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Development of multi-target dsRNAs targeting *PcNLP* gene family to suppress *Phytophthora capsici* infection in *Nicotiana benthamiana*

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Abstract

Phytophthora capsici, which causes diseases in solanaceous crops, secretes necrosis and ethylene-inducing peptide 1-like proteins (NLPs) that induce plant defense responses and leaf necrosis. In this study, we used RNA interference (RNAi) technique, a proven strategy for crop protection and gene regulation in plants, to suppress *P. capsici* infection through the inhibition of *PcNLPs*. In the RNAi mechanism, Dicer processes double-stranded RNA (dsRNA) into smaller entities known as small interfering RNAs (siRNAs). These siRNAs subsequently integrate into the RNA-induced silencing complex to form sequence-specific base pairing with complementary regions of the target mRNA. This interaction effectively initiates the degradation process of the target mRNA. We designed and synthesized dsRNAs targeting the "AIMY" and "GHRHDWE" conserved motifs of *PcNLP* gene family, which are predicted to be key elements for the expression of NLPs and pathogen infection. After infiltration of dsRNAs targeting the motifs and inoculation with *P. capsici*, we confirmed a significant suppression of *P. capsici* infection and downregulation of the *PcNLP* gene family. These findings imply that the dsRNA-mediated RNAi technique holds potential for mitigating a wide range of pathogens, while simultaneously suppressing the expression of a particular gene family using dsRNA targeting functional conserved motifs in the gene family.

Keywords RNA interference, *Phytophthora capsici*, NLP, Multi-target double-stranded RNA

Introduction

RNA interference (RNAi) has emerged as a promising tool for crop protection and gene regulation in plants. Following the exogenous introduction of double-stranded RNA (dsRNA) molecules, the RNAi pathway triggers a gene silencing mechanism, offering a potential means to control plant genes and pathogens [1]. In the cellular system of plants, dsRNA is cleaved by Dicer-like endonucleases into small interfering RNAs (siRNAs). These siRNAs are then incorporated into the Argonaute protein, forming the RNA-induced silencing complex, which can specifically target and silence complementary sequences in the target mRNA [1–3].

The oomycete plant pathogen *Phytophthora capsici* (*P. capsici*) targets solanaceous crops, including pepper,

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which causes root rot and results in substantial yield losses [4, 5]. The secretion of necrosis and ethylene-inducing peptide 1-like proteins (NLPs) by oomycetes induces both the plant defense response and leaf necrosis [6, 7]. In *P. capsici*, significant upregulation of *PcNLP2* and *PcNLP6* was observed during the infection stage. Furthermore, when *PcNLP2* and *PcNLP6* were introduced in agroinfection assays, *Capsicum annuum* and *Nicotiana benthamiana* leaves showed the most extensive necrotic areas, indicating the crucial role of these genes in promoting virulence throughout the infection phases [8]. Most of the NLPs contain the "AIMY" motif and highly sequence-conserved "GHRHDWE" motif. Mutations in the "AIMY" motif in NLP1 reduce the production of reactive oxygen species and suppress *Colletotrichum orbiculare* infection in cucumber [9]. Additionally, the "GHRHDWE" motif is a requirement for the activity of NLPs. In addition, this motif is critically involved in cavity formation on the protein surface, which is important for the necrosis-inducing activity of *Pectobacterium carotovorum* [10]. These results suggest that the "AIMY" and "GHRHDWE" motifs in *PcNLP2* and *PcNLP6* may play a particularly important role in *P. capsici* infection.

In our previous studies, we suppressed pathogens, including the pepper mottle virus and *P. capsici*, using dsRNAs varying position of their targets [11–13]. We confirmed that *P. capsici* infection was suppressed by dsRNAs targeting *PcNLP2* and *PcNLP6* of *P. capsici*. Significant differences in the suppression of *P. capsici* infection were observed depending on the regions of *PcNLP2* and *PcNLP6* that were targeted by the dsRNA [13]. Interestingly, the dsRNAs that effectively suppressed *P. capsici* infection targeted regions containing the "AIMY" and "GHRHDWE" motifs. Therefore, to investigate the role of the "AIMY" and "GHRHDWE" motifs in *P. capsici* infection, we used dsRNAs specifically targeting regions containing these motifs in *PcNLP2* and *PcNLP6*, as well as dsRNAs targeting regions not containing the motifs. Furthermore, we examined whether dsRNAs targeting regions containing the motifs also suppressed the expression of other *PcNLPs*. Through this approach, we aimed to ascertain whether our designed multi-target dsRNA, which targets the functional conserved motifs of a particular gene family, can effectively regulate the expression of that gene family.

