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NEP1 orthologs encoding necrosis and ethylene inducing proteins exist as a multigene family in *Phytophthora megakarya*, causal agent of black pod disease on cacao

Hanhong BAE[1], John H. BOWERS[1], Paul W. TOOLEY[2] and Bryan A. BAILEY[1]

[1] *USDA/ARS, Plant Sciences Institute, Sustainable Perennial Crops Laboratory, Beltsville Agricultural Research Center-West, Beltsville, MD 20705, USA.*
[2] *USDA/ARS, Foreign Disease-Weed Science Research Unit, Ft. Detrick, MD 21702-5023, USA.*
E-mail: *rbae@asrr.arsusda.gov*

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Phytophthora megakarya is a devastating oomycete pathogen that causes black pod disease in cacao. *Phytophthora* species produce a protein that has a similar sequence to the necrosis and ethylene inducing protein (Nep1) of *Fusarium oxysporum*. Multiple copies of *NEP1* orthologs (*PmegNEP*) have been identified in *P. megakarya* and four other *Phytophthora* species (*P. citrophthora*, *P. capsici*, *P. palmivora*, and *P. sojae*). Genome database searches confirmed the existence of multiple copies of *NEP1* orthologs in *P. sojae* and *P. ramorum*. In this study, nine different *PmegNEP* orthologs from *P. megakarya* strain Mk-1 were identified and analyzed. Of these nine orthologs, six were expressed in mycelium and in *P. megakarya* zoospore-infected cacao leaf tissue. The remaining two clones are either regulated differently, or are nonfunctional genes. Sequence analysis revealed that six *PmegNEP* orthologs were organized in two clusters of three orthologs each in the *P. megakarya* genome. Evidence is presented for the instability in the *P. megakarya* genome resulting from duplications, inversions, and fused genes resulting in multiple *NEP1* orthologs. Traits characteristic of the *Phytophthora* genome, such as the clustering of *NEP1* orthologs, the lack of CATT and TATA boxes, the lack of introns, and the short distance between ORFs were also observed.

INTRODUCTION

Black pod of cacao is caused by *Phytophthora* species and can result in complete yield loss under some conditions (Hoopen *et al.* 2001, Bennett 2003). This loss of yield has a devastating effect on local agricultural economies. Several *Phytophthora* species, such as *P. megakarya*, *P. palmivora*, *P. citrophthora*, and *P. capsici*, attack the tropical tree *Theobroma cacao* (cacao) causing seedling blights, stem cankers, and pod rots. *P. megakarya* is the most aggressive among the four species on cacao and attacks all plant parts.

The necrosis and ethylene inducing protein 1 (Nep1), is a 24 kDa extracellular protein that is produced by *Fusarium oxysporum* in liquid cultures (Bailey 1995, Bailey, Jennings & Anderson 1997). Nep1 causes necrosis in dicotyledonous plants, but is inactive in monocotyledonous plants (Bailey 1995, Jennings *et al.* 2000, Veit *et al.* 2001). Nep1-like proteins (NLPs), such as necrosis-inducing protein PsojNIP (GenBank accession no. AAK01636, isolated from *P. sojae*), necrosis-inducing *Phytophthora* protein 1 NPP1 (AAK19753, isolated from *P. parasitica*), and necrosis-inducing protein NIP1 (AAK25828, isolated from *P. infestans*), range from 24 to 26 kDa and have been identified in many different plant pathogens including fungi and bacteria (Veit *et al.* 2001, Fellbrich *et al.* 2002, Qutob, Kamoun & Gijzen 2002, Pemberton & Salmond 2004). Nep1 and NLPs cause induction of pathogen-related (PR) genes, K^+ and H^+ channel fluxes, callose apposition, accumulation of reactive oxygen species (ROS) and ethylene, altering cell respiration, creating a hypersensitive response (HR), and localized cell death (Jennings *et al.* 2001, Veit *et al.* 2001, Fellbrich *et al.* 2002, Keates *et al.* 2003). Nep1 also induced the breakdown of the cuticle, chloroplast envelope and internal membrane structures after 1–4 h of treatments in *Arabidopsis thaliana*, spotted knapweed (*Centaurea maculosa*), and dandelion (*Taraxacum officinale*) (Keates *et al.* 2003). In addition to disease resistance genes, Nep1 treatment induced genes putatively involved in plant stress responses, including wounding, drought and senescence (Keates *et al.* 2003). When Nep1 protein was applied in combination with the pathogen *Pleospora papaveracea*, it enhanced disease development and death of opium poppy (*Papaver somniferum*; Bailey *et al.* 2000). Qutob *et al.* (2002) demonstrated that PsojNIP was produced by *P. sojae*, a

pathogen of soybean (*Glycine max*), during the necrotrophic phase of the disease. Therefore, NLPs might function as necrosis inducing toxins rather than inducers of functional plant resistance responses (Jennings *et al.* 2000).

In *P. sojae*, two additional predicted open reading frames (*PsojORF II* and *PsojORF III*) related to *PsojNIP* were found clustered with *PsojNIP* within a 3515-bp region (AF511649; Qutob *et al.* 2002). *PsojNIP* and *PsojORF II* did not have introns, while the *PsojORFIII* had a 25-bp intron. The three ORFs had shared identities between 67% and 69%. The size of two intergenic sequences between the three ORFs was 674 bp and 713 bp, respectively. According to RT-PCR, only *PsojNIP* (AAK01636) was transcriptionally active. Alignment of NLPs of *Phytophthora* species revealed that the encoded proteins were highly similar to each other (69–89% identity). However, the sequence identity to *Fusarium oxysporum* Nep1 was relatively low (34–37%).

This study was initiated to characterize the gene family encoding NLPs in *Phytophthora* species causing black pod, a disease that severely reduce cacao yields around the world. *NEP1* orthologs were cloned and characterized in the *P. megakarya* strain Mk-1. Multiple copies of *NEP1* orthologs in *P. megakarya* were identified, as well as in four other *Phytophthora* species. Of nine *NEP1* orthologs cloned, seven of them were transcriptionally active in *P. megakarya*, both in mycelia and during infection of the cacao leaf.

MATERIALS AND METHODS

Plant growth and inoculation of leaf disks

Seeds of *Theobroma cacao* cv. 'Comun' (Lower Amazon Amelonado type) were planted in pots filled with a soilless mix (2:2:1, sand:perilte:promix). Seedlings were grown in a greenhouse up to 9 months with ambient light, 75% humidity, and a temperatures of 20–29 °C. Leaf development was separated into four stages: (1) unexpanded leaves (UE), less than 1 cm long with limited pigmentation; (2) young red leaves (YR), 5–10 cm long and pliable; (3) immature green leaves (IG), 10–20 cm long and pliable; and (4) mature green leaves (MG), 10–20 cm and rigid. For the zoospore infection study, leaves that were between IG and MG stage were used.

The cacao leaf disks were inoculated with zoospores of *P. megakarya* strain Mk-1 (Pierre Tondje, IRAD, Youande, Cameroon) that were synchronously produced in axenic culture (Bowers & Mitchell 1991). Identification of the *P. megakarya* strain Mk-1 was confirmed by morphology and phylogenetic analysis of the ribosomal DNA ITS region (data not shown). Leaf disks (85 mm) were cut from detached leaves and placed abaxial side up on a moist No. 2 Whatman paper in Petri dishes (100×15 mm). The disks were inoculated with multiple drops of 20 μl of water containing zoospores of *P. megakarya* (3×10^5 zoospores ml^{-1}), and control leaf disks were treated with multiple 20 μl drops of distilled water. The Petri dishes were sealed with parafilm and incubated at room temperature for up to 3 d under ambient light conditions. Three replications of each Petri dish were harvested 3 d after inoculation.

PCR and RACE (Rapid Amplification of cDNA Ends)

DNA and RNA were extracted from *Phytophthora megakarya* mycelia grown in clarified V8 (Campbell Soup Company, Camden, NJ) liquid culture (Martin & Tooley 2003) using DNeasy Plant Maxi Kit and RNeasy Mini Kit, respectively (Qiagen, Valencia, CA). Total RNA from *P. megakarya* zoospore-infected and control leaf disks was extracted by the adaptation of several established methods including those of Chang, Puryear & Cairney (1993), McKenzie *et al.* (1997), and the RNeasy Mini Kit (Qiagen) (Verica *et al.* 2004). The extracted RNA was treated with DNase I (Roche, Indianapolis, IN) according to manufacturer's recommendation and was quantified using a spectrophotometer and the integrity was verified with agarose gels.

