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Methodology

Identification of 18 genes encoding necrosis-inducing proteins from the plant pathogen *Phytophthora capsici* (Pythiaceae: Oomycetes)

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ABSTRACT. *Phytophthora capsici* is an aggressive plant pathogen that affects solanaceous and cucurbitaceous hosts. Necrosis-inducing *Phytophthora* proteins (NPPs) are a group of secreted toxins found particularly in oomycetes. Several NPPs from *Phytophthora* species trigger plant cell death and activate host defense gene expression. We isolated 18 *P. capsici* NPP genes, of which 12 were active during hypha growth from a *Phytophthora* stain isolated from pepper (*Capsicum annuum*) plants in China. The 18 predicted proteins had a sequence homology of 46.26%. The 18 *Pcnpp* sequences had a conserved GHRHDWE motif and fell into two groups. Eleven sequences in group 1 had two conserved cysteine residues, whereas the other seven sequences in group 2 lacked these two cysteine residues. A phylogenetic tree was constructed on the basis of the alignment of the predicted protein sequences of 52 selected NPP genes from oomycetes, fungi and bacteria from Genbank. The tree did not rigorously follow the taxonomic classification of the species; all the NPPs from oomycetes formed their own clusters, while fungal sequences were grouped into

two separate clades, indicating that based on NPPs, we can separate oomycetes from fungi and bacteria, and that expansion of the NPP family was a feature of *Phytophthora* evolution.

Key words: Oomycete; Necrosis-inducing *Phytophthora* protein; *Phytophthora capsici*; Horizontal gene transfer

INTRODUCTION

Like fungi, oomycetes have a global distribution and prosper in diverse environments. The oomycetes belong to the kingdom Stramenopile and contain a lot of pathogenic species (Baldauf et al., 2000). The genus *Phytophthora* contains a large number of phytopathogens, such as *P. capsici*, *P. infestans*, *P. sojea*, and so on. And most *Phytophthora* species are the cause of blight, crown rot, as well as stem, leaf, and fruit lesions on many plants. Moreover, the *Phytophthora* pathogen has been identified as a devastating pathogen on solanaceous and cucurbitaceous hosts including pepper, cucumber, eggplant, squash, pumpkin, tomato, melon, and zucchini (Lamour and Hausbeck, 2004). Sporangia and/or oospores develop in the lesions, resulting in a powdered-sugar appearance on the surfaces of fruits. Infected fruits quickly break down both in the field and postharvest.

Many pathogens have been reported to secrete elicitor proteins, which 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). In fact, elicitors are connected to a series of proteins that cause cell death at the infection site, thereby limiting or slowing down disease (Garcia et al., 2007). Although the necrosis can prevent biotrophic growth of pathogens, the induction of such condition can be beneficial to saprotrophic pathogens (Qutob et al., 2002).

NPPs, namely necrosis-inducing *Phytophthora* proteins, are also called NLPs (Nep1-like proteins), because the protein shares substantial sequence similarity with the first discovered member (named Nep1), which was defined as the necrosis and ethylene-inducing protein isolated from *Fusarium oxysporm* f.sp. *erythroloxyli* in liquid cultures (Bailey, 1995). NPPs represent a new class of necrotic elicitors.

Genes encoding NPPs, or the proteins themselves, have been detected in superfamilies in eukaryotic and prokaryotic organisms. The NPPs are not found in plants, animals, but they often occur in fungi, bacteria and also in oomycetes. Oomycetes, particularly many *Phytophthora* species such as *P. infestans*, *P. parasitica*, *P. aphanidermatum*, *P. sojea*, and *P. ramorum* form NPPs (Pemberton and Salmond, 2004). Although NPPs are present in phylogenetically distant organisms, the NPP sequence has remained a remarkably conserved domain called the necrosis-inducing *Phytophthora* protein 1 (NPP1) (Fellbrich et al., 2002). There is a hepta-peptide (GHRHDWE) and some conserved cysteine residues are present in every sequence. According to the cysteine residue number and position, NPPs are classified into two groups (Gijzen and Nurnberger, 2006). Dean et al. (2005) found that *Magnaporthe grisea* has the NPP genes of these two groups. To date, monocots are apparently not affected by NPPs, but it has been reported that many NPPs are able to trigger numerous dicotyledonous plant defense responses, necroses and cell death (Fellbrich et al., 2002; Qutob et al., 2002; Bailey et al., 2005). Some organisms, like *Verticillium dahliae* can secrete NPPs (VdNEP) that induce cotton wilting (Wang et al., 2004). In tobacco leaves, Nep1 caused an increase of 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 may be involved. Studies with *P. parasitica* showed that Nep1 induced superoxide anions production and salicylic acid-dependent resistance gene PR1 expression (Fellbrich et al., 2002). Additionally PsojNIP was linked to the transition from biotrophic to the saprotrophic phase in *P. sojea* (Qutob et al., 2002). Remarkably, it was observed that the crystal structure of an NLP from *Pythium aphanidermatum* exhibited structural similarities to cytolytic toxins produced by marine organisms, suggesting that this protein contributed to host infection by plasma membrane destruction and cytolysis (Ottmann et al., 2009). However, the reason why *Phytophthora* species have a large NPP gene family and the roles these genes play during infection process are still unclear.