Materials and methods

Plant growth conditions and *P. capsici* maintenance

The wild-type *Nicotiana benthamiana* was grown at 25 ℃ and 50% humidity, exposed to a daily photoperiod of 16 h of light followed by 8 h of darkness, all within a growth chamber. For the experiments, *N. benthamiana* leaves were selected when plants were 3 weeks old. The KACC 40476 strain of *P. capsici*, generously supplied by Dr. Doil Choi's Laboratory (Seoul National University, Seoul, Republic of Korea), was grown on V8 juice agar medium for 8 days, maintained in continuous darkness at 23 ℃.

Design and synthesis of dsRNAs

Two dsRNAs, each 200 bp in length, were designed to target the regions containing the "AIMY" and "GHRHDWE" motifs of *PcNLP2* and *PcNLP6*, and regions not containing the motifs, respectively (Fig. 1a, b). The conservation of amino acids and RNA sequences was measured using Jalview software [14]. A 200 bp dsRNA, designed to target the *Renilla luciferase* gene, was employed as a mock in the experiments. As reported in our previous study [13], for the synthesis of dsRNAs, we first prepared the corresponding DNA templates, incorporating T7 promoter sequences (5′-TAATAC GACTCACATATAAGAGAG-3′). This was achieved through a polymerase chain reaction (PCR) utilizing Phusion High-Fidelity DNA polymerase (Thermo Scientific, United States), in accordance with the manufacturer's protocol. The resulting PCR products were subsequently employed in the dsRNA synthesis process utilizing MEGAscript RNAi Kit (Invitrogen, United States), in accordance with the manufacturer's protocol. The purity of dsRNAs was shown in Additional file 1: Fig. S1. The primers used for PCR are listed in Additional file 1: Table S1.

Administration of dsRNAs and *P. capsici* inoculation in *N. benthamiana*

As reported in our previous study [13], 2 days prior to *P. capsici* inoculation, we introduced 500 µL of dsRNAs (200 nM) into the abaxial side of *N. benthamiana* leaves using a needle-free syringe. A day before the *P. capsici* inoculation, the mycelium of *P. capsici*, cultured on V8 juice agar medium, was scrapped and left to incubate overnight under continuous exposure to light at 23 ℃ to facilitate sporangia formation. On the day of inoculation, the culture plate was flooded with 10 mL of distilled water, followed by a 1 h incubation at 4 ℃ to collect the zoospores. When the concentration of the zoospores reached at the 5×10^4 zoospores mL^{-1}, 12 µL of collected zoospore suspension was inoculated in the abaxial side of the *N. benthamiana* leaves. Following inoculation, the leaves were incubated in darkness at 23 ℃ for approximately 24 h. The confirmation of *P. capsici* infection was conducted through phenotypic observation prior to sampling of leaves.

