

Article

Defensin-like Protein Homologues Participate in Antiviral Resistance in *Nicotiana benthamiana*

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Abstract

Plant defensin-like proteins, as the core effector molecules of innate immunity, play an important role in resistance against fungi and bacteria. However, their function in plant antiviral resistance remains unclear. Here, NbDLP, a defensin-like protein from *Nicotiana benthamiana* (*N. benthamiana*), is identified through transcriptome analysis. NbDLP is up-regulated upon viral infection of Tobacco Vein Mottling Virus (TVMV). Then, we cloned NbDLP into plant expression vector by gateway recombination to obtain pEAQ-NbDLP. We found that the transient expression of NbDLP in *N. benthamiana* could significantly inhibit TVMV, TuMV and TMV infection. Further, silencing NbDLP contributed to TuMV and TMV infection. In conclusion, the results indicate that NbDLP participates in the plant antiviral resistance against plant viral infection and might be used for defining antiviral strategies in application points.

Keywords: defensin-like protein; *Nicotiana benthamiana*; tobacco vein mottling virus; tobacco mosaic virus; turnip mosaic virus; antiviral resistance

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1. Introduction

Natural antimicrobial peptides (AMPs) produced by plants and other organisms have emerged as critical for resisting specific pathogens. They serve a critical role in plant innate immunity and animal adaptive immunity. As a class of AMPs, plant defensin-like proteins (PDLs) are a family of small-molecule polypeptides distributed in the plant, which function as key defense molecules in the plant innate immune system [1]. PDLs are small, basic peptides with a distinctive three-dimensional folding pattern fixed by eight disulfide-linked cysteines [2]. The molecular weight of PDLs typically ranges from 5 to 10 kDa, and their amino acid sequences are rich in cysteine residues (usually 8–10 per

protein). Through the formation of 4–5 disulfide bonds, PDLs adopt a stable three-dimensional structure, specifically, and include the cysteine-stabilized $\alpha\beta$ (CS $\alpha\beta$) motif. This structural feature confers robust thermal stability and resistance to proteolytic hydrolysis, allowing them to retain biological activity in complex biological environments [3]. PDLs exert distinct anti-pathogenic mechanisms against pathogens, including bacteria, fungi and viruses [4,5]. Specifically, PDLs act as effector molecules of the natural immune system, making a significant contribution to the host's defense against diverse pathogens—encompassing enveloped and non-enveloped viruses, fungi, and bacteria in the human system [6].

In terms of classification, PDLs can be categorized into distinct subclasses based on their origin or biological function, such as seed defensins, leaf defensins, and floral organ defensins. Seeds represent the most abundant source of defensins [7]. Specifically, seed defensins exhibit inhibitory activity against fungal growth and enhance seedling survival rates [8]. Previous study explored the defensive effects of defensins on seeds and plants using radish (*Raphanus sativus*) seeds and defensins [9]. When radish seeds germinated in a medium containing *Fusarium graminearum*, an inhibitory circle formed around them to suppress fungal growth. This indicates that seeds can secrete antimicrobial substances, with defensins being the primary candidate components [9]. The results showed that the radish seeds contain two paralogous 5 kDa proteins rich in cysteine, the characterized *Raphanus sativus* antifungal protein 1 (RsAFP1) and RsAFP2, both displaying robust in vitro antifungal activity [9]. Induced proteins in leaves designated RsdFP3 and RsdFP4 were isolated and shown to share homology with seed-sourced RsAFPs, while also exerting comparable antifungal activity in an in vitro setting. Transgenic tobacco plants expressing a recombinant chimeric RsAFP2 gene driven by the constitutive cauliflower mosaic virus (CMV) 35S promoter set out reinforced resistance to the foliar pathogen *Puccinia longicolla*. Collectively, these findings indicate that plant defensins represent key defensive molecules in *Raphanus* species, with prominent antimicrobial properties [9]. Additionally, PDLs have also been characterized in other plant tissues, including leaves, floral organs, tuber structures, fruits, and roots systems [7]. In *Arabidopsis thaliana*, a minimum of 13 predicted plant defensin genes have been characterized, which encode 11 distinct plant defensin peptides. These defensins suppress the proliferation of a wide spectrum of fungal strains yet appear to be non-toxic to both mammalian and plant cells. The antifungal activity of these peptides is dependent on specific binding to membrane-associated molecular targets [2]. A 454 bp cDNA fragment isolated from sweet pepper (*Capsicum annuum*) encodes a novel fruit-specific defensin [10]. The plant defensin SPD1, cloned from the roots of the sweet potato (*Ipomoea batatas*), exhibits antimicrobial properties and weak inhibitory effects on bovine pancreatic trypsin [11].

Other studies have further confirmed the role of PDLs in plant defense. For instance, transgenic rice plants with constitutive expression of *wasabia* defensins showed enhanced resistance to the fungal pathogen *Magnaporthe oryzae* [12]. Similarly, transgenic Papaya harboring the Dm-AMP1 defensin gene exhibited elevated defense against the oomycete pathogen *Phytophthora palmivora* [13]. Under both greenhouse and field conditions, transgenic potato plants expressing *alfalfa* defensins were found to have enhanced resistance to the fungal pathogen *Verticillium dahlia* [14]. It was reported that Cabbage leaf curl virus (CaLVCV) induces six defensin family genes, suggesting the potential antiviral activity of defensins and defensin-like peptides (DEFLs) [15]. Meanwhile, certain plant defensin-like peptides have been shown to exhibit anti-HIV-1 activity [16]. In addition, only mild modulation of certain defensin genes was observed in tomato plants challenged with Cauliflower mosaic virus (CaMV) as well as Potato virus Y (PVY) [17].