To amplify the transcriptionally active *NEP1* ortholog, 2 μg of DNase I treated total RNA, extracted from zoospore-infected cacao leaf disks, were used to generate cDNA using the QIAGEN OneStep RT-PCR Kit (Qiagen) with 11F/16R primer set. primer sequences were designed based on the consensus sequence of the *NEP1* orthologs of *P. sojae* (AAK01636), *P. infestans* (AK25828), and *P. parasitica* (AAK19753) (11F 5′-AACGGCTGCCACCCGTACCCTGC-3′, 16R 5′-CGTGAG(T/C)TGGTCCCACATGATCAG-3′). Transcripts were amplified using the following conditions: reverse transcription at 50 °C for 30 min, initial activation of HotStar *Taq* DNA polymerase and inactivation of the reverse transcriptase at 95 ° for 15 min, denaturation for 30 s at 95 °, annealing for 30 s at 57 °, extension for 1 min at 72 °, and an additional 10 min extension at 72 °, 40 cycles. The PCR product was electrophoresed in 0.8% agrose gel, purified by using QIAEX II Gel Extraction System (Qiagen), cloned into pCR2.1-TOPO vector for small inserts or pCR-XL-TOPO vector for large inserts (Invitrogen, Calsbad, CA), and prepared for sequencing. DNA sequences were determined by the University of Maryland, DNA Sequencing Facility (http://www.umbi.umd.edu/~cbr/dna.html). Half μg of mycelial DNA was also used as a template for PCR amplification. This was performed using the following conditions: initial activation of *Taq* DNA Polymerase at 94 ° for 5 min, denaturation for 30 s at 94 °, annealing for 30 s at 55 °, extension for 1 min at 72 °, and an additional 10 min extension at 72 °, with 30 cycles. In order to clone the full length of *NEP1* orthologs, both 5′ and 3′ RACE were performed using the RACE system (Invitrogen).

Semiquantitative RT-PCR was used to detect the transcripts of *NEP1* orthologs in *P. megakarya* mycelia and in *P. megakarya* zoospore-infected cacao leaf

Table 1. Ortholog specific primer sequences for nine *PmegNEP* clones and the *ACTIN* gene. F, forward primer: R, reverse primer.

Clone name (GenBank accession no.)	Sequences (5′ to 3′)	Expected size (bp)
PmegNEP1 (AY741082)	F: TTCGACCTGGTATAACGGA	300
	R: CGTCTCACCACTATCCGAT	
PmegNEP2 (AY741083)	F: CACGTACAAAGGGGTCTAC	300
	R: GGTCTCTCCCACATCTGTG	
PmegNEP3 (AY741084)	F: GAAAGGAGCCCCTAGTGCTA	250
	R: CAGAATCTTGGGGTTTTCA	
PmegNEP4 (AY741085)	F: AGCCCCCACCGGGTCGGAG	250
	R: CGATGTTTTGGCTGGTCAAC	
PmegNEP4-2 (AY741086)		
PmegNEP5 (AY741087)	F: GACTGGTGCTCCTAGTTCA	250
	R: GATAATCTTCGGCTCCGGA	
PmegNEP6 (AY741088)	F: ATGAATCTTCGAGCCTT	320
	R: CACCATGAGTACATGAAAGCGTA	
PmegNEP6-2 (AY741089)	F: ATGCATCTTCGAGCCTC	320
	R: TACCATGAGTACATGAAAGCATT	
PmegNEP7 (AY741090)	F: TCATCAATCACGATGGAGTC	245
	R: CCAGACGTCATTGAA	
ACTIN	F: CAAGGAGAAGCTGACGTACA	457
	R: TGTACGTCAGCTTCTCCTTG	

tissues. Eight sets of *PmegNEP* ortholog-specific primers are listed in Table 1. Four μg of DNase I treated total RNA was used to generate the first strand complementary DNA (cDNA) using the oligo $(dT)_{20}$ primer. The first strand cDNA was then synthesized from the total RNA, using SuperScript III™ RNase H^- reverse transcriptase, according to the manufacturer's instructions (Invitrogen). Then the first strand cDNA was used as a template for semiquantitative RT-PCR. This was performed using the following conditions: initial activation of *Taq* DNA Polymerase at 94 °C for 5 min, denaturation for 30 s at 94 °, annealing for 30 s at 55 °, extension for 1 min at 72 °, and an additional 10 min extension at 72 °, with 30 cycles for *PmegNEP* orthologs and 22 cycles for *P. megakarya ACTIN*. Mycelial DNA was used as a PCR template to verify the existence of nine *PmegNEP* ortholgs in the genome of *P. megakarya* using the same set of ortholog-specific primers mentioned above (Table 1). The primer sequences for 459-bp *ACTIN* fragment were designed based on *P. infestans* (M59715) and was used as a PCR control. RT-PCR was repeated at least three times with different RNA extractions from separate plants or sources of *P. megakarya* mycelia.

DNA and RNA blot analyses

For Southern blot analysis, 2.5 μg of the mycelial DNA was digested with restriction enzymes *Bgl*II and *Pst*I at 37 ° overnight, separated in a 0.8 % agarose gel, blotted onto a membrane, and hybridized with the ^{32}P-labeled *PmegNEP2* (450-bp DNA PCR product amplified with 11F/16R). For Northern blot analysis, 10 μg of total mycelial RNA was denatured at 50 ° for 30 min in an equal volume of NorthernMax-Gly™ loading dye (Ambion, Austin, TX), and was separated by electrophoresis in a 1.3 % agarose gel and hybridized with the ^{32}P-labeled *PmegNEP2*. Northern blot analyses were repeated two times on separate samples to confirm the reproducibility of the results. Blots were washed and imaged at 200 μm resolution on a Typhoon 8600 Variable Mode Imager (Molecular Dynamics/Amersham-Pharmacia Biotech, Sunnyvale, CA). Probe hybridization and washing blots were performed under medium stringency condition according to Keates *et al.* (2003). Three other *Phytophthora* species pathogenic on cacao (Edna Luz, CEPLAC, Itabuna, Brazil) were used for Southern blot analyses: *P. ctirophthora 62E*, *P. capsici* 197E and *P. palmivora* 252E.

Data analyses

DNA and protein sequence analyses were performed with Biology WorkBench (http://workbench.sdsc.edu/). Genome database searches of *Phytophthora sojae* and *P. ramorum* were performed with *Phytophthora* Molecular Genetics Research Collaboration Network (http://pmgn.vbi.vt.edu/). The sequences were examined for signal peptides using SignalP v. 3.0 (http://www.cbs.dtu.dk/services/SignalP/). The degree of glycosylation of PmegNep clones was assessed using motif search programs NetOGlyc 3.1 Server (http://www.cbs.dtu.dk/services/NetOGlyc/). A phylogenetic tree was constructed from amino acid sequences, using neighbor-joining method of ClustalW with the number of differences model as implemented in Mega2 (http://www.megasoftware.net/).

RESULTS

Multiple copies of NEP1 orthologs detected

The universal primer set (11F and 16R) was used for PCR and the PCR products were cloned into

Table 2. Percent identity between amino acid sequences of PmegNep clones. Partial amino acid sequences of 11F-16R PCR products were used to compare PmegNep clones. For PmegNep6-2 and PmegNep7, partial sequences were used to compare with other PmegNep clones due to lack of sequence information.

