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Review

Nep1-like proteins from plant pathogens: Recruitment and diversification of the NPP1 domain across taxa

Mark Gijzen [a,*], Thorsten Nürnberger [b]

[a] *Agriculture and Agri-Food Canada, 1391 Sandford Street, London, Ont., Canada N5V 4T3*
[b] *Zentrum fur Molekularbiologie der Pflanzen, Auf der Morgenstelle 5, Eberhard-Karls Universität, Tübingen 72076, Germany*

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This review is dedicated to Prof. Rodney Croteau, for the occasion of his 60th birthday.

Abstract

An emerging group of proteins found in many plant pathogens are related to their ability to cause plant cell death. These proteins may be identified by the presence of a common NPP1 (necrosis-inducing *Phytophthora* protein) domain, and have collectively been named NLPs (Nep1-like proteins). The NLPs are distinguished by their wide distribution across taxa and their broad spectrum of activity against dicotyledonous plants. The function of NLPs is not known but there is strong evidence that they may act as positive virulence factors, accelerating disease and pathogen growth in plant hosts. Interest in NLPs is gaining momentum as more members of this protein family are discovered in more species of plant pathogens.

Keywords: Bacteria; Cell death; Defence; Disease; Elicitor; Fungus; Oomycete; Pathogen; Toxin; Virulence

Contents

1. Introduction

The characterization of pathogen molecules that cause plant cell death has been a subject of longstanding interest among plant pathologists. As a defensive measure, host perception of key pathogen molecules may initiate cell death processes that arrest pathogen spread and limit infection (Nimchuk et al., 2003). There is gathering evidence that these cell death programs may also be commandeered by pathogens during infection and subverted in ways that actually work against the host (Greenberg and Yao, 2004). Among the elements that influence interactions between plants and pathogens are molecules that have

* Corresponding author. Tel.: +1 519 457 1470; fax: +1 519 457 3997.
E-mail address: gijzenm@agr.gc.ca (M. Gijzen).

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traditionally been known as elicitors and toxins. These terms and other used in this review have been defined in Table 1.

It was recognized early on that pathogens may produce toxins that kill plant cells and promote disease (Luke and Wheeler, 1955). These have been classified into two basic groups, host-selective and non-selective toxins (Agrios, 2005), including a wide array of large and small molecules, such as polyketides, terpenoids, peptides and proteins (Daly and Deverall, 1983; Walton, 1996; Kimura et al., 2001). Toxin production is apparently a common phenomenon that contributes to the virulence of many fungal and bacterial plant pathogens, especially facultative parasites that engage in a necrotrophic lifestyle.

There are also pathogen molecules not considered to be toxins that nonetheless may trigger host cell death, such as elicitors. The study of these molecules arose from Flor's descriptions of complementary genetic inheritance of plant resistance and pathogen virulence, the gene-for-gene hypothesis (Flor, 1971). Thus, pathogen elicitors are molecules that are recognized by plant cells and that activate defence responses, and sometimes cell death programs, in the host (Nürnberger, 1999). Elicitors are regarded as factors that may limit or slow-down disease. A cultivar-specific elicitor that negatively and qualitatively affects virulence corresponds to an avirulence (Avr) determinate in the pathogen.

As our understanding of plant immunity and pathogen virulence has become more sophisticated, it has emerged that pathogen elicitors encompass a great variety of molecules that vary in their structure and activity. For example, many bacterial Avr products correspond to protein effectors that are delivered into host cells via specialized transport systems (Büttner and Bonas, 2003), whereas pathogen-associated molecular patterns (PAMPs) have conserved molecular motifs or signatures that are recognized by plant immune systems (Nürnberger et al., 2004). The idea that pathogen Avr-proteins have original roles as positive virulence factors was controversial in the past, but now an overwhelming amount of evidence indicates that this is often the case (Espinosa and Alfano, 2004). It has also become clear that host responses to certain elicitors, such as PAMPs, can be mediated by the same class of receptors that participate in Avr-protein recognition (Gomez-Gomez and Boller, 2000).