Although PDLs have a well-established functional role in defending against bacteria (*Clavibacter insidiosus* and *Pseudomonas* spp.), fungi (*Phoma medicaginis* and *Rhizoctonia*),

and oomycetes (*Pythium* spp.) [18], their role in plant–virus interactions remain rarely reported. In this study, we identified the NbDLP gene from *N. benthamiana*. We investigated the role of NbDLP in viral infection by Tobacco vein mottling virus (TVMV), Turnip mosaic virus (TuMV) and Tobacco mosaic virus (TMV).

2. Materials and Methods

2.1. Plant Material Preparation

N. benthamiana seeds were sown into potting soil, and the resulting seedlings were grown in a greenhouse under controlled conditions: a temperature range of 22–26 °C and a photoperiod of 16 h light/8 h dark. For virus infection experiments, *N. benthamiana* (the fourth and fifth leaves from the top) is taken from plants of similar size [19].

The infection clone of TVMV has been previously described [20]. TuMV-GFP infection clone was obtained from Xiaoming Zhang's laboratory at the Institute of Zoology at the Chinese Academy of Sciences. TMV-GFP infection clones were described previously [21,22]. Competent *Escherichia coli* DH5α cells were obtained commercially from TaKaRa Bio Inc., Osaka, Japan. The vectors pDONR207 and pEAQ-HT-DEST3, along with *Agrobacterium tumefaciens* strain C58C1, were generously gifted by Professor Juan Antonio García of the National Centre for Biotechnology (CNB) in Spain.

2.2. Vector Construction

Based on the sequence of the NbDLP gene, specific cloning primers targeting this gene were designed (Table S1). The PCR reaction volume totaled 50 µL (primers 2 µL, Ex Taq Version 2.0 plus dye 25 µL, cDNA 1 µL). PCR amplification was conducted at 98 °C for 3 min, then, 98 °C for 10 s, 60 °C for 30 s, and 72 °C for 30 s by 29 cycles, with a final extension step at 72 °C for 5 min. The PCR products were obtained by 1% agarose gel electrophoresis and subsequently purified, after which the NbDLP fragment was ligated with pMD19-T vector, and the recombinant plasmid was transformed into *Escherichia coli* DH5α. Plasmids were extracted from positive colonies, and the correctness of the inserted fragment was verified by Sanger sequencing. (Figures S1 and S2).

The expression vector pEAQ-NbDLP was generated via the Gateway cloning system. A BP recombination reaction was conducted to fuse pMD19-NbDLP with the pDONR207 vector at 25 °C for 8 h, and the resulting pDONR207-NbDLP plasmid was validated through double restriction enzyme digestion with *EcoR* V and *Pst* I. Subsequent construction of the binary expression vector pEAQ-NbDLP was achieved by means of an LR recombination reaction, and positive pEAQ-NbDLP clones were identified via PCR amplification combined with restriction enzyme digestion using *Apa*I and *Xho* I.

In addition, gene-specific primers directed at the silencing fragment were designed on the basis of the NbDLP coding sequence (Table S1). Utilizing a PCR reaction volume of 50 µL, with 2 µL of primers included, 25 µL of Premix Taq (2 × PCR Buffer for KOD FX), and 1 µL of cDNA, the PCR amplification procedure was set as follows: pre-deformation at 94 °C for 2 min, followed by 29 cycles (98 °C for 10 s, 62.2 °C for 30 s, 68 °C for 15 s), and a final cycle at 68 °C for 5 min. The construction of the gene silencing vector was accomplished through restriction endonuclease digestion and the connection/recombination between pTRV2 and the NbDLP fragment. The silence fragment (207bp) of NbDLP was obtained. After the amplification products were digested with *Xba* I/*Xho* I enzymes, they were then ligated with the products of the pTRV2 vector using T4 DNA ligase. The reaction system included 7 µL of the recovered product after digestion with pTRV2, 2 µL of the product after digestion with NbDLP silencing fragment, 1 µL of 10 × T4 DNA ligase buffer, and 0.1 µL of T4 DNA ligase. Then, the ligation product was transformed into *E.*

coli DH5 α . After plasmid extraction, PCR and Sanger sequencing were performed to verify positive clones of pTRV2-NbDLP.

2.3. Sequence and Structure Analysis

Heatmaps of structural similarity matrix for defensins across species were generated using TBtools-II (v2.390). Multiple sequence alignment of defensins from different species was performed with ESPript 3.x and Snap Gene software tools (<https://www.snapgene.com/>), and phylogenetic trees were constructed using Molecular Evolutionary Genetics Analysis version 11 (MEGA 11) [23]. The physicochemical characteristics of NbDLP were determined via the ProtParam tool on the ExPaSy platform. NbDLP's secondary structural features were predicted utilizing the NovoPro bioinformatics platform. The conserved domains of the NbDLP were predicted using the subcellular localization prediction from NCBI, and the three-dimensional (3D) structural model of NbDLP was predicted by Swiss-Model server [24].