PmegNep clones	PmegNep clones							
	Nep2 (193 aa)[a] 133 aa[b]	Nep3 (132 aa) 132 aa	Nep4 (174 aa) 131 aa	Nep4-2 (235 aa) 131 aa	Nep5 (175 aa) 132 aa	Nep6 (233 aa) 131 aa	Nep6-2 (138 aa) 79 aa	Nep7 (82 aa) 52 aa
Nep1[c]	71	62	65	64	50	66	68	57
Nep2		59	64	64	42	64	67	46
Nep3			52	53	64	52	56	63
Nep4				96	42	81	83	46
Nep4-2					43	79	82	46
Nep5						43	51	57
Nep6							96	46
Nep6-2								44

[a] number of total amino acids detected. PmegNep4-2 and PmegNep6 have putative full length of ORF.
[b] number of amino acid used for alignment.
[c] number of total amino acids detected = 175 aa, number of amino acid used for alignment = 132 aa.

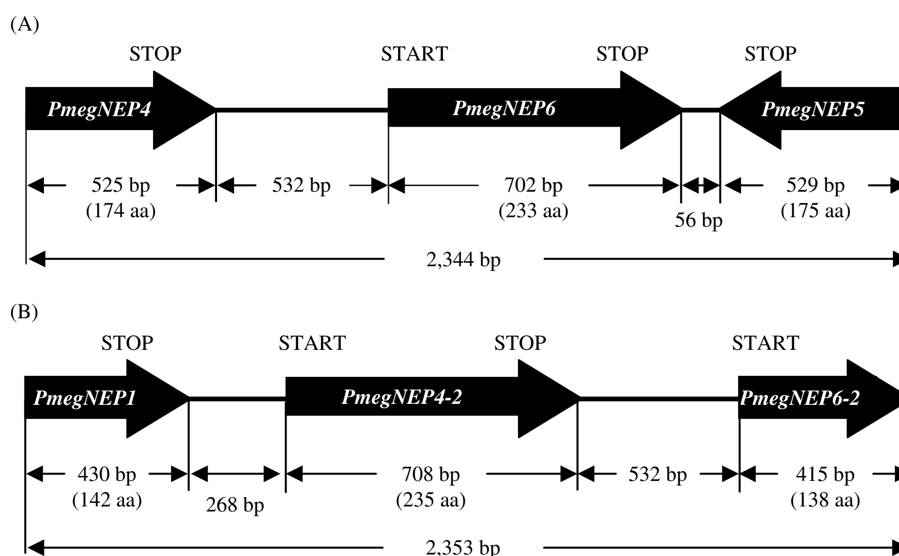

Fig. 1. Schematic diagrams of the two clusters of *PmegNEP* clones amplified from DNA using PCR with the 11F primer (A), and *PmegNEP1* Forward and *PmegNEP6* Reverse primers (B). Each cluster contained two partial *NEP1* orthologs and one full length *NEP1* ortholog. The size of the PCR product does not include a 23-bp primer sequence on both ends.

pCR2.1-TOPO cloning vector for sequencing. According to sequence results, three different PCR products were identified from two different PCR templates (DNA and RNA from mycelia). The size of PCR fragments were 400, 400 and 397 bp without primer sequences. The three partial *NEP1* orthologs were named as *P. megakarya* necrosis and ethylene inducing proteins, PmegNep1 (AY741082), PmegNep2 (AY741083), and PmegNep3 (AY741084), respectively (Table 2). The deduced amino acid sequences of *PmegNEP1*, *2* and *3* shared identities between 53 and 84% with database sequences of three Nep1-like proteins (NLPs) in *Phytophthora* species (*P. sojae*, AAK01636; *P. infestans*, AAK25828; *P. parasitica*, AAK19753).

The same primer set (11F and 16R) was used to amplify additional *NEP1* orthologs in the genome of *P. megakarya* with longer extension time (10 min instead of 1 min at 72 °). More than one DNA PCR products were detected on the gel. A 2390-bp (2344 bp without primer sequences) PCR fragment was cut out of the gel and was cloned into pCR-XL-TOPO vector for sequencing. Sequencing results revealed that the 2390-bp PCR fragment was only amplified by the 11F primer, and contained an additional three *NEP1* orthologs (Fig. 1A). The fragment contained two partial copies and one full length copy of *NEP1* orthologs, and the three orthologs were named as *PmegNEP4* (AY741085), *PmegNEP6* (AY741088) and *PmegNEP5* (AY741087), respectively. Because one *NEP1* ortholog (*PmegNEP5*) was inverted, one primer could anneal to 5′ and 3′ regions resulting in the amplification of 2390-bp PCR fragment. To verify the existence of the 2390-bp PCR product in the genome of *P. megakarya*, ortholog specific primer set for *PmegNEP1* Forward and *PmegNEP6* Reverse were designed (Table 1) and

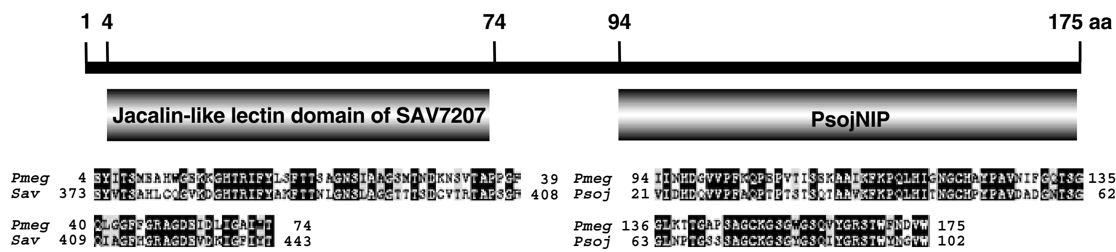

Fig. 2. Schematic diagram of the fused genes, *SAV7207/PmegNEP7*, identified using 5′ RACE. Primers were designed based on the 3 *PmegNEP* consensus sequences (1, 2 and 3) in the middle of the *PmegNEP* sequences. During the process of 5′ RACE, a ninth clone of a *NEP1* ortholog (*PmegNEP7*, Accession No. AY741090) was identified. The 525-bp 5′ RACE product predicted to encode 175 amino acids and consisted of two partial genes that were fused together. The N-terminal region of the 5′ RACE product matched with the C-terminal region of the unknown functional protein of SAV7207 of *Streptomyces avermitilis* MA-4680 (NP828383). The sequence of the 5′ RACE product ranging from amino acids 4 to 74 corresponded to the Jacalin-like lectin domain of SAV7207, and the sequence between amino acids 94 to 175 matched the partial sequences of the necrosis-inducing-like protein of *P. sojae* (AAM48171).

PCR was performed with mycelial DNA. The PCR products around 2.4 kb were cloned into pCR-XL-TOPO and more than one colonies were picked for sequencing. The existence of the 2390-bp PCR product was confirmed by sequence result. In addition, a 2353-bp PCR product (without primer sequences) that contained new *NEP1* orthologs was detected. The 2353-bp PCR fragment contained three *NEP1* orthologs (Fig. 1B). The fragment contained two partial and one full length copies of *NEP1* orthologs. The first partial copy was the same as the previously detected *PmegNEP1*. However, the full length and the other partial length copies were not the same as previously detected copies. The two copies had 96 % identity with the amino acid sequences of PmegNep4 and PmegNep6, and were named as PmegNep4-2 (AY741086) and PmegNep6-2 (AY741089), respectively.

For 5′ RACE, primers were designed based on the three *PmegNEP* consensus sequences (1, 2 and 3) in the middle of the *PmegNEP* sequences. During the process of 5′ RACE, a ninth clone of a *NEP1* ortholog (*PmegNEP7*, Accession No. AY741090) was identified. According to a blast search, the 525-bp 5′ RACE product consisted of two partial genes that were fused together. The N-terminal region had a portion that contained an area of unknown function, and the remaining regions of the amino acid sequences matched with the partial sequence of PsojNIP (*NEP1* ortholog in *P. sojae*) (Fig. 2). The N-terminal region of the 5′ RACE product (AY741091) had a 57 % identity (*E*-value 9e-15) with the C-terminal region of the SAV7207 from *Streptomyces avermitilis* MA-4680 (445 amino acids, NP 828383). SAV7207 has two conserved domains according to NCBI Conserved Domain Search (http://www3.ncbi.nlm.nih.gov/BLAST/), Endonuclease/ exonuclease/phosphatase domain (amino acid sequence between 33 and 297 in SAV7207) and Jacalin-like lectin domain (amino acid sequence between 373 and 443 in SAV7207). The amino acid sequences of 4–74 in the 5′ RACE product matched with the sequences of 373–443 in SAV7207. A blast search of the upper region of the 5′ RACE product (the amino acid sequences corresponding to the Jacalin-like lectin domain in SAV7207), when compared to *P. sojae* and *P. ramorum* genome database revealed that there were similar sequences in both genomes, however, the fused gene was not present. The amino acid sequences 94–175 in 5′ RACE product had a 69 % identity (*E*-value 4e-28) and matched with the sequences that ranged from 21 to 102 of the necrosis-inducing-like protein of *P. sojae*, PsojNIP (236 amino acids, Accession No. AAM48171).

