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Molecular characterization and functional analysis of the Nep1-like protein-encoding gene from *Phytophthora capsici*

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ABSTRACT. *Phytophthora capsici* is an aggressive plant pathogen that affects solanaceous and cucurbitaceous hosts. Nep1-like proteins (NLPs) are a group of effectors found particularly in oomycetes and considered important virulence factors. We identified an NLP gene (*phcnlp1*) from the highly virulent *P. capsici* strain Phyc12 that had an encoded polypeptide of 476-amino acid residues and a predicted molecular mass of 51.75 kDa. We performed quantitative reverse transcription-polymerase chain reaction to detect the expression pattern of *phcnlp1* during various phases of interaction with the host plant, and the results showed that *phcnlp1* was increasingly expressed during symptom development after *P. capsici* infection of pepper leaves. We also confirmed that *phcnlp1* caused significant necrosis on tobacco plants when expressed based on potato virus agroinfection. All results indicated that *phcnlp1* belongs to the NLP gene family and is important for the pathogenesis of *P. capsici* in its hosts.

Key words: *Phytophthora capsici*; Necrosis-inducing activity; Inoculation; Pathogenesis

INTRODUCTION

Like fungi, oomycetes are globally distributed and prosper in diverse environments. The oomycetes belong to the kingdom Stramenopila and contain a large number of pathogenic species (Baldauf et al., 2000). The genus *Phytophthora* contains a large number of phytopathogens such as *P. capsici*, *P. infestans*, and *P. sojae*. *Phytophthora* species are the predominant causative agents of blight, crown rot, and stem, leaf, and fruit lesions in many plants. Moreover, *Phytophthora* pathogens have been identified as devastating pathogens on solanaceous and cucurbitaceous hosts, including pepper, cucumber, eggplant, squash, pumpkin, tomato, melon, and zucchini (Lamour and Hausbeck, 2004). Infected fruits quickly break down both in the field and postharvest.

Many pathogens have been reported to secrete elicitor proteins that are recognized by the defense system in the plant. Elicitors are signaling molecules that play an important role in determining host specificity and disease development for compatible and incompatible interactions (Pare et al., 2005). These elicitors are known as effectors and are described as pathogen proteins and small molecules that alter host-cell structure and function. These alternations either facilitate infection (virulence factors and toxins) or trigger defense responses (avirulence factors and elicitors), or both (Hogenhout et al., 2009). Effectors target distinct sites in the host plant and are generally separated into two classes. Some are apoplasmic effectors that interact with plant extracellular targets and surface receptors. By contrast, cytoplasmic effectors are secreted into the plant intracellular space, presumably through specialized structures such as infection vesicles and haustoria that invigilate living host plant cells (Kamoun, 2006). Plant pathogens secreting effectors have been documented in bacteria, fungi, oomycetes, and nematodes (Huang et al., 2003; Chisholm et al., 2006; Kamoun, 2007). Notably, fungal and oomycete effectors reportedly have virulence functions, but they are difficult to demonstrate experimentally during infection (Ellis et al., 2009). Overall, the function of effectors remains largely unknown.

NLPs (Nep1-like proteins) are a new class of necrotic elicitors. They share substantial sequence similarity with the first discovered member of the class (named Nep1), which was defined as a necrosis- and ethylene-inducing protein isolated from *Fusarium oxysporm* f.sp. *erythroloxyli* in liquid cultures (Bailey, 1995). Genes encoding NLPs, or the proteins themselves, have been detected in super-families of eukaryotic and prokaryotic organisms. NLPs are absent in plants and animals, but they often occur in fungi, bacteria, and oomycetes. Oomycetes, particularly, many *Phytophthora* spp such as *P. infestans*, *P. parasitica*, *Pythium aphanidermatum*, *P. sojae*, and *P. ramorum* have NLPs (Pemberton and Salmond, 2004). Although necrosis-inducing *Phytophthora* proteins (NPPs) are present in phylogenetically distant organisms, their sequences have remarkably remained in a conserved domain called the NPP1 domain (Fellbrich et al., 2002). A hepta-peptide and some conserved cysteine residues are present in every sequence. NPPs are classified into two groups according to cysteine residue number and position (Gijzen and Nürnberger, 2006). Dean et al. (2005) found that *Magnaporthe grisea* have NLP genes from both groups. To date, no NLP effects on monocots have been documented, but many NLPs reportedly trigger numerous dicotyledonous plant defense response, necrosis, and cell death (Fellbrich et al., 2002; Qutob et al., 2002; Bailey et al., 2005).

