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Activity and Phylogenetics of the Broadly Occurring Family of Microbial Nep1-Like Proteins

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Abstract

Necrosis- and ethylene-inducing peptide 1 (Nep1)-like proteins (NLP) have an extremely broad taxonomic distribution; they occur in bacteria, fungi, and oomycetes. NLPs come in two forms, those that are cytotoxic to eudicot plants and those that are noncytotoxic. Cytotoxic NLPs bind to glycosyl inositol phosphoryl ceramide (GIPC) sphingolipids that are abundant in the outer leaflet of plant plasma membranes. Binding allows the NLP to become cytolytic in eudicots but not monocots. The function of noncytotoxic NLPs remains enigmatic, but the expansion of *NLP* genes in oomycete genomes suggests they are important. Several plant species have evolved the capacity to recognize NLPs as molecular patterns and trigger plant immunity, e.g., *Arabidopsis thaliana* detects nlp peptides via the receptor-like protein RLP23. In this review, we provide a historical perspective from discovery to understanding of molecular mechanisms and describe the latest developments in the NLP field to shed light on these fascinating microbial proteins.

INTRODUCTION

Microbial plant pathogens have an extensive toolbox to manipulate host processes, enabling them to colonize and thrive on plant tissues (87). Pathogenic microbes evolved effector proteins that are highly specific in targeting host molecules, e.g., to suppress plant immune responses, thereby supporting the intimate interaction with living plant cells (93). Additionally, a large number of pathogens kill host cells during infection, allowing them to feed from dead plant tissues. This is the main strategy of necrotrophic pathogens. On the other extreme are obligate biotrophic pathogens that fully depend on living host plant tissue for their growth. Hemibiotrophic pathogens initially grow as biotrophs but later during infection switch to a necrotrophic lifestyle. To kill host cells, these microbes use different types of toxins that can either have broad activity or be host specific. Pathogen-produced toxins can be secondary metabolites, small peptides, or proteinaceous effectors (83, 98). These toxins can target intracellular host processes or act extracellularly on the exposed host plasma membrane or proteins therein (98). Here, we discuss the activity and phylogenetic distribution of necrosis- and ethylene-inducing peptide 1 (Nep1)-like proteins (NLPs), which can act as cytolytic toxins, immunogenic patterns, and/or noncytotoxic proteins with unknown function.

The first experimentally characterized member of the NLP family, the protein Nep1, was purified from culture filtrates of *Fusarium oxysporum* f. sp. *erythroxyli* (8), the fungus causing vascular wilt disease of coca (*Erythroxylum coca*). Nep1 was found to be a major constituent in culture filtrates of this fungus and induced necrosis as well as the production of the plant hormone ethylene when applied to coca leaves. Notably, when tested on a larger collection of plants, Nep1 induced necrosis on eudicot plants but not on monocots (8). Protein sequences obtained from the Nep1 N terminus and internal peptide fragments subsequently allowed the cloning of the *Nep1* gene of *F. oxysporum* (58). An N-terminal signal peptide (SP) of 24 amino acids was identified that mediates Nep1 export through the classical secretion route. However, the mature protein in the culture filtrate was found to start at amino acid position 32, suggesting that the N terminus is further processed, possibly by secreted fungal proteases, similar to the secreted AVR9 effector of *Cladosporium fulvum* (91). Similarly, NLPs were subsequently identified in the oomycetes *Pythium aphanidermatum* (95) and several *Phytophthora* species (29, 30). Because NLPs lack similarity to any previously described proteins or functionally characterized domains (33, 58), NLPs constitute a novel family that can be defined by a conserved protein domain. Therefore, a Pfam domain named the NPP1 domain (PF05630) was developed (30). Pfam domains contain information on amino acid conservations, which are derived from sequence comparison, and thus can be used to rapidly discover protein sequences with similar domains in proteomes. Two major amino acid patterns are conserved in NLPs: (*a*) a heptapeptide motif with the sequence GHRHDWE in the central region of the protein, of which -HRH-W- (where the hyphens indicate any amino acid) is the conserved core (**Figure 1*a***), and (*b*) two highly conserved cysteine residues in the N-terminal half of NLPs. We outline the role of these features in the NLP protein structure and corresponding biochemical activity below.