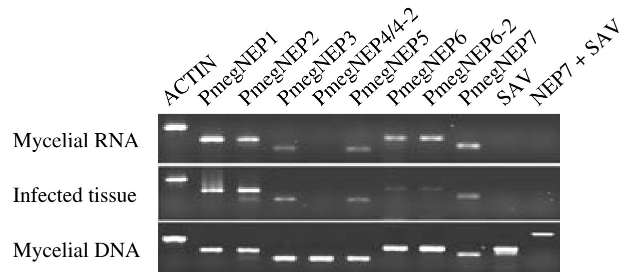

Fig. 3. DNA- and RT-PCR analyses of *Phytophthora megakarya* were performed using *PmegNEP* ortholog-specific primers. RNA was isolated from *P. megakarya* zoospore-infected cacao leaf disks (3 d after inoculation) and from mycelia grown in liquid culture. The *ACTIN* gene was used as a control.

An alignment of the deduced amino acid sequences of the partial *NEP1* orthologs (region corresponding to the primer set of 11F and 16R) revealed that the nine *PmegNEP* clones were different from each other (Table 2). The deduced amino acid sequences of nine clones shared identities between 42 and 96 % with each other. The existence of the nine clones of *NEP1* orthologs in the *P. megakarya* genome was confirmed by conducting PCR using *P. megakarya* mycelial DNA as a template, and the *ACTIN* gene as a control (Fig. 3). PCR with *P. megakarya* DNA amplified the expected size band for each *PmegNEP* clone, confirming the existence of nine clones in the *P. megakarya* genome including the fused gene *PmegNEP7*. PCR using DNA from other *Phytophthora* species showed different band

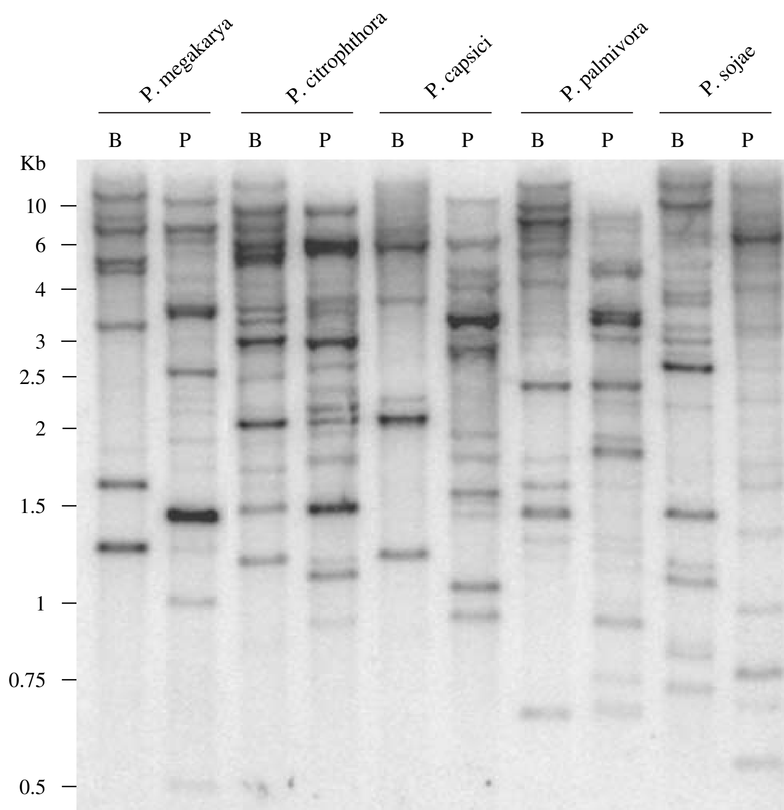

Fig. 4. Southern blot analysis of *NEP1* orthologs in five *Phytophthora* species. Two and half μg of DNA was digested with restriction enzyme *Bgl*II (B) and *Pst*I (P), then separated in a 0.8 % agrose gel, and blotted onto a nylon membrane. The blot was hybridized with a 450-bp *PmegNEP2* probe and washed under medium stringency conditions.

patterns or was unable to amplify any fragment (data not shown). To test whether each *PmegNEP* clone was transcriptionally active, RT-PCR was performed (Fig. 3). Total RNA from *P. megakarya* zoospore-infected cacao leaf tissue and *P. megakarya* mycelia were used as templates for RT-PCR. The common primer set for *PmegNEP4* and *PmegNEP4-2* was used, while clone specific primer sets were used to amplify *PmegNEP6*, *PmegNEP6-2*, and the remaining orthologs (Table 1). Six *PmegNEP* clones (*1*, *2*, *3*, *5*, *6*, *6-2*) were transcriptionally active in leaf during infection (3 days after inoculation) and in mycelial tissue. RT-PCR products of *PmegNEP1*, *2*, *6* and *6-2* had a greater intensity than the intensity of *PmegNEP3* and *5* in mycelial tissue. On the other hand, in *P. megakarya* zoospore-infected tissue, the intensity of *PmegNEP1*, *2*, *6* and *6-2* was higher than any other *PmegNEP* clones. For *PmegNEP7*, we tried three different primer sets that covered: (1) the two fused genes (forward primer 5′-AAGTACCAAGAGTACATTAC-3′; reverse primer 5′-CCAGACGTCATTGAACCAAG-3′); (2) the Jacalin-like lectin domain of SAV7207 (forward primer 5′-GTACATTACGTCGATGGAAG-3′; reverse primer 5′-CAAATCGCACCGATGAGGTC-3′); and (3) the PsojNIP domain (PmegNep7, primers sequences in Table 1). When the primer set that covered the entire region of two fused genes (SAV7207+PsojNIP) was used, transcript was not detected by RT-PCR. However, the transcript for *PmegNEP7* was detected when the primer set that covered only the NLP domain was used. The transcript of the Jacalin-like domain of SAV7207 was not detected by RT-PCR. The PCR fragments from DNA and RT-PCR were the same size indicating there were no introns in *PmegNEP* orthologs.

In order to determine the genomic characteristics of the *NEP1* orthologs, Southern blot analysis was conducted. The genomic DNA of five *Phytophthora* species was digested with two restriction enzymes, *Bgl*II and *Pst*I, and was probed with *PmegNEP2*. Multiple bands with different patterns were detected in all species tested, confirming that there were multiple copies of *NEP1* orthologs (Fig. 4). Band intensities varied which may be due to the length of the hybridized region, the degree of homology to the probe, or to the presence of more than one band.