The phylogenetic relationship between NPP sequences from different organisms has been analyzed by several authors. Notably, all these authors suggested that horizontal gene transfer occurred during NPP gene evolution, because bacterial NPP genes within a phylogram were not monophyletic, but interspersed with fungal sequences (Pemberton and Salmond, 2004; Gijzen and Nurnberger, 2006; Garcia et al., 2007). On the other hand, an alternative explanation was raised. Garcia et al. (2007) indicated that NPPs were ancient genes, and underwent duplication and evolution, resulting in the division of two types of genes (types I and II) before the division in eukaryotes and prokaryotes. In this case, some organisms that did not use NPP protein such as plants and animals may have lost these genes.

In this study, we showed the presence of NPPs in a high-virulent *P. capsici* strain SD33 isolated from China. A total of 18 NPP genes were cloned and characterized in this strain, 12 of which were apparently active in SD33 mycelia. The sequence alignment of these 18 NPPs delineated two groups. Eleven members in group 1 had two conserved cysteine residues, whereas the other seven sequences in group 2 were lacking these two cysteine residues. All the 18 predicted function proteins displayed high sequence homology with a conserved GHRHDWE motif. The phylogenetic tree was constructed on the basis of the alignment of predicted protein sequences of 52 selected NPP genes from oomycetes, fungi and bacteria. All the NPPs of oomycetes were clustered together, while fungal sequences were grouped into two clades, indicating that NPPs were able to separate oomycetes from fungi and bacteria, and that fungi and bacteria would have acquired these genes by horizontal gene transfer.

MATERIAL AND METHODS

Phytophthora capsici strain

A highly virulent *P. capsici* strain, SD33, was used as described previously (Feng et al., 2010) and maintained at 25°C on 10% V8 juice agar medium as described in Tyler et al. (1995).

Isolation of *Pcnpp* genes and sequence analysis

The GHRHDWE motif was searched for in the released *P. capsici* genome sequence (http://genome.jgi-psf.org/PhycaF7/PhycaF7.download.html). The candidate amino sequences were determined through manual revision and checked by submitting sequences to the online SMART software (http://smart.embl-heidelberg.de/) for conserved function domain analysis. The orthologs of the reported NPP gene in other oomycte genomes, including *P. sojea*, *P.*

infestans, and *P. parasitica*, were downloaded from NCBI. Also NPP genes from reported species in fungi and bacteria were downloaded from NCBI. Most of the available complete NPP amino acid sequences were multialigned using Clustal X 2.0.

To amplify NPP orthologous in the SD33 strain, a series of primers were designed using the Primer Express 3.0 software according to the identified sequences in the JGI database (as shown in Table 1). Genomic DNA of SD33 was extracted from hyphae grown in 10% V8 liquid medium according to the protocol described by Tyler et al. (1995). 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, 72°C for 1 min, and a final extension at 72°C for 10 min, and minor adjustments were made to amplify different genes. The PCR products were cloned in T3-vector and confirmed by sequencing.

Nucleotide and amino acid sequence homology searches were compared with the sequences in the NCBI-BLAST program (http://www.ncbi.nlm.nih.gov/).

Table 1. Primers used for *Pcnpp* gene cloning.

Primer name	Primer sequence	Application
7756PF	ATGCAACTACGTGCCTTCATCTCT	*Pcnpp*1
7756PR	TTAAGTGTAGTACGCGTTAGCTAGTTTA	
23292PF	ATGAAATTCGTCGTTTTCCTCTGTG	*Pcnpp*2
23292PR	CTAGAAGGGCCAGGCCTTGTCCAG	
71103PF	ATGAACCTTCTGGGATTCCTCGCC	*Pcnpp*3
71103PR	TTAGAATGGCCAAGCCTTACCCAA	
43883PF	ATGTACTCTTGGTACTTCCCCAAG	*Pcnpp*4
43883PR	ATTCAGATTCCACTGTGGAAAGAAGC	
70852PF	ATGAAGTTCAAACCCCAACTGCAC	*Pcnpp*5
70852PR	CTAGAAGGGCCAGGCCTTGTCCAG	
24573PF	ATGAGGTTTACCACCATCTTCTGG	*Pcnpp*6
24573PR	TTAAAACGGCCAGGCGTTTTCAAT	
68295PF	ATGTACATGTGCACCTTTGCCATC	*Pcnpp*7
68295PR	TTAGAACGGCCAAGCCTTGTCCAGT	
73591PF	ATGAGGCTCAGTATCGCCTTGGGCG	*Pcnpp*8
73591PR	TCATTGAAAGGGCCAAGCTTTGGC	
78535PF	ATGAGGCTCTTCGCTTTCCTATGG	*Pcnpp*9
78535PR	TTAGAACGGCCAAGCCTTGTCCAGT	
23459PF	ATGTTCAAGACGTTCATTATCGCTG	*Pcnpp*10
23459PR	CTACTGGTACCAGGCGTTCGCGAGC	
20844PF	ATGGAGCCCCTGAATATAAG	*Pcnpp*11
20844PF	TTAGAAGGGCCAAGCCTTC	
21024PF	ATGACCGACAGTAAAAACACCGTTACAGCT	*Pcnpp*12
21024PR	CTATTTTTTTTCGCCAAATGGCCAGGCTTTAC	
69004PF	ATGTACTCGTGGTACTTCCCTAAAGATTCG	*Pcnpp*13
69004PR	CTATTTTTTTTCGCCAAATGG	
70605PF	ATGTACTCTTGGTATTTCCCCAAGGATTCT	*Pcnpp*14
70605PR	TTAATCAAACGGCCAGGCCTTGTTGAGTTT	
78817PF	ATGTACTCGTGGTACTTCCCAAAGGACTCA	*Pcnpp*15
78817PR	TTAGAAGGGCCAAGCCTTCTCCAGCTTCGG	
86540PF	ATGTACTCGTGGTATTTCCCCAAGGATTCC	*Pcnpp*16
86540PR	CTAAAAGGGCCAAGCCTTGTCCAACTTGG	
68053PF	ATGTACTCGTGGTACTTCCC	*Pcnpp*17
68053PR	TTAGAAGGGCCAAGCCTTCTCCAGCTTCG	
72101PF	ATGTACTCTTGGTACTTCCCGAAAGACTCA	*Pcnpp*18
72101PR	TTAAAACGGCCAGGCGTTTTCAATTTTCG	