Some organisms, such as *Verticillium dahliae*, can secrete NLPs that induce cotton wilting (Wang et al., 2004). In tobacco leaves, Nep1 increases ethylene production, indicating

that necrosis could be an indirect effect of this hormone (Jennings et al., 2000). However, in some plants, necrosis induction was not accompanied by ethylene emission, suggesting that other mechanisms are involved. Studies with *P. parasitica* have shown that Nep1 induced superoxide anion production and salicylic acid-dependent resistance pathogenesis-related 1 gene expression (Fellbrich et al., 2002). Additionally, *P. sojae* necrosis-inducing protein has been linked to transition from the biotrophic to the saprotrophic phase (Qutob et al., 2002). Remarkably, the crystal structure of an NLP from *P. aphanidermatum* exhibits structural similarities to cytolytic toxins produced by marine organisms, suggesting that this protein contributes to host infection through plasma membrane destruction and cytolysis (Ottmann et al., 2009). However, the reason *Phytophthora* spp have a large NLP gene family and the roles these genes play during infection are still unclear.

In this study, we characterized the *phcnlp1* gene in *P. capsici* and evaluated its role in host infection. We investigated *phcnlp1* expression patterns in pepper leaves after inoculation with *P. capsici* and detected necrosis-inducing activity after the treatment of host leaves with the recombinant protein resulting from heterologous expression. Our results help clarify the role of the *phcnlp1* gene in the development of *Phytophthora* blight in pepper.

MATERIAL AND METHODS

P. capsici strain

Strains of *P. capsici* were isolated from *P. capsici*-infected pepper plants collected in a greenhouse at a Xinzhou pepper production base in Shanxi Province. To isolate the pathogen, we treated diseased plant tissues with 70% ethanol for 30-50 s and then soaked them in 0.1% mercuric chloride for 5 min, rinsed them in sterilized distilled water twice, and plated them on 10% V8 plate at 28°C (Feng et al., 2010). Single-zoospore isolates were derived from hyphae grown on plates and subcultured on V8 plates for 7 days in darkness. To induce the formation of sporangia, we removed agar pieces (1-1.5 cm^2) from the culture plates and soaked them in distilled water at 25°C for 3-4 days. Sporangia were dislodged and harvested from the agar pieces. The morphological characteristics of sporangia were determined, including width and length of pedicels. The length and width ratios were calculated. Strains were identified according to previous descriptions of *P. capsici* (Leonian, 1922; Gerrettson-Cornell, 1989). Sixteen single-zoospore isolates of *P. capsici* were obtained. Strains were stored at 4°C and transferred at 4- to 6-month intervals.

Virulence study

To induce zoospore release, the hyphae of *P. capsici* strains were inoculated in 20 mL sterile 10% V8 juice on Petri dishes. After 4 days of stationary culture, the sporulating hyphae were washed with sterile distilled water three times and inoculated at 4°C for 1 h. Detached leaves of the pepper Tianying during the 5th to 6th leaf stage were placed on Petri dishes containing 1.5% water agar (w/v). Each leaflet was spot-incubated with 2.5 µL zoospore suspension (1 x 10^5 zoospores/mL) and kept in darkness at 28°C. Control pepper leaves were inoculated with distilled water. The length and width of the lesions were measured at 3 days post-inoculation (dpi). Pathogenicity tests were conducted three times for all 16 strains.