Phytophthora megakarya *Nep1 clones share high homology with NLPs of other organisms*

Recently, a number of NLPs have appeared in public databases, including representatives from various oomycete and bacterial species. A phylogenetic tree and the deduced amino acid sequence alignment of seven *PmegNEP* clones containing the region that corresponded with the primer set 11F and 16R, and 14

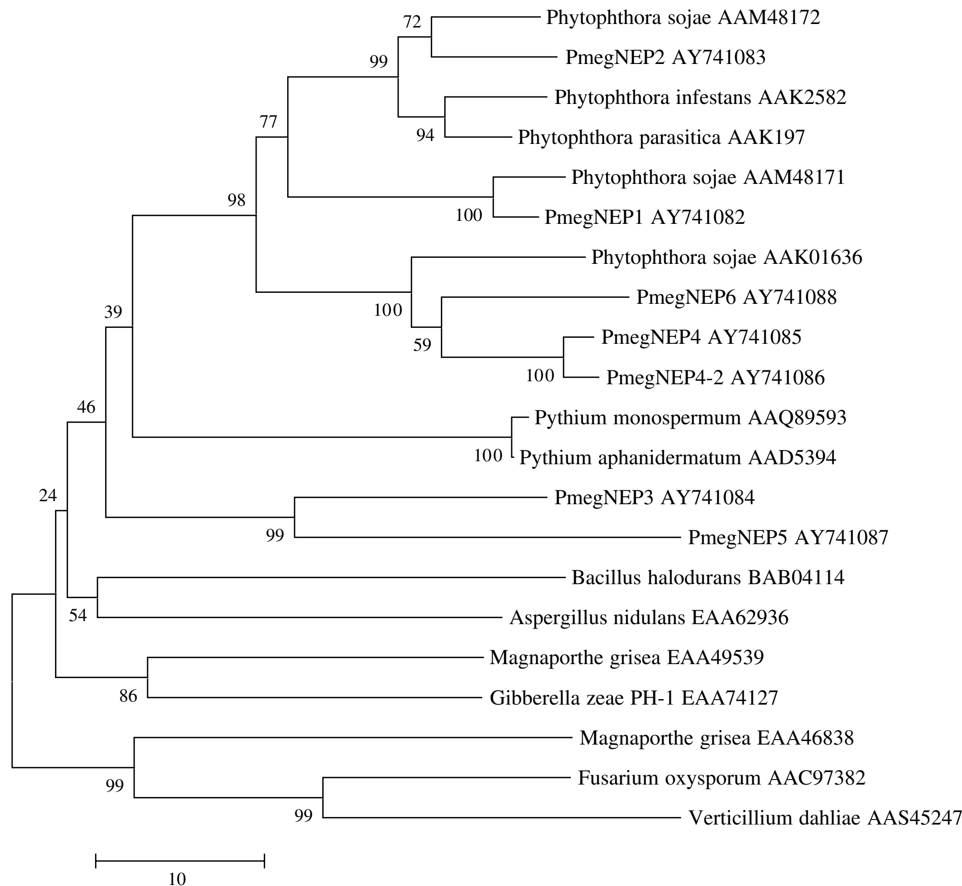

Fig. 5. Phylogenetic analysis of the partial sequences of Nep1 orthologs from oomycetes and other organisms. The 11F and 16R region of Nep1 orthologs was used to create a phylogenetic tree. The tree was constructed from amino acid sequences by the neighbor-joining method of ClustalW and Mega2 (http://www.megasoftware.net/). Numbers at the nodes are boostrap values from 100 replications and represent weighted amino acid substitutions, with the scale bar represents 10% weighted sequence divergence. GenBank accession numbers are shown to the right of the organism.

other NLPs were generated (Figs 5–6). The alignment of the partial deduced amino acid sequences of *PmegNEP* clones revealed that seven *PmegNEP* clones and 14 NLPs of the *Phytophthora* species clustered together (Fig. 5). The percentage identities between the deduced amino acid sequence of *PmegNEP* and other NLPs ranged from 24–93%. The highest matches existed with NLPs of *Phytophthora*, 93% between PmegNep1 and *P. sojae* (AAM48171, necrosis-inducing-like protein), 87% between PmegNep2 and *P. sojae* (AAM48172), 84% between PmegNep2 and *P. parasitica* (AAK19753), and 82% between PmegNep2 and *P. infestans* (AAK25828, necrosis-inducing protein). The identities between *Fusarium oxysporum* (AAC97382) and the deduced amino acid sequences of *PmegNEP* clones were relatively low (34 to 39%).

The deduced sequence encoding the full length of PmegNep4-2 and PmegNep6 precursor proteins consisted of 235 and 233 amino acids, respectively. Algorithms for the prediction of secondary structures did not reveal any characteristic domains within the two deduced amino acid sequences of *PmegNEP* clones (data not shown). Two cysteine residues were present in all of the deduced amino acid sequences of *PmegNEP* orthologs (C55 and C81, C56 and C82, C54 and C80, and C54 and C80 of the pre-protein in PmegNep2, PmegNep4-2, PmegNep6, and PmegNep6-2, respectively); although PmegNep4, PmegNep4-2, PmegNep6, and PmegNep6-2 were distinguished by the existence of the third and/or fourth cysteine residues (e.g. cysteine 126 of PmegNep6) of the pre-protein (Fig. 6). The third cysteine was also detected in PsojNIP at position 128 (AAK01636; Qutob *et al.* 2002). The GC contents of PmegNep4-2 and PmegNep6 were 54.7% and 50.9%, respectively, which were lower than other *Phytophthora* NLPs, such as 56.9% for *P. infestans*, 59.3% for *P. sojae*, and 56.6% for *P. parasitica*.

Using SignalP v.3.0, signal peptide sequences were analyzed for PmegNep2, 4-2, 6, 6-2 and other NLPs of *Phytophthora* species (Bendtsen *et al.* 2004) (Table 3). Nineteen to 17 amino acid sequences were predicted as signal peptides for the PmegNep clones, while nineteen signal peptides were predicted for other *Phytophthora* NLPs.

Transposable-like sequences are abundant in the *Phytophthora* genome. A *copia*-like retrotransposable element was amplified during the PCR process with *PmegNEP3* Forward and *PmegNEP5* Reverse primers,