2.4. RT-qPCR to Detect Gene Expression

In order to assess the expression of NbDLP in *N. benthamiana*, total RNA was extracted from infiltrated leaf tissue samples and subsequently used for cDNA synthesis. Briefly, 1 μ g of total RNA was reverse-transcribed into cDNA using the Evo M-MLV RT Mix Kit with gDNA Clean for qPCR Ver.2. (Accurate Biology (Changsha, China), Cat. No. AG 11728), which efficiently removes genomic DNA contamination.

Real-time quantitative PCR (qPCR) experiments were conducted on the QuantStudio™ 3/5 real-time quantitative PCR instrument (Thermo Fisher Scientific, Waltham, MA, USA, MAN0010407) using the SYBR Green Premix Pro Taq HS qPCR Kit (Rox Plus; Accurate Biology, Cat. No. AG11718), following the manufacturer's recommended protocols. Each 10 μ L reaction system was subjected to the following thermal cycling program: 95 °C for 30 s, followed by 40 cycles consisting of 95 °C for 5 s and 60 °C for 30 s.

Gene-specific primers were designed for NbDLP and the reference gene (*N. benthamiana* ubiquitin, NtUB), with primer sequences listed in Table S1. The efficiency of NbDLP qPCR primers and raw Ct values for all samples are provided in Supplementary Figure S4. The relative expression level was calculated. Δ Ct = Ct (internal reference) – Ct (target gene/control). $\Delta\Delta$ Ct = Δ Ct (target gene) – Δ Ct (control). Then, the values of $2^{\Delta\Delta$ Ct} were calculated.

All experiments were conducted with three biological repetitions, each consisting of three technical replicate groups. The data were analyzed using GraphPad Prism (v8.3.0) software [25].

2.5. Viral Inoculation

The constructed plasmid pEAQ-NbDLP was transferred into Agrobacterium C58C1. Collect Agrobacterium in a 50 mL centrifuge tube and centrifuge at 4000 rpm for 15 min. The supernatant was then decanted. Then, 20 mL of induction solution (0.01 mole of magnesium chloride, 0.01 mole of MES solution, and 0.15 mL of acetic acid mixed solution) was added. The bacterial precipitate was suspended and washed to remove the residual antibiotics and bacterial impurities, and centrifuged at 4000 rpm for 10 min. The supernatant was discarded, and 5 mL buffer and vortex mix was added, followed by incubation at room temperature for 5 h.

The Agrobacterium strains carrying the pEAQ-NbDLP, pEAQ-HT-DEST3, viral infectious clone pLX-TVMV, TMV-GFP and TuMV-GFP vectors were respectively cultured in an oscillating incubator until the OD₆₀₀ value of the bacterial suspension reached 1.0. The pEAQ-NbDLP was mixed with TVMV, pEAQ-NbDLP with TMV-GFP, and pEAQ-NbDLP with TuMV-GFP to form equal volume (1:1, *v:v*) mixed suspensions. The empty

vector pEAQ-HT-DEST3 was mixed with each corresponding virus at a 1:1 (*v:v*) ratio and used as the negative control group. Then, 1 mL syringes were used to infiltrate and inoculate the *Agrobacterium* into the leaves (4–5 leaves) of *N. benthamiana*. From 3 to 11 days post-agroinfiltration (dpa), the disease symptoms induced by different viral strains on inoculated plants were visually observed and documented by photography. Additionally, *agrobacteria* carrying pTRV1, pTRV2 and pTRV2-NbDLP were cultured to an OD₆₀₀ of 1.0 following the same method; a 1:1 (*v:v*) mixed suspension of pTRV1 and pTRV2-NbDLP was then prepared, while a 1:1 (*v:v*) mixture of pTRV1 and empty pTRV2 was used as the control for VIGS assays. This *agrobacterial* mixture was infiltrated into the leaves (4–5 leaves) of *N. benthamiana* by 1 mL syringes. When typical albino phenotypes were exhibited on the leaves of *N. benthamiana* plants infiltrated with pTRV1 + pTRV2-PDS, the wild-type TVMV, TMV-GFP and TuMV-GFP viruses were subsequently inoculated onto the plants for further infection assays.

2.6. Western Blot Analysis for Viral Detection

Approximately 0.2 g of virus-infected leaf tissue was obtained and snap-frozen in liquid nitrogen. The tissue was ground into extremely fine powder, and 5% SDS solution was added in two successive aliquots, mixing thoroughly until the tissue was completely homogenized. The mixture was kept at 95 °C for 5 min, then centrifuged at 12,000 rpm at 4 °C for 10 min (Wang, Zhang et al. 2025 [26]). Next, 2x loading buffer (containing 62.5 mM Tris-HCl (pH 6.8), 25% glycerol, 2% SDS, 0.01% bromophenol blue and 250 mM dithiothreitol) was added at a 1:1 ratio (*v:v*) to the protein extract. The mixture was incubated at 95 °C for 5 min, placed in an ice bath for 2 min, then centrifuged at 12,000 rpm and 4 °C for 10 min, providing the supernatant for Western blotting analysis (Yang et al., 2024 [27]). A 5 µL sample of the protein extract was loaded onto an SDS-polyacrylamide gel (SDS-PAGE) followed by electrophoresis at a constant voltage. The gel image was captured using a GelDoc Imaging System (Thomas Fisher Scientific, Waltham, MA, USA) and used as the loading control for subsequent normalization. Subsequently, separated proteins were transferred onto a nitrocellulose (NC) membrane by wet electroblotting. The membrane was incubated in blocking buffer for 2 h at room temperature, and subsequently rinsed three times with Tris-buffered saline containing Tween-20 (TBST), with each rinse lasting 2 min. Immunodetection was performed using anti-TVMV coat protein (CP) and anti-GFP antibodies to detect TVMV, TMV-GFP and TuMV-GFP.