Mating type

Strains were tested for mating type by placing culture plugs 5 cm apart from known mating type strains (A1 and A2) on V8 agar. The presence of oospores was scored after incubation at 28°C in darkness for 15 days. The mating type tester strains A1 (CBS 521.77) and A2 (CBS 370.72) were obtained from CBS Fungal Diversity Center (Utrecht, the Netherlands).

Isolation of *phcnlp1*, rapid amplification of complementary DNA (cDNA) ends (3'-RACE), and sequence analysis

The orthologs of the reported NLP gene in other oomycete genomes, including *P. sojae*, *P. infestans*, and *P. parasitica*, were downloaded from the National Center for Biotechnology Information (NCBI) along with NLP genes from reported species in fungi and bacteria. Most of the available complete NLP amino acid sequences were multialigned using ClustalX 2.0.

To amplify NLPs orthologous in strain Phyc12, we designed primers using the Primer Express 3.0 software according to the identified sequences in the Joint Genome Institute database (http://www.jgi.doe.gov/; Table 1). Genomic DNA of Phyc12 was extracted from hyphae grown in 10% V8 liquid medium according to the protocol described above. Polymerase chain reaction (PCR) parameters were as follows: 94°C for 4 min; 35 cycles of 94°C for 1 min, 55°C for 30 s, and 72°C for 1 min; and a final extension at 72°C for 10 min. The PCR products were confirmed as 3' incomplete fragments through sequencing.

Table 1. Primers used in this study.

Primer	Sequence (5'-3')	Purpose
P1	TT(G/A/C)GGA(C/T)CAC(T)GACTGGGA	*phcnlp1* cloning
P2	CCACATG(A)ATG(C)A(G)A(G)A(G)T	
P3	CGGACTTTGGAGACGCCAATCCCATAGGGACAC	3'-RACE for *phcnlp1*
P4	GTGTTCCCAGTCGTGACGGTGACCGAAGCCCG	
qPCR1	CCCATAGGGACACCGATCAAGCT	Real-time PCR for *phcnlp1*
qPCR2	TGTAGCCGCTATGGGCCGACG	
18SrRNAF	TTTCGGTCCTATTACGTTGG	*Capsicum annuum* 18S ribosomal RNA
18SrRNAR	TTCGCAGTTGTTCGTCTTTC	
PVXF	CC*ATCGAT*GCTGTTATCGACCACGACCAGGTCGT	Potato virus X (PVX)
PVXR	TCC*CCCGGG*CTGGTACCAGGCGTTCGCGAGCT	

3'-RACE = rapid amplification of complementary DNA ends; PCR = polymerase chain reaction; PVXF (*Cla*I in italics); PVXR (*Sma*I in italics).

For incomplete gene fragments, we performed 3'-RACE on the total RNA of the strain using a cDNA Amplification Kit (Clontech, USA). Phyc12 mycelia grown in 10% V8 liquid medium for 3 days were collected and ground in liquid nitrogen. Total RNA was extracted using a fungal RNA kit (OMEGA) following manufacturer recommendations. The RNA was quantified by measuring absorbance at 260/280 nm with a spectrophotometer, and the quality was examined with electrophoresis on a 1.2% formaldehyde agarose gel. Ten micrograms of total RNA was treated with 4 U Rnase-free DNaseI (Takara, Japan) at 37°C for 30 min and used for reverse transcription (RT) with an Omniscript RT kit (Qiagen). Synthesized cDNA was used for PCR.

We performed 3'-RACE on 1 µg total RNA of Phyc12 using the SMART™ RACE cDNA Amplification Kit (Clontech, USA) according to the manufacturer protocol. Specific primers were designed to amplify the 3'-end of the candidate gene (see Table 1). The PCR

products were verified via sequencing. Nucleotide and amino acid sequence homology search results were compared with the sequences using the NCBI Basic Local Alignment Search Tool (http://www.ncbi.nlm.nih.gov/).