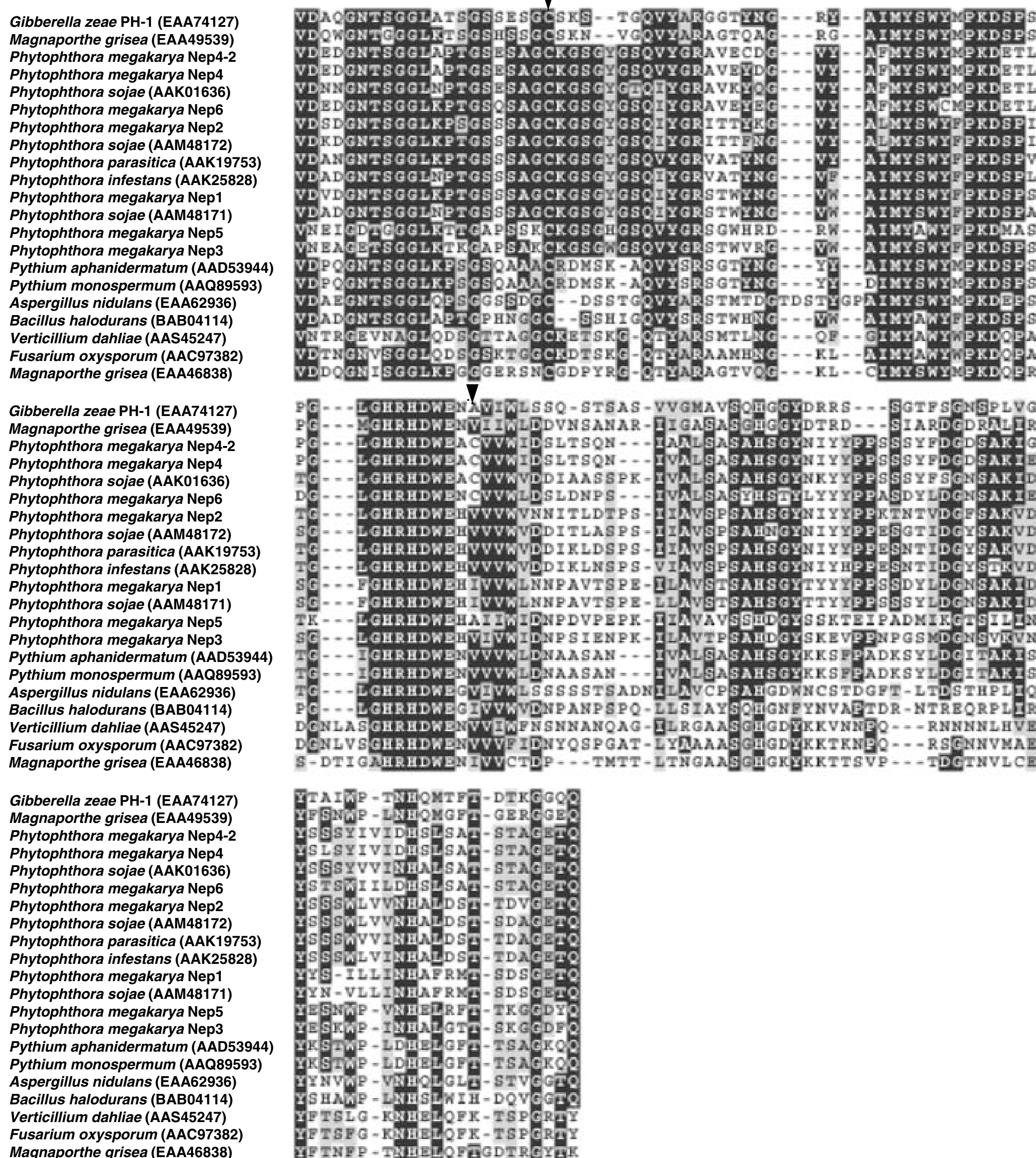

Fig. 6. Alignment of the partial amino acid sequences of Nep1 orthologs from oomycetes and other organisms. Alignment of 11F and 16R region of Nep1 orthologs was performed using ClustalW and BOXSHADE sequence alignment program (http://seqtool.sdsc.edu /CGI/BW.cgi#!). Identical amino acids are highlighted with black. Dashes indicate gaps that were introduced to optimize the alignment. The arrow head indicates the conserved cysteine residue (C80) and a third cysteine residue (C128) of PmegNep4-2. GenBank accession numbers are shown in parenthesis to the right of the organism.

yielding a product size of 1,283 bp (Accession No. AY741092). However, no evidence of recombination of *PmegNEP* sequence with the transposable element was found. In other words, the sequence of the *NEP1* ortholog was not found in the PCR fragment.

Multiple Nep1 orthologs in Phytophthora sojae *and* P ramorum

Recently, full genome sequence information has been made available for *Phytophthora sojae* and *P. ramorum.* Searches of the genome databases of *P. sojae* and *P. ramorum* for 'necrosis-inducing protein' detected many putative NLPs (*Phytophthora* Molecular Genetics Research Collaboration Network, http://pmgn.vbi.vt.edu/). In *P. sojae*, out of 54 putative NLPs, 19 open reading frames had an identity greater than 50% in comparison with 3 previously reported PsojNIPs (AAK01636, AAM48171 and AAM48172) or PmegNep6 amino acid sequences using Biology WorkBench (http://workbench.sdsc.edu/). Ten out of the 19 sequences were found clustered together with the

Table 3. Predicted signal peptide sequences of necrosis and ethylene inducing proteins from *Phytophthora* species.

GenBank accession no.	Species	Signal sequence[a]	Predicted glycosylated residue[b]
AY 741083 (PmegNep2)	*P. megakarya*	**M**NLLVFLVAALSFAVTQA↓SV	33, 35, 36, 37, 67
AY741086 (PmegNep4-2)	*P. megakarya*	**M**NLRVIIFAGFAYLSGVQA↓AV	34, 75
AY741088 (PmegNep6)	*P. megakarya*	**M**NLRAFVAAIAFSGVQA↓AV	32, 73
AY741089 (PmegNep6-2)	*P. megakarya*	**M**HLRASVAAIAFSGVQA↓AV	32, 35, 73
AAK25828	*P. infestans*	**M**NILQLFASAAAALTVAHA↓DV	34, 35, 36, 37, 38, 39, 76
AAK19753	*P. parasitica*	**M**NVLTFLIAAAVSLAVVQA↓DV	34, 36, 37, 38, 75
AAM48171	*P. sojae* (NIP-like)	**M**NLRRLVVAAVAFLTASHA↓SV	34, 36, 38, 41, 68, 75, 147, 154, 155
AAM48172	*P. sojae*	**M**NLRAFFTAAAASLALTQA↓DV	56, 63
AAM48170	*P. sojae* (NIP)	**M**NLRPALLATLASFAYVSA↓SV	None

[a] Inititator methionine is in boldface type. The small uncharged residues, defining the signal peptidase cleavage site at the -1, -3 positions are under lined. The predicted cleavage site is indicated by an arrow.

[b] A match to an extracellular peptide was assumed when the scores for the SignalP NN Mean S score and the SignalP HMM Output was greater than 0.500 and 0.700, respectively.

known PsojNIPs in the phylogenetic tree, and were selected for alignment with the known PsojNIPs and PmegNep6 (Fig. 7). In a search of the *P. ramorum* genome, 61 putative NLPs matched with a necrosis-inducing protein. 30 of the 61 putative necrosis-inducing proteins shared over a 50% identity with 3 previously reported PsojNIP (AAK01636, AAM48171 and AAM48172) or PmegNep6 amino acid sequences. Nine putative necrosis-inducing proteins clustered together with the known PsojNIPs in the phylogentic tree, and were selected for further alignment (Fig. 7). The highest identities were 85% (*P. sojae* 18) and 88% (*P. ramorum* 38) between the PmegNep6 and the putative NLPs from *P. sojae* or *P. ramorum*, respectively.

DISCUSSION

NEP1 orthologs constitute a multigene family

Analysis of the *Phytophthora megakarya* genome using Southern blot and PCR analyses showed that there are multiple *NEP1* orthologs. *PmegNEP2* probe was cross-hybridized with multiple DNA fragments under medium stringency condition on Southern blots. The resulting DNA hybridization patterns showed that polymorphisms are present in five species of *Phytophthora*. That is, the copy number and gross structural features of the *NEP1* multigene family are not conserved among *Phytophthora* species. In previous studies, one major band was detected in Southern blot analyses in *P. parasitica* and *P. sojae* under high stringency condition (Fellbrich *et al.* 2002, Qutob *et al.* 2002). However, multiple weakly hybridizing bands were detected when genomic DNA was probed under medium stringency (data not shown, Qutob *et al.* 2002). Fellbrich *et al.* (2002) were able to detect up to four bands in five *Phytophthora* species (*P. parasitica*, *P. infestans*, *P. palmivora*, *P. cactorum*, and *P. nicotianae*) as well as *Pythium vexans* under medium stringency condition. In this study, a complex gene family of *NEP1* orthologs was detected in *P. megakarya* and *P. sojae* as well as three other species studied using Southern blot analysis. In addition to Southern blot analysis, genome database searches of the *P. sojae* and the *P. ramorum* also confirmed the existence of a *NEP1* multigene family. While the identities between PmegNep6 and the three PsojNIPs were around 70%, two of the putative PsojNIPs (*P. sojae* 14 and 15 in Fig. 7) showed identities over 80% with PmegNep6. In addition, there are three putative NLPs that have identities over 80% with PmegNep6 in the genome *P. ramorum*. These results strongly suggest that there are additional PsojNIPs and that there are multiple copies of NLPs in the genome of other *Phytophthora* species.

Multiple PmegNEPs expressed during the infection of cacao leaves and mycelia growth

Nine *PmegNEP* orthologs were verified in the genome of *Phytophthora megakarya* when mycelial DNA was used as a PCR template. The accumulation of *PmegNEP* transcripts during the infection of cacao leaf disks and during mycelial growth was investigated by Northern blot analysis, however, no transcript was detected (data not shown). The greater sensitivity of RT-PCR analysis allowed for the detection of *PmegNEP* transcripts both in inoculated cacao leaf tissue and in mycelia. Expression of only six of nine orthologs was detected in both tissues. The remaining three clones (*PmegNEP4*, *PmegNEP4-2*, and *PmegNEP7*) were not detected by RT-PCR, which may be due to differences in regulation or possibly that they are non-functional. The intensity of the RT-PCR products of *PmegNEP1* and *2* was higher than *PmegNEP3*, *5*, *6*, *6-2* and *7* in the infected tissue; while the intensity of *PmegNEP1*, *2*, *6*, *6-2* was higher than *PmegNEP3*, *5* and *7* in the mycelia. The intensity of the RT-PCR product of *PmegNEP6* and *6-2* were distinctly higher in the mycelia compared to the infected tissue, which may be due to differences in transcriptional activity between the tissues. The transcript that is similar to *PmegNEP7* was detected only when the primer set that covered PsojNIP domain was used, which indicates that an ortholog similar to *PmegNEP7* exists in another location of the *P. megakarya* genome and is transcriptionally