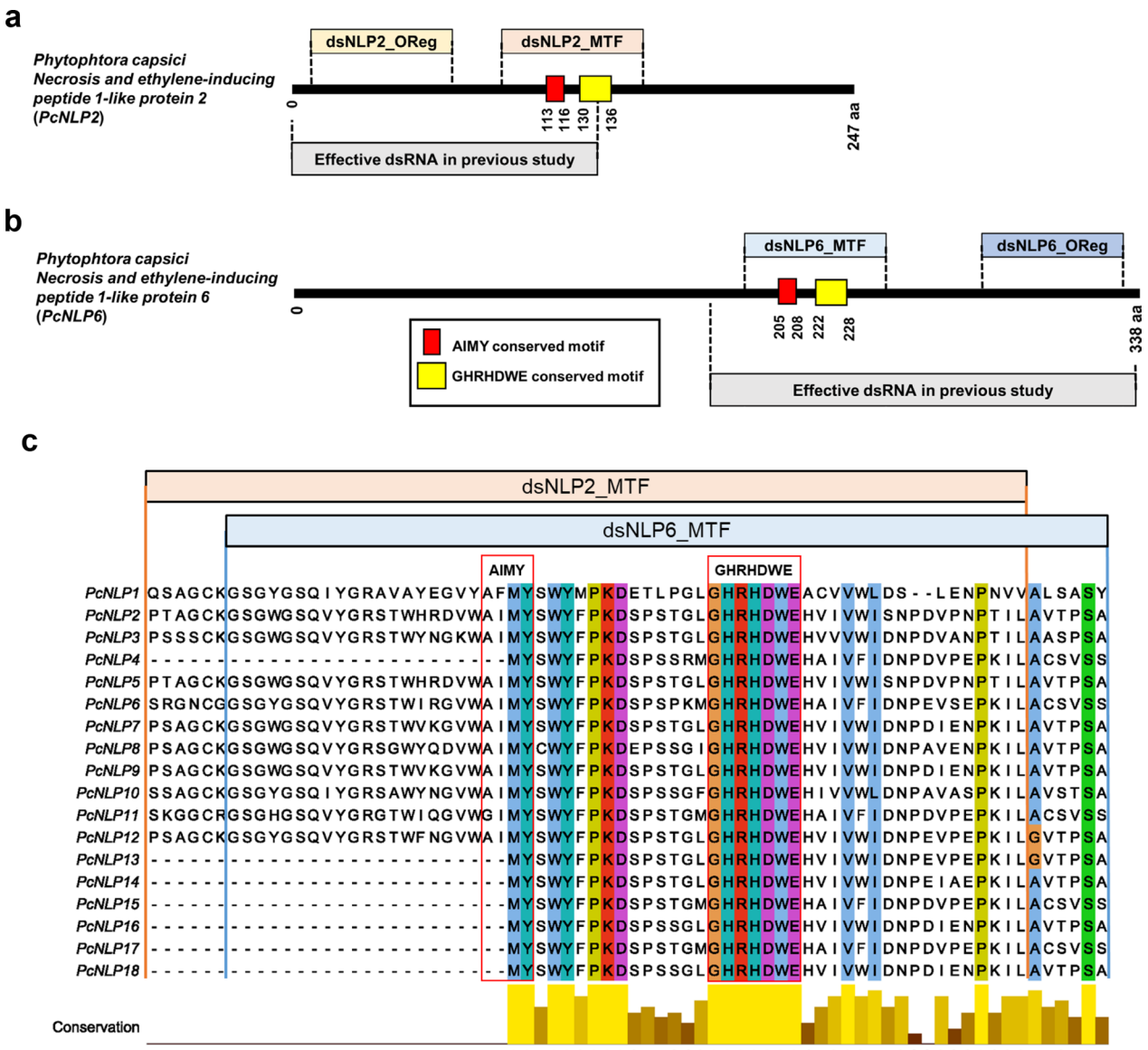

Fig. 1 Design of double-stranded RNAs (dsRNAs) targeting the "AIMY" and "GHRHDWE" motifs of *PcNLP2* and *PcNLP6*. **a** dsNLP2_MTF and dsNLP2_OReg were designed to target the regions containing the "AIMY" and "GHRHDWE" motifs of *PcNLP2*, and the outer region not containing the motifs, respectively. The length of each dsRNA was 200 bp. **b** dsNLP6_MTF and dsNLP6_OReg were designed to target the region containing the "AIMY" and "GHRHDWE" motifs of *PcNLP6*, and the outer region not containing the motifs, respectively. The length of each dsRNA was 200 bp. **c** Conservations of amino acids in *PcNLPs* in the regions targeted by dsNLP2_MTF and dsNLP6_MTF was measured using Jalview software. MTF: Motif, OReg: Outer region. Red boxes: "AIMY" and "GHRHDWE" motifs region

Assessment of chlorophyll fluorescence expression in *N. benthamiana* leaves

The infection of *P. capsici* on *N. benthamiana* leaves was confirmed by chlorophyll fluorescence expression using FOBI fluorescence in vivo imaging system (Neoscience, Republic of Korea), as previously described [13]. ImageJ was used to quantify the size of lesion [15].

Total RNA extraction and complementary DNA (cDNA) synthesis

Following assessment of chlorophyll fluorescence expression, *N. benthamiana* leaves were collected and ground in liquid nitrogen. Total RNA extraction was performed using RiboEx (GeneAll, republic of Korea), and the extracted total RNA was treated with recombinant

DNase I (Takara, Japan) to remove both single-stranded and double-stranded DNAs, in accordance with the manufacturer's instructions.

To synthesize cDNA, we utilized 1 μg of each RNA sample. This process was executed using PrimeScript Reverse Transcriptase (Takara, Japan) with oligo (dT) primers (Thermo Scientific, United States), in accordance with the manufacturer's instructions.

Quantitative real-time PCR (qRT-PCR)

Light Cycler 480 (Roche, United States) with AccuPower 2X GreenStar qPCR Master Mix (Bioneer, Republic of Korea) was used for performing qRT-PCR, along with cDNA and gene-specific primers. The Ct values for the target genes were normalized to those of the housekeeping gene *NbEF1a*, as a control. To calculate relative expression levels, the ΔΔCt method was employed. Statistical analysis was performed using Student's *t*-test (*$P<0.05$; **$P<0.01$; ***$P<0.001$). Due to high homology among *PcNLPs*, we designed qRT-PCR primers considering potential off-target effects and the motif regions affected by dsRNAs. The primer sequences are listed in Additional file 1: Table S2. The qRT-PCR target regions within *PcNLPs* are depicted in Additional file 1: Fig. S2.