To verify the NLP gene amino acid sequence, we analyzed sequence data using the GCG software package version 10.0 (Genetics Computer Group, Wisconsin, USA). Nucleotide and amino acid sequence homology searches were compared using the NCBI Basic Local Alignment Search Tool. Available complete NLP amino acid sequences, including oomycete pathogens, fungi, and bacteria were multialigned using ClustalX 1.83 (Thompson et al., 1997) and GeneDoc (version 2.6.002) (Nicholas et al., 1997). The NLP sequences of *P. capsici* and *P. sojae* were downloaded from the Joint Genome Institute. Genes for hypothetical protein analysis were obtained using the NCBI Open Reading Frame Finder (http://www.ncbi.nlm.nih.gov/projects/gorf/).

Production of infected pepper leaf tissue

The pepper cultivar Tianying was selected for inoculation with a zoospore suspension of strain Phyc12. Seedlings were grown as previously described (Feng et al., 2010). *P. capsici* sporangia and zoospores were produced as previously described (Feng et al., 2010). Detached leaves obtained during the 5th and 6th leaf stage were inoculated with 2.5 µL zoospore suspension (1 x 10^5 zoospores/mL) and placed on 1.5% (w/v) water agar plates in darkness for 7 days at 28°C. Control leaves were inoculated with distilled water.

RNA extraction and primer design for real-time PCR

RNA from infected leaves was extracted using an Rneasy Plant Mini Kit (Qiagen, Maryland, USA) according to manufacturer instructions and then treated with RNase-free DNase (Takara). RNA concentrations were quantified with a spectrophotometer (SpectraMax plus 384; Molecular Devices, Sunnyvale, CA, USA), and RT was performed using a RETRO Script Kit (Ambion) according to manufacturer instructions. The specific primer of *phcnlp1* was designed by avoiding conserved regions using ClustalX 1.83 (Gabriel, 1989). Pepper 18S ribosomal RNA (rRNA) was chosen as the internal control (Rozen and Skaletsky, 2000). The specific primers for real-time RT-PCR were designed using the Primer Express 3.0 software (Applied Biosystems, Foster City, USA; see Table 1).

Real-time PCR

Real-time quantitative PCR was performed in the ICycler IQ real-time PCR detection system (Bio-Rad, Denmark) using an SYBR primer Script RT-PCR kit (TaKaRa). For PCR, 2.5 µL cDNA template was added to 12.5 µL 2X SYBR Green PCR Master Mix, 800 nM of each primer, and double-distilled water to a final volume of 25 µL. After a denaturation step at 95°C 10 min, the cycle profile used was 10 s at 95°C, 55 s at 60°C, and 45 s at 72°C for 45 cycles. All reactions were performed in triplicate, and negative controls (with no template) were included for each gene. The threshold cycle (C_T) values were determined automatically by the instrument, and the fold-changes of each gene were calculated using the equation:

$2^{-\Delta\Delta C_T}$, where $\Delta\Delta C_T = (C_T$ target - C_T 18S rRNA) sample X - (C_T target - C_T

18S rRNA) sample 1 (Feng et al., 2009). In this study, sample 1 of the candidate gene acted as the mock infection, whereas sample X was the PCR product of the gene at 2-day intervals from 1 to 7 dpi.

Plant cultivation and construction of recombinant *Agrobacterium tumefaciens* binary potato virus X (PVX) vectors

Tobacco plants (*Nicotiana benthamiana*) were grown on a 1:1 soil/sand mix in a greenhouse at 23° to 25°C (16-h light period). To obtain the mature protein, we used *phcnlp1* cDNA as a template with primers PVXF (including that for *Cla*I in italics) and PVXR (including that for *Sma*I in italics), listed in Table 1. PCR parameters were as follows: 94°C for 4 min, 35 cycles of 94°C for 1 min, 62°C for 30 s, 72°C 1 min, and a final extension at 72°C for 10 min. The PCR products were digested with *Cla*I and *Sma*I, and then ligated into PVX pGR106 (Jones et al., 1999). The recombinant plasmids were verified by DNA sequence analysis in company. Recombinant binary plasmids were maintained and propagated in *Escherichia coli* DH-5α grown in the presence of 50 mg/mL kanamycin and 12.5 mg/mL tetracycline.

