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Expression of a *Phytophthora sojae* necrosis-inducing protein occurs during transition from biotrophy to necrotrophy

Dinah Qutob[1,2], Sophien Kamoun[3] and Mark Gijzen[1,*]
[1]*Agriculture and Agri-Food Canada, 1391 Sandford Street, London, Ontario, Canada N5V 4T3,*
[2]*Department of Microbiology and Immunology, University of Western Ontario, London, Ontario, Canada N6A 5C1, and*
[3]*Department of Plant Pathology, The Ohio State University-OARDC, Wooster, OH 44691, USA*

Received 27 May 2002; revised 8 July 2002; accepted 16 July 2002.
*For correspondence (fax +1 519 457 3997; e-mail gijzenm@agr.gc.ca).

Summary

Phytophthora sojae **is an oomycete that causes stem and root rot on soybean plants. To discover pathogen factors that produce disease symptoms or activate plant defense responses, we identified putative secretory proteins from expressed sequence tags (ESTs) and tested selected candidates using a heterologous expression assay. From an analysis of 3035 ESTs originating from mycelium, zoospore, and infected soybean tissues, we identified 176 putative secreted proteins. A total of 16 different cDNAs predicted to encode secreted proteins ranging in size from 6 to 26 kDa were selected for expression analysis in** ***Nicotiana benthamiana*** **using an** ***Agrobacterium tumefaciens*** **binary potato virus X (PVX) vector. This resulted in the identification of a 25.6-kDa necrosis-inducing protein that is similar in sequence to other proteins from eukaryotic and prokaryotic species. The genomic region encoding the** ***P. sojae*** **necrosis-inducing protein was isolated and the expression pattern of the corresponding gene determined by RNA blot hybridization and by RT-PCR. The activity of this** ***P. sojae*** **protein was compared to proteins of similar sequence from** ***Fusarium oxysporum***, ***Bacillus halodurans***, **and** ***Streptomyces coelicolor*** **by PVX-based expression in** ***N. benthamiana*** **and by transient expression via particle bombardment in soybean tissues. The** ***P. sojae*** **protein was a powerful inducer of necrosis and cell death in both assays, whereas related proteins from other species varied in their activity. This study suggests that the** ***P. sojae*** **necrosis-inducing protein facilitates the colonization of host tissues during the necrotrophic phase of growth.**

Keywords: cell death, ***Glycine max*****, hemibiotroph, pathogen, toxin.**

Introduction

A common feature of most plant–pathogen interactions is the occurrence of host cell death. This response can be mediated as part of an active defense against the aggressor or deliberately instigated by the invader (Jackson and Taylor, 1996). During a compatible interaction, obligate biotrophs have evolved the means to evade recognition or to suppress plant defense responses while parasitizing living tissue (Greenberg, 1997). For these organisms, cell death becomes an unwanted consequence of the disease process that occurs late in infection. Conversely, elicitation of plant resistance during an incompatible interaction occurs with host recognition of pathogen-specific signal molecules that trigger a multifaceted defense response (Dangl *et al.*, 1996). Commonly associated with plant resistance is the induction of a hypersensitive response (HR), a rapid and localized cell death that restricts microbial growth to the site of ingress. Whereas biotrophic pathogens require living host cells to support their obligate parasitism, necrotrophic plant pathogens are facultative parasites that may actively kill their host during the disease process by secretion of toxins (Agrios, 1997). It has been proposed that necrotrophs may even manipulate plant cell death programs such as HR for their own advantage (Dickman *et al.*, 2001; Mayer *et al.*, 2001; Richael *et al.*, 2001). The host plant may also develop capabilities to recognize pathogen-secreted toxins early during infection, whereupon the molecules become liabilities rather than weapons (Rohe *et al.*, 1995).

Oomycete plant pathogens have a variety of parasitic lifestyles and include necrotrophic and biotrophic species, as well as intermediate types called hemibiotrophs (Drenth and Goodwin, 1999). Hemibiotrophs establish themselves in host cells by eluding detection and forming associations with living cells, much like biotrophs. Later during the infection process, these pathogens more closely resemble necrotrophs, since they may spread rapidly and actively kill host cells. Generally, necrotrophs have a wider host range compared to the more specialized hemibiotrophs and biotrophs (Erwin and Ribeiro, 1996). Within the oomycetes, biotrophy is considered a more highly evolved form of parasitism that arose from previous necrotrophic or hemibiotrophic modes. The obligate biotrophic *Peronospora* and *Bremia* genera are considered to be derived from *Phytophthora* lineages that lost the ability to produce zoospores (Cooke *et al*., 2000). The *Phytophthora* genus itself is monophyletic and comprises some 60 species of plant pathogens, including necrotrophs, hemibiotrophs, and biotrophs (Cooke *et al*., 2000; Erwin and Ribeiro, 1996). Thus, *Phytophthora* and related oomycetes are regarded as a group of very successful plant pathogens that exploit a spectrum of parasitic strategies.

Phytophthora sojae is an aggressive hemibiotroph that causes root and stem rot on soybean plants. Resistance or susceptibility of soybean to *P. sojae* is attributed to a complex exchange of signaling cues between the pathogen and the host that results in colonization and disease, or recognition of the invading pathogen by the host and abrogation of the infection process (Ward, 1990). In this system, the primary determinates of compatibility among different *P. sojae* races and soybean cultivars are pathogen avirulence (*Avr*) genes and host resistance (*Rps*) genes (Gijzen *et al*., 1996; Tyler *et al*., 1995; Whisson *et al*., 1995). Thus, a *P. sojae Avr* gene product directly or indirectly interacts with a corresponding soybean *Rps* gene product, resulting in rapid host activation of defense responses and plant resistance, in accord with contemporary interpretations of Flor's hypothesis (Flor, 1971; Staskawicz *et al*., 1995). In susceptible interactions, there is no longer recognition between host resistance factors and pathogen avirulence determinants, and host cell death is not a feature of the early infection process. Nonetheless, as the disease progresses, *P. sojae* causes severe lesions that lead to necrosis, browning, and death of host cells (Ward *et al*., 1979, 1989; Ward, 1990). Hence, the timing and control of host cell death is as important as the process itself in mediating the outcome of this parasitic interaction. It also follows that *P. sojae* will possess a number of factors that trigger cell death, ranging from *Avr* gene products that absolutely restrict the pathogen to toxins that aid its colonization of host tissues.

Most of the 13 or more *P. sojae Avr* genes have not been isolated, although recent work has shown that *Avr* loci may be successfully approached by positional cloning methods (Tyler, 2001). This has led to the isolation of the *Avr1a* and *Avr1b/Avr1k* loci, and the identification of the Avr1b protein (MacGregor *et al*., 2002; Tyler, 2001). Other *P. sojae* molecules have been described that elicit host and non-host responses, although these do not seem to be determinants of *Avr* gene-mediated race specificity (Cheong *et al*., 1991; Nürnberger *et al*., 1994). *P. sojae* also produces small cysteine-rich proteins, called elicitins, that are active in provoking an HR-like response when infiltrated into tobacco leaves (Becker *et al*., 2000; Kamoun *et al*., 1994). Despite these examples, the existing repertoire of *P. sojae* molecules that are known to elicit plant defense responses or cell death, especially in its soybean host, is almost wholly incomplete.