The terms Avr-protein, elicitor, toxin, PAMP, and virulence-factor all imply a particular positive or negative outcome of the disease interaction. However, problems always arise using these terms with their implied meaning, such as for Avr-proteins that have a virulence function. This is why the word 'effector' has been adopted where 'Avr-protein' would have been used in the past. Likewise, the definitional overlap between elicitors and toxins has always been apparent (van't Slot and Knogge, 2002; Wolpert et al., 2002). Nonetheless, the interchangeable nomenclature does not apply to all such molecules and it remains useful to distinguish between the perceived functional roles of elicitors and toxins. The relative contributions to pathogen virulence or host resistance are useful criteria to consider in assessing elicitors and toxins. How the molecule is perceived by the plant immune system is important, but in many cases this is not known. Some knowledge of the mode-of-action is necessary to gain an understanding of the molecule's true purpose or role in the disease process.

The difficulties that may arise in distinguishing between elicitors and toxins is exemplified by a new class of proteins that are reviewed here. The purification of a 24-kD necrosis and ethylene inducing protein, named Nep1, from culture filtrates of *Fusarium oxysporum* resulted in the identification of a new type of protein capable of triggering plant cell death (Bailey, 1995). When the Nep1 sequence was determined, it was shown to be unrelated to any known protein or functional domain (Nelson et al., 1998). Many other Nep1-like proteins (NLPs) have since been discovered in a variety of organisms. This has led to a growing interest in determining their function and role in plant–pathogen interactions (Pemberton and Salmond, 2004). A new domain, called the necrosis-inducing *Phytophthora* protein 1 (NPP1) domain is present in all NLPs (Fellbrich et al., 2002). This review summarizes our knowledge of NLPs, and presents new information on the evolution of this protein family in *Phytophthora* and other organisms.

Table 1
Definition of terms

Molecule	Definition[a]
Avr protein	Avirulence protein; a protein coded for by an *Avr* gene, acting as an elicitor of defense reactions
Elicitor	Molecules produced by a pathogen that induce a defense response in the host
PAMP	Pathogen associated molecular pattern; molecules produced by a microbe that bind to pattern recognition receptors in the host and trigger defense reactions
Toxin	A compound produced by a microorganism; being toxic to a plant or animal
Virulence factor	Coded for by virulence genes that are helpful but not essential for induction and development of disease

[a] The definitions are from Agrios (2005) or from Nürnberger et al. (2004).

2. The occurrence of NLPs in fungi, oomycetes, and bacteria

With the spread of genomic and proteomic methods of analysis, the NLP family is expanding rapidly in size and distribution. Genes encoding NLPs, or the proteins themselves, have been detected in eukaryotic and prokaryotic organisms. The NLPs occur in at least two major branches of the eukaryotic tree, the fungi and the oomycetes, and also in Gram-positive and Gram-negative bacteria. Most species with NLPs are plant pathogens but there are exceptions to this trend, since genes encoding NLPs have been detected in fungal and bacterial species that are not known to be pathogenic on plants.