The membrane was incubated with the primary antibody for 1 h at room temperature and then washed three times with TBST. It was subsequently incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G (IgG; Cat. No. ab205718; Abcam, New Albany, OH, USA). Following three additional washes with TBST buffer, the membrane was incubated in an enhanced chemiluminescence (ECL) reagent (Beyotime Biotechnology, Hercules, CA, USA), and the target protein signals were detected by the ECL method.

Image J (v1.54g) software was used to perform gray value analysis of NbDLP and loading control bands for Western blot quantification. Relative expression levels of NbDLP were calculated by normalizing the gray value of each NbDLP band to that of its corresponding loading control band (i.e., dividing the gray value of the target protein band by the gray value of the loading control band). Subsequently, the normalized values obtained were imported into GraphPad Prism for statistical analysis and plot generation.

3. Results

3.1. The Identification of NbDLP During TVMV Infection in *N. benthamiana*

N. benthamiana plants inoculated with TVMV showed typical viral infection symptoms, such as shrinkage and vein mottling (Figure 1A). Viral accumulation was verified via Western blot analysis utilizing an antibody specific to the TVMV coat protein (CP), which showed high levels of viral infection of TVMV in infected samples compared to healthy plants (Figure 1B). From the previously reported transcriptome sequencing data [28], we identified that DLP homologue (NbDLP) was upregulated in TVMV-infected *N. benthamiana* compared to healthy plants (Figure 1C,D). The alignment of NbDLP amino acid sequences from 10 species, including *N. benthamiana*, *Nicotiana attenuata*, *Datura stramonium*, *Capsicum annuum*, *Quillaja saponaria*, *Vitis vinifera*, *Gerbera hybrida*, *Agave sisalana*, *Brassica napus*, and *Lycium barbarum*, was performed using the ESPript tool (Figure 1E). Specifically, regions with high conservation (e.g., yellow-highlighted areas) exhibited minimal amino acid variation, suggesting these residues may be critical for protein structure or function. In contrast, divergent regions (red-highlighted areas and unmarked regions with amino acid differences) reflected species-specific sequence variations, which may correlate with evolutionary adaptation and functional specialization.

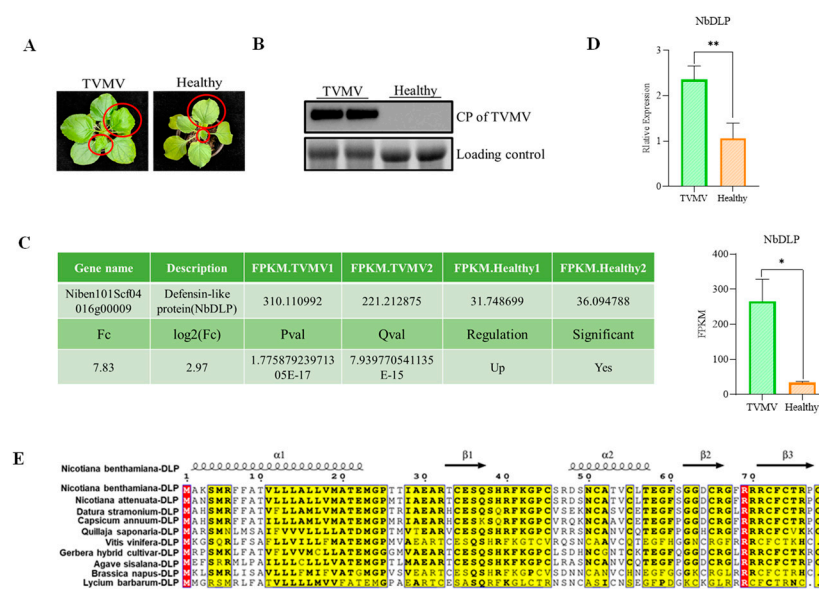


Figure 1. NbDLP is upregulated during TVMV infection. (A) The symptoms of TVMV-infected plant and healthy *N. benthamiana*; (B) Western blot detection of viral accumulation of TVMV by anti-CP. The protein band of rubisco used as loading control; (C) Transcriptomic analysis of NbDLP. The FPKM values, the fold changes (Fc), *p*-values, log2(fc), Q value, regulation, significant Niben101Scf04016g00009 (NbDLP) in TVMV-infected plants and healthy plants. (D) The relative expression of NbDLP homologue in TVMV-infected plant and healthy *N. benthamiana* (E). Sequence alignment of NbDLP across different species by ESPript. The conserved amino acids are marked with red color. The relevant conserved domains are shown with yellow color. * represents significance at $p < 0.05$. ** represents significance at $p < 0.01$. In the multiple sequence alignment results generated by ESPript, the green numbers represent the sequence numbers of the secondary structure elements of proteins, and their specific meanings can be expressed as: In this multi-sequence alignment diagram, green numbers are used to consecutively number the secondary structural elements such as α -helix ($\alpha 1$, $\alpha 2$) and β -fold ($\beta 1$, $\beta 2$, $\beta 3$) that appear in the target protein (NbDLP), thereby clearly marking the positions and arrangements of different secondary structural units in the amino acid sequence.