OCCURRENCE AND PHYLOGENETICS OF *NLP* GENES

NLP Genes Have a Broad Taxonomic Distribution

Members of the NLP family have previously been identified to occur in bacterial and eukaryotic microbes (33, 60, 62). To determine the taxonomic distribution of NLPs, we queried the proteomes of 9,090 Bacteria, 479 Archaea, and 1,095 eukaryotes (**Figure 1** caption for details). In total, we identified 1,794 NLP homologs (**Supplemental Table 1**), of which the majority (~80%) contain

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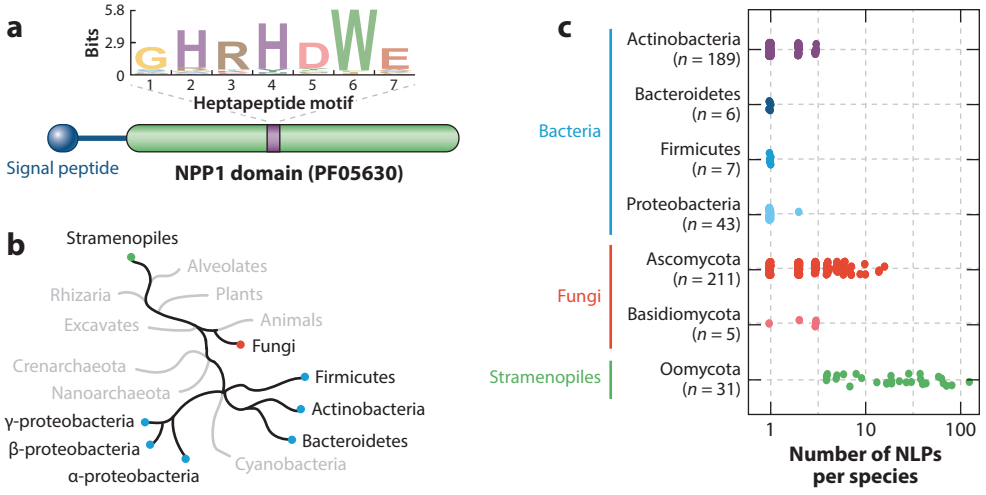

Figure 1

Nep1-like proteins (NLPs) occur in three different kingdoms of life. (*a*) Schematic overview of an NLP protein indicating the presence of an N-terminal signal peptide and the location of the conserved heptapeptide motif within the NPP1 domain (PF05630). (*b*) The taxonomic distribution of NLP proteins was determined by systematically searching an extensive protein database covering the reference proteomes of 10,664 species throughout the tree of life from the UniProt database (89) (release February 28, 2018; could also include predicted proteins from pseudogenes) and of 29 additional oomycete predicted proteomes (55). The database was queried for the occurrence of NLP domains using a previously defined hidden Markov model (profile HMM) of the NPP1 domain (PF05630.10), which mainly covers type I and II NLPs (60), as well as a profile HMM based on previously identified mature type III NLPs (60) to query the predicted proteins using HMMER (3.1b2; e-value cutoff 1e-3) (27). Proteins containing matches to these two profile HMMs were collected; overlapping and/or local profile HMM matches were manually curated. The occurrence of NLPs within species belonging to major taxonomic groups is indicated (different taxonomic groups highlighted by different colors: stramenopiles in green, fungi in red, and bacteria in blue). Groups without identified NLPs are shown in light gray. (*c*) The number of identified NLP proteins per species within different taxonomic groups is plotted (for groups with five or more species; *n*, number of species per group), revealing large differences between species. Note that the *x* axis is in log scale.

a predicted SP that directs proteins to the secretory pathway for delivery into the extracellular environment (**Figure 1*a***). In the proteomes of more than ten thousand species NLPs could be identified in only 497 species (<5%), in line with previous observations (33, 60, 62). These species belong to multiple bacterial classes, including actinobacteria and α-, β-, and γ-proteobacteria, as well as to multiple fungal taxa and species within the oomycetes (**Figure 1*b***). Thus, NLPs have a broad taxonomic distribution, occurring in major bacterial and eukaryotic microbial phyla but are absent from archaea (Crenarchaeota and Nanoarchaeota) and higher eukaryotes such as animals and plants (**Figure 1*b***).