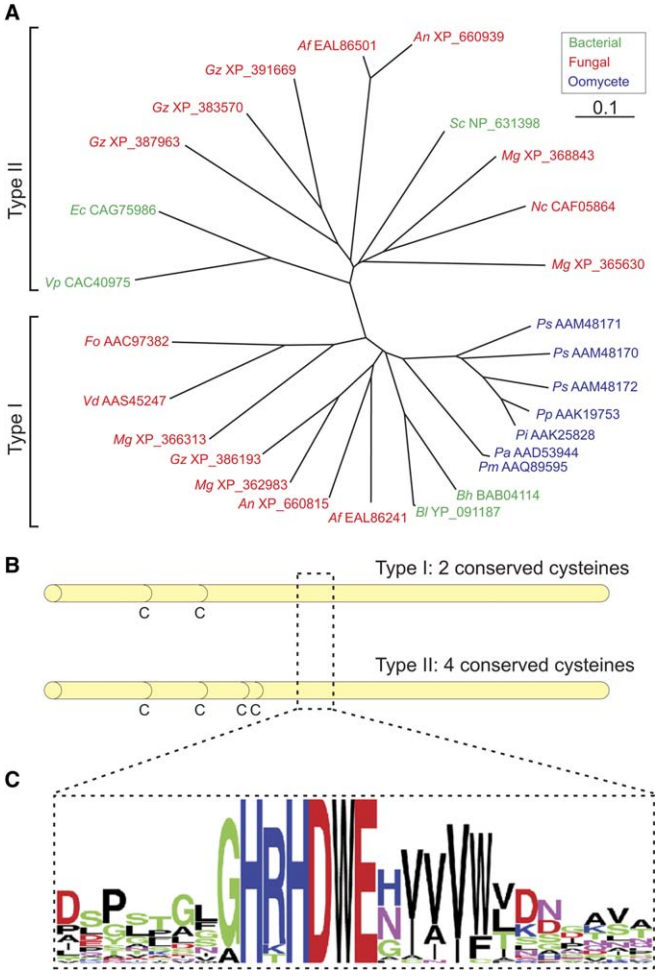

Fig. 1. Comparison of sequences encoding NLPs. (A) An un-rooted phylogenetic tree constructed from NLP sequences from GenBank. Protein sequences were aligned using ClustalX (1.81) and the phylogram was drawn in TreeView. The NLPs containing Type I or Type II NPP1 domains is shown on the left. The 27 deduced protein sequences were from bacterial, fungal, and oomycete species, as indicated by colour. The scale bar represents 10% weighted sequence divergence. The protein sequences are coded as follows: the first two letters in italics identify the organism, followed by the GenBank protein accession number. *An*, *Aspergillus nidulans*; *Af*, *Aspergillus fumigatus*; *Bh*, *Bacillus halodurans*; *Bl*, *Bacillus licheniformis*; *Ec*, *Erwina caratovora*; *Fo*, *Fusarium oxysporum*; *Gz*, *Gibberella zeae*; *Mg*, *Magnaporthe grisea*; *Nc*, *Neurospora crassa*; *Pa*, *Pythium aphanidermatum*; *Pi*, *Phytophthora infestans*; *Pm*, *Pythium monospermum*; *Pp*, *Phytophthora parasitica*; *Ps*, *Phytophthora sojae*; *Sc*, *Streptomyces coelicolor*; *Vd*, *Verticillim dahlia*; *Vp*, *Vibrio pommerensis*. (B) A schematic illustration of the Type I and Type II NPP1 domains, showing the number and position of conserved cysteine residues. C, A graphic representation of amino acid conservation within the central region the NPP1 domain, showing the position of a hepta-peptide motif "GHRHDWE". Amino acids are represented as single letters, the height which corresponds to the frequency at a particular position, among the 27 NLP sequences from GenBank. The image was drawn using WebLogo and the letters are coloured according to default values of the program, based upon the properties of the amino acid side chains (Crooks et al., 2004).

The spectrum and relatedness of NLP sequences from various organisms is illustrated in Fig. 1A. This phylogram was constructed from sequences currently retrievable from GenBank. Most of the protein sequences are predicted from gene calling programs, without other evidence, so some may be pseudogenes. Nonetheless, the comparison provides new information about the evolution of NLPs. By examining the sequence alignments used to construct the phylogram, it is apparent that the NPP1 domain may contain either two or four conserved cysteine residues, as shown in Fig. 1B. We refer to these as Type I and Type II, respectively. At the deepest node, two main branches of the tree separate proteins with Type I or Type II NPP1 domains, indicating that it was an ancient event in NLP evolution that led to this fundamental bifurcation. Type I domains are present in fungi, oomycetes, and bacteria. The Type II domain occurs only in fungi and bacteria, and is thus far absent from oomycetes. Representative genes encoding both Type I and Type II domains may occur in a single fungal species. For example, the genome of *Magnaporthe grisea* encodes four open reading frames (ORFs) with NPP1 domains, two each of Type I and Type II.