In this study, we have used data from *P. sojae* expressed sequence tags (ESTs) coupled with a viral-based expression system to assay for biologically active peptides. This led to the identification of a cDNA encoding a *P. sojae* necrosis-inducing protein (PsojNIP) that shares sequence similarity with proteins purified from other oomycete and fungal plant pathogens, as well as predicted proteins from eubacteria species. This *P. sojae* protein was active in causing spreading necrosis in *Nicotiana benthamiana* plants when expressed using a viral vector based on potato virus X (PVX). PsojNIP was also active in soybean tissues when transiently expressed by particle bombardment. The expression pattern of PsojNIP and the genomic region encoding this protein were characterized, and activities of related proteins from *Fusarium oxysporum, Bacillus halodurans*, and *Streptomyces coelicolor* were assayed and compared to PsojNIP. Overall the results indicate that PsojNIP is a potent member of an emerging group of conserved proteins that are variable in their ability to cause plant cell death. The expression of PsojNIP during infection accompanies the transition from biotrophy to necrotrophy in *P. sojae*, and we propose that the protein accelerates host cell death during this phase of pathogen growth.

Results

Putative secreted proteins are identified from EST data by in silico *analyses*

Candidate cDNA clones were selected from ESTs using PexFinder, an algorithm developed for the identification of extracellular proteins from *Phytophthora* (T.A. Torto, A. Styer, and S. Kamoun, unpublished; http://www.oardc.ohio-state.edu/pexfinder). A set of 3035 ESTs from *P. sojae* (race 2, strain P6497) mycelium or zoospores, or from a susceptible interaction of *P. sojae* (race 2, strain P6497) and soybean (cv Harosoy) 48 h after inoculation were used for this analysis (Qutob *et al*., 2000). The edited sequences

were translated into three forward reading frames and the corresponding 9105 translations were compiled in FASTA format (Pearson and Lipman, 1988). Those sequences possessing translational start sites within the first 105 nucleotides of the cDNA were edited to remove the 5′ UTR. All other sequences considered to be lacking the full open reading frames were discounted from further study. The predicted open reading frames of putative full length clones were queried against SignalP v. 2.0 (Nielsen *et al.*, 1997) for the presence or absence of a signal peptide sequence. The sequences were assessed as probable extracellular factors when two criteria were met. First, a SignalP-HMM Output score of >0.7 (Hidden Markov Model) with a signal peptide prediction was required. Secondly, a SignalP-NN (Neural Network) mean score of >0.5, with an indication of a probable cleavage site within the first 10–35 amino acids, was also necessary for further assessment.

Redundancy within the set of ESTs assumed to encode anchored or secreted factors was considered by screening each sequence by BLASTN searches against the original *P. sojae* data set of 3035 ESTs, publicly available at the Phytophthora Genome Consortium (PGC) (https://xgi.ncgr.org/pgc; Waugh *et al.* 2000). Similarity searches were also performed with *Phytophthora infestans* EST data also registered at PGC (Kamoun *et al.*, 1999). From this analysis, 322 ESTs, representing 202 non-redundant sequences, were predicted to contain 176 secreted and 26 membrane-associated proteins. Among the 202 non-redundant set of clones predicted to encode extracellular factors, 72 originated from zoospore ESTs, 46 from mycelial ESTs, and 70 from infected-plant ESTs, while 14 represented ESTs present in more than one cDNA library.

The 202 translations predicted as extracellular factors were re-evaluated for classification of putative function using the BLASTX algorithm for potential matches to recent GenBank sequence depositions (Altschul *et al.*, 1990). A total of 122 were similar to existing protein sequences (*E*-values $< 10^{-5}$), including 36 that were predicted to encode extracellular and membrane-associated factors, and 24 that were similar to unknown or hypothetical proteins. Thus, half (104/202) of the non-redundant set of ESTs encoding putative extracellular factors could not be assigned a putative biological function.

The non-redundant set of 79 ESTs with predicted signal transit sequences from the infected plant library had an average G + C content of 58.5%. This suggests that the majority of secreted or anchored factors in the mixed library are *P. sojae* specific, since soybean and *P. sojae* transcripts differ significantly in mean G + C content (Qutob *et al.*, 2000). Assembly of all ESTs predicted to encode extracellular factors from the three different cDNA libraries allowed for the positive identification of tissue source when ESTs from the infected plant library matched mycelial or zoospore-specific transcripts. Tissue source of candidate ESTs derived from the 'mixed' library were verified by PCR analysis of genomic DNA from soybean (cv Harosoy) or *P. sojae* (race 2, strain P6497) (data not shown).

Identification of a necrosis-inducing protein by PVX expression analysis in N. benthamiana

The degree of hydrophobicity and glycosylation of each predicted extracellular factor was assessed using motif search programs TopPred2 (von Heijne, 1992) and NetOGlyc 2.0 (http://www.cbs.dtu.dk/services/NetOGlyc/ Hansen, *et al.*, 1998), respectively. A total of 16 candidate cDNAs that encoded 6–26 kDa proteins without extensive glycosylation or hydrophobic domains were selected for functional assays (Table 1). Three candidates chosen for further analysis were similar to elicitors identified in other plant pathogens, as follows: 3-3c-MY, similar to β-cryptogein, a basic elicitin from *Phytophthora cryptogea* (Ricci *et al.*, 1989); 11-11c-ZO, similar to Vex1, a secreted acidic elicitin from *Pythium vexans* (Huet *et al.*, 1995); and 11-4e-HA, similar to a necrosis- and ethylene-inducing peptide from the coca pathogen *Fusarium oxysporum* f. sp. *erythroxyli* (Nelson *et al.*, 1998). Five ESTs (6-10a-HA, 8-9g-HA, 1-7c-MY, 9-9a-MY and 7-10d-ZO) were selected because they encoded small cysteine-rich proteins, a common feature of secreted elicitors identified in other host–pathogen interactions (Templeton *et al.*, 1994). Eight putative proteins matching unknown factors or proteins associated with cell structure were additionally chosen.

Candidates were tested for the ability to elicit a plant defense response by introducing cDNAs corresponding to selected ESTs into the potato virus X (PVX) genome in the form of a binary plant transformation vector, pGR107 (Jones *et al.*, 1999). Assessing candidate cDNAs for HR-inducing activity was compared to pGR107 containing no insert and to a recombinant pGR107 construct containing *inf*1, a previously identified elicitor from the potato pathogen *P. infestans* (Kamoun *et al.*, 1997). Plants were observed for up to 14 days post-inoculation for the development of mosaic symptoms, or the presence of local or systemic necrotic lesions. Representative phenotypes are shown in Figure 1 and results are summarized in Table 1. Plants inoculated with *Agrobacterium tumefaciens* carrying pGR107 displayed systemic mosaic symptoms typical of PVX infection at 5–9 days post inoculation (dpi) while pGR107::*inf*1 caused localized necrosis without mosaic symptoms. Most (13/16) of the pGR107 recombinant plasmids with cDNA inserts selected from the EST analysis had no noticeable effects on PVX symptom development, whereas three of the cDNAs resulted in altered phenotypes. A cDNA originating from the mycelium ESTs (1-7c-MY) attenuated mosaic lesion development in most plants tested with this construct. Another cDNA encoding a protein similar to β-elicitins (3-3c-MY) caused localized yellowing or mild

Table 1 Functional classification and signal peptide sequence of 16 candidate secreted factors from *Phytophthora sojae*

