAi) is a gene regulatory mechanism that involves the interaction of small interfering RNAs (siRNAs) and RNA-induced silencing complex (RISC). Dicer cleaves exogenous double-stranded RNA (dsRNA) into siRNAs, which get incorporated into RISC and bind to complementary sequences on the target mRNA to induce its degradation. In this study, we adopted RNAi technology using dsRNAs to suppress *Phytophthora capsici*, which causes diseases in solanaceous crops, including pepper. We designed and synthesized dsRNAs targeting the *P. capsici* effector genes *PcNLP2* and *PcNLP6*, respectively. These genes encode necrosis and ethylene-inducing peptide 1-like proteins in *P. capsici*, which are known to promote oomycete infection. *Nicotiana benthamiana* leaves were first infiltrated with dsRNAs and inoculated with *P. capsici* 2 days later. We confirmed significant suppression of *P. capsici* and *PcNLP2*, *PcNLP6* expression in dsRNA-treated leaves. In addition, we found that downregulation of *PcNLP2* and *PcNLP6* distinctly affected the expression of some defense-related genes. These results suggest that dsRNA mediated RNAi technology can be used to suppress various pathogens, and may contribute toward crop protection.

Keywords *Phytophthora capsici*, Double-stranded RNA, Exogenous RNA application, RNA interference

Introduction

Pepper is an important crop used worldwide as a vegetable, spice, and a source of pharmacological agents. *Phytophthora capsici* is an oomycete plant pathogen that infects solanaceous crops like pepper, causing root rot leading to yield losses [1, 2]. Criollo de Morelos 334 is a pepper landrace known to be resistant to *P. capsici* but it is not used commercially [2, 3]. Moreover, the continuous use of chemical fungicides containing mefenoxam cause *P. capsici* to develop resistance to them [4, 5]. Therefore, new strategies are necessary to manage *P. capsici*.

RNA interference (RNAi), induced by exogenous double-stranded RNA (dsRNA), can be used for crop protection and other processes as a gene silencing mechanism in plants [6]. Dicer-like endonucleases cleave dsRNA into 20–25-nucleotide small interfering RNAs (siRNAs) in plant cellular system. The guide strand of siRNAs is incorporated into the Argonaute protein to form RNA-induced silencing complex (RISC), which post-transcriptionally silences target genes through sequence-specific base pairing [6–8]. Target gene silencing via exogenous dsRNA is influenced by the length of dsRNA, regions in the target mRNA, and the method of application [9–14]. In previous studies, we suppressed green fluorescent protein and pepper mottle virus in plants using dsRNAs varying in their length and position of their targets [12–14].

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These results suggest that *P. capsici* can be effectively suppressed via exogenous dsRNA.

Plant pathogens carry various effector proteins that contribute to microbial fitness and pathogen virulence by manipulating plant immune responses at infection interface [15–18]. Necrosis and ethylene-inducing peptide 1-like proteins (NLPs) secreted by oomycetes induce leaf necrosis distinct from immune-related programmed cell death in plants. They have been suggested to play roles of both an immune response trigger and a toxin-like virulence factor [19]. Most of the NLPs in *P. sojae* are highly expressed during cyst germination and infection stages [20]. In *P. capsici*, *PcNLP2* and *PcNLP6* were highly expressed at infection stage. Moreover, the largest necrotic area in *Capsicum annuum* and *Nicotiana benthamiana* leaves were produced in agroinfection assays that delivered *PcNLP2* and *PcNLP6*, respectively, indicating the importance of these genes for virulence during infection stages [21]. Thus, inhibiting *PcNLP2* and *PcNLP6* may suppress *P. capsici* infection in plants.

The aim of this study was to suppress *P. capsici* via RNAi using dsRNAs targeting *PcNLP2* and *PcNLP6*. For efficient application of dsRNAs, location on the target gene and time of application are crucial factors [12, 13]. Therefore, we designed and synthesized two distinct dsRNAs targeting *PcNLP2* and *PcNLP6*, respectively, and applied them at the optimum time for effective *P. capsici* suppression.