RNA extract and RT-PCR analysis

SD33 mycelium, grown in 10% V8 liquid medium for three days, was collected and ground in liquid nitrogen. Total RNA was extracted using a fungal RNA kit (OMEGA) fol-

lowing manufacturer recommendations. The RNA was quantified by measuring absorbance at 260/280 nm with a spectrophotometer and the quality was examined by electrophoresis on a 1.2% formaldehyde agarose gel. Ten micrograms total RNA was treated with 4 U Rnase-free DNaseI (TaKaRa) at 37°C for 30 min, and then was used for reverse transcription using the Omniscript RT kit (Qiagen). Synthesized cDNA was used for PCR, and the specific primers for PCR were designed using the Primer Express 3.0 software (Table 2). The PCR parameters were as follows: 94°C for 4 min, then 30 cycles of 94°C for 1 min, 53°C for 30 s, and 72°C for 30 s, and then 72°C for 10 min for a final extension. *P. capsici* actinA gene was used as control. The PCR products were electrophoresed on 1% agarose gels and visualized by ethidium bromide staining. Results were obtained from three repeated experiments.

Table 2. Primers used for RT-PCR.

Primer name	Primer sequence	Application	Expected size
ARTF	GTTGGACTCACGGA AAATCC	*Pcnpp*1	147
ARTR	GTGGTCAAGGATAACCCAACT		
BRTF	ATGAAATTCGTCGTTTTCCTC	*Pcnpp*2	179
BRTR	AGTTGGGGTTTGAACTTCATC		
FRTF	TCCAAGCTGGAACTATCGAT	*Pcnpp*3	166
FRTR	AGTCCACCACTGGTCTCTCC		
GRTF	ACGACGGCTACAAGAAGT	*Pcnpp*4	117
GRTR	AGTATCCAGATCGTGGTTGA		
HRTF	GGGCTTGAAAACTACCGG	*Pcnpp*5	114
HRTR	CAAGAGTACATGATGGCCCAG		
JRTF	ACCACCATCTTCTGGATCAG	*Pcnpp*6	165
JRTR	AGAGTCTTGAGTCTGCGGTT		
KRTF	GACCCAAGATGACAGCAGTA	*Pcnpp*7	196
KRTR	TAGGAGCTGGAGTAGGTGAC		
LRTF	GTTCGCAGGTACAACAAATT	*Pcnpp*8	202
LRTR	AGATAGTGACAGGTTCAGGTTG		
MRTF	GACCCAAGATGACAGCAGTA	*Pcnpp*9	180
MRTR	TGACAGGGTAGGCAAATATG		
ORTF	CTGAGCGATCATGTACTCCT	*Pcnpp*10	171
ORTR	AGGATAGTAGACCGTGTAGCC		
QRTF	ACCACCAACTCCAGAACCAA	*Pcnpp*11	177
QRTR	TTTCAACTTCACACCAGCCTT		
RRTF	GGTCGTGATGGAGATGAAAT	*Pcnpp*12	118
RRTR	GATTATCTTT TCCGTCCAT		
TRTF	GTCGGCTCTTAGTGGTTATT	*Pcnpp*13	156
TRTR	AGTCCACATGATGAGGTCTTG		
URTF	GGAAGATCTTGGCTGTCA	*Pcnpp*14	165
URTR	GGTCCTGGTAGTCCCCTC		
XRTF	GCATGCTGGCTACAAGAA	*Pcnpp*15	137
XRTR	AGGTCCTGAAAAGTCCCA		
YRTF	AGCGTCTACTCCAAGCAA	*Pcnpp*16	140
YRTR	CATGATGAGGTCCTGGAAGTC		
SRTF	AAAGGACTCACCGTCTACTG	*Pcnpp*17	150
SRTR	GGCGGGCACGGATTGTACTT		
VRTF	CCGTCATGACTGGGAGCACG	*Pcnpp*18	162
VRTR	GTCCAGGTCGTGGTTGTAAG		
ActinRTF	GTACTGCAACATCGTGCTGTCC	Actin gene	250
ActinRTR	TTAGAAGCACTTGCGGTGCACG		

Phylogenetic analysis

To generate the phylogenetic tree, 34 reported NPP genes (including 23 type I genes and 11 type II genes) (Pemberton and Salmond, 2004; Gijzen and Nurnberger, 2006) and 18

Pcnpp genes were used (Table 3). Multiple alignment was performed on these 52 sequences by use of Clustal X (2.0). Phylogenetic trees were generated by neighbor-joining, as implemented in PAUP* 4.0 Beta (Sinauer Associates, Sunderland, MA, USA) with the default parameters. Nodal support of the trees was estimated by bootstrapping, with 1000 pseudoreplicate data sets.