Clone ID[a]	Best match	Species of best match	*E*-value[b]	Signal sequence[c]	NN[d] Mean S	HMM[d] Output	Predicted[e] mass (kDa)	PVX screen result
3-9f-HA	Unknown/hypothetical	*Leishmania major*	10^{-1}	**M**RVSSLLVIAAGFLLASSEA↓FS	0.895	1.000	21.4	Mosaic
4-12d-HA	Unknown/hypothetical	*TT virus*	10^{-5}	**M**QGIFTLALLLLNGWLGHAEA↓VD	0.855	0.999	6.6	Mosaic
6-10a-HA	At1g05070	*Arabidopsis thaliana*	10^{-43}	**M**ALNQSKAVLLRVSLVVVALFIAGYIVG↓PP	0.849	0.750	20.6	Mosaic
8-9g-HA	HP8 peptide	*Homo sapiens*	10^{-7}	**M**NSRSMNVLAVAVTLLAACCALALG↓GS	0.910	0.988	11.5	Mosaic
11-4e-HA	Necrosis-/ethylene-inducing peptide	*Fusarium oxysporum*	10^{-42}	**M**NLRPALLATLASFAYVSA↓SV	0.843	0.998	25.6	Local/systemic necrosis
1-7c-MY	RNA binding protein	*Homo sapiens*	10^{-7}	**M**KIFAVAAVAIAALSTVDA↓VE	0.819	0.999	15.1	Asymptomatic
2-5g-MY	Unknown/hypothetical	*Homo sapiens*	10^{0}	**M**HRHIYILLLVAIVLVSSTNA↓TL	0.947	0.999	15.3	Mosaic
3-3c-MY	β-Elicitin	*Phytophthora cinnamomi*	10^{-44}	**M**NFTALLAAIAAALVGSANA↓TA	0.909	1.000	12.2	Mild/delayed necrosis
9-9a-MY	Ampullate silk protein	*Nephila clavipes*	10^{-50}	**M**KFAAVFVLASLVIVGISA↓DD	0.917	1.000	8.3	Mosaic
10-11f-MY	Unknown/hypothetical	*Homo sapiens*	10^{-2}	**M**RLSTRMGAALAALLAAARPSLTVA↓RG	0.789	1.000	7.6	Mosaic
1-6b-ZO	Unknown/hypothetical	*Caenorhabditis elegans*	10^{0}	**M**KLLHHLLVSAACVSVLA↓AT	0.922	1.000	22.5	Mosaic
2-9a-ZO	Kexin	*Pneumocystis carinii*	10^{-5}	**M**VRLLALASLLVVAVVSLSPAAA↓IP	0.945	1.000	7.1	Mosaic
2-9b-ZO	Proteophosphoglycan	*Leishmania major*	10^{-8}	**M**KLLHHLLSSAVCVAVLAATTVANVEG↓KV	0.769	0.993	22.3	Mosaic
6-12b-ZO	Transmembrane protein	*Streptomyces coelicolor*	10^{-11}	**M**AYIKTSIALLLVLAAGISDA↓TN	0.893	0.998	18.4	Mosaic
7-10d-ZO	Unknown/hypothetical	*Homo sapiens*	10^{-14}	**M**KTACLSALALAGAIAYSSA↓AD	0.768	1.000	20.9	Mosaic
11-11c-ZO	Acidic elicitin	*Phytophthora cinnamomi*	10^{-4}	**M**PSFTSIVLLGLAMASPVRA↓AD	0.816	1.000	16.7	Mosaic

[a]Clone identifications HA were isolated from the *P. sojae*-infected soybean cDNA library; MY were from the mycelial library; and ZO were from the zoospore library.
[b]*E*-value from BLASTX search.
[c]Initiator methionine is in boldface type. Hydrophobic residues are underlined and basic amino acids are in red type. The small uncharged residues, defining the signal peptidase cleavage site at the −1, −3 positions are in blue type. The predicted cleavage site is indicated by an arrow.
[d]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. SignalP v.2.0 is available at (http://www.cbs.dtu.dk/services/SignalP-2.0/).
[e]Mass of predicted pre-protein shown in kilodaltons (kDa).

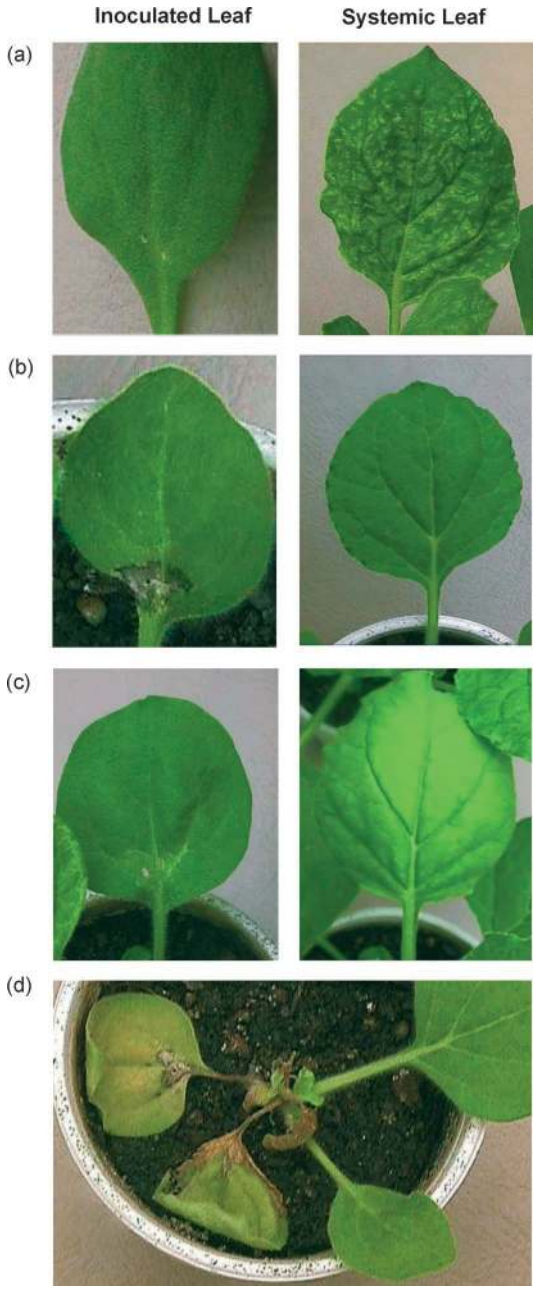


Figure 1. Representative symptoms observed on *Nicotiana benthamiana* plants following inoculation of lower leaf with recombinant PVX virus. Photographs were taken 10–14 days post-inoculation. Comparative phenotypes include the following.
(a) Typical mosaic disease symptoms on upper leaf, lack of a plant response at the site of inoculation.
(b) Necrosis localized to the site of inoculation. Upper leaves displaying no mosaic disease symptoms.
(c) Asymptomatic.
(d) Spreading necrosis.

necrosis at 7–9 dpi but otherwise did not affect the spread of mosaic symptoms. Among the 16 cDNAs tested, the most dramatic change in phenotype occurred in plants inoculated with pGR107 containing 11-4e-HA. These plants displayed necrosis around the site of inoculation at 5 dpi and lacked mosaic symptoms on the upper leaves. The necrosis became more severe by 8–9 dpi and eventually caused the entire inoculated leaf to collapse. In some cases, necrosis was observed to spread to upper leaves. The cDNA corresponding to 11-4e-HA was chosen for further characterization because it was most active in the PVX screening assay. It also originated from the infected plant library and thus was considered more likely to have a role in the infection process than transcripts isolated from axenically grown tissues.

PsojNIP *is highly expressed and temporally regulated during infection*

Analysis of soybean and *P. sojae* DNA by PCR and Southern blot hybridization confirmed that 11-4e-HA represented a *P. sojae* cDNA. The protein encoded by 11-4e-HA was named *P. sojae* necrosis-inducing protein (PsojNIP). Representative results shown in Figure 2(a) indicate that *PsojNIP* is a single copy gene that does not significantly cross-hybridize with other sequences under high-stringency conditions. However, multiple weakly hybridizing bands were noted in some analyses (not shown). Hybridization of the *PsojNIP* cDNA probe to genomic DNA isolated from 12 different *P. sojae* races was also performed to determine whether there are significant polymorphisms at this locus among *P. sojae* isolates. These results showed that the hybridization pattern was monomorphic, indicating that the copy number and gross structural features of this gene are conserved among different *P. sojae* race types (not shown).