Materials and methods

Plant materials and growth conditions

The wild-type *N. benthamiana* was used in this study. Its seeds were sown in 200-plug tray filled with horticultural soil (Seoulbio, Republic of Korea). Two-week-old *N. benthamiana* plants were transplanted into pots and grown at 25 ℃ under 16 h of light per day and 50% humidity in a growth chamber. Three-week-old *N. benthamiana* plants were selected for the following experiments.

Design and synthesis of dsRNAs

For each of *PcNLP2* and *PcNLP6*, two dsRNAs were designed to target the 5′ and 3′ regions of the mRNA. They had a length of 400 bp and 540 bp with overlaps of 59 bp and 63 bp between each dsRNA, respectively (Fig. 1). A 500 bp-long dsRNA targeting the *Renilla luciferase* gene was used as a mock. For dsRNA synthesis, their corresponding DNA templates, including the T7

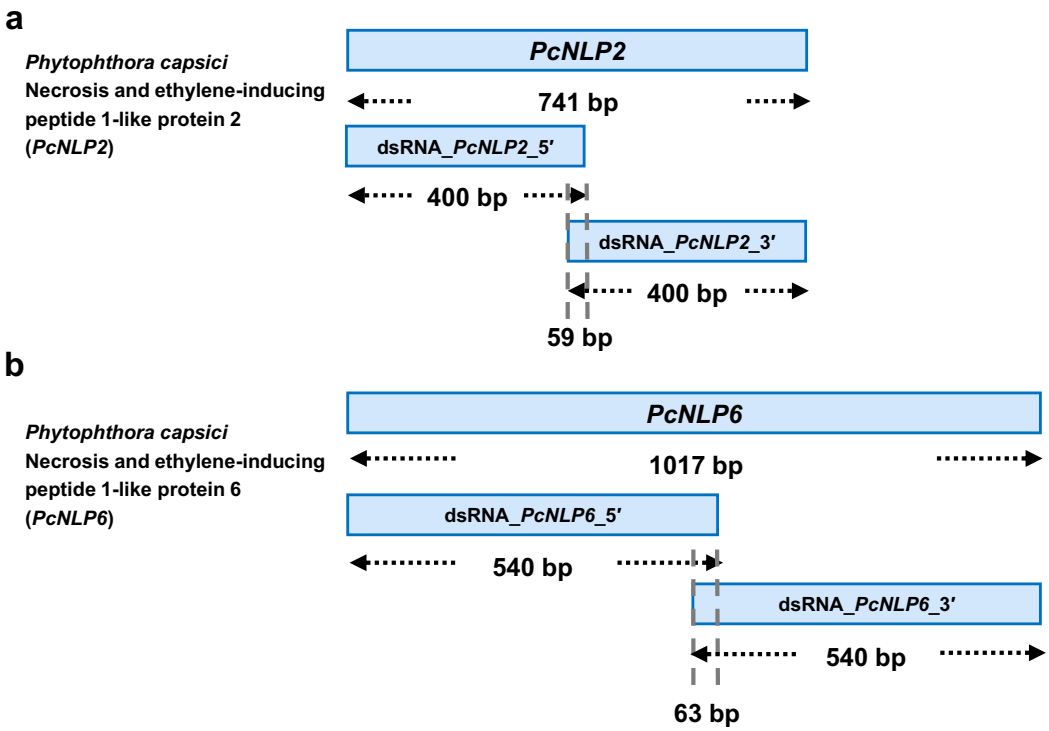

Fig. 1 Design of double-stranded RNAs (dsRNAs) targeting *PcNLP2* and *PcNLP6*. **a** dsRNA_*PcNLP2*_5′ and dsRNA_*PcNLP2*_3′ were designed to target the 5′ and 3′ regions of the *PcNLP2* mRNA sequence, respectively. Each dsRNA was 400 bp in length, with a 59 bp overlap between them. **b** dsRNA_*PcNLP6*_5′ and dsRNA_*PcNLP6*_3′ were designed to target the 5′ and 3′ regions of the *PcNLP6* mRNA sequence, respectively. Each dsRNA was 540 bp in length, with a 63 bp overlap between them

promoter sequences (5′-TAATACGACTCACATATA AGAGAG-3′), were synthesized by polymerase chain reaction (PCR) using Phusion High-Fidelity DNA polymerase (Thermo Scientific, United States), according to the manufacturer's protocol. The PCR products were then used for dsRNA synthesis using the MEGAscript RNAi Kit (Invitrogen, United States), following the manufacturer's protocol (Additional file 1: Fig. S1). The primers for PCR are listed in Additional file 1: Table S1.