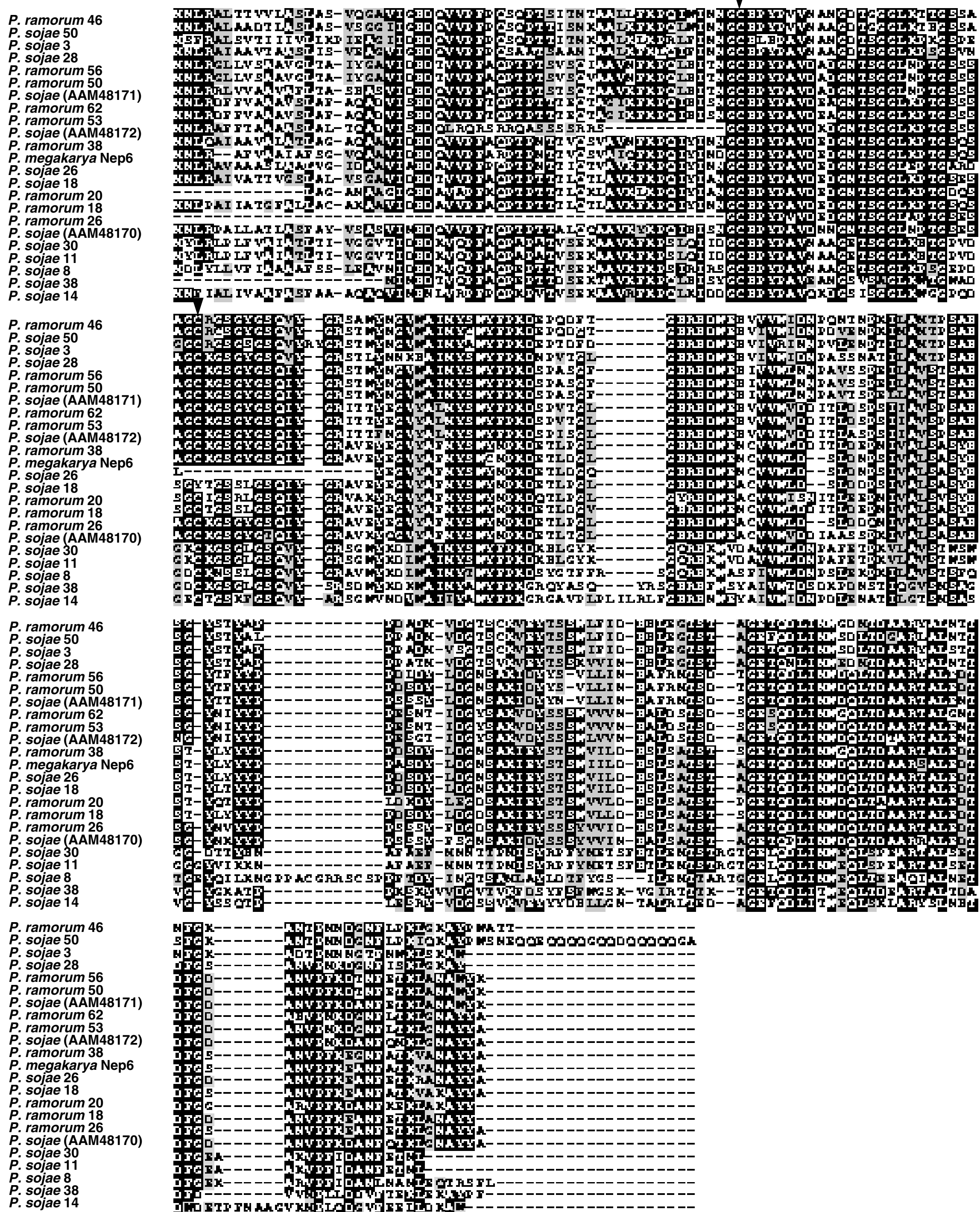

Fig. 7. Alignment of putative NLPs from *Phytophthora sojae* and *P. ramorum* with three of *P. sojae* PsojNIPs and PmegNep6. Alignment of the amino acid sequences was performed using ClustalW and BOXSHADE sequence alignment program (http://seqtool.sdsc.edu/CGI/BW.cgi#!). Identical amino acids are highlighted with black. Dashes indicate gaps that were introduced to optimize the alignment. The arrow head indicates the conserved cysteine residue (C56) and a second cysteine residue (C82) of PsojNIP (AAM48171). GenBank accession numbers for PsojNIP are in parenthesis. The putative Nep1 orthologs derived from *Phytophthora* Molecular Genetics Research Collaboration Network (http://pmgn.vbi.vt.edu/) were numbered randomly for *P. sojae* and *P. ramorum*.

active. The same possibility can also apply to the other PCR products in Fig. 3. That is, even the expected sizes of the PCR products were detected, the products could be new orthologs that have homologous sequences to the primers used. Qutob *et al.* (2002) detected the *PsojNIP* transcripts during the transition from biotrophy to necrotrophy, that is, the transcript was not detected during the early stage of infection. These results suggest differential induction of *NEP1* orthologs in *Phytophthora* species.

PmegNEP orthologs show characteristics of the genome structure of **Phytophthora** *spp.*

Some characteristics of the *Phytophthora* genome organization were revealed under further analysis of the *P. megakarya* genome for *PmegNEP* orthologs. Two cassettes were found that contained at least three *PmegNEP* orthologs each. A characteristic of the *Phytophthora* genome is a short distance between ORF's (Kamoun 2003). For example, the distance between *PmegNEP4* and *PmegNEP6* (also between *PmegNEP4-2* and *PmegNEP6-2*) was 532 bp, and the distance between *PmegNEP1* and *PmegNEP4-2* was 268 bp. The distance between the two stop codons of *PmegNEP6* and *PmegNEP5* was only 56 bp. Similar observations were made in *P. sojae*. In *P. sojae* the average distance between ORFs was 497 bp (range 102 to 714 bp) in the region of seven genes including three genes encoding PsojNIP and PsojNIP-like proteins clustered in a 10835-bp *Bam*HI fragment (Qutob *et al.* 2002). Analysis of three promoter regions showed that there were no motifs corresponding to CAAT and TATA boxes in *P. megakarya*, which is a typical phenomenon in the *Phytophthora* genome (Judelson & Michelmore 1991, Judelson, Tyler & Michelmore 1992, Kamoun 2003). The clustering of genes and the short distance between ORFs also matches the characteristics of the highly compact transcripts with short untranslated regions found in *Phytophthora* spp. (Pieterse *et al.* 1994, Pesole *et al.* 2000, Kamoun 2003). Introns were not found in any of the *PmegNEP* orthologs, which is also a common characteristic of the genome of other *Phytophthora* spp. (Kamoun 2003).

The existence of genes in small clusters has been reported previously in other *Phytophthora* species. For examples, two clusters of *in planta-induced* (*ipi*) genes were detected in *P. infestans* (Pieterse *et al.* 1994). Three similar *ipiB* genes were arranged start-to-stop codons with short intergenic sequences (820 bp) and two *ipiO* genes were arranged start-to-start codons with a 2200-bp intergenic sequence. In *P. cinnamomi*, there are at least six polygalacturonase genes in several clusters; in addition, at least three pyruvate phosphate dikinase genes were arranged in a small cluster (Marshall *et al.* 2001).

PmegNep clones share sequence characteristics with other Nep1-like proteins (*NLPs*)

The structural features of the PmegNep clones include a hydrophobic signal peptide at the N-terminus. The signal peptides for PmegNeps had 17–19 amino acid sequences, while 19 amino acid signal peptides were predicted for other *Phytophthora* NLPs. A conserved amino acid sequence could not be detected for the signal peptides. Unlike typical elicitins, PmegNep proteins are predicted to have a glycosylated region, suggesting localization to the cell wall or membranes. According to the result obtained from transmission electron microscopy, Nep1 treatment induces rapid breakdown of thylakoid membrane structures. This result might indicate that the Nep1 protein is localized to membrane structures. However, no glycosylated residue was found in the active PsojNIP of *P. sojae* (AAK01636, Qutob *et al.* 2002). This result indicates that glycosylation by *Phytophthora* spp. is not important in the ability of NLPs to induce necrosis.