As a first approach to investigate the contribution of PsojNIP to the infection cycle of *P. sojae*, the accumulation of mRNA transcripts during nine stages of life cycle development was analyzed by RNA blot hybridization. Total RNA isolated from zoospores, germinating zoospores, axenically grown mycelia or from etiolated soybean hypocotyls harvested at different time points after infection with *P. sojae* was probed with *PsojNIP* cDNA. In consideration of the changing ratio of *P. sojae* and soybean tissue biomass during the course of infection, a cDNA encoding a *P. sojae* actin gene (EST 7–2 g-MY) was used as a probe to estimate the proportion of *P. sojae* mRNA in each of the samples. As shown in Figure 2(b), the expression of the *PsojNIP* gene was exclusively detected during the latest sampling point (48 h) of the infection process. No *PsojNIP* expression was detected in axenically grown tissues nor during the early stages of infection. A more sensitive RT-PCR analysis could detect *PsojNIP* transcripts in axenically grown mycelium and during growth on soybean tissues from 12 h post-inoculation and onward, but not in zoospores nor early infection stages (not shown).

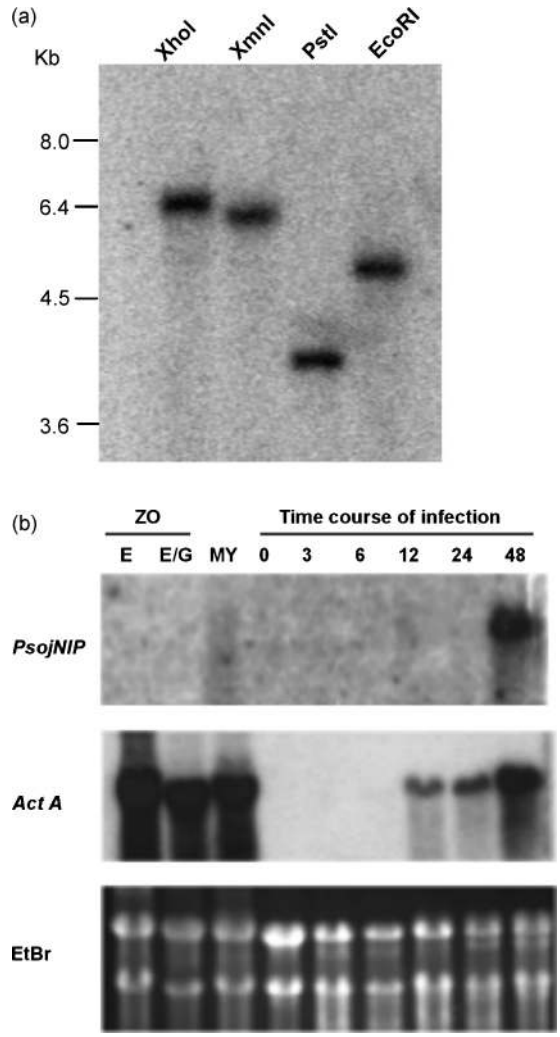


Figure 2. Structure and expression analysis of the *PsojNIP* gene.
(a) Genomic *Phytophthora sojae* DNA (5 μg) was digested with the restriction enzymes *Xho*I, *Xmn*I, *Pst*I, *EcoR*I, fractionated on a 0.8% agarose gel, transferred to a Hybond N membrane and hybridized with ^{32}P-labelled *PsojNIP* cDNA. The migration of the molecular mass markers are shown at the left.
(b) Total RNA (25 μg) was isolated from: zoospores (ZO) that had encysted (E) or encysted and germinated (E/G); axenically grown mycelia (MY); or from *P. sojae*-infected soybean tissue at 0, 3, 6, 12, 24 and 48 h after infection. The RNA blot was sequentially hybridized with probes derived from cDNAs encoding the *P. sojae* necrosis-inducing protein (*PsojNIP*) and a *P. sojae* actin protein (*ActA*). The corresponding ethidium bromide-stained agarose gel prior to transfer is also shown (EtBr).

Isolation of the genomic region encoding PsojNIP

To isolate the genomic region encoding *PsojNIP*, a bacterial artificial chromosome (BAC) library of 13 056 clones was screened by PCR using primers specific for the gene. Two positive, overlapping BAC clones were identified, and a region common to each of the clones containing *PsojNIP* was localized to a 10.8-kb *Bam*HI fragment. Sequencing of this 10.8-kb *Bam*HI fragment revealed a gene-dense region containing seven predicted open reading frames (ORFs) (GenBank accession number AF511649). The sequence of ORF I contains no introns and exactly matches the *PsojNIP* cDNA sequence. Immediately upstream of *PsojNIP* are two predicted ORFs that encode proteins similar to PsojNIP, although ORF III contains a single intron. The *PsojNIP*-like paralogs encoded by ORF II and ORF III have sequence similarity to *P. infestans* ESTs MY-24-C-03 and MY-19-A-05 (https://xgi.ncgr.org/pgc). ORF IV matches a hypothetical protein from *Mus musculus* (*E*-value = 10^{-6}). The ORF V predicted protein sequence is similar to UDP-glucose 4-epimerase from *Saccharomyces cerevisiae* (*E*-value = 10^{-50}) and the DNA sequence matches *P. sojae* ESTs 6-12e-ZO and 2-8f-HA, indicating that this gene is actively expressed in zoospores and during pathogenesis. ORF VI was similar (84% nucleotide sequence identity) to a *P. sojae* EST but is otherwise unknown. ORF VII is nearly identical to ORF IV in nucleotide and predicted protein sequence, since these genes are encoded within a near perfect inverted repeat that flanks ORF V and ORF VI.

To determine whether the *PsojNIP* paralogs corresponding to ORF II and ORF III are transcriptionally active, samples of RNA from treatments identical to those shown in Figure 2(b) were tested by RT-PCR. No amplification products were detected for these predicted genes in any of the samples, indicating that ORF II and ORF III are regulated differently than *PsojNIP*, or that they may be pseudogenes (not shown).

The P. sojae *necrosis-inducing protein shows sequence similarity to known proteins*

From the EST and cDNA sequences corresponding to PsojNIP, it was clear that the predicted protein was similar to a necrosis- and ethylene-inducing peptide, named NEP1, from the fungus *F. oxysporum* f.sp. *erythroxyli* (Nelson *et al.*, 1998). More recently, a number of other proteins with similarity to PsojNIP have appeared in public databases, including representatives from oomycete species and from bacteria. A phylogenetic tree based on these sequences is shown in Figure 3(a). All of the oomycete protein sequences clustered together and were most similar to a sequence from *B. halodurans*. The NEP1 protein from *F. oxysporum* displayed greater sequence divergence, but nonetheless was more similar to PsojNIP than sequences from *S. coelicolor* and *Vibrio* sp. Thus, there was no clear delineation between prokaryotic and eukaryotic sequences by this analysis.

The deduced sequence encoding the PsojNIP precursor protein consists of 237 amino acids, as shown in Figure 3(b). Cleavage of the predicted 19 amino acid signal peptide sequence results in a mature protein of 23.6 kDa with a pI of 6.2. Two cysteine residues within the N-terminal half of the protein are present in all of the PsojNIP-like