3.2. The Cloning of NbDLP from *N. benthamiana*

To further validate the specific functions of NbDLP, an NbDLP fragment (298 bp) was amplified from the cDNA of TVMV-infected tissue (Figures 2A and S1). Subsequently, the amplified NbDLP fragment was cloned into the pMD19-T vector. Sanger sequencing of the pMD19-T-NbDLP plasmid confirmed successful construction (Figure S2). The NbDLP fragment was ligated into the pDONR207 vector by BP recombination reaction, obtaining the recombinant plasmid pDONR-NbDLP. Specific fragments of 2814 bp and 793 bp were obtained by restriction endonuclease digestion using *EcoR* V/*Pst* I (Figure 2B). Then, the NbDLP fragment was introduced into the expression vector pEAQ-HT-D3 by LR reaction to create pEAQ-NbDLP. The fragments of 8632 bp and 1634 bp were obtained by restriction enzyme digestion of pEAQ-NbDLP with *Apa* I/*Xho* I, indicating the successful construction of pEAQ-NbDLP (Figure 2C).

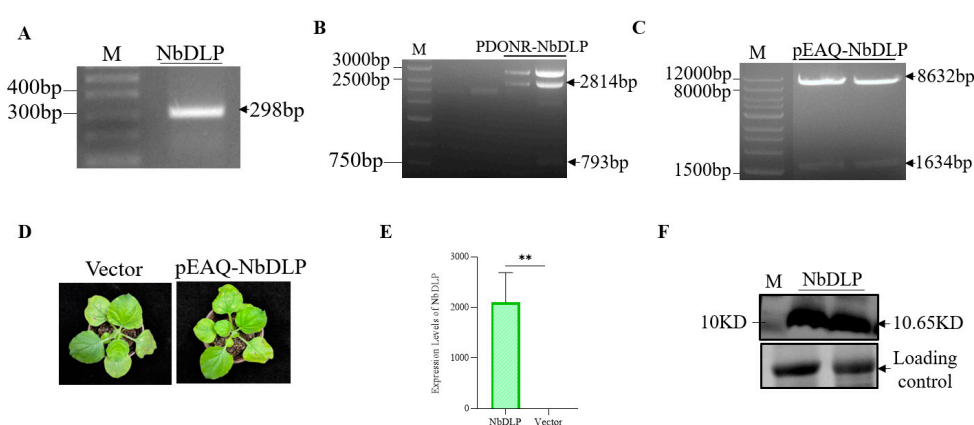


Figure 2. Cloning and NbDLP from *N. benthamiana*. (A) PCR amplification of NbDLP fragment (298 bp); M, Marker. (B) pDONR-NbDLP digestion by *EcoR* V and *Pst* I digestion. M, Marker. Target bands of 2814 bp and 793 bp were successfully amplified and obtained. (C) pEAQ-NbDLP digestion by *APA* I and *Xho* I yielded specific bands of 8632 bp and 1634 bp. (D) Phenotypic characterization of *Nicotiana benthamiana* at 3 days post-agroinfiltration with agrobacterial cultures harboring pEAQ-NbDLP and the empty pEAQ-HT-DEST3 vector. (E) Expression of NbDLP detected by qRT-PCR. ** $p < 0.01$. (F) Western blot detection of DLP protein expression. A 10.65 kD band was detected by anti-His.

To verify that the transient expression of NbDLP influences the plant phenotype, the pEAQ-NbDLP expression vector was introduced into *Agrobacterium* strain C58C1, and the result agroinfiltrated into the leaves of *N. benthamiana*. The empty pEAQ-HT-DEST3 vector was used as the negative control. No significant phenotypic differences were observed in *N. benthamiana* plants agroinfiltrated with pEAQ-NbDLP relative to the control group (Figure 2D). RT-qPCR analysis confirmed successful transient expression of NbDLP in *N. benthamiana*, with significantly higher relative expression levels compared to the control (Figure 2E). Western blot analysis further verified NbDLP protein expression (10.65 kDa) (Figure 2F), which confirmed successful expression of NbDLP in *N. benthamiana*.

3.3. Structural Modeling and Characteristics of NbDLP

In order to clarify the phylogenetic relationships of the defensin family, a phylogenetic tree was generated by MEGA 11 (Figure 3A). The NbDLP sequence (marked with a red triangle) showed the closest genetic relationship with *N. attenuata*, supported by a bootstrap value of 99, indicating a recent common ancestor and close evolutionary affinity. In contrast, the *N. benthamiana* exhibited more distant relationships with other species,

structure of NbDLP was predicted by Swiss-Model. The corresponding domain of NbDLP is shown; colors by prediction confidence scores.

3.4. NbDLP Inhibits TVMV Infection in *N. benthamiana*

To test whether NbDLP is involved in plant viral infection, the co-infiltration assay of pEAQ-NbDLP and pLX-TVMV infectious clone in *N. benthamiana* was performed. Symptoms manifested on the upper systemic leaves at 3 dpi, 6 dpi, 9 dpi and 12 dpi, with no viral symptoms detected at 3 dpi, whereas the control group displayed significantly more severe symptoms (Figure 4A). Western blot analysis further revealed that TVMV accumulation in the NbDLP overexpression plant was significantly lower than that of the control (Figure 4A). Quantification of viral CP protein levels confirmed that NbDLP significantly inhibited TVMV infection (Figure 4B).