Results

Design of dsRNAs targeting "AIMY" and "GHRHDWE" motifs of *PcNLP2* and *PcNLP6*

In our previous study [13], plants treated with dsRNAs targeting 5′ and 3′ regions of *PcNLP2* and *PcNLP6* sequence yielded different effects on the target gene and *P. capsici* infection. The dsRNAs that targeted the 5′-region of *PcNLP2* and the 3′-region of *PcNLP6* resulted in effective suppression of target gene expression and *P. capsici* infection. Interestingly, the target regions of the effective dsRNAs contained the "AIMY" and "GHRHDWE" motifs (Fig. 1a, b), which are predicted to be key elements in *P. capsici* infection and the expression of *PcNLPs*, based on previous studies [9, 10]. Therefore, we designed two dsRNAs for each gene: dsNLP2_MTF and dsNLP6_MTF (MTF; Motif) targeting both the "AIMY" and "GHRHDWE" motifs of *PcNLP2* and *PcNLP6*, respectively, and dsNLP2_OReg and dsNLP6_OReg (OReg; Outer region) targeting regions that do not contain motifs, which were the outer regions of effective dsRNA in previous study [13], respectively (Fig. 1a, b). dsNLP2_MTF and dsNLP6_MTF are designed to target the "AIMY" motif on nine *PcNLPs* and the "GHRHDWE" motif on all *PcNLPs* (Fig. 1c). In addition, the RNA sequences of "AIMY" and "GHRHDWE" motifs, which are targets of dsRNA, are identical in most *PcNLPs* (Additional file 1: Fig. S3). In total four dsRNAs were synthesized to evaluate ability to suppress *P. capsici* infection and alter the expression of *PcNLPs*.

Suppression of *P. capsici* infection via dsRNAs targeting "AIMY" and "GHRHDWE" motifs of *PcNLP2* and *PcNLP6*

Consistent with the previous study [13], we introduced dsRNAs targeting the motif regions and the outer region of *PcNLP2*, *PcNLP6*, or mock into the *N. benthamiana* leaves to assess the effect of dsRNAs on *P. capsici* infection (Figs. 2a, 3a). We measured the size of the infected lesions and the expression of *PcNLP2* and *PcNLP6* in *P. capsica*-inoculated leaves at 24 h post-inoculation (hpi) using FOBI and qRT-PCR, respectively. At 24 hpi, infected lesions were significantly suppressed in dsNLP2_MTF-treated leaves compared with mock-treated leaves (Fig. 2b, d), but there was less suppression in dsNLP2_OReg-treated leaves than the dsNLP2_MTF-treated leaves (Fig. 2c, d). Similarly, there was significant suppression of lesions in dsNLP6_MTF-treated leaves (Fig. 3b, d), whereas this did not occur in dsRNA6_OReg-treated leaves (Fig. 3c, d).

Additionally, we analyzed the levels of *PcNLP2* and *PcNLP6* transcript in leaves treated with dsRNAs, respectively. Compared with mock-treated leaves, *PcNLP2* expression was 57-fold lower in dsNLP2_MTF-treated leaves, and *PcNLP6* expression was 6,133-fold lower in dsNLP6_MTF-treated leaves (Figs. 2e, 3e). These results indicate that dsRNAs targeting the motifs suppress *P. capsici* infection and suppress the expression of *PcNLP2* and *PcNLP6*. However, dsRNAs targeting the outer region did not significantly affect the expression of *PcNLP2* and *PcNLP6* ($P>0.05$), causing only a slight reduction (Figs. 2e, 3e). In addition, they had no effect on *P. capsici* infection (Figs. 2d, 3d).