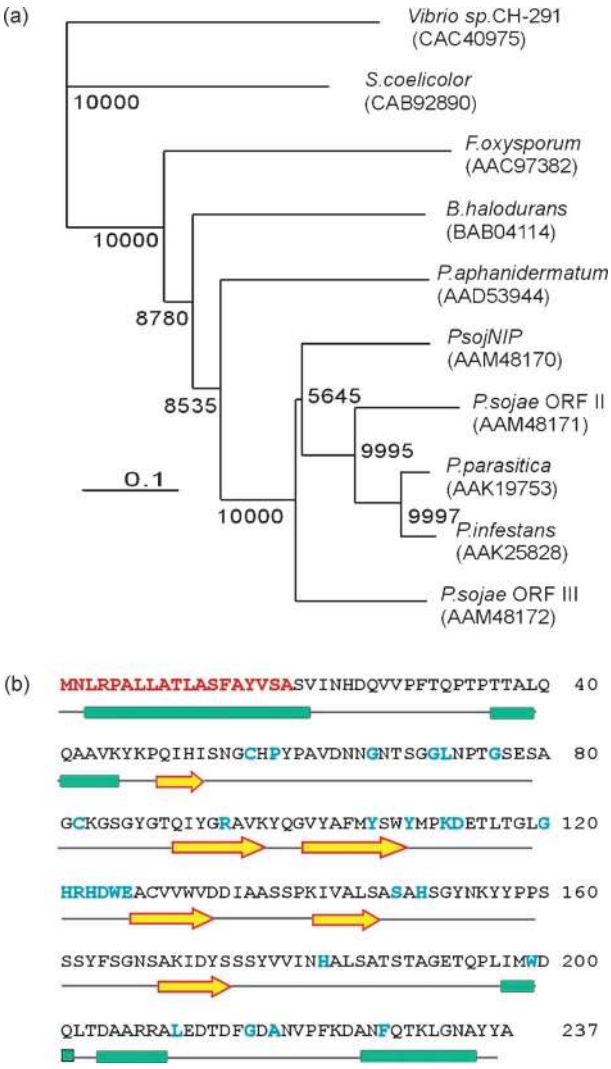


Figure 3. Analysis of the PsojNIP deduced amino acid sequence.
(a) The rooted phylogenetic tree was constructed from protein sequences with N-terminal transit peptides removed, using the neighbour-joining method of Clustal X (DNA STAR, MEGALIGN). Numbers at the nodes are bootstrap values from 10 000 replications. Branch lengths represent weighted amino acid substitutions. The scale bar represents 10% weighted sequence divergence. The tree was rooted with the *Vibrio* sp. CH-291 protein as the outgroup. GenBank accession numbers are shown in parenthesis.
(b) Deduced amino acid sequence of the PsojNIP precursor protein. Residues shown in red correspond to the predicted signal peptide; residues shown in blue are absolutely conserved among all 10 proteins from the phylogenetic analysis. Also shown below the amino acid sequence is the predicted secondary structure as determined by 3D-PSSM (version 2.6.0, http://www.bmm.icnet.uk/~3dpssm/); green bars are predicted α-helical regions and yellow arrows are predicted extended sheet.

sequences described to date, although PsojNIP is distinguished by an additional cysteine residue, cysteine 128 of the pre-protein. The most highly conserved regions of this group of proteins lie between the first two cysteine residues and within the central portion of the peptide chain, especially the area encompassing histidine 121 and histidine 123. The predicted secondary structure of PsojNIP is roughly symmetrical around these two centrally located histidine residues.

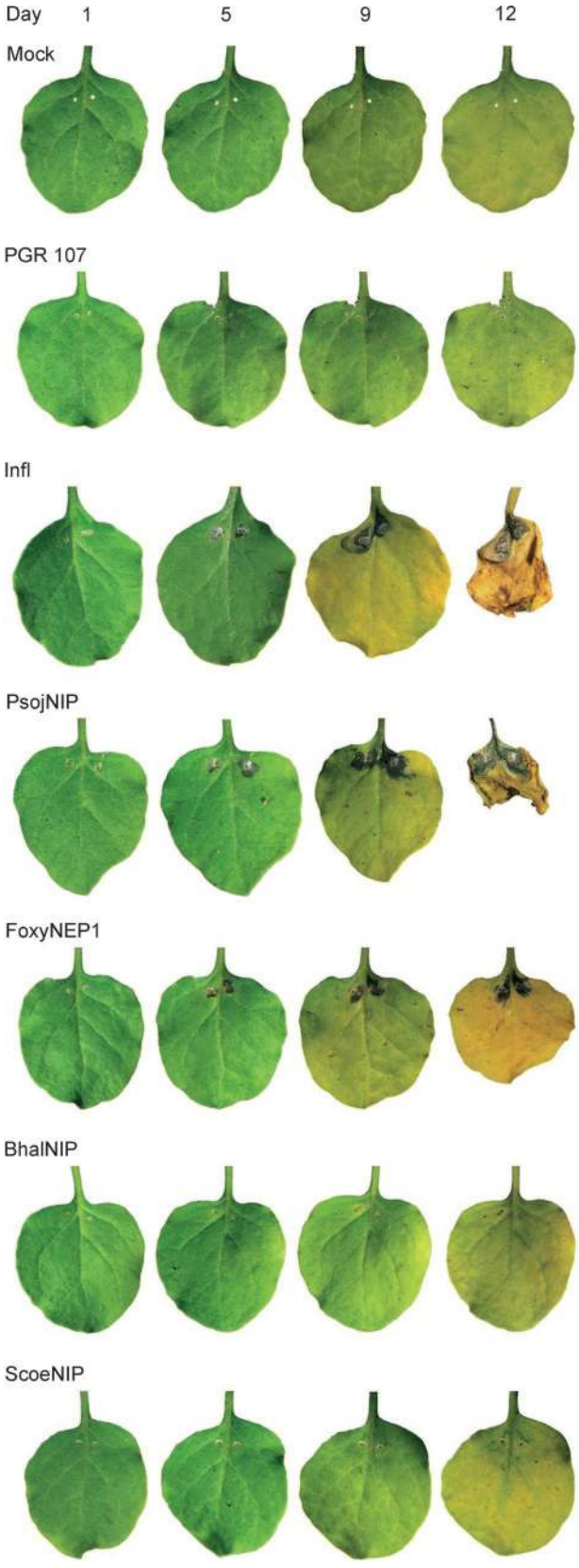
Proteins with sequence similarity to PsojNIP vary in necrosis-inducing activity

To assess the conservation of necrosis-inducing activity of similar proteins from other species, PsojNIP orthologs present in *F. oxysporum*, *B. halodurans,* and *S. coelicolor* were tested by PVX expression in *N. benthamiana*. The *P. infestans* elicitin gene *inf1* provided an independent reference, since it is known to induce an HR when expressed in *N. benthamiana* (Kamoun *et al.*, 1998). Typical results are shown in Figure 4. By this assay, *PsojNIP* and *inf1* were comparable in their ability to produce visible necrotic lesions within 5 days. These lesions rapidly spread and caused the entire inoculated leaf to wither and collapse within 12 days. *NEP1* from *F. oxysporum* was also active in causing necrosis in *N. benthamiana* when transiently expressed using PVX; however, symptoms were less severe and slower to develop than in leaves inoculated with the *PsojNIP* or *inf1* constructs. In contrast, the *B. halodurans* and *S. coelicolor PsojNIP*-orthologs were inactive or weak in their ability to induce necrosis or alter mosaic symptom development. In some experiments, the *S. coelicolor PsojNIP*-like gene was active in causing small necrotic lesions and accelerating senescence of the inoculated leaf.

Considering the possibility that the corresponding prokaryotic genes are not efficiently translated in plants, we determined codon usage in each of the three genes and compared this to known codon frequencies occurring in tobacco using EMBOSS (http://www.uk.embnet.org/Software/EMBOSS/). By this analysis, *PsojNIP* (CAI = 0.48) was less suited for expression in tobacco than the *B. halodurans* (CAI = 0.68) or *S. coelicolor* (CAI = 0.50) genes, suggesting that variation in translational efficiency is not the cause of the different activities of these genes in the PVX assay.