Maintenance of *P. capsici*

The KACC 40476 strain of *P. capsici* was kindly provided by Dr. Doil Choi's Laboratory (Seoul National University, Seoul, Republic of Korea). It was grown on V8 juice agar media for 8 days in the dark at 23 °C.

Infiltration of dsRNAs and infection of *N. benthamiana* with *P. capsici*

According to our previous study [12], 2 mL of dsRNAs (25 ng/μL) were infiltrated 2 days before *P. capsici* infection into the abaxial side of *N. benthamiana* leaves using a 1 mL needle-free syringe. One day later, the mycelium of *P. capsici* on V8 juice agar media was scraped and incubated overnight at 23 °C under continuous light to form sporangia. The next day, the plate was filled with 10 mL of distilled water and incubated at 4 °C for 1 h to harvest the zoospores. The zoospore suspension was counted with a hemocytometer and adjusted to a concentration of 5×10^4 zoospores mL^{-1}. The abaxial side of detached *N. benthamiana* leaves was infected with a 12 μL droplet of zoospore suspension. Infected leaves were incubated in the dark at 23 °C for about 24 h and *P. capsici* infection was confirmed via phenotypic observation before sampling the leaves.

Observation of chlorophyll fluorescence expression

Chlorophyll fluorescence was confirmed by the fluorescence in vivo imaging system FOBI (Neoscience, Republic of Korea) under blue light combined with a yellow filter. This method was used to confirm *P. capsici* infection because infected lesions do not exhibit chlorophyll fluorescence. The size of infected lesions was quantified using ImageJ [22].

Total RNA extraction

After observing chlorophyll fluorescence, *N. benthamiana* leaves were sampled and ground in liquid nitrogen. Total RNA was isolated using RiboEx (GeneAll, Republic of Korea) and treated with recombinant DNase I (Takara, Japan) to eliminate single-stranded and double-stranded DNA, according to the manufacturer's instructions.

Quantitative real-time PCR (qRT-PCR)

Complementary DNA was synthesized from 1 μg of each RNA sample using PrimeScript Reverse Transcriptase (Takara, Japan) and oligo (dT) primers (Thermo Scientific, United States), according to the manufacturer's instructions. qRT-PCR was performed using Light Cycler 480 SYBR Green I Master (Roche, United States) with SYBR Green detection and gene-specific primers. The Ct values for genes were obtained using *NbL23* as a control [23], and relative expression values were calculated using the ΔΔCt method. The primers for defense-related genes were kindly provided by Dr. Doil Choi's Laboratory (Seoul National University, Seoul, Republic of Korea) and sequences of all the primers are listed in Additional file 1: Table S2.

Results

Design of dsRNAs targeting *PcNLP2* and *PcNLP6* in *P. capsici*

In our previous study [12, 13], treating plants with dsRNAs targeting different regions in the target mRNA sequence yielded different effects on the target gene. Likewise, we first examined which region of the *PcNLP2* and *PcNLP6* mRNAs should be targeted to most effectively suppress *P. capsici* infection. We designed two dsRNAs for each gene: dsRNA_*PcNLP2*_5′ and dsRNA_*PcNLP2*_3′ targeting the 5′ and 3′ regions of *PcNLP2*, dsRNA_*PcNLP6*_5′ and dsRNA_*PcNLP6*_3′ targeting the 5′ and 3′ regions of *PcNLP6*, respectively (Fig. 1). A total of four dsRNAs were synthesized to evaluate their suppressive effects on *P. capsici* infection.

Determination of the treatment time of dsRNAs and *P. capsici*

In our previous study, pepper mottle virus was suppressed the most when dsRNAs were infiltrated 2 days before viral inoculation [12], suggesting that pre-treatment of dsRNAs provides sufficient time for siRNA production and RISC formation to counteract virus infection. Hence, we injected dsRNAs into *N. benthamiana* leaves 2 days before *P. capsici* inoculation as well (Additional file 1: Fig. S2a).