Besides the cysteine residues, a characteristic feature of the NPP1 domain in both Type I and Type II sequences is a central conserved region with the hepta-peptide motif 'GHRHDWE', as shown in Fig. 1C. Other residues scattered throughout the NPP1 domain also show high conservation. With a few possible exceptions, all NLPs appear to have amino terminal signal peptides targeting them for secretion.

Horizontal gene transfer has been suggested as one mechanism to account for the occurrence of NLPs in diverse organisms, such as fungi and bacteria. The distribution of bacterial NLP sequences within the phylogram shows that the bacterial proteins are not monophyletic but are interspersed with sequences from fungal proteins. The nucleotide composition of certain bacterial NLP genes has also been cited as evidence of acquisition of these genes from other organisms (Pemberton and Salmond, 2004). Thus, it remains a reasonable and likely possibility that bacterial species obtained their NLPs via horizontal gene transfer.

3. Genome sequencing in *Phytophthora* reveals large NLP gene families

There are approximately 65 species in the genus *Phytophthora*. Every species in this genus is a plant pathogen. Collectively, *Phytophthora* species are among the most damaging plant pathogens on the globe, by any measure (Erwin and Ribeiro, 1996; Tyler, 2002; Kamoun, 2003). These organisms are oomycetes and are now classified separately from fungi (Cooke et al., 2000). The resemblance of oomycetes to fungi results from convergent evolution rather than from close ancestry (Latijnhouwers et al., 2003). Oomycetes are grouped together with Stramenopiles and are most closely related to heterkont algae (Baldauf et al., 2000).

Recently, the *Phytophthora sojae* and *Phytophthora ramorum* genomes have been fully sequenced and deter-

mined to have sizes of 95 and 65 Mbp, respectively (http://www.jgi.doe.gov). Both of these organisms are destructive plant pathogens; *P. sojae* causes stem and root rot of soybean, while *P. ramorum* is able to infect many different woody plants and is responsible for the sudden oak death epidemic currently spreading through California.

Analysis of the genome sequences demonstrates that the NLP family is large and diverse, and is encoded by 50–60 loci in each species, although more than half of the predicted genes are likely pseudogenes. The protein family appears to be evolving fast in *Phytophthora*, since protein sequence alignments result in many species-specific clusters of NLPs in *P. sojae* and *P. ramorum*.

Although the *Phytophthora* NLPs are exceedingly diverse the proteins nonetheless form a monophyletic group when compared to all other known proteins of this class, from omycetes, fungi, and bacteria. Thus, *Phytophthora* NLPs all contain Type I NPP1 domains and phlyograms constructed from all known NLP protein sequences place the *Phytophthora* proteins on a single, densely articulated branch. A new class of NLPs with an additional hydrophilic domain inserted between the signal peptide and NPP1 domain were also discovered to be present in the *P. sojae* and *P. ramorum* genomes. An example of a predicted *P. sojae* protein with a hydrophilic domain in shown in Fig. 2.The domain is characterized by a Gln-rich region followed by a Pro- and Thr-rich stretch, its total length varying from 80 to 130 aa. At least three different predicted genes encoding NLPs matched to *P. sojae* ESTs, including one with a Gln- and Pro-rich hydrophilic domain, providing evidence that multiple and varied NLPs may be expressed by a single *Phytophthora* species.

Recent work has also identified an expressed gene from *Hyaloperonospora parasitica* that encodes an NLP with a similar Gln-rich hydrophilic domain (G. Van den Ackerveken, personal communication). This plant pathogen causes downy mildew of Arabidopsis plants (Slusarenko and Schlaich, 2003). *H. parasitica* is an oomycete but it is different in its lifestyle and reproduction from *Phytophthora* species; *H. parasitica* is a biotroph whereas most *Phytophthora* species are considered hemibiotrophs (Birch and Cooke, 2004; Gijzen, 2004).