The binary expression constructs were electro-transformed into *A. tumefaciens* strain GV3101 and the cells were allowed to grow for 2 days at 28°C on Luria-Bertani agar plates supplemented with 50 mg/mL kanamycin and 12.5 mg/mL tetracycline as selective agents.

Transient co-expression of recombinants was performed as follows. *A. tumefaciens* strains carrying the respective constructs were mixed in a 2:1 ratio in MMA induction buffer (10 mM $MgCl_2$; 150 mM acetosyringone; 10 mM MES (2-morpholineethanesulfonic acid, pH 5.6), to a final optical density at 600 nm of 0.4. All suspensions were incubated for 1-3 h followed by infiltration into leaf tissue using 1-mL plastic syringes. Routinely, infiltrations were performed on 4-week-old tobacco leaves. Symptoms were documented for 10 days after inoculation.

RESULTS

Isolated strains and virulence test

Sixteen single-zoospore isolates of *P. capsici* were obtained from infected pepper plants according to previous descriptions of *P. capsici* (Leonian, 1922; Gerrettson-Cornell, 1989). Only one strain, Phyc12, was defined as highly virulent, and it was selected for further study because it showed obvious symptoms when used to treat pepper leaves (data not shown). All 16 strains belonged to the A1 mating type. Oospores were released when each strain was cultured with the A2 mating type.

Gene cloning

One complete-sequence NLP gene (*phcnlp1*) was identified and submitted to GenBank (accession No. JQ780443). In the multiple sequence alignment of *phcnlp1* with each of the existing 18 *P. capsici* NLP genes from GenBank (accession No. HM543167-84), amnion acid similarities ranged from 43 to 85%, and none was identical to *phcnlp1*.

The open reading frame of *phcnlp1* contains 1431 bp and encodes a polypeptide of

476-amino acid residues with a predicted molecular mass of 51.75 kDa. It contains a signal peptide of 18-amino acid residues and has a 1 N-glycosylation site (N313) but exhibits no intron. Figure 1 shows the structure of *phcnlp1*, which was analyzed using the NCBI online database to confirm that its sequence was homologous to that of NLPs from fungi, bacteria, and other oomycete pathogens. Alignment of most of the amino acid sequences showed that *phcnlp1* contains two repeat conserved sequence segments (121_RHDWE and 362_RHDWE), which differ from other well-known NLP sequences. Moreover, the former segment nearly exists in other well-known oomycete pathogens.