Table 3. NPP sequences used in the phylogenetic analysis.

Organism	Accession number	Gene type
Aspergillus nidulans	XP_660815	I
A. nidulans	XP_660939	II
A. fumigatus	XM_743186	I
A. fumigatus	XM_743446	II
Bacillus halodurans	BAB04114	I
B. licheniformis	YP_091187	I
Fusarium oxysporum	AAC97382	I
Gibberella zeae	XP_387963	II
G. zeae	XP_391669	II
G. zeae	XP_383570	II
G. zeae	XP_386193	I
Magnaporthe grisea	XP_362893	I
M. grisea	XM_366313	II
M. grisea	XM_365630	II
M. grisea	XM_368843	II
M. oryzae	XM_362983	I
Moniliophthora perniciosa	EF114673	I
M. perniciosa	EF109894	I
Neurospora crassa	XM_954671	II
Phytophthora infestans	AY961417	I
P. parasitica	AF352031	I
P. megakarya	AY741083	I
P. megakarya	AY741082	I
P. megakarya	AY741086	I
P. megakarya	AY741088	I
P. sojea	AF320326	I
P. sojea	AAM48171	I
P. sojea	AAM48172	I
Pythium aphanid	AF179598	I
P. aff. vanterpoolii	AAQ89595	I
P. middletonii	AY389162	I
Verticillium dahliae	AAS45247	I
Vibrio pommerensis	CAC40975	II
Streptomyces coelicolor	AL939131	II

NPPs were divided into two types (I and II) according to numbers of cysteine residues in their sequences as described in previous studies (Pemberton and Salmond, 2004; Gijzen and Nurnberger, 2006).

RESULTS

Isolation of *Pcnpp* genes and sequence analysis

Genome-wide identification of NPPs was performed by searching for the GHRHDWE motif in the released *P. capsici* genome sequence. We identified 53 candidate genes containing the conserved GHRHDWE motif. Among these NPPs, 14 were single copies, while the rest were multicopies ranging from 2-12 (see Table S1). A series of primers were designed using the released *P. capsici* genome sequence (JGI). The PCR product of expected length was ligated with T3-vector in DH5α and then sequenced in company. A database search confirmed that it was homologous to NPP genes from oomycete, fungi and bacteria.

A total of 18 *Pcnpp* gene sequences designated *Pcnpp*1 to *Pcnpp*18 were identified and submitted to Genbank (accession Nos. HM543167 to HM543184). They were all complete genes, without an intron. As shown in Figure 1, most of them were expressed in hypha, whereas six genes (*Pcnpp*4, *Pcnpp*11, *Pcnpp*12, *Pcnpp*16, *Pcnpp*17, and *Pcnpp*18) appeared to be inactive in hypha grown by RT-PCR analysis.

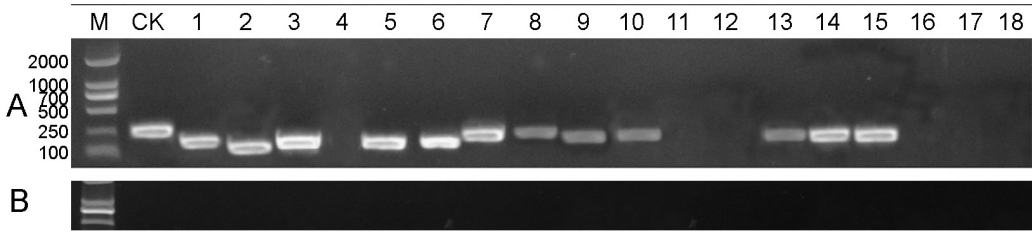

Figure 1. A. RT-PCR analysis of the 18 *Pcnpp* genes expressed in hyphae RNA. M = DNA marker DL-2000; CK = actinA used as control; *lanes 1-18* = RT-PCR products: *Pcnpp*1 to *Pcnpp*18. **B.** RNA used as template for RT-PCR analysis to detect DNA contamination. *Lanes 1-19* = there were no bands present, indicating no DNA.

Amino acid sequences were deduced from the open reading frames. The predicted proteins varied in length from 132- to 338-amino acid residues, as a result of N-terminal extension variation in length (Figure 2). Eight genes (*Pcnpp*1, *Pcnpp*2, *Pcnpp*3, *Pcnpp*6, *Pcnpp*7, *Pcnpp*8, *Pcnpp*9, and *Pcnpp*10) contained a signal peptide predicted using SignaLP 3.0 Server as shown in Table 4. Putative monobasic propeptide cleavage sites were present between 17- and 20-amino acid residues in the eight sequences as shown in Table 4.