We performed qRT-PCR at 3, 6, 12, 24, 27, 30, and 36 h post-inoculation (hpi) of *P. capsici* (without dsRNAs) to examine the kinetics of *PcNLP2* and *PcNLP6* expression during the infection stage in *N. benthamiana* leaves. The highest *PcNLP2* and *PcNLP6* expressions were observed at 24 hpi (Additional file 1: Fig. S3). Therefore, we measured the size of infected lesions and *PcNLP2*/*PcNLP6* expression in *P. capsica*-inoculated leaves at 24 hpi using FOBI and qRT-PCR, respectively (Additional file 1: Fig. S2).

P. capsici via suppression dsRNAs targeting *PcNLP2* and *PcNLP6*

dsRNAs targeting *PcNLP2*, *PcNLP6*, or mock (50 µg each) were infiltrated into the abaxial side of 3 week-old *N. benthamiana* leaves, which were inoculated with *P. capsici* zoospore drops 2 days later. At 24 hpi, infected lesions were significantly suppressed in dsRNA_*PcNLP2*_5′-treated leaves compared with mock-treated leaves (Fig. 2a, b), but no significant change in lesion size was observed in dsRNA_*PcNLP2*_3′-treated leaves (Additional file 1: Fig. S4a). On the contrary, dsRNA_*PcNLP6*_3′-treated leaves showed significantly suppressed lesions (Fig. 3a, b), while dsRNA_*PcNLP6*_5′-treated leaves did not (Additional file 1: Fig. S4b). These results indicate that dsRNA-mediated gene suppression varies with the region targeted, highlighting the importance of dsRNA design.

Additionally, we analyzed *PcNLP2* and *PcNLP6* transcript levels in leaves-treated with dsRNA_*PcNLP2*_5′ and dsRNA_*PcNLP6*_3′, respectively. Compared with mock-treated leaves, *PcNLP2* and *PcNLP6* expression were more than two-fold lower in dsRNA_*PcNLP2*_5′-treated leaves and dsRNA_*PcNLP6*_3′-treated leaves, respectively (Figs. 2c and 3c). Therefore, the exogenous application of these designed dsRNAs could significantly suppress *P. capsici* infection and inhibit *PcNLP2* and *PcNLP6* expression in *N. benthamiana*.

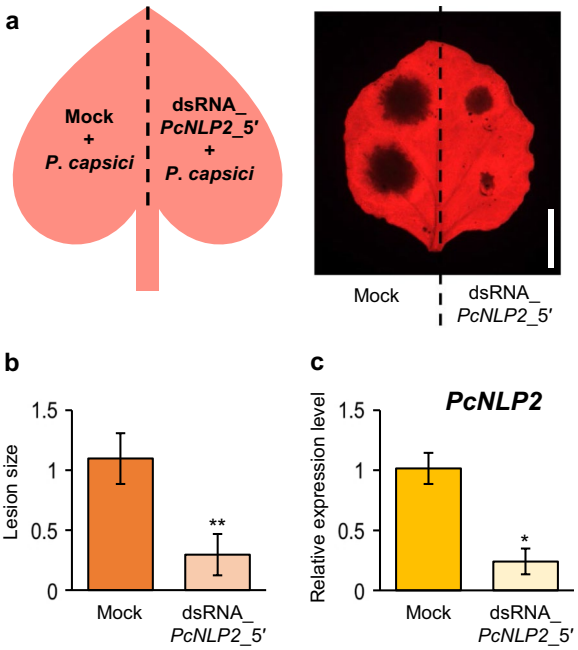

Fig. 2 Suppression of *Phytophthora capsici* infection and *PcNLP2* expression via *PcNLP2*-targeting dsRNA. **a** The left figure shows the scheme of dsRNA treatment and *P. capsici* infection, and the right figure shows the phenotype result of *P. capsici* infection after treatment with mock and dsRNA_*PcNLP2*_5′, respectively, in 3 week-old *Nicotiana benthamiana* leaves using a fluorescence in vivo imaging system FOBI. Scale bar = 1 cm. **b** Quantification of *P. capsici* infection lesion size using ImageJ. **c** Quantification of *PcNLP2* transcript level using quantitative real-time PCR (qRT-PCR). Mock: Treated with dsRNA targeting *Renilla luciferase*; dsRNA_*PcNLP2*_5′: Treated with dsRNA_*PcNLP2*_5′. Data represent mean ± standard error of mean (SEM; $N = 3$). Significance is determined by Student's *t*-test, $^{*}P < 0.05$ and $^{**}P < 0.01$