Two full length *PmegNEP* orthologs were cloned: *PmegNEP4-2* and *PmegNEP6* predicted to yield proteins with 235 and 233 amino acids, respectively. Cysteine residues have important roles in folding, structure, and function of proteins (Boissy *et al.* 1999, Qutob *et al.* 2002). When cysteine residues are exposed to air, the sulphydryl (SH) groups oxidize and are joined by a disulphide bond. Deletion mutation analysis in *P. parasitica* NPP1 indicated the elicitor activity was dependant upon the tertiary structural characteristics of the protein and the pattern of spacing of the conserved cysteine residue (Fellbrich *et al.* 2002). The importance of cysteine residues has also been reported for other secreted elicitors (Templeton, Rikkerink & Beever 1994). In this study, it was determined that the second cysteine residue (C82 and C80 in PmegNep4-2 and PmegNep6, respectively) was conserved in the predicted amino acid sequences of all nine *PmegNEP* orthologs. Only partial sequences (corresponding to 11F and 16R) were used for alignment due to the lack of sequence information regarding the sequences in some of *PmegNEP* orthologs. Additional information about the N-terminal region for PmegNep2 and the C-terminal region for PmegNep1 was gained through 5′ and 3′ RACE. The first cysteine residue (C55, 56, 54, and 54 in PmegNep2, PmegNep4-2, PmegNep6 and PmegNep6-2, respectively) was also conserved in all the predicted amino acid sequences of *PmegNEP* orthologs when sequence data was available (Fig. 7). Most notably, there are 25 amino acids between the first and second cysteines in most NLPs except in *Neurospora crassa* (26 amino acids, EAA30528) and *Magnaporthe grisea* (63 amino acids, EAA54347). This indicates that spacing between the two cysteines may be important for activity. The importance of the spacing between cysteines was also observed in the deletion mutation of *P. parasitica* NPP1 (Fellbrich *et al.* 2002) and in other secreted elicitors (Templeton *et al.* 1994). Interestingly, additional cysteine residues were predicted in some of the *PmegNEP* orthologs, for example, the third or/and fourth cysteine residues in PmegNep4, C98 and C128 in PmegNep4-2, C107 and C126 in PmegNep6, and C126 in PmegNep6-2. Further analysis is required to examine the importance of these additional cysteine residues.

Genetic diversity and genome stability

Of the multiple copies of *PmegNEP*, nine clones that were analyzed in this study had unique ORFs, encoding

different forms of PmegNep. Comparisons of the two cassettes (Fig. 1) that contain *PmegNEP* orthologs suggest that duplication and mutation has occurred in the *P. megakarya* genome. Based on comparisons within the cassettes (Fig. 1), polymorphisms in the *PmegNEP* ORFs indicate that the amplification of some *PmegNEP* orthologs might be a remote event so that there has been sufficient time for genetic variation to accumulate. Alternatively, there is mechanism for rapid genetic variation in *Phytophthora* species, such as transposable elements, gene conversion, mitotic recombination, and/or dispensable chromosomes (Martin 1995, Goodwin 1997, Chamnanpunt *et al.* 2001, Judelson 2002). The overall identities among *PmegNEP* orthologs ranged from 42–83%. In contrast, the second cassette (Fig. 1B) contained three putative *PmegNEP* orthologs and two out of three were only slightly different from previously found *PmegNEP* orthologs. That is, while *PmegNEP1* was identical with a previously detected sequence, *PmegNEP4-2* and *PmegNEP6-2*, although different, were closely related (96% identity) to the previously detected clones *PmegNEP4* and *PmegNEP6*, respectively. In addition, while the length of the intergenic sequences between *PmegNEP4* (*PmegNEP4-2*) and *PmegNEP6* (*PmegNEP6-2*) were the same (532 bp) they had slightly different sequences (14 bp out of 532 bp were different). Based on the sequence comparison between the two cassettes, the duplication of *PmegNEP* from one cassette to the other cassette is a relatively recent event, resulting in the generation of *PmegNEP* orthologs with high homology. In addition, the existence of the DNA ortholog *PmegNEP7* that contains two genes fused together suggests instability in the genome of *P. megakarya*.

The survival of an organism depends on the ability to adapt to various environments and new or modified gene functions are a means of generating such adaptability. Duplication and divergence is the classical model of the generation of these novel genes (Hooper & Berg 2003). The dosage effect might be advantageous primarily for weakly expressed genes (Hooper & Berg 2003). Rapid generation of genetic variation has been reported in *Phytophthora* species grown in culture (Fry 1982) and under field conditions (Chamnanpunt, Shan & Tyler 2001, Judelson 2002). The mechanisms of generating such variation are not clear, but possible sources may be transposable elements, gene conversion, mitotic recombination, and/or dispensable chromosomes (Martin 1995, Goodwin 1997, Chamnanpunt *et al.* 2001, Judelson 2002). Gao *et al.* (2002) postulated that the copy number diversity of the Telomere-Linked Helicase (*TLH*) gene family in *Magnaporthe grisea* isolates could be explained by unequal crossing over between repetitive elements associated with the *TLH* gene family. Repetitive sequences are abundant in the genomes of *Phytophthora* species. For example, five families of tandemly repeated sequences were found in *P. sojae* (Mao & Tyler 1996) and, 33 distinct families of repeated DNA have been detected in *P. infestans* (Judelson & Randall 1998). The repeated sequences cover at least 50% of the genome and copy numbers ranged from 70–8400 per haploid genome. A role for transposable elements in gene amplification has been reported in *Pyrenophora tritici-repentis*, the causal agent of tan spot in wheat, which produces a host-selective toxin, ToxB (Gao *et al.* 2002, Martinez, Oesch & Ciuffetti 2004). It was found that ToxB loci were associated with truncated retrotransposon-like sequences. The association of a retrotransposon-like sequence with ToxB provided for the opportunity of unequal crossing over with the same or similar sequences. Serendipitously, we amplified a *copia*-like retrotransposable element from *P. megakarya* DNA using the specific primer set of *PmegNEP6* Forward/ *PmegNEP3* Reverse primers. The DNA PCR fragment was 1283-bp long and had a moderate homology with the *copia*-like retrotransposable element of *Arabidopsis thaliana* (41% identity with *E*-value 3e-46, BAB01972). However, no trace of *PmegNEP* sequence could be found in the PCR fragment.

Based on two characteristics, Qutob *et al.* (2002) suggested that PsojNIP is a virulence factor rather than an avirulence factor: (1) PsojNIP was active in a host cultivar that was susceptible to most *P. sojae* isolates; and (2) PsojNIP-like proteins are found in taxonomically different organisms with different hosts. A similar mechanism to PsojNIP has been proposed for Nep1 from *Fusarium oxysporum* (Jennings *et al.* 2001), and for various non-proteinaceous toxins from *Alternaria alternata* f. sp. *lycopersici* (Wang, Bostock & Gilchrist 1996), *Cochliobolus victoriae* (Navarre & Wolpert 1999), and *Fusarium moniliforme* (Stone *et al.* 2000). In addition, the existence of a complex multigene family encoding NLPs may also support the hypothesis that NLPs are virulence factors.

The data presented indicates that the *NLP* gene family structure and its regulation are much more complex than previously thought. At least nine copies of *PmegNEP* orthologs were detected in *P. megakarya*. Multiple copies of *PmegNEP* were expressed during the infection of cacao leaves and mycelial growth, and were regulated differently. The *P. megakarya* genome is unstable, resulting in the generation of duplications, inversions, and fused genes. The multiple and ubiquitous existence of the NLP gene family in the *Phytophthora* species, as well as many other organisms suggests a significant role of the gene in pathogenicity of microbes. The existence of a gene family containing NLPs may be advantageous for a wide range of plant pathogens. Certainly, efforts aimed at understanding the function(s) of NPLs in microbial biology and plant microbe interactions, whether as avirulence or virulence factors, will be impacted by the complex nature of the NPL gene family and the potential for generation of genes with new functions due to the instability of the *Phytophthora* genome.

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