Expression of *PcNLPs* in *P. capsici* treated with dsRNAs targeting "AIMY" and "GHRHDWE" motifs of *PcNLP2* and *PcNLP6*

We performed qRT-PCR on *PcNLPs* of *P. capsici* to investigate the multi-target effects of dsNLP2_MTF and dsNLP6_MTF. Interestingly, compared with mock-treated leaves, the expression of *PcNLPs* decreased from 2-fold to 362-fold in dsNLP2_MTF-treated leaves (Fig. 4a), and from 5-fold to 6,133-fold in dsNLP6_MTF-treated leaves (Fig. 4b). However, there was no significant decrease in the expression of *PcNLPs* in dsNLP2_OReg and dsNLP6_OReg-treated leaves (Additional file 1: Fig. S4). A Ct value for *PcNLP8* was not detected in mock-treated and dsNLP6_MTF-treated leaves (Fig. 4b). This was attributed to the significantly lower expression level of *PcNLP8* at the *P. capsici* infection stage compared with other *PcNLPs*, resulting in the non-detection of *PcNLP8* using qRT-PCR. (Additional file 1: Fig. S5). Overall, these

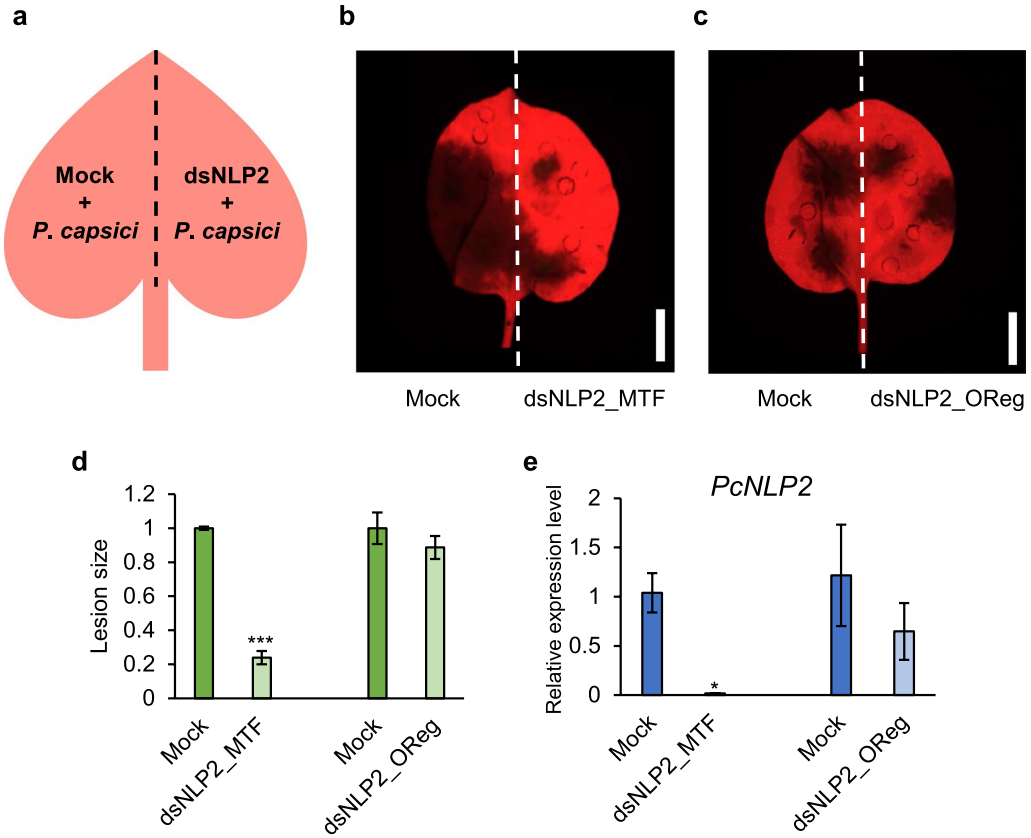

Fig. 2 Regulation of *Phytophthora capsici* infection and *PcNLP2* expression via dsNLP2_MTF and dsNLP2_OReg. **a** The experimental scheme for dsRNA treatment and *P. capsici* infection. **b** The phenotype of *P. capsici* infection lesion introduced with mock and dsNLP2_MTF, respectively, in 3-week-old *Nicotiana benthamiana* leaves, determined using a FOBI in vivo fluorescence imaging system. Scale bar = 1 cm. **c** The phenotype of *P. capsici* infection lesion introduced with mock and dsNLP2_OReg, respectively. Scale bar = 1 cm. **d** Quantification of *P. capsici* infection lesion size using ImageJ. **e** Relative expression level of *PcNLP2* using quantitative real-time PCR (qRT-PCR). Mock: Treatment with dsRNA targeting *Renilla luciferase*; dsNLP2_MTF: Treatment with dsNLP2_MTF; dsNLP2_OReg: Treatment with dsNLP2_OReg. MTF: Motif, OReg: Outer region. Data represent the mean ± standard error of mean (SEM; $N = 3$). Statistical significance is determined by Student's *t*-test (*$P < 0.05$ and ***$P < 0.001$)

results suggest that multi-target dsRNAs targeting the "AIMY" and "GHRHDWE" motifs of *PcNLPs* not only suppress *P. capsici* infection, but also reduce the expression of *PcNLPs*.