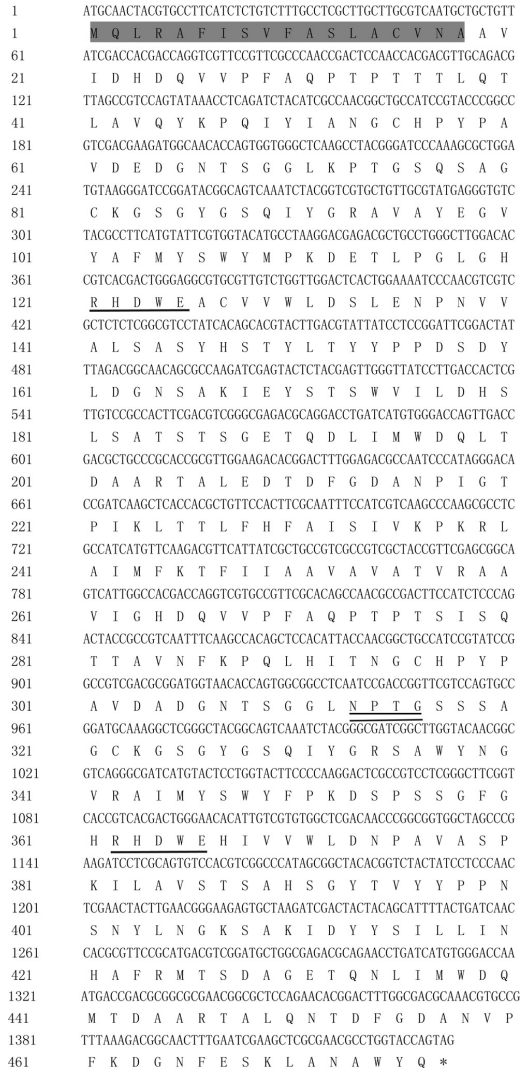

Figure 1. Nucleotide and deduced amino acid sequences of *phcnlp1*. Highly conserved domains that exist in Nep-like protein genes (*nlp*) are underlined. One potential *N*-glycosylation site is doubly underlined. Signal peptides are gray.

Expression pattern of *phcnlp1* in infected pepper leaves

Pepper leaves exhibited increasingly severe lesions or decay from 1 to 7 dpi (data not shown). The expression levels of *phcnlp1* were estimated with real-time RT-PCR. After analysis as previously described (Vandesompele et al., 2002), 18s rRNA was selected as an appropriate internal standard based on low variation among the various samples (data not shown).

The expression pattern of *phcnlp1* was investigated every day from 1 to 10 dpi (Figure 2). Various expression levels were elicited in inoculated tissues up to 7 dpi, and the expression levels of *phcnlp1* showed an increasing trend during the infection process and eventually reached definite peaks at 7 dpi. The *phcnlp1* gene exhibited lower expression from 1 to 3 dpi (see Figure 2) and then showed an obvious increase at 5 dpi, peaking at 7 dpi. In summary, *phcnlp1* genes showed insignificant expression level changes during the infection process, suggesting that this gene is critical to the capability of *P. capsici* to cause observable necrotic lesions.

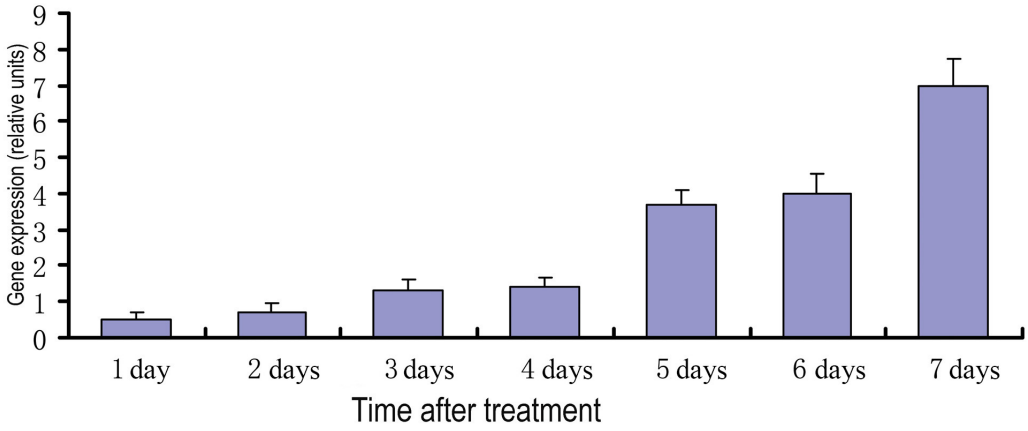

Figure 2. Real-time polymerase chain reaction analysis of *phcnlp1* expression in pepper leaves inoculated with *Phytophthora capsici* from 1 to 7 days post-inoculation. 18S ribosomal RNA was chosen as an endogenous control. Data represent the average of three independent experiments with standard error.

Functional analysis of *phcnlp1* through PVX agroinfection assay in pepper and tobacco plants

Mature *phcnlp1* (with predicted signal peptides removed) was introduced into PVX pGR106 (Wu et al., 2008). Test plants were observed for up to 10 dpi for the development of symptoms. Representative phenotypes are shown in Figure 3.