Transient expression of PsojNIP *in soybean tissues*

To determine the activity of PsojNIP in soybean tissues, a transient expression assay used to detect cell death or HR in plant cells was performed (Mindrinos *et al.*, 1994). The assay relies on the expression of the reporter gene β-glucuronidase (*GUS*) as an indicator for the viability of the transformed cells. In this technique, independent constructs containing *GUS* and the test gene are co-introduced into plant cells. Transient expression of a test insert that causes cell death reduces or suppresses *GUS* expression in the transformed cells. For this study, *PsojNIP* cDNA or cDNA corresponding to EST 6-12b-ZO was cloned into the transformation vector pFF19 (Timmermans *et al.*, 1990)



for transient expression in soybean cv Harosoy. This cultivar is susceptible to most *P. sojae* isolates, including P6497. The *F. oxysporum* NEP1, and the PsojNIP orthologs present in *B. halodurans,* and *S. coelicolor* were also tested by this method to provide a comparison to the PVX expression assays. Results shown in Figure 5 demonstrate that soybean hypocotyls transformed with pFF19::GUS alone or co-introduced with the pFF19::6-12b-ZO construct displayed strong GUS activity. In contrast, little or no GUS activity was observed in hypocotyls when PsojNIP was co-expressed with the *GUS* reporter construct. This suppression of GUS activity by *PsojNIP* was only observed in plants transformed with full-length constructs encoding the entire precursor protein. Plants co-bombarded with pFF19::GUS and a pFF19::PsojNIP construct lacking the signal peptide displayed GUS activity, suggesting that the protein is active outside of the plant cell (not shown).

The *F. oxysporum* NEP1, and the PsojNIP orthologs from *B. halodurans* and *S. coelicolor* were also active in reducing levels of reporter gene expression, as measured by histochemical staining and by a fluorometric assay of GUS enzyme activity. These results are in contrast to those observed using the PVX-based assay in *N. benthamiana,* wherein the various PsojNIP-like proteins varied in necrosis-inducing activity.

Discussion

The PVX-based binary plant transformation vector is an effective molecular tool for transient expression of foreign genes in a plant system. It has also been used to post-transcriptionally silence both transgenes and endogenous plant genes by co-suppression (Jones *et al.*, 1999; Thomas *et al.* 2001). This system has been applied to assay two secreted elicitors from the tomato pathogen, *Cladosporium fulvum* (ECP1 and ECP2) against a collection of tomato genotypes to identify plants that respond with an HR. Application of this technique led to the isolation of the corresponding resistance gene of *ECP2* designated, *Cf-ECP2* (Laugé *et al.*, 1998). More recently, this system has been exploited as a high-throughput functional screening tool. For example, cDNAs generated from RNA of *in vitro*

Figure 4. Comparative analysis of necrosis-inducing activity of various proteins by PVX expression in *Nicotiana benthamiana* leaves. The leaf base flanking the main vein was inoculated, as described in Experimental procedures. Control plants were mock-inoculated with water (Mock), or with *Agrobacterium tumefaciens* harboring the PVX expression vector (pGR107). Test plants were inoculated with *A. tumefaciens* harboring the PVX expression vector containing a DNA construct encoding an open reading frame, as follows: *Phytophthora infestans* elicitin (inf1); *Phytophthora sojae* necrosis-inducing protein (PsojNIP); *Fusarium oxysporum* necrosis- and ethylene-inducing peptide (FoxyNEP1); *Bacillus halodurans* protein with sequence similarity (BhalNIP); and *Streptomyces coelicolor* protein with sequence similarity (ScoeNIP). Shown are photographs of individual, representative leaves taken at 1, 5, 9, and 12 days after inoculation.

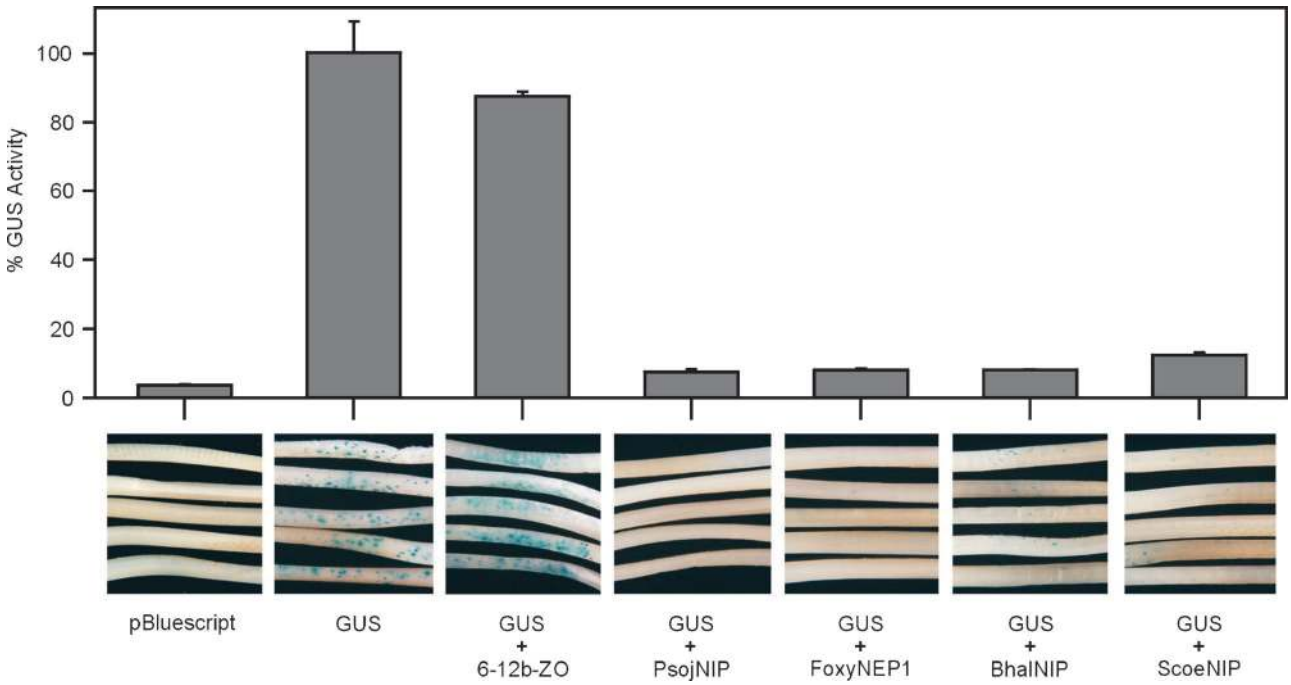


Figure 5. Activity of PsojNIP and other proteins in soybean tissues as determined by a co-bombardment and transient expression assay. The photographs show soybean hypocotyls after bombardment and histochemical staining for GUS activity. The histogram provides a quantitative measure of GUS activity as determined by a fluorometric assay. Tissues were bombarded with control plasmid without insert (pBluescript) or with pFF19 containing a GUS expression cassette (GUS), as described in Experimental procedures. Tissues co-bombarded with pFF19 containing the GUS expression cassette in combination with a second pFF19 construct containing a test insert are also shown as follows: zoospore EST 6-12b-ZO (GUS + 6-12b-ZO); *Phytophthora sojae* necrosis-inducing protein (GUS + PsojNIP); *Fusarium oxysporum* necrosis- and ethylene-inducing peptide (GUS + FoxyNEP1); *Bacillus halodurans* protein with sequence similarity (GUS + BhalNIP); and *Streptomyces coelicolor* protein with sequence similarity (GUS + ScoeNIP).

grown, nutrient-starved *C. fulvum* were cloned into a PVX-based expression vector and randomly screened for elicitor activity by testing for the development of necrotic lesions around inoculation sites (Takken *et al.*, 2000).

This established technique was used here in combination with the *in silico* based screening algorithm, PexFinder, to assay putative extracellular factors from *P. sojae*. Of the 16 candidates screened, expression of 13 cDNAs resulted in mosaic disease symptoms typical of that observed during infection by the wild-type virus. This outcome was unexpected with expression of transcript 11-11c-ZO since the corresponding protein sequence has significant similarity to secreted acidic elicitins and, therefore, had been expected to evoke a plant response. However, unlike typical elicitins, this protein is predicted to have a glycosylated, hydrophobic region, suggesting that it may be localized to the cell wall or membrane. This feature may account for its lack of activity. It is also not clear why plants inoculated with the construct pGR107::1-7c-MY were asymptomatic, not even displaying typical mosaic lesions. It is possible that this gene interferes with viral replication or spread without resulting in visible necrosis or HR-like symptoms, although there are other explanations that cannot be excluded. Further work is required before any conclusions can be drawn regarding the activity of this gene.