Discussion

Traditional fungicides have conventionally controlled *P. capsici* [5, 16], but their prolonged use and accumulation may induce fungicide-resistant pathogens and unknown mutations in the pathogen. dsRNA-mediated RNAi technology is now extensively employed to target plant genes, insects, viruses, and fungi [1]. This approach allows the fastest response to the mutation of plant pathogens as dsRNAs can be targeted to the specific mutated gene.

In a previous study [13], we showed the effective suppression of *P. capsici* by dsRNAs targeting specific regions of the *P. capsici* effector genes *PcNLP2* and *PcNLP6*. In this study, we confirmed that the "AIMY" and "GHRHDWE" motifs were included in the target regions of the effective dsRNAs used in the previous study. The dsRNAs were designed and synthesized by dividing the target regions of the effective dsRNAs used in the previous study into regions containing motifs (dsNLP2_MTF, dsNLP6_MTF) and regions not containing motifs (dsNLP2_OReg, dsNLP6_OReg) (Fig. 1a, b). dsNLP2_MTF and dsNLP6_MTF, which targeted the motif regions, effectively suppressed *P. capsici* infection (Figs. 2, 3) and the expression of other *PcNLPs* (Fig. 4), including *PcNLP2* and *PcNLP6*. However, dsNLP2_OReg and dsNLP6_OReg did not significantly affect the expression of *PcNLP2*, *PcNLP6*, and *P. capsici* infection, respectively (Figs. 2, 3). The region targeted by dsNLP2_OReg does not contain conserved motifs and the RNA sequences of *PcNLPs* compared with the region targeted by dsNLP2_MTF (Additional file 1: Figs. S6, S7). However, the region targeted by dsNLP6_OReg contains