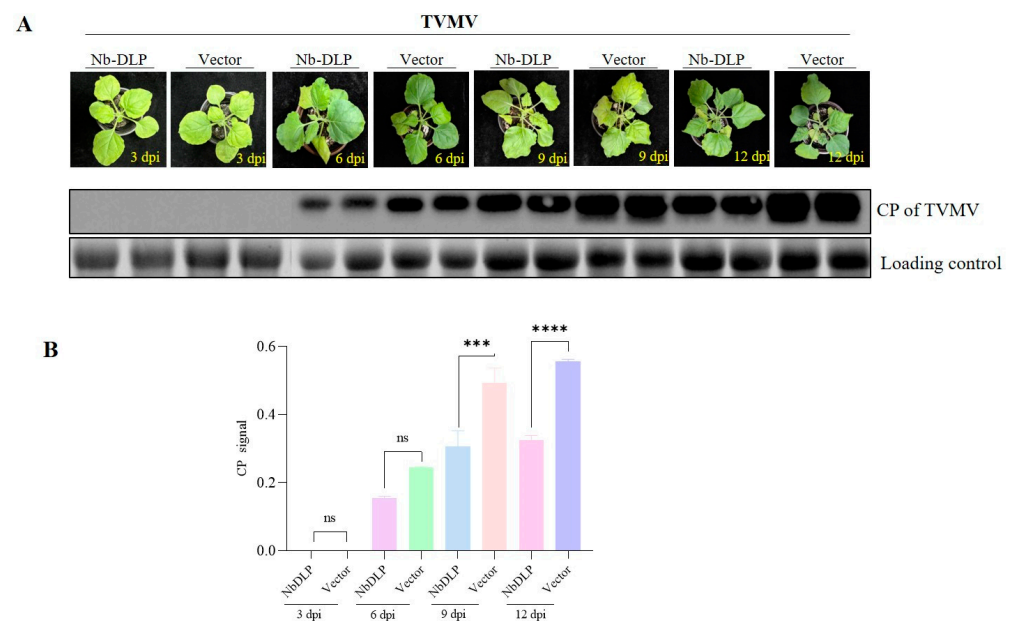


Figure 4. The impact of transient NbDLP expression on TVMV infection. **(A)** Symptoms of TVMV infection in *N. benthamiana* with transient expression of NbDLP at 3 dpi, 6 dpi, 9 dpi and 12 dpi. TVMV viral accumulation assayed via Western blot (ant-CP). The protein band of rubisco used as loading control. **(B)** The gray value used to analyze the accumulation amount of the virus was Image J (version 1.54g); “ns” denotes no statistically significant difference between the experimental and control groups. *** represents significance at $p < 0.001$. **** represents significance at $p < 0.0001$.

3.5. NbDLP Inhibits TuMV Infection in *N. benthamiana*

To validate the antiviral activity of NbDLP against other potyviruses, *Agrobacterium* cultures carrying pEAQ-NbDLP or the empty vector were co-infiltrated separately into *N. benthamiana* leaves with the TuMV-GFP infectious clone at a volume ratio of 1:1 (v/v). At 2 dpi or 3 dpi, plants treated with NbDLP did not show any fluorescence of green fluorescent protein (GFP) in the inoculated leaves, while the inoculated leaves of the control plants were filled with GFP; in the following 4–5 days, the viral infection of the inoculated leaves had reached saturation, and the green fluorescence phenomenon in the systemic leaves of the plants treated with NbDLP was less than that in the systemic leaves of the control plants (Figure 5A). TuMV viral accumulation was assayed via Western blot with an anti-GFP antibody. The findings revealed an absence of viral accumulation at 2 dpi and only a small amount of viral accumulation at 3 dpi, in comparison with the levels observed in control plants (Figure 5B). Quantification of the GFP signal also showed a significant

difference between NbDLP treatment and the control (Figure 5C). At 4, 5 and 6 dpi, viral accumulation showed no significant difference relative to the control group (Figure 5D). Additionally, the quantitative detection of the GFP signal revealed no notable variation between the NbDLP treatment group and the vector group (Figure 5E).

To further confirm the antiviral resistance of NbDLP, TRV-mediated silencing vector (pTRV-NbDLP) was constructed (Figure S3A–C). The empty vector pTRV2 and pTRV2-PDS (VIGS reporter vector carrying the PDS gene) were used as the controls. When bleaching phenotypes were observed on the upper leaves of pTRV2-PDS-infiltrated plants (Figure S3D), upper leaves of plants infiltrated with pTRV-NbDLP were collected. RT-qPCR analysis was performed to assess the silencing efficiency of pTRV2-NbDLP (Figure S3E).

To investigate the effect of NbDLP silencing on TuMV infection, once the typical bleaching phenotype was observed in pTRV2-PDS-infiltrated plants, TuMV-GFP infection clone was infiltrated into the systemic leaves of plants. Western blot analysis of upper leaves detected viral accumulation with anti-GFP. The results showed that TuMV accumulation was significantly increased in NbDLP-silenced plants compared with those of the control (Figure 5F,G). Therefore, our results reveal that transient expression of NbDLP specifically inhibits TuMV infection in *N. benthamiana*.