Two cDNAs were identified that caused visible necrosis or HR-like symptoms when expressed in *N. benthamiana* together with recombinant PVX. The product of one transcript (3-3c-MY) was similar to basic elicitins from *Phytophthora* species and displayed a weak necrosis at the site of inoculation, eventually becoming systemic. The second, more robust necrosis-inducing cDNA, encoded PsojNIP. The PsojNIP sequence is similar to that of NEP1, a protein purified from culture filtrates of the coca pathogen *F. oxysporum* f.sp. *erythroxyli* that elicits ethylene production and necrosis when applied to leaves of *Erythroxylum coca* (Bailey, 1995; Nelson *et al.*, 1998). Additional orthologs of PsojNIP have since been identified in the eubacteria *B. halodurans*, *S. coelicolor,* and *Vibrio* sp., and in other oomycete plant pathogens *P. infestans, P. parasitica,* and *Pythium aphanidermatum* (Bentley *et al.*, 2002; Fellbrich *et al.*, 2002; Takami *et al.*, 2000; Veit *et al.*, 2001). A matching EST from a *P. medicagins*/*M. truncatula* interaction library has also been reported (GenBank accession number AW559250).

It is not clear how these related proteins arose in such taxonomically disparate organisms, including prokaryotes and two distinct groups of crown eukaryotes, but horizontal gene transfer most likely played a role. Evidence to date also indicates that these proteins are not common or

necessary features of bacterial or fungal genomes, since their known distribution is restricted to the aforementioned species. The occurrence of these proteins in unrelated organisms is perhaps associated with shared saprophytic and parasitic lifestyles.

Sequencing of the genomic region encoding PsojNIP revealed that this protein is part of a gene family in *P. sojae*. Previous work has also shown that a DNA marker linked to the *Avr1a* gene occurs within a PsojNIP-like pseudogene (MacGregor *et al.*, 2002). Overall, the prevalence of PsojNIP-like sequences in *P. sojae* and the presence of orthologous genes in other plant pathogenic oomycetes suggests that these organisms have recruited the proteins to aid their parasitic mode of life.

It is unknown how proteins like PsojNIP cause necrosis in plant cells but evidence is accumulating that they act by manipulating intrinsic cell death programs of the host (Fellbrich *et al.*, 2002; Jennings *et al.*, 2001; Veit *et al.*, 2001). The possibility that PsojNIP is a secreted protein that aids in colonization is supported by results from the expression analysis. The detection of *PsojNIP* transcripts during the transition from biotrophy to necrotrophy, and the high level of expression during the late stages of a compatible interaction, suggests that this protein is an elicitor-toxin that accelerates plant cell death during the necrotrophic phase of disease development. No expression of *PsojNIP* was detected in encysted or germinating zoospores, nor during the early stages of infection of plant tissues.

From analyses of the *F. oxysporum* (Bailey, 1995), *P. aphanidermatum* (Veit *et al.*, 2001), and *P. parasitica* (Fellbrich *et al.*, 2002) proteins, it has been determined that dicot plants are generally susceptible and monocots insensitive to the necrosis-inducing activity of these proteins. Despite sequence similarities, our results show that PsojNIP and related proteins from other species are variable in their necrosis-inducing activity when expressed in *N. benthamiana* using an *A. tumefaciens* binary PVX vector. Among four different proteins tested from oomycete, fungal, and bacterial species, the most powerful inducer of necrosis was PsojNIP followed by NEP1 from *F. oxysporum*. Similar proteins from *B. halodurans* and *S. coelicolor* were inactive or comparatively weak in their necrosis-inducing activity. This was surprising because the *B. halodurans* protein sequence shares greater similarity with PsojNIP than does NEP1 from *F. oxysporum*. The results from the PVX-based assays show an association between necrosis-inducing activity and plant pathogenesis, since *P. sojae* and *F. oxysporum* are successful plant pathogens while *B. halodurans* and *S. coelicolor* are soil-dwelling microbes that are not known to be pathogenic on any plants. However, the different proteins showed less variation in activity when assayed by co-bombardment and transient expression in soybean tissues. The host plant tissues employed in each assay may be differentially sensitive to the effects of these proteins. Particle bombardment also causes extensive tissue damage, possibly making the cells more sensitive to the effects of the *B. halodurans* and *S. coelicolor* proteins in comparison to the viral-based expression system.

Regardless, the effectiveness of PsojNIP in abrogating *GUS* expression by co-bombardment demonstrates that the protein is fast acting and potent in soybean tissues. In these assays, *PsojNIP* activity compares to previously characterized avirulence genes in other systems when expressed in the presence of the complementary resistance gene (Jia *et al.*, 2000; Mindrinos *et al.*, 1994). However, at least two important characteristics of PsojNIP distinguish it from known avirulence gene products. First, PsojNIP is active in a host cultivar that is susceptible to most *P. sojae* isolates, including the race 2 strain P6497 from which PsojNIP was isolated. This indicates that PsojNIP is unlikely to be an avirulence determinant in the soybean–*P. sojae* interaction. Secondly, PsojNIP-like proteins are present in taxonomically diverse organisms and may be active in causing necrosis in a broad range of dicotyledonous plants. These observations lead us to suggest that PsojNIP is a virulence factor that manipulates host cell death programs for the pathogen's advantage. This mechanism has also been proposed for NEP1 from *F. oxysporum* (Jennings *et al.*, 2001), and for various non-proteinaceous toxins produced by necrotrophic plant pathogens *Alternaria alternata* f. sp. *lycopersici* (Wang *et al.*, 1996), *Cochliobolus victoriae* (Navarre and Wolpert, 1999), and *F. moniliforme* (Stone *et al.*, 2000). The concept also has parallels in studies of bacterial pathogenesis of animal cells (Navarre and Zychlinsky, 2000). Thus, it is reasonable to extend this model to PsojNIP. It also raises the possibility of creating plants with greater resistance to hemibiotrophic or necrotrophic plant pathogens by lessening their susceptibility to toxic-elicitor proteins such as PsojNIP.

Experimental procedures

Plant material P. sojae cultures

Soybean (*Glycine max* [L] Merr) cv Harosoy (*Rps7*) seeds were obtained from a collection at Agriculture and Agri-Food, Canada. Etiolated soybean seedlings were grown in water-soaked vermiculite at 22°C for 7 days prior to harvest for experimental use. *N. benthamiana* plants were grown in a greenhouse at 27°C, in styrofoam cups containing a soil-less mix (Pro-Mix 'BX', Premier Horticulture Ltd, Rivière-du-Loup, Canada). Plants were challenged with recombinant PVX constructs at approximately 4–6 weeks after seed germination. *Phytophthora sojae* race 2 strain P6497, virulent on cv Harosoy, was used throughout this study (Förster *et al.*, 1994). Cultures were routinely grown on V8-juice medium at 25°C in the dark (Ward *et al.*, 1979). Axenically grown mycelia and zoospores were prepared as described by Qutob *et al.* (2000). To produce encysted zoospores, free-swimming zoospores were agitated by vortex mixing and collected by centrifugation at 2000 *g*.

Cysts were allowed to germinate at 22°C for 6 h. Infection assays with *P. sojae* were performed by inoculation of soybean plants with 10-μl water droplets, each containing approximately 10^3 free-swimming zoospores, placed along the hypocotyl length (Gijzen *et al.*, 1996; Ward *et al.*, 1979).