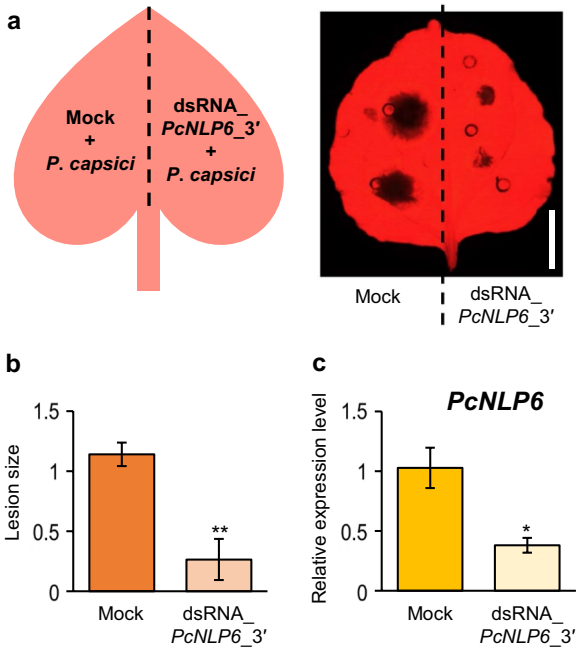

Fig. 3 Suppression of *P. capsici* infection and *PcNLP6* expression via *PcNLP6*-targeting dsRNA. **a** The left figure shows the scheme of dsRNA treatment and *P. capsici* infection, and the right figure shows the phenotype result of *P. capsici* infection after treatment with mock and dsRNA_*PcNLP6*_3′, respectively, in 3 week-old *N. benthamiana* leaves using FOBI. Scale bar = 1 cm. **b** Quantification of *P. capsici* infection lesion size using ImageJ. **c** Quantification of *PcNLP6* transcript level using qRT-PCR. Mock: Treated with dsRNA targeting *Renilla luciferase*. dsRNA_*PcNLP6*_3′: Treated with dsRNA_*PcNLP6*_3′. Data represent mean ± SEM ($N = 3$). Significance is determined by Student's *t*-test, $^{*}P < 0.05$ and $^{**}P < 0.01$

Expression of defense-related genes in *N. benthamiana* treated with dsRNAs

We investigated the expression of some well-known plant defense-related genes in response to targeting *PcNLP2* and *PcNLP6* with dsRNAs. We performed qRT-PCR on three defense-related genes in *N. benthamiana*: *Pathogenesis-related 1* (*PR1*), a defense factor that responds to pathogen infection; *WRKY8*, a disease-associated gene that interacts with mitogen-activated protein kinase involved in plant innate immunity; and *Harpin-induced 1* (*Hin1*), a marker gene for hypersensitive response [24–26].

Compared with mock-treated leaves, the expression of *NbPR1* and *NbHin1* decreased by six-fold each and that of *NbWRKY8* by two-fold in dsRNA_*PcNLP2*_5′-treated leaves. Interestingly, dsRNA_*PcNLP6*_3′ treatment only reduced the expression of *NbHin1* by three-fold, while the expressions of *NbPR1* and *NbWRKY8* were not affected significantly (Fig. 4). These results suggest that suppression of *PcNLP2* and *PcNLP6* by dsRNAs may play distinct roles in the expression of *NbPR1* and *NbWRKY8*.

Discussion

Chemical fungicides have been traditionally used to control *P. capsici* [2, 4], but their long-term use and accumulation may induce unknown mutations in the pathogen. dsRNA-mediated RNAi technology is now being widely used to target plant genes, insects, viruses, and fungi [6]. This approach enables us to respond to plant diseases faster than any other technology, especially in an ecosystem where disease-related mutations are frequent. When a specific gene mutation leads to the emergence of a new plant disease that resists existing chemical pesticides, it can be quickly managed by using dsRNAs targeting the specific mutated gene.