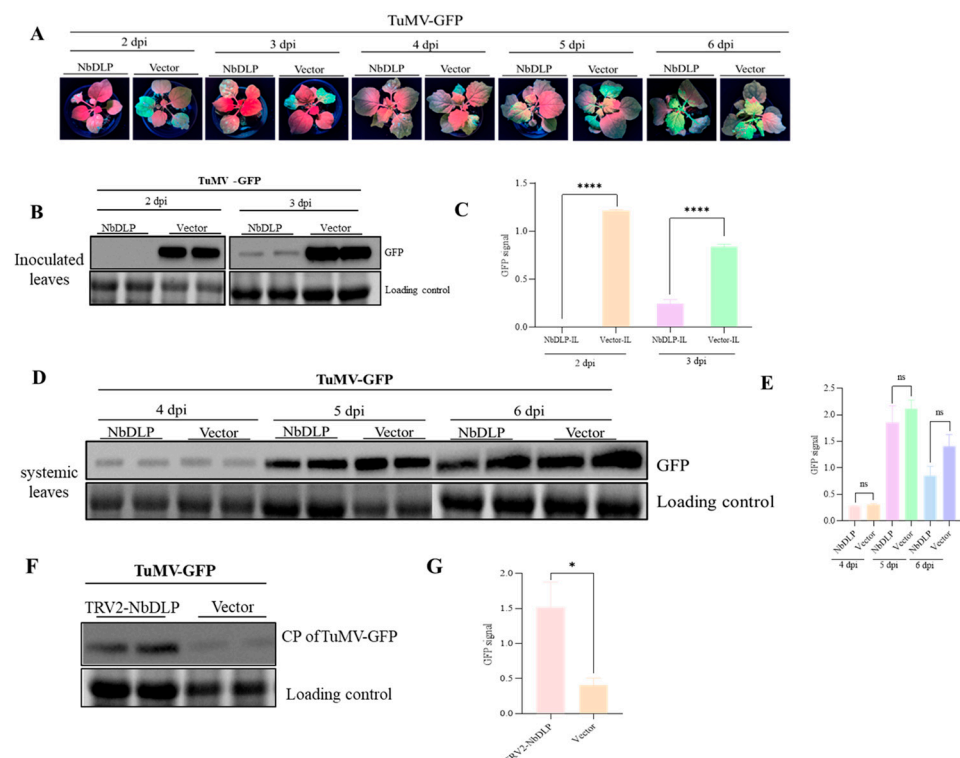


Figure 5. The effect of transiently expressed NbDLP on TuMV infection. (A) After 2 days to day 6, the symptom phenotypes induced by TuMV in the plants expressing NbDLP. (B) Detection of virus accumulation in the leaves injected with TuMV on the second and third days by Western blot (anti-GFP). The protein band of rubisco was used as the loading control. (C) Quantification of virus accumulation on the second and third days using Image J (V.1.54g). **** $p < 0.0001$. (D) Detection of virus accumulation in the systemic leaves of TuMV-GFP on the fourth to sixth days by Western blot. The protein band of rubisco was used as the loading control. (E) Quantification of virus accumulation on the fourth, fifth, and sixth days using Image J (V.1.54g). ns indicates that the statistical results did not reach a significant level. (F) Western blot analysis of TuMV-GFP virus accumulation in NbDLP silenced plants. The vector represents TRV1 + TRV2. (G) Quantitative results of GFP signal. * $p < 0.05$.

3.6. NbDLP Inhibits TMV Infection in *N. benthamiana*

To analyze the antiviral effect of NbDLP against TMV infection, co-infiltration of pEAQ-NbDLP and TMV-GFP infection clone was performed in *N. benthamiana*. At 4 dpi, we found that GFP fluorescence in NbDLP-treated plants had decreased compared with the control (Figure 6A). Correspondingly, viral accumulation of TMV in NbDLP-treated plants was significantly decreased ($p < 0.05$) (Figure 6B,C). When we inoculated the NbDLP-silenced plants with TMV-GFP, viral accumulation was elevated in comparison with the control group (Figure 6D,E).

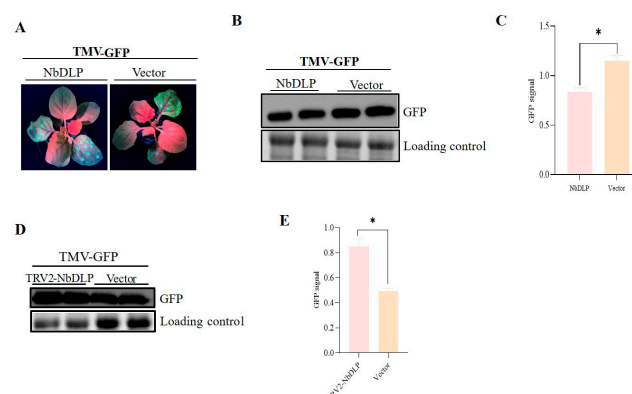


Figure 6. The impact of transiently expressed NbDLP on TMV invasion. **(A)** TMV-induced symptomatic phenotypes in NbDLP-transiently expressed *N. benthamiana* at 4 dpi. **(B)** TMV viral accumulation assayed via Western blot (anti-GFP). The protein band of rubisco used as loading control. **(C)** Quantification of GFP signal by Image J. * $p < 0.05$. **(D)** The Western blot analysis of viral accumulation of TMV-GFP in NbDLP-silenced plants. Vector represents TRV1 + TRV2. **(E)** The quantification of GFP signal is shown. * $p < 0.05$.