The large size of the NLP family discovered in *Phytophthora* species is in contrast to NLP gene copy number in other organisms. Whole genome sequencing of *M. grisea* and *Gibberella zeae* indicates that each of these fungal plant pathogens possesses four genes encoding NLPs. The genomes of *Aspergillus nidulans* and *Aspergillus fumigatus* each contain two NLPs, and a single gene is present in the *Neurospora crassa* genome; these fungal species are not known to be plant pathogens but *A. fumigatus* causes more opportunistic infections in humans than any other fungus (Denning, 1998). Genes encoding NLPs have also been detected in single copy in a number of plant pathogenic and saprophytic bacterial species, but NLPs are certainly not a common feature of bacterial genomes. Overall, the evidence indicates that the *Phytophthora* NLP family arose from a single gene ancestor then greatly expanded and diversified during the evolution of this genus. It is noteworthy that *Phytophthora* species are primarily pathogens of dicotyledonous plants (Erwin and Ribeiro, 1996), mirroring the activity of NLPs. In any case, it is likely that there is an important role for NLPs in the pathogenic lifestyle of *Phytophthora*.

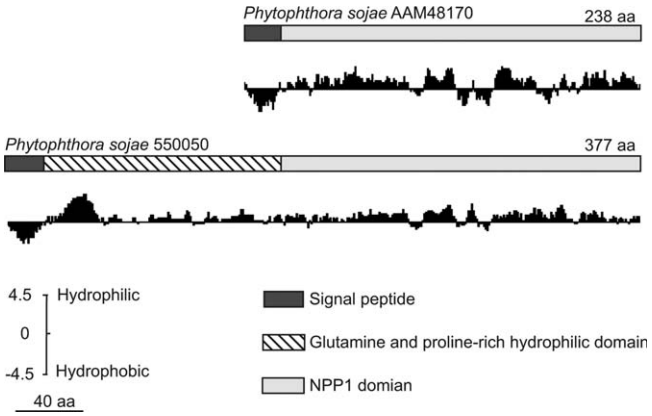

Fig. 2. A schematic illustration of domain structure and hydrophilic character of two NLPs from *Phytophthora sojae*. The upper protein corresponds to PsojNIP, identified by its GenBank accession number. The lower protein is predicted from the sequencing of the *P. sojae* genome (*Phytophthora sojae* v 1.0, http://www.jgi.doe.gov/), as a representative NLP containing a glutamine- and proline-rich hydrophilic domain. The total length of each protein in amino acid (aa) residues is indicated, and Kyle–Doolittle hydrophilicity plots are shown below the protein domain illustrations.

4. The activity of NLPs on plant cells: rapid activation of defence and death

A characteristic of many NLPs is their ability to trigger numerous plant defence responses, necrosis, and cell death in dicotyledonous plants (Jennings et al., 2001; Veit et al., 2001; Fellbrich et al., 2002; Keates et al., 2003; Verica et al., 2004; Bailey et al., 2005). Wilting is also a symptom that has been associated with the application of NLPs (Wang et al., 2004). Plant sensitivity to NLPs may vary with development (Bailey et al., 1997, 2005). Monocots are apparently not affected by NLPs (Bailey, 1995; Fellbrich et al., 2002), but more testing needs to be done with a greater variety of species. The responses of conifers, ferns, mosses, algae, and other plant groups to NLPs have not been documented and should also be examined. Likewise, the sensitivity of animal and fungal cells, and bacteria, to NLPs has not been thoroughly investigated but unpublished experiments and anecdotal reports suggest that cells from these species are not affected by NLPs, with one possible exception. The haemolytic activity of *Vibrio pommerensis* against human and animal erythrocytes could be mapped to a region that contained a gene encoding an NLP but it is uncertain whether the NLP is necessary for cell haemolysis (Jores et al., 2003).