Table 4. Identities of 18 *Phytophthora capsici* sequences and signal peptide predictions of the translated proteins.

Gene	GenBank No.	Extracellular protein[a]	SignalP-HMM probability[b]
*Pcnpp*1	HM543167	Yes	0.999
*Pcnpp*2	HM543168	Yes	1.000
*Pcnpp*3	HM543169	Yes	1.000
*Pcnpp*4	HM543170	No	0.000
*Pcnpp*5	HM543171	No	0.000
*Pcnpp*6	HM543172	Yes	1.000
*Pcnpp*7	HM543173	Yes	1.000
*Pcnpp*8	HM543174	Yes	0.999
*Pcnpp*9	HM543175	Yes	1.000
*Pcnpp*10	HM543176	Yes	0.999
*Pcnpp*11	HM543177	No	0.038
*Pcnpp*12	HM543178	No	0.000
*Pcnpp*13	HM543179	No	0.000
*Pcnpp*14	HM543180	No	0.000
*Pcnpp*15	HM543181	No	0.000
*Pcnpp*16	HM543182	No	0.000
*Pcnpp*17	HM543184	No	0.000
*Pcnpp*18	HM543185	No	0.000

[a]A protein sequence is assigned a 'Yes' for extracellular protein if the protein is predicted to be a signal peptide by the SignalP Hidden Markov Model (SignalP-HMM) with a probability greater than 0.900 and the SignalP Network predicted cleavage site between 10- and 40-amino acid residues. Otherwise, it is assigned a 'No'. [b]Calculated by the SignalP Hidden Markov Model algorithm.

Figure 2. Sequence alignment of the 12 potential *Pcnpp* genes. Signal peptides of each sequence are in grey, the two conserved cysteines are in Box1 and Box2, the hepta-peptide motif 'GHRHDWE' is in Box3, the C-terminal relatively conserved motif 'QDLIMWDQ' is in Box4. Arrowheads indicate potentially active sites. Signal peptides for each *Pcnpp* gene are underlined. Dark highlights indicate that the residues are conserved in all NPPs compared, whereas other color highlights denote sequences only conserved in some NPPs. The consensus line shows only those residues that are identical in 100% of the sequences.

Sequences and RT-PCR transcripts for these 18 genes, confirmed that 12 of these genes were expressed during *in vitro* growth. All the 18 predicted proteins displayed sequence homology of 46.26%. The 18 genes fell into two groups. Group 1 had 11 sequences (*Pcnpp*1, *Pcnpp*2, *Pcnpp*3, *Pcnpp*5, *Pcnpp*6, *Pcnpp*7, *Pcnpp*8, *Pcnpp*9, *Pcnpp*10, *Pcnpp*11, and *Pcnpp*12) and had two highly conserved cysteine residues, which were numbered C^{55} and C^{80} in *Pcnpp*1 at the N-terminal extension (Figure 2). By contrast, sequences in group 2 (*Pcnpp*4, *Pcnpp*13, *Pcnpp*14, *Pcnpp*15, *Pcnpp*16, *Pcnpp*17, and *Pcnpp*18) were lacking the conserved cysteine residues, and the average length was ca. 400 bp. However, all these 18 genes had a conserved GHRHDWE motif and a relatively conserved bexakis-residue QDLIMW at the C-terminal extension. All *Pcnpp* have four potential enzyme activity sites according to the crystal model by Ottmann et al. (2009). Numbered according to their positions in *Pcnpp*1, these residues were D^{112}, H^{120}, D^{123}, and E^{125} (Figure 2). These four residues have been shown to be involved in necrosis lesion formation in NLPs from *P. aphanidermatum* (Ottmann et al., 2009). Additionally, the *Pcnpp* had a GC content of 56.6%, which was similar to other *Phytophthora* NPPs, such as 56.9% for *P. infestans*, 59.3% for *P. sojae* and 56.6% for *P. parasitica* (Bae et al., 2005). The identities between *Pcnpp* sequences and fungi and bacteria were relatively low (15-44.73%).

Phylogenetic analysis

To generate the phylogenetic tree, 34 reported NPP genes (including 23 type I genes and 11 type II genes) and 18 *Pcnpp* genes were used.

The phylogenetic tree (as shown in Figure 3) showed the relationships among the selected 52 NPPs. The tree was constructed on the basis of alignment of the predicted protein sequences of the selected NPP genes. All oomycete genes were clustered together, while fungal sequences were grouped into two separate clades (Fungi 1 and 2). Bacterial sequences were selected as the outgroup. Unexpectedly, two bacterial sequences (AL939131 and CAC40975) were interspersed with fungal sequences in clade Fungi 2. In our phylogenetic tree, all the genes in clade Fungi 1 belonged to type I as previously described (Pemberton and Salmond, 2004; Gijzen and Nurnberger, 2006); however, in clade Fungi 2 most genes belonged to type II, except four genes (EF114673, EF109894, AAC97382, and AAS45247). This result was somewhat different from the previous conclusion, which showed that NPPs could be grouped strictly according to the gene type. Remarkably, all the oomycete NPP genes were attributed to type I as described above, and *Pcnpp* were grouped together with other NPP protein sequences from oomycetes, which was consistent with previous results (Pemberton and Salmond, 2004; Gijzen and Nurnberger, 2006; Garcia et al., 2007).