Most tobacco (*N. benthamiana*) leaves showed yellowing around the inoculated sites at 3 dpi (Figure 3b). As time passed, the chlorotic areas gradually turned brown and then showed necrosis spots of various degrees at 7 dpi (see Figure 3). None of the plants treated with empty vector pGR106 showed a necrotic response.

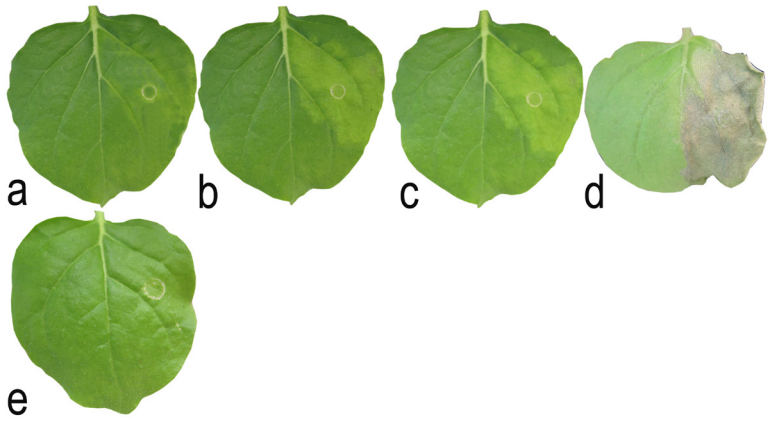

Figure 3. Symptoms appeared on *Nicotiana benthamiana* leaves treated with *phcnlp1* expressed by potato virus X (PVX) in *Agrobacterium tumefaciens*. **a.-d.** Photographs of inoculated leaves taken at 1, 3, 5, and 10 dpi. **e.** Represents control plants that were mock inoculated with *A. tumefaciens* harboring the PVX expression vector (pGR106).

DISCUSSION

In this study, we characterized *phcnlp1* in *P. capsici* and evaluated its role in the infection of host plants. We analyzed the expression profiles of *phcnlp1* after inoculation of pepper leaves with *P. capsici.* Results showed that *phcnlp1* expression increased during infection. Furthermore, transient expression in tobacco plants showed that *phcnlp1* caused necrosis symptoms when expressed based on PVX agroinfection. From these results, we inferred that *phcnlp1* was involved in the pathogenicity of *P. capsici*. We also speculate that other genes in the *phcnlp* family play roles in the host-pathogen interaction in *P. capsici* infection. These roles remain an important target for further research.

Typical symptoms on plants caused by NLPs were necrosis, cell death, and wilting (Fellbrich et al., 2002; Wang et al., 2004). The *phcnlp1* gene caused significant symptoms on two plant species, as shown in Figure 3. Studies have shown that some NLP genes - for example, 2 orthologous genes (MpNEP1 and MpNEP2) in *Moniliophthora perniciosa* - cause no changes in plants and show different characteristics when applied to plants (Garcia et al., 2007). These findings are reminiscent of a study that showed that eight Pg genes caused four types of significantly different changes (Wu et al., 2008). This phenomenon suggests that members of a gene family can have diversified functions.

Using a series of *P. capsici*-infected pepper leaves enabled the study of transcriptional changes at distinct phases of interaction. Real-time RT-PCR analysis of *phcnlp1* expression during infection demonstrated it to be specifically expressed in plants, which is consistent with the expression profile reported for other *nlps* in *M. perniciosa* and *P. sojae*, in which peak expression is associated with the appearance of disease symptoms at the final phases of the interaction (Motteram et al., 2009). The expression trend of *phcnlp1* increases obviously in this phase of interaction, and the typical symptoms on plants when applied through PVX agroinfection allowed us to speculate possible contributions of *phcnlp1* to pathogenicity or virulence.

Although it remains unclear whether the identified response to PVX agroinfection should be characterized as a general plant defense or the result of another activity in the *Phytophthora*-pepper host system, our results indicated that the potential function of *phcnlp1* as a virulence gene on pepper hosts should be explored further. Other future research could address RNA silence of *phcnlp1* in *P. capsici* and the function of additional members of the *nlp* family.

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