To date, no study has demonstrated the suppression of *P. capsici* via the suppression of *PcNLPs*. Our study showed that *P. capsici* was effectively suppressed using dsRNAs targeting the *P. capsici* effector genes *PcNLP2* and *PcNLP6*. In particular, dsRNAs targeting different mRNA regions in *PcNLP2* and *PcNLP6* brought about different effects on *P. capsici* suppression (Figs. 2, 3, and Additional file 1: File. S4). In our previous study [12], differential cleavage tags of target genes were observed in dsRNA-treated samples. Therefore, the different effects of dsRNAs on *P. capsici* may be because different amounts of siRNAs are produced from distinct dsRNA sequences, which can be confirmed by analyzing the pool of small RNAs in dsRNA-treated samples.

Since *NLPs* are known to affect plant defense and immune responses [19], we explored the expression of three well-known defense-related genes in response to *PcNLPs* suppression using qRT-PCR [27]. Interestingly, inhibition of *PcNLP2* significantly downregulated *NbPR1*, *NbWRKY8*, and, *NbHin1*, whereas inhibition of *PcNLP6* downregulated *NbHin1* only (Fig. 4). The expression of *Hin1*, a hypersensitive response marker gene [24], was expected to change in the same pattern as the size of *P. capsica*-infected lesions (Figs. 2b, 3b, and 4c). *PR* genes have been shown to induce systemic acquired resistance, increasing the defensive capacity of plants against necrotizing infections [25]. Silencing of *WRKY8*, which interacts with mitogen-activated protein kinase, has been shown to increase the burden of pathogen-induced disease [26]. Therefore, we suggest that dsRNA_*PcNLP6*_3′ is more effective than dsRNA_*PcNLP2*_5′ in maintaining the expression of *NbWRKY8* and *NbPR1* to retain the defense capacity of plants against other pathogen-induced diseases. We also suggest that *PcNLP2* and *PcNLP6* have distinct functions in the plant immune