DISCUSSION

In this study, we confirmed that there was a large NPP gene family comprising at least 18 NPP genes in *P. capsici* strain SD33. Although many NPP genes occurred in several *Phytophthora* species, little was known about these genes at the molecular and biological level in *P. capsici*. Other plant pathogens contained multiple NPP genes, but the size and structure of this gene family was diverse. For example, *Magnaporthe grisea* and *Gibberella zeae* possess four NPPs (Dean et al., 2005; Gijzen and Nurnberger, 2006), whereas *Aspergillus nidulans* and

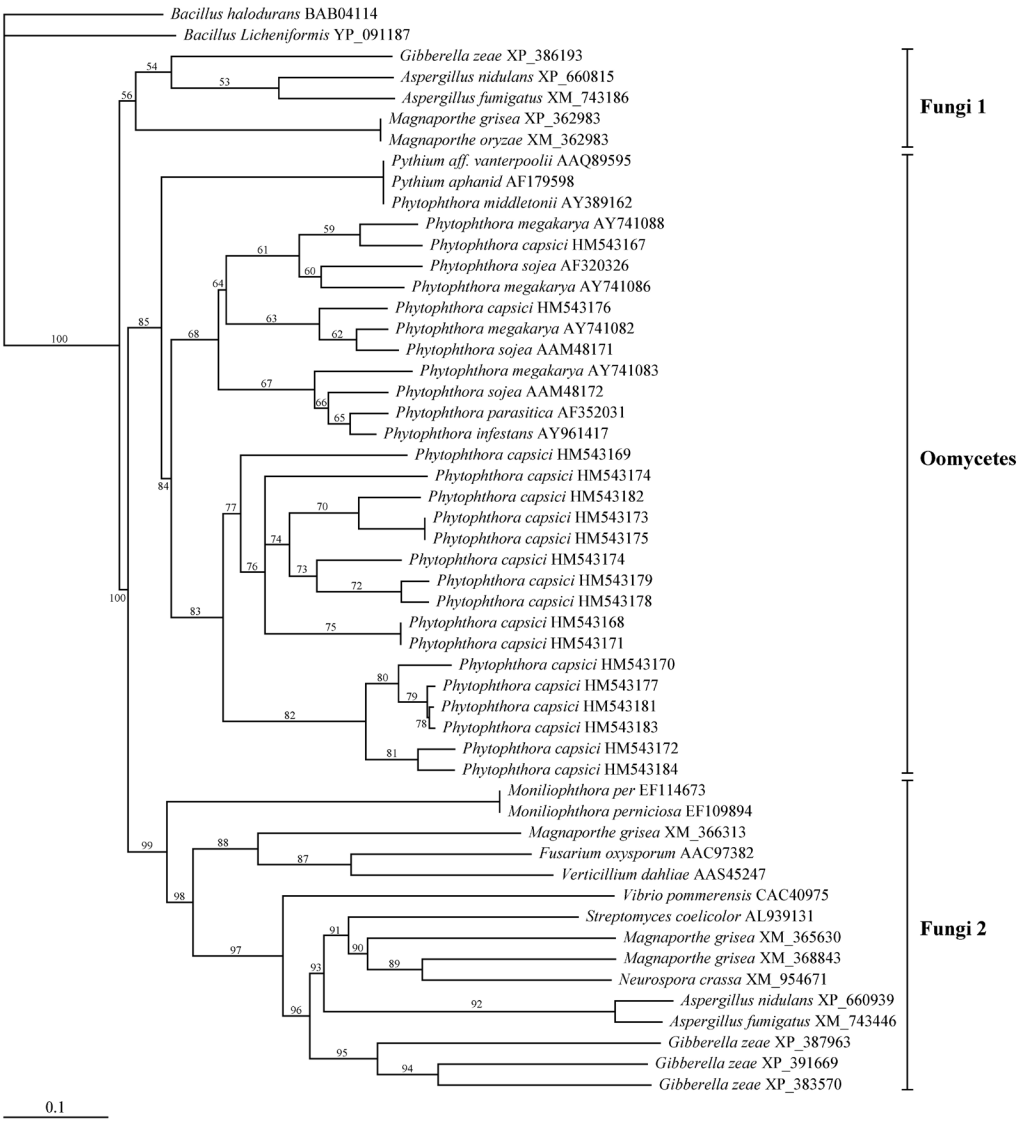

Figure 3. Phylogenetic analysis of the sequences of NPP orthologs from oomycetes and other organisms. The phylogram was generated by the neighbor-joining method as implemented in PAUP* 4.0 Beta. Numbers beside each node indicate bootstrap values as a percentage of 1000 replicates, with the scale bar representing 10% weighted sequences divergence. The accession number of each gene is shown to the right of the organism.

A. fumigatus each contain two NPP genes (Galagan et al., 2005), and a single gene is present in the *Neurospora crassa* genome (Galagan et al., 2003). Many bacterial species contain only 1-4 NPP genes in their genome (Pemberton and Salmond, 2004). Genome sequences demonstrate that around 50-60 NPP genes are present in *P. sojae* and *P. ramorum*, respectively; but it is believed that more than half of the predicted genes are likely pseudogenes (Tyler et al., 2006).