There is also evidence that different NLPs may vary in their ability to cause necrosis or cell death in a given plant species, and it is possible that certain NLPs may not have any activity in this regard. In a test of four different NLPs from *Phytophthora infestans*, only one (PiNPP1.1) was found to cause necrosis when expressed in plants using a binary potato virus X (PVX) based expression system (Kamoun, personal communication). Similarly, no necrosis-inducing activity can be associated with an NLP recently isolated from *H. parasitica* (G. Van den Ackerveken, personal communication). Finally, comparison of four different NLPs by PVX-based expression in *Nicotiana benthamiana* resulted in highly variable symptoms (Qutob et al., 2002). The NLPs tested were from an oomycete, a fungus, and two different bacteria, and the effects they caused when expressed in *N. benthamiana* ranged from a severe, spreading necrosis to no visible difference from negative-control inoculations. However, all the NLPs were effective in killing soybean cells when tested by transient expression via particle bombardment, so one should be cautious in comparing results from different assays. Different levels of protein expression or tissue wounding associated with each of the methods may have caused differences in the plant responses to the NLPs.

Although different assay methods may sometimes produce contradictory results, a variety of approaches to test the activities of NLPs can yield valuable information. For example, expression assays and protoplast experiments have implicated the outer surface of the plasma membrane of the plant cell as the site of action of NLPs. Transient expression of NLPs via particle bombardment of plant tissues has demonstrated that these proteins require a signal peptide to kill cells (Gijzen and Nürnberger, unpublished results). Exposure of protoplasts to purified NLPs also shows that isolated plant cells devoid of their cell wall retain their sensitivity to NLPs, by mounting defence responses and dying shortly after treatment (Fellbrich et al., 2002).

The structural requirements for the activity of NLPs were investigated by testing various peptides and site-directed mutants derived from the *Phytophthora parasitica* NPP1 sequence (Fellbrich et al., 2002). These results illustrate the importance of the conserved cysteine pair in Type I NLPs. This study also indicates that NLP activity cannot be reduced to a peptide motif or span of residues within the protein. The only alteration that was tolerated, without loss of activity, was a small deletion from the carboxy-terminal end of NPP1 (Fellbrich et al., 2002).

The genetic requirements for the activity of NLPs were investigated using mutants of Arabidopsis impaired in defined signalling pathways and by virus-induced gene silencing in *N. benthamiana*. By monitoring the induction of *PR1* gene expression as a measure of NLP responsiveness in Arabidopsis plants, it was shown that the *NDR1* and *PAD4* mutants were deficient in their ability to respond to NLPs (Fellbrich et al., 2002). Cell death and necrosis in *N. benthamiana* caused by expression of *P. infestans* NPP1.1 appeared to be reduced after virus-induced gene silencing of the defence signalling proteins SGT1, COI1, MEK2, NPR1 and TGA2.2, and the chaperone HSP90 (S. Kamoun, personal communication).

5. Are NLPs elicitors or toxins or both?

Plant cells actively responding to NLPs appear to enter into a hyper-defensive state prior to death. They release ethylene, activate MAP kinases, synthesize phytoalexins, induce PR gene transcription, elevate cytoplasmic Ca^{2+} levels, and display numerous other rapid and prolonged changes in physiology and gene expression (Jennings et al., 2001; Veit et al., 2001; Fellbrich et al., 2002; Keates et al., 2003; Verica et al., 2004, Bailey et al., 2005). These rapid and numerous defence responses that are activated in dicotyledonous plant cells in response to NLPs are comparable to the activities of well characterized elicitors, or PAMPs, such as flagellin protein from *Escherichia coli* (Felix et al., 1999) or the 42-kD transglutaminase from *Phytophthora* (Nürnberger et al., 1994; Brunner et al., 2002). There are also significant differences between NLPs and these well-known PAMPs, as summarized in Table 2. This shows that certain characteristics of NLPs are closer to those of enzymes or protein toxins than to elicitors or PAMPs. A key question is whether NLPs interact with pattern recognition receptors in host plants. An elicitor-mediated action of NLPs that is indirect, via the generation of active molecules, should also be considered as a possibility since this mode of action has been proposed for other PAMPs (Fliegmann et al., 2004; Ron and Avni, 2004).