4. Discussion

The antibacterial and antifungal functions of plant defensins have been widely demonstrated. Their mode of action mainly involves direct damage to the cell membranes of pathogens, resulting in increased membrane permeability and the leakage of intracellular components [28]. For example, the defensin MtDef4 from *Medicago truncatula* (barrel clover) induces dose-dependent gene expression of the lipopolysaccharide aminoarabinose modification and surface polycation spermidine production operon. Fluorescence microscopy also visually confirmed the ability of MtDef4 to damage the bacterial outer membrane, and MtDef4 exhibits specific and potent antibacterial activity against *Pseudomonas aeruginosa* strains.

On the other hand, defensin can also enter the interior of pathogen cells and interact with intracellular targets to inhibit protein synthesis or induce cell autolysis [29]. PtDef from *Populus trichocarpa* (black cottonwood) possesses a canonical defensin structure and displays antifungal activity against fungal pathogens. This protein not only has inherent antifungal properties but is also suggested to trigger the plant defense response by regulating salicylic acid (SA) and jasmonic acid (JA) signaling pathways, as well as reactive oxygen species (ROS) metabolism [30]. PtDef may also induce autolysis in *Escherichia coli*, which in turn may activate a two-component system (TCS) to adapt to the stress. Transgenic plants with enhanced disease resistance can be generated by transforming defensin-encoding genes. For instance, PtDef-overexpressing poplars show strong resistance to the poplar stem-boring pest *Scolytus populiperda*.

In addition, the success of heterologously expressing defensins in transgenic plants confirms their biotechnological potential. For instance, constitutive expression of the NmDef02 defensin in transgenic soybeans significantly enhanced field resistance against both fungal pathogens *Phakopsora pachyrhizi* and *Colletotrichum truncatum* without impairing beneficial symbiosis with rhizobia [30]. Compared with the non-transgenic control, the transgenic soybean showed significantly lower morbidity and disease severity, as well as a substantial reduction in fungal biomass, ultimately increasing yield. Importantly, NmDef02 expression did not negatively affect symbiotic nitrogen fixation between soybeans and rhizobia, highlighting its environmental compatibility [31].

Although it is well known that defensin could induce the defense response in fungi and bacteria, the role of defensin in plant viral infection remains unknown. In this study, we identified a defensin homologue from *N. benthamiana*, NbDLP, which was upregulated upon TVMV infection. Then, we cloned and characterized the NbDLP at the protein level. The core of the global fold of NbDLP is similar to those of reported defensins from plant defensins, which includes a cysteine-stabilized α -helix β -sheet (CSab) motif [2]. This indicates that NbDLP might act through similar activities. We investigated the role of NbDLP in plant viral infections by transient expression assay. We found that plants expressing NbDLP inhibit two potyviruses (TVMV and TuMV-GFP) and TMV-GFP infection, followed by obviously decreasing viral accumulation or GFP fluorescence. This suggests that defensin might be used for anti-viral strategy in field applications. However, attention should be focused on exploring the mechanism of defensin in antiviral resistance in future studies.

Previous studies have reported that defensin-encoding genes are downstream markers of jasmonic acid (JA) signaling. JAs are lipid-derived hormones that play a key role in regulating plant defense responses. Upon pathogen infection or insect herbivory, plants rapidly accumulate JAs, which trigger large-scale transcriptional reprogramming of defense- and metabolism-related genes, including defensin-encoding genes such as the plant defensin genes PDF1.2a and PDF1.2b [30]. Defensins not only have direct antibacterial activity, they also possess the capacity to modulate endogenous defense signaling pathways as well as reactive oxygen species (ROS) metabolism [30].

Since viruses lack a cellular membrane, the antiviral activity of NbDLP suggests a mechanism distinct from the canonical membrane permeabilization described for defensins like MtDef4. The induction of NbDLP by a viral pathogen adds complexity to defense signaling. Defensin genes are well-established as downstream markers of the JA pathway [30], whereas viral infections typically activate the SA pathway. Therefore, the upregulation of NbDLP by TVMV suggests potential cross-talk between SA and JA signaling. Next, the signaling pathway for NbDLP induction will be elucidated.

5. Conclusions

A defensin-like protein-encoding gene (NbDLP) was identified from *N. benthamiana*, and was significantly upregulated upon TVMV infection. Validation via transient expression and virus-induced gene silencing (VIGS) demonstrated that overexpression of NbDLP in *N. benthamiana* effectively inhibited viral infections of TVMV, TuMV and TMV. NbDLP silencing significantly enhanced plant susceptibility to TuMV and TMV. Our data revealed a plant defensin in antiviral defense and involving possible regulatory networks of plant SA and JA pathways in viral infection. This provides a new genetic resource and theoretical basis for the development of plant antiviral strategies.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/life16020286/s1>, Table S1. Primer sequences used in PCR. Figure S1. Sequence of NbDLP. Figure S2. Sequence of NbDLP from pMDP19-T-NbDLP. Figure S3.

Construction of *NbDLP* silencing vector. Figure S4. Raw data of qPCR for detecting *NbDLP* expression. Figure S5. Alignment of *NbDLP* with previously reported plant defensins. Figure S6. Expression of *NPR1* and *LOX1* associated with SA and JA signaling pathways in *N. benthamiana* co-infiltrated with *NbDLP* and TVMV infectious clone by qPCR.

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