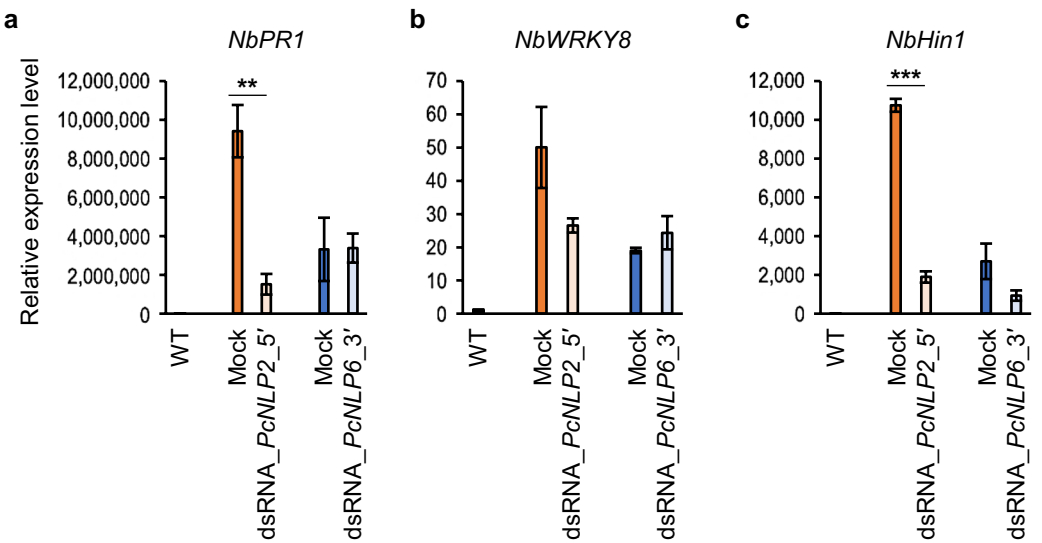

Fig. 4 Changes in expression of defense-related genes after treatment with dsRNAs targeting *PcNLP2* and *PcNLP6*, respectively. Quantification of the expression of defense-related genes **a** *NbPR1*, **b** *NbWRKY8*, and **c** *NbHin1* using qRT-PCR. WT: Uninfected wild-type *N. benthamiana*; Mock: *P. capsica*-infected *N. benthamiana* treated with dsRNA targeting *Renilla luciferase*; dsRNA_*PcNLP2*_5′: *P. capsica*-infected *N. benthamiana* treated with dsRNA_*PcNLP2*_5′; dsRNA_*PcNLP6*_3′: *P. capsica*-infected *N. benthamiana* treated with dsRNA_*PcNLP6*_3′. Data represent mean ± SEM ($N = 3$). Significance is determined by Student's *t*-test, $^{**}P < 0.01$ and $^{***}P < 0.001$

system after *P. capsici* infection, since their suppression distinctly influenced the expression of *NbWRKY8* and *NbPR1*. In the future, a transcriptome-wide analysis of dsRNA-treated plants would allow us to profile disease-relevant genes, including other defense-related genes, following suppression of *PcNLPs* by dsRNAs. This approach would help uncover the relationship between *PcNLP* effector genes of *P. capsici* and plant defense genes.

In conclusion, this study demonstrated the successful suppression of P. capsici using dsRNAs. In addition, we suggest that PcNLP2 and PcNLP6 have distinct relationships with plant defense-related genes. Based on these results, RNAi applications using dsRNAs can be used to replace chemical pesticides as well as to screen the function of a gene of interest.

Abbreviations

RNAi	RNA interference
dsRNA	Double-stranded RNA
siRNA	Small interfering RNA
RISC	RNA-induced silencing complex
NLP	Necrosis and ethylene-inducing peptide 1-like protein
qRT-PCR	Quantitative real-time polymerase chain reaction
hpi	Hours post-inoculation

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13765-023-00768-4.

Additional file 1: Table S1. Primer sequences used in dsRNA synthesis. **Table S2**: Primer sequences used in qRT-PCR. **Figure S1.** The scheme of double-stranded RNA (dsRNA) synthesis. **Figure S2.** Diagram of the dsRNA infiltration, *Phytophthora capsici* infection and screening of the phenotype. (a) Diagram of the dsRNA infiltration and *P. capsici* infection in *Nicotiana benthamiana* leaves. Two days after dsRNA syringe infiltration, *P. capsici* infection was performed. For the *P. capsici* infection, detached leaf assay was performed. (b) Diagram of the phenotype screening. The infection of the *P. capsici* was confirmed using a fluorescence *in vivo* imaging system FOBI. Blue light with yellow filter was used for the screening of the lesion size. After screening the *P. capsici* infection, quantification of the mRNA expression level was performed using quantitative real-time PCR (qRT-PCR). **Figure S3.** Transcript expression levels of effector *PcNLP2* and *PcNLP6*. (a) Transcript expression level of *PcNLP2* using qRT-PCR. (b) Transcript expression level of *PcNLP6* using qRT-PCR. Relative expression levels were calculated by the ΔΔCt method. Data represent mean ± standard error of mean (SEM; $N = 3$). Significance is determined by Student's *t*-test, $^{***}P < 0.001$. **Figure S4.** Phenotype of *P. capsici* infection in *N. benthamiana* leaves treated with dsRNA_*PcNLP2*_3′ and dsRNA_*PcNLP6*_5′. (a) The left figure shows the scheme of dsRNA treatment and *P. capsici* infection, and the right figure shows the phenotype result of *P. capsici* infection after treatment with mock and dsRNA_*PcNLP2*_3′, respectively, in 3 week-old *N. benthamiana* leaves using FOBI. Scale bar = 1 cm. (b) The left figure shows the scheme of dsRNA treatment and *P. capsici* infection, and the right figure shows the phenotype result of *P. capsici* infection after treatment with mock and dsRNA_*PcNLP6*_5′, respectively, in 3 week-old *N. benthamiana* leaves using FOBI. Scale bar = 1 cm.

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Author contributions
CS conceived the project. MP, YK, DL performed experiments. MP, YK, and CS wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
Not applicable.

Declarations

Competing interests
The authors declare that they have no competing interests.

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