Table 2
A comparison of the characteristics of Nep1-like proteins (NLPs) with two other proteins that are considered pathogen associated molecular patterns (PAMPs)

Characteristic	NLPs	Transglutaminase[a]	Flagellin[a]
Causes rapid activation of plant defence responses	Yes	Yes	Yes
Acts outside of plant cell on plasma membrane surface	Yes[b]	Yes[b]	Yes
Causes tissue necrosis and plant cell death	Yes	No	No
Activity is heat labile	Yes	No	No
Activity can be reduced to peptide fragment	No	Yes	Yes
		Pep-13	*flg 22*
Spectrum of host plant species sensitive to protein	Wide[c]	Narrow[c]	Narrow[c]
	Dicots	*Parsley*	*Arabidopsis*
		Potato	*Tomato*
			Tobacco

[a] Transglutaminase is a 42-kD glycoprotein isolated from *Phytophthora parasitica* (Brunner et al., 2002), and flagellin is a flagella protein from *E. coli* (Zipfel and Felix, 2005).

[b] The receptors or molecular targets for transglutaminase and NLPs have not yet been identified, but existing evidence indicates that these proteins act on the outer plasma membrane surface.

[c] The full spectrum of plants sensitive to each protein is not known.

Most investigators in the field seem to favour the elicitor or PAMP-meditated mode of action for NLPs. But the possibility that the proteins are directly toxic to plant cells by forming pores or by interacting with crucial molecular targets in the plasma membrane should not be ignored. These primary toxic effects may, in certain cases, rapidly activate endogenous cell death programs. However, few proteinaceous toxins from plant pathogenic organisms have been described (Walton, 1996). Those that have been identified are host-selective toxins and their molecular targets are not known (Quayyum et al., 2003; Manning et al., 2004; Oka et al., 2005). Nonetheless, there are numerous examples of well-characterized bacterial protein toxins that target animal cells. The mode-of-action of these toxins is diverse but it has been estimated that one-third of all such toxins assemble into pore-forming structures within host membranes (Parker and Feil, 2005). Host range and specificity of these pore-forming toxins may be dictated by molecules that occur on the surface of the target cell membrane. For example, the Cry family of *Bacillus thuringiensis* insecticidal proteins require membrane glycolipid receptors for toxin action (Griffitts et al., 2005). These toxins and many others display a modular structure, with separate domains for receptor binding and toxicity functions (Falnes and Sandvig, 2000; Griffitts and Aroian, 2005). The NLPs need to be closely examined to determine whether they share any features with well-characterized protein toxins. Attempts to date to demonstrate pore forming activity of NLPs has failed (Gijzen and Nürnberger, unpublished). These negative results may raise some doubts but do not eliminate pore formation as a mechanism of action of NLPs since the conditions or biological materials used in the experiments may not have been appropriate. We believe that it remains an open question as to whether NLPs may form pores or otherwise damage biological membranes.

6. A positive role for NLPs in the virulence of plant pathogens

The first *Nep1* genetic transformation experiments on *F. oxysporum* suggested that expression of *Nep1* did not affect the aggressiveness or virulence of this pathogen towards its host (Bailey et al., 2002). But now there is mounting evidence indicating a positive role of NLPs in virulence. There is both direct and indirect evidence that comes from a variety experiments on NLPs from fungal, bacterial, and oomycete species.

Transformation of the fungus *Colletotrichum coccodes* with *Nep1* from *F. oxysporum* dramatically increased the virulence and expanded the host range of this plant pathogen (Amsellem et al., 2002). The *Nep1* transformants of *C. coccodes* caused ''rapid withering death'' when inoculated onto seedlings of *Abutilon theophrasti*, whereas the untransformed *C. coccodes* cultures produced only mild disease symptoms (Amsellem et al., 2002). The *Nep1*-transformants could also infect and kill tomato and tobacco plants that were resistant to wild-type cultures of the pathogen. The authors concluded that engineering *Nep1* expression into *C. coccodes* created a new hypervirulent form of the pathogen.