In *P. megakarya*, NPP orthologs have been found organized in clusters and at least six of them seem to be expressed (Bae et al., 2005).

Although a number of possibilities have been proposed about the roles of NPPs, the biological significance of multiple NPPs in plant pathogens is not fully understood. It is likely that NPPs play an important role in the pathogenic progress of *Phytophthora* (Qutob et al., 2006). Some NPPs are related to pathogenicity during pathogen infection of plants, but NPPs in many fungi and bacteria species are not known to be pathogenic in plants. This suggests that NPPs may have other physiological functions within the pathogen other than their role in plant infection. In contrast, NPP sequences are not found in plant or animal, which suggests that NPPs are involved in the physiological or pathogenic processes of microorganisms.

All *Pcnpp* grouped together by phylogenetic comparison with NPPs from oomycetes, fungi and bacteria. The results showed that the fungal NPPs containing two or four conserved cysteines did not form separate clades, which were different from previous studies that classified all the fungus NPP genes as type I and Ⅱ. Fungal NPP sequences were divided into two different groups (Fungi 1 and 2), the type I in Fungi 1 and both types in Fungi 2 (Figure 3). This result was different from a former study indicating that all NPPs could be clearly separated according to the number and position of conserved cysteins (Gijzen and Nurnberger, 2006; Garcia et al., 2007). However, the tree was able to better group oomycete NPPs, as shown in Figure 3. The members of the oomycete clade belonged to *Phytophthora* and *Phythium* species. They formed three main branches in the oomycete clade, in which all *Pcnpp* formed a compact cluster, except *Pcnpp*1 (HM543167) and *Pcnpp*10 (HM543176). This result suggested that the NPP family was a character of oomycete organisms, and was suitable for oomycete classification. On the contrary, it seemed that the NPP genes were not suitable for fungi or bacteria classification. Also there was no monophyletic group containing fungal NPPs or bacterial NPPs, respectively (Figure 3). The phylogenetical distribution of fungi and bacteria might be explained by the horizontal gene transfer mechanism. In fact, the nucleotide composition of certain bacteria was unequal to the genomic average, which suggested that these bacteria got NPP genes from other organisms (Pemberton and Salmond, 2004). In addition, NPPs existed in certain basidiomycetes like MpNEPs in *Moniliophthora perniciosa*, but could not be detected in other basidiomycete genomes, indicating discontinuity in the evolution of these genes. Moreover, although the codon usage of MpNEPs was similar to that of other *M. perniciosa* genes, the GC content was much higher than that of other genes, suggesting that these NPP genes were recently introduced in the species by horizontal gene transfer from other organisms (Garcia et al., 2007). On the contrary, *Phytophthora* NPPs had a GC content and codon usage that were the same as those of NPP genes in other organisms, indicating that these NPPs were ancient gene families in the *Phytophthora* species. Taken together, these data indicated that they were able to separate oomycete from fungi and bacteria, and that the occurrence of NPPs in fungi and bacteria might be involved in horizontal gene transfer.

Although NPPs showed relatively high homology, these sequences had intraspecific and interspecific diversity in structure and biological performance. In fact, 18 *Pcnpp* showed structural diversity in this study, and a case in point was that two NPP genes (MpNEP1 and MpNEP2) in *M. perniciosa* had high similarity but showed different characteristics when applied to tobacco and cacao leaves (Garcia et al., 2007). Maybe this was connected to birth-and-death evolution that had spent enough time for the divergence of gene duplicates and/or their orthologs in different organisms (Nei and Rooney, 2005). In addition, the percentage identities

between the deduced amino sequence of *Pcnpp* and other NPPs ranged from 15 to 84.19%. The highest matches were found with NPPs of *Phytophthora*, 84.19% between *Pcnpp*1 (HM543167) and *P. megakarya* (AY741088), and 83.9% between *Pcnpp*10 (HM543176) and *P. sojae* (AAM48171). And many NPP genes were apparently pseudogenes and occurred in more than one copy in some species. Additionally, these phenomena agreed with the birth-and-death theory that gene duplication and mutation emerged in an evolutionary process to adapt to different niches. As a result of duplication and mutation process, genes would diverge either in structure or in expression pattern, or be rendered non-functional as pseudogenes, and would be eventually removed from the genome (Garzón-Ospina et al., 2010).

In summary, we reported that an NPP multigene family existed in *P. capsici*, and that these paralog proteins had diverse physical properties and would be differentially expressed. We also did primary phylogenetic analysis of NPPs from different organisms, indicating that NPP expansion was a feature of *Phytophthora*. It is possible that the *Pcnpp* genes play an important role in pepper blight. We are currently studying the necrosis mechanisms, and developing protocols for silencing these genes in *P. capsici*.

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REFERENCES

Bae H, Bowers JH, Tooley PW and Bailey BA (2005). NEP1 orthologs encoding necrosis and ethylene inducing proteins exist as a multigene family in *Phytophthora megakarya*, causal agent of black pod disease on cacao. *Mycol. Res.* 109: 1373-1385.

Bailey BA (1995). Purification of a protein from culture filtrates of *Fusarium oxysporm* that induces ethylene and necrosis in leaves of *Erythroloxylum coca*. *Phytopathology* 85: 1250-1255.