Construction of recombinant A. tumefaciens *binary PVX vectors*

Candidate cDNAs were PCR-amplified using high-fidelity DNA polymerase (Expand™, Roche Diagnostics, Laval, PQ) and subcloned into the PVX vector pGR107 (Jones *et al.*, 1999). Gene-specific primers complementary to the 5′ and 3′ ends of each respective open reading frame were designed to include restriction site overhangs for cloning into pGR107 (all primer sequences are available upon request). Upstream primers contained sequences corresponding to the native signal peptide for extracellular targeting, with the exception of clones 3-3c-MY and 11-11c-ZO for which the sequence encoding the signal peptide was replaced by that of the secreted protein PR-1a of *N. tabacum* (Hammond-Kosack *et al.*, 1994). To facilitate post-translational processing of *P. sojae* signal peptides in a plant system, the codon encoding the amino acid immediately downstream of the start codon (within the signal peptide sequence) was altered to incorporate a neutral charged valine or alanine residue.

Amplification products were digested with appropriate restriction enzymes, size-fractionated and purified from 0.8% agarose gels (QIAprep gel extraction kit, Qiagen Inc., Mississauga, ON) prior to ligation into pGR107. Orientation and sequence of the cloned inserts were verified by DNA sequence analysis. Recombinant binary plasmids were maintained and propagated in *Escherichia coli,* strain XL1-Blue MFR′, grown in the presence of 50 μg ml^{-1} kanamycin, 12.5 μg ml^{-1} tetracycline. The binary expression constructs were electro-transformed into *A. tumefaciens* strain GV3101 and the cells allowed to grow for 2 days at 28°C in LB agar plates supplemented with tetracycline (12.5 μg ml^{-1}) and kanamycin (50 μg ml^{-1}) as selective agents. Individual colonies were toothpick-inoculated onto the lower leaves of *N. benthamiana* plants (Takken *et al.*, 2000). The development of disease symptoms was recorded for up to 14 days following plant inoculations.

Biolistic transformation of soybean

The expression vector pFF19 containing a β-glucuronidase (*GUS*) reporter gene driven by the cauliflower mosaic virus 35S promoter (pFF19::GUS) was used for particle bombardment studies of etiolated soybean hypocotyls (Timmermans *et al.*, 1990). pBluescript (Stratagene, La Jolla, CA) was used as a negative control. Transformation cassettes were constructed by PCR amplification of coding sequences using primers designed to include flanking adapters for cloning. Bombardment was performed using a particle delivery system described by Buckley *et al.* (1995). DNA samples were prepared for each transformation by coating 1 mg of tungsten particles (Bio-Rad Laboratories, Hercules, CA) with 0.8 μg DNA of a chimeric pFF19 construct. In the case of co-bombardment assays, 0.8 μg DNA of the reporter plasmid pFF19::GUS was also added to the tungsten/DNA suspension. Following bombardment, seedlings were incubated in the dark at 25°C for 48 h.

GUS activity was assayed by histochemical staining with 5-bromo-4-chloro-3-indoyl-β-D-glucuronic acid (X-Gluc, Sigma, Oakville, ON) as described by Jefferson (1987). Plant tissues were submerged in GUS-staining buffer (0.5 mg ml^{-1} X-Gluc, 0.1 M sodium phosphate buffer pH 7.0, 0.01 mM EDTA, 0.1% Triton X-100, 0.5 mM ferrocyanide, 0.5 mM ferricyanide), vacuum infiltrated for 30 min, and incubated overnight at 37°C in the dark. GUS activity was measured fluorometrically using the substrate 4-methylumbelliferone glucuronide (MUG, Sigma, Oakville, ON) according to Jefferson (1987), with some modifications. Three hundred milligrams of bombarded hypocotyl tissue was ground in liquid nitrogen, transferred to a tube containing 900 ml of GUS extraction buffer (GEB: 50 mM $NaP0_4$ pH 7.0, 10 mM β-mercaptoethanol, 10 mM Na_2EDTA pH 8.0, 0.1% Triton X-100) and homogenized for 2 min. After centrifugation, the supernatant was collected and stored at −80°C. Reactions were performed by transferring from 50 to 100 μl of each sample into 500 μl GEB containing 1 mM MUG and 20% methanol. Enzymatic assays were carried out at 37°C in the dark and stopped at intervals. In each case, 100 μl of reaction mix was transferred into a tube containing 1.9 ml 0.2 M Na_2CO_3. Fluorescence was measured in a spectrofluorimeter (excitation 365 nm, emission 450 nm) calibrated with 4-methylumbelliferone (MU) in stop buffer.

Nucleic acid extraction and nucleotide sequencing

Methods for the isolation of total RNA and genomic DNA from *P. sojae* tissues and soybean plants have been described (Qutob *et al.*, 2000). Plasmid DNA was purified from *E. coli* cultures by alkaline lysis and adsorption chromatography (Qiagen Inc., Mississauga, ON), according to the manufacturers' instructions. The complete sequence of cDNAs selected for functional analyses was obtained using vector and gene-specific primers. Automated sequencing was carried out using dye-termination chemistry and electrophoresis (Applied Biosystems 377).

RNA and DNA gel blot analysis

Samples of 25 μg of RNA were denatured and fractionated by electrophoresis through agarose gels containing 1.0% formaldehyde. Samples of 5 μg of *P. sojae* DNA were digested with restriction enzymes, size-separated in 0.8% (w/v) agarose gels, and transferred to nylon membranes (Hybond N, Amersham Pharmacia Biotech, Baie d'Urfé, PQ). Probes corresponding to *PsojNIP* or *ActA* (*P. sojae* actin A) were excised from a plasmid cloning vector (BK-CMV, Stratagene, La Jolla, CA), isolated from agarose gels (QIAquick Gel Extraction Kit, Qiagen Inc., Mississauga, ON), labeled with [α-^{32}P]-dCTP by random primer extension (*redi*primell, Amersham Pharmacia Biotech, Bai d'Urfé, PQ), and purified (Nick Column, Amersham Pharmacia). The purified, labelled probes were denatured by boiling for 1 min, cooled on ice, and then added to the pre-hybridization solution. Both pre-hybridization and hybridization steps were each performed at 65°C for 16 h in a buffer containing 0.25 M Na_2HPO_4 (pH 7.2), 1% bovine serum albumin (BSA), 7% SDS, and 1 mM EDTA (pH 8.0). Filters were washed four times at 68°C and once at 22°C during 30-min intervals to a final stringency of 0.02 M Na_2HPO_4 (pH 7.2), 1% SDS, and 1 mM EDTA (pH 8.0) before exposure to Kodak X-AR film (Eastman Kodak, Rochester, NY). RT-PCR was carried out using 5 μg total RNA from encysted zoospores, encysted/germinating zoospores, axenically grown mycelia and from *P. sojae*-infected soybean tissue at 0, 3, 6, 12, 24, 48 h post-infection (Thermoscript RT-PCR system, Life Technologies, Gaithersburg, MD). First strand cDNA products were used as templates for PCR amplification of *PsojNIP* and two *PsojNIP*-like paralogs using primers flanking the corresponding open reading frames.

Isolation of BAC clones encoding PsojNIP

A *P. sojae* (P6497) genomic bacterial artificial chromosome (BAC) library (Brett Tyler, unpublished) was subjected to a hierarchical pooling strategy to recover clones that covered the genomic region encoding *PsojNIP.* The ordered library, propagated in the pBeloBACII, was screened by PCR using primer pairs encoding the 5′ and 3′ most exons of the target gene of interest. A 10.8-Kb *Bam*HI fragment containing *PsojNIP* was subcloned from BAC 26B15 into a plasmid vector (pBluescript, Stratagene, La Jolla, CA) for shotgun sequencing by transposon insertion (GPS-1, New England Biolabs, Beverly, MA). A total of 422 individual sequence runs was assembled into a single contig of 10 829 bp.

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