The virulence of the *Erwinia carotovora* is also affected by expression of its endogenous NLP. This bacteria is a plant pathogen that causes soft rot of host tissues. Independent experiments by separate investigators on the *E. carotovora* necrosis inducing protein (Nip) yielded similar results. The expression of the *E. carotovora Nip* gene leads to larger disease lesions and more rot of host tissues compared to identical strains that lack the gene (Mattinen et al., 2004; Pemberton et al., 2005). In one case, these experiments were performed on two different subspecies of the pathogen (Pemberton et al., 2005). This work additionally shows that the *E. carotovora Nip* gene is under control of the quorum sensing signal molecule, *N*-(3-oxohexanoyl)-L-homoserine lactone, providing more evidence that the NLP gene product has a role in virulence.

Patterns of NLP gene expression in oomycetes also suggest that these proteins facilitate infection. After inoculation of soybean plants with zoospores of *P. sojae*, transcripts encoding the necrosis-inducing protein (PsojNIP) reached their highest level after transition from biotrophy to necrotrophy (Qutob et al., 2002). The destructive necrotrophic phase of growth occurs when the pathogen rapidly spreads and kills host cells, and coincides with the expression of many pathogen genes encoding secreted hydrolytic enzymes (Qutob et al., 2000; Moy et al., 2004).

Finally, it is important to consider the experiments and field trials using purified Nep1 from *F. oxysporum*. This work indicates that Nep1 can kill plants when applied together with surfactants as a foliar spray (Bailey et al., 2000b, Jennings et al., 2000; Gronwald et al., 2004). Nep1 also enhances disease and promotes the effectiveness of live biocontrol agents when it is included in a formulation designed to kill targeted plant species (Bailey et al., 2000a).

Taken together, the results from these many different experiments provide a strong argument for a positive role of NLPs as virulence factors in plant–pathogen interactions. This hypothesis is compelling, but it also raises other questions to the forefront. How do NLPs contribute to the virulence of pathogens? Do NLPs also have a role in the virulence of pathogens that infect monocotyledonous plants? Why are genes encoding NLPs also found in saprophytic organisms, biotrophic plant pathogens, and even opportunistic human pathogens? Most importantly, what is the molecular basis of NLP activity and specificity?

7. Summary

It has been 10 years since the report of Nep1 from *F. oxysporum* but widespread interest in NLPs has only occurred in the last few years. The realization that NLPs are prevalent, especially in plant pathogenic organisms, and that the proteins may contribute to virulence has fostered this

interest. Different approaches to investigating NLPs, pursued by plant pathologists, microbiologists, geneticists, and biochemists, are now beginning to yield new information. However, the mechanism of action of NLPs on plant cells is an unresolved and pressing issue. The NLPs could very well function as both elicitors and toxins depending on the pathosystem or even the developmental stage of the infection. Biochemical components of NLP mediated cell death seem to overlap with those mediated by plant resistance (R) proteins but evidence so far is preliminary. In our view, there are two key experiments that are most likely to broaden and deepen our understanding of NLP function. First, the isolation of plant mutants insensitive to NLPs and the identification of the corresponding genetic loci could be enormously useful and informative. Genetic loci that condition insensitivity to NLPs could point directly to the molecular targets of NLP action, or implicate certain pathways or structures. Second, the determination of the three dimensional structure of an NLP could provide guidance regarding NLP function. The structural data may reveal folds or other features that would indicate affinity for certain substrates or ligands. Both of these approaches are currently under active investigation. Thus, we are hopeful that a model for the molecular action of NLPs will emerge in the near future.

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Mark Gijzen is a Research Scientist at Agriculture and Agri-Food Canada in London Ontario. He completed his Ph.D. in Biochemistry at Washington State University in the laboratory of Professor Rodney Croteau. His current research includes work on *Phytophthora sojae*, the cause of root rot in soybean, to identify and characterize pathogen factors that determine host range and virulence.

Thorsten Nürnberger is Chair of Plant Biochemistry at the University of Tübingen, Germany. He completed his PhD in Biochemistry at Martin-Luther-University Halle-Wittenberg in the laboratory of Prof. Dieter Schlee/Dr. Konrad Glund. His current research focuses on the identification and characterization of pathogen-associated molecular patterns and their mode of perception by plant cells.