Bailey BA, Bae H, Strem MD, Antunez de Mayolo G, et al. (2005). Developmental expression of stress response genes in *Theobroma cacao* leaves and their response to Nep1 treatment and a compatible infection by *Phytophthora megakarya*. *Plant Physiol. Biochem.* 43: 611-622.

Baldauf SL, Roger AJ, Wenk-Siefert I and Doolittle WF (2000). A kingdom-level phylogeny of eukaryotes based on combined protein data. *Science* 290: 972-977.

Dean RA, Talbot NJ, Ebbole DJ, Farman ML, et al. (2005). The genome sequence of the rice blast fungus *Magnaporthe grisea*. *Nature* 434: 980-986.

Fellbrich G, Romanski A, Varet A, Blume B, et al. (2002). NPP1, a *Phytophthora*-associated trigger of plant defense in parsley and *Arabidopsis*. *Plant J.* 32: 375-390.

Feng B, Li P, Wang H and Zhang X (2010). Functional analysis of *Pcpme6* from oomycete plant pathogen *Phytophthora capsici*. *Microb. Pathog.* 49: 23-31.

Galagan JE, Calvo SE, Borkovich KA, Selker EU, et al. (2003). The genome sequence of the filamentous fungus *Neurospora crassa*. *Nature* 422: 859-868.

Galagan JE, Calvo SE, Cuomo C, Ma LJ, et al. (2005). Sequencing of *Aspergillus nidulans* and comparative analysis with *A. fumigatus* and *A. oryzae*. *Nature* 438: 1105-1115.

Garcia O, Macedo JA, Tiburcio R, Zaparoli G, et al. (2007). Characterization of necrosis and ethylene-inducing proteins (NEP) in the basidiomycete *Moniliophthora perniciosa*, the causal agent of witches' broom in *Theobroma cacao*. *Mycol. Res.* 111: 443-455.

Garzón-Ospina D, Cadavid LF and Patarroyo MA (2010). Differential expansion of the merozoite surface protein (msp)-7 gene family in *Plasmodium* species under a birth-and-death model of evolution. *Mol. Phylogenet. Evol.* 55: 399-408.

Gijzen M and Nurnberger T (2006). Nep1-like proteins from plant pathogens: recruitment and diversification of the NPP1

domain across taxa. *Phytochemistry* 67: 1800-1807.
Jennings JC, Apel-Birkhold PC, Bailey BA and Anderson JD (2000). Induction of ethylene biosynthesis and necrosis in weed leaves by a *Fusarium oxysporum* protein. *Weed Sci.* 48: 7-14.
Lamour KH and Hausbeck MK (2004). *Phytophthora capsici* on vegetable crops: research progress and management challenges. *Plant Dis.* 88: 1292-1303.
Nei M and Rooney AP (2005). Concerted and birth-and-death evolution of multigene families. *Annu. Rev. Genet.* 39: 121-152.
Ottmann C, Luberacki B, Küfner I, Koch W, et al. (2009). A common toxin fold mediates microbial attack and plant defense. *Proc. Natl. Acad. Sci. U. S. A.* 106: 10359-10364.
Pare PW, Farag MA, Krishnamachari V, Zhang H, et al. (2005). Elicitors and priming agents initiate plant defense responses. *Photosynth. Res.* 85: 149-159.
Pemberton CL and Salmond GP (2004). The Nep1-like proteins-a growing family of microbial elicitors of plant necrosis. *Mol. Plant. Pathol.* 5: 353-359.
Qutob D, Kamoun S and Gijzen M (2002). Expression of a *Phytophthora sojae* necrosis-inducing protein occurs during transition from biotrophy to necrotrophy. *Plant J.* 32: 361-373.
Qutob D, Kemmerling B, Brunner F, Küfner I, et al. (2006). Phytotoxicity and innate immune responses induced by Nep1-like proteins. *Plant Cell* 18: 3721-3744.
Tyler BM, Forster H and Coffey MD (1995). Inheritance of avirulence factors and restriction fragment length polymorphism markers in outcrosses of the oomycete *Phytophthora sojae*. *Mol. Plant Microbe Interact.* 8: 515-523.
Tyler BM, Tripathy S, Zhang X, Dehal P, et al. (2006). Phytophthora genome sequences uncover evolutionary origins and mechanisms of pathogenesis. *Science* 313: 1261-1266.
Wang JY, Cai Y, Gou JY, Mao YB, et al. (2004). VdNEP, an elicitor from *Verticillium dahliae*, induces cotton plant wilting. *Appl. Environ. Microbiol.* 70: 4989-4995.

Supplementary Table 1

Table S1. Candidate genes identified in the JGI *Phytophthora capsici* genome.

Single copy genes	Multicopy genes	
	Genes	Identical members in genome
20406	23459	70850
20844	70852	122619
21024	76138	113086
24573	82430	116399
68295	114323	114326
68503	7756	23286; 70849
69004	23292	7613; 37194
70605	71103	23660; 7723
72101	74207	27731; 27732; 74207
73591	8760	86961; 39481; 118625; 124767
75230	43883	43884; 43885; 43886; 43887; 66543;
78535		91548; 91549; 91460; 91461; 91462;
78817		119696; 41934; 41935; 41936; 41937;
82067		65858
86540		