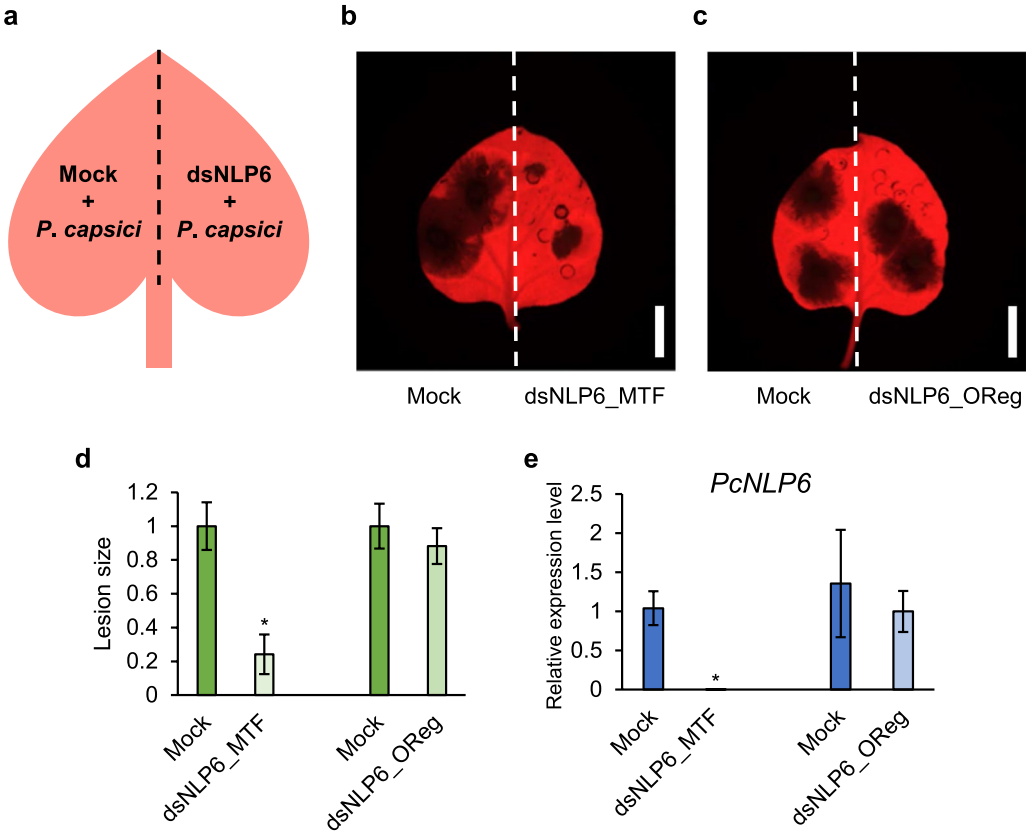

Fig. 3 Regulation of *Phytophthora capsici* infection and *PcNLP6* expression via dsNLP6_MTF and dsNLP6_OReg. **a** The experimental scheme for dsRNA treatment and *P. capsici* infection. **b** The phenotype of *P. capsici* infection lesion introduced with mock and dsNLP6_MTF, respectively, in 3-week-old *Nicotiana benthamiana* leaves, determined using a FOBI in vivo fluorescence imaging system. Scale bar = 1 cm. **c** The phenotype of *P. capsici* infection lesion introduced with mock and dsNLP6_OReg, respectively. Scale bar = 1 cm. **d** Quantification of *P. capsici* infection lesion size using ImageJ. **e** Relative expression level of *PcNLP6* using quantitative real-time PCR (qRT-PCR). Mock: Treatment with dsRNA targeting *Renilla luciferase*; dsNLP6_MTF: Treatment with dsNLP6_MTF; dsNLP6_OReg: Treatment with dsNLP6_OReg. MTF: Motif, OReg: Outer region. Data represent the mean ± standard error of mean (SEM; $N = 3$). Statistical significance is determined by Student's *t*-test (*$P < 0.05$)

conserved amino acid residues and RNA sequences except for the "AIMY" and "GHRHDWE" motifs (Additional file 1: Figs. S8, S9). The regulation of the target region except for the motifs by dsNLP6_OReg suggests that it cannot control *P. capsici* infection (Fig. 3c, d) or the expression of other *PcNLPs* (Additional file 1: Fig. S4b), even if some conserved amino acid residues or RNA sequences are regulated by dsRNA. These results showed that using only dsNLP2_MTF and dsNLP6_MTF to target the "AIMY" and "GHRHDWE" motifs can suppress *P. capsici* infection and the expression of *PcNLPs* (Figs. 2, 3, 4). In addition, when dsNLP2_OReg and dsNLP6_OReg were used, the suppression of *PcNLP2* and *PcNLP6* expression was weaker than when dsNLP2_MTF and dsNLP6_MTF were used (Figs. 2e, 3e), suggesting that "AIMY" and "GHRHDWE" motifs are key elements for *P. capsici* infection and *PcNLPs* expression, similar to previous studies [9, 10]. In future research, it will be necessary to separate the dsRNA targeting both the "AIMY" and "GHRHDWE" motifs into dsRNAs targeting only one of these motifs to compare the degree of suppression of *P. capsici* infection and *PcNLPs* expression. This approach will help to uncover the roles of the "AIMY" motif and "GHRHDWE" motif, respectively, in regulating *P. capsici* infection and *PcNLPs* expression.

In conclusion, this study demonstrated that multi-target dsRNAs targeting conserved motifs in the *PcNLP* gene family could suppress *P. capsici* infection and the

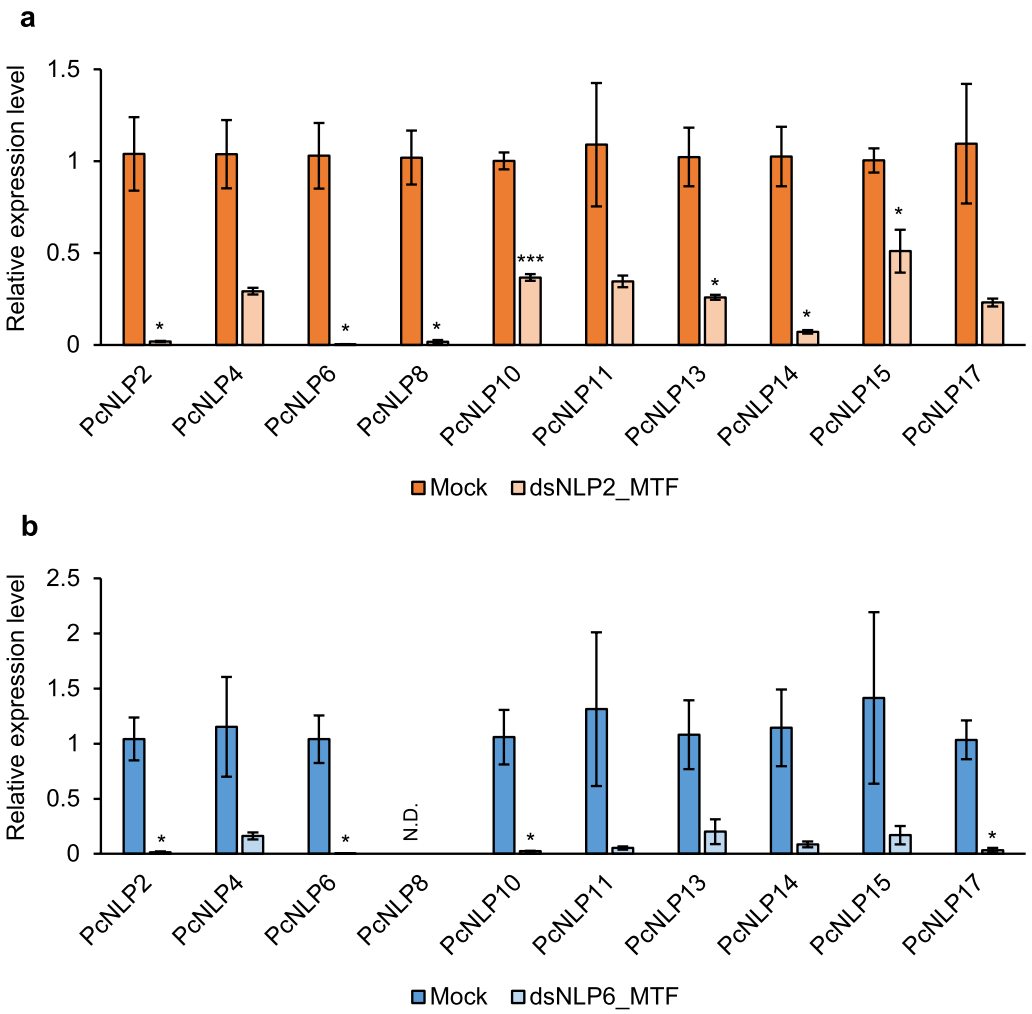

Fig. 4 Changes in the expression of the *PcNLP* gene family after treatment with dsRNAs targeting dsNLP2_MTF and dsNLP6_MTF. Quantification of the expression of the *PcNLP* gene family after treatment with mock, **a** dsNLP2_MTF, and **b** dsNLP6_MTF using qRT-PCR. Mock: Treatment with dsRNA targeting *Renilla luciferase*; dsNLP2_MTF: Treatment with dsNLP2_MTF; dsNLP6_MTF: Treatment with dsNLP6_MTF. MTF: Motif. N.D.: Not detected. Data represent the mean ± SEM ($N = 3$). Statistical significance is determined by Student's *t*-test (*$P < 0.05$ and ***$P < 0.001$)

expression of *PcNLPs*. In addition, we provided further evidence that the "AIMY" and "GHRHDWE" motifs could be key elements regulating *P. capsici* infection and the expression of *PcNLPs*. These results can contribute to the development of RNAi technology that can control the entire gene family using one specific multi-target dsRNA.

Abbreviations

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

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13765-023-00828-9.

Additional file 1: Table S1. Primer sequences used in dsRNA synthesis. **Table S2.** Primer sequences used in qRT-PCR. **Figure S1.** The purity of Mock, dsNLP2_MTF, dsNLP2_OReg, dsNLP6_MTF, and dsNLP6_OReg. M: 100 bp marker, 1: After transcription, 2: After nuclease treatment, 3: After purification. MTF: Motif, OReg: Outer region. **Figure S2.** Schematic diagram of target regions in PcNLPs using qRT-PCR. **Figure S3.** RNA sequences conservation of the region containing the "AIMY" and "GHRHDWE" motifs of PcNLPs targeted by dsNLP2_MTF and dsNLP6_MTF. MTF: Motif. **Figure S4.** Quantification of the expression of PcNLP family genes after treatment with mock, (a) dsNLP2_OReg, and (b) dsNLP6_OReg using qRT-PCR. Mock: Treated with dsRNA targeting Renilla luciferase; dsNLP2_OReg: Treated with dsNLP2_OReg; dsNLP6_OReg: Treated with dsNLP6_OReg. OReg: Outer region. Data represent mean ± SEM (N = 3). **Figure S5.** Dot plot representation of the delta Ct values of PcNLPs in P. capsica-infected wild-type leaves using qRT-PCR. The dot represents

outliers of replicated samples, and whiskers represent standard deviation. of mean. **Figure S6.** Amino acid sequences conservation of the region targeted by dsNLP2_OReg. OReg: Outer region. **Figure S7.** RNA sequences conservation of the region targeted by dsNLP2_OReg. OReg: Outer region. **Figure S8.** Amino acid sequences conservation of the region targeted by dsNLP6_OReg. OReg: Outer region. **Figure S9.** RNA sequences conservation of the region targeted by dsNLP6_OReg. OReg: Outer region.

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Author contributions
CS conceived the project. MP, YK, JE, MO 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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