Microbial Nep1-like protein presence is correlated with plant-associated lifestyles. The number of species that encode NLPs differs considerably between different taxonomic groups (**Figure 1*c***). For instance, only a subset of actinobacteria encodes NLP proteins (189 of 1,215 reference proteomes). NLPs have been previously observed in many different fungal species (33, 60, 62): 60% of all ascomycete fungi (211 of 360 reference proteomes) contain NLPs, yet only ∼4% of the analyzed basidiomycete fungi do (**Figure 1*c***). Importantly, many species encoding NLPs have a plant-pathogenic lifestyle. In oomycetes, all NLP-containing species are

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phytopathogenic, whereas in fungi ~35% of NLP-containing genome-sequenced species are considered phytopathogenic, including well-known plant pathogens (**Supplemental Table 2**). Nevertheless, NLPs are also found in saprophytic (~45%) and animal-pathogenic fungi (~15%) (**Supplemental Table 2**), most of which are found in soil or compost and are associated with dead plant material. NLPs occur in multiple species belonging to the mainly saprophytic fungal genera *Aspergillus*, *Penicillium*, and *Neurospora*. For example, in nature, the model fungus *Neurospora crassa* can be found on burned vegetation (38) and the human-opportunistic fungus *Aspergillus fumigates* is ubiquitous in compost (84). Similarly, many NLP-containing bacteria, e.g., species within the Actinomycetes (15), are associated with the decay of plant material. The broad taxonomic distribution of NLPs (bacteria, fungi, and oomycetes) and their occurrence in species with distinct lifestyles therefore suggest roles of these proteins beyond pathogen virulence on plants.

Phylogenetic support for three Nep1-like protein types. Amino acid sequence and phylogenetic analyses could distinguish three distinct NLP types: type I, type II (33), and the more recently described type III (60). Type I and type II NLPs have been historically defined based on the occurrence of either two or four cysteine residues, of which the disulfide bridge formed by the conserved cysteines, present in both type I and type II, is essential for NLP activity (30). The second disulfide bridge in type II NLPs is not required for necrosis induction by the *Pectobacterium* (previously named *Erwinia*) *Pcc*NLP (also known as NLP_{Pcc} or Nip_{Ecc}) (60). A more striking difference is the presence of a predicted calcium-binding site, which is required for cytotoxicity as tested in *Pcc*NLP, in type II, but not in type I, NLPs (60). Phylogenetic analysis clearly distinguishes type I and II NLPs (33, 60) (**Figure 2**). Type I NLPs are the most abundant NLP type and can be found in bacteria, fungi, and, especially, oomycetes (**Figure 2**), where they subsequently have been divided into type I and type Ia based on the occurrence of characteristic amino acid substitutions (60). In contrast, type II NLPs have initially only been observed in bacteria and fungi (33, 60). However, recent analyses of 37 oomycete genomes and transcriptomes have led to the identification of a type II subclade that contains sequences of the oomycetes *Phytopythium vexans*, *Pilasporangium apinafurcum*, and *Pythium oligandrum* (36, 55), indicating that type II NLPs are also present in oomycetes (**Figure 2**). Previous phylogenetic analysis revealed the presence of an additional group of NLPs, type III, that occurred exclusively in ascomycete fungi (60). In contrast to type I and II NLPs, type III NLPs are less conserved and most contain six cysteine residues that potentially form three disulfide bridges (60). However, we observed an additional clade associated with ascomycete type III NLPs that also contains bacterial sequences (**Figure 2**), suggesting that type III NLPs might have a broader taxonomic distribution than previously thought. Notably, only a limited number (8%) of ascomycete species encode NLPs belonging to all three types, a phenomenon that occurs only in fungi (60). The occurrence of all three NLP types in ascomycetes, occasionally within the same species, suggests that the evolutionary origin of NLPs lies within this phylum (60).

Horizontal *NLP* gene transfer. The broad taxonomic distribution and phylogenetic association of *NLP* genes suggest that horizontal gene transfer (HGT)—the transfer of genetic material between unrelated species—contributed to their evolution (33, 60, 62, 72, 85). From ascomycetes, NLPs seem to have been distributed to different taxa by HGT. For example, the interspersed occurrence of bacterial NLPs within ascomycetes suggests that these bacterial taxa acquired their NLPs from ascomycete donors (**Figure 2**). The nucleotide composition of bacterial *NLP* sequences is often similar to the genome-wide average and thus suggests a more ancient acquisition by HGT (62). In a few cases, however, the nucleotide composition supports a recent acquisition. For instance, the nucleotide sequence of *Pca*NLP (also known as NIP_{Eca}) of the blackleg soft rot pathogen of potato *Pectobacterium carotovorum* subsp. *atroseptica* has a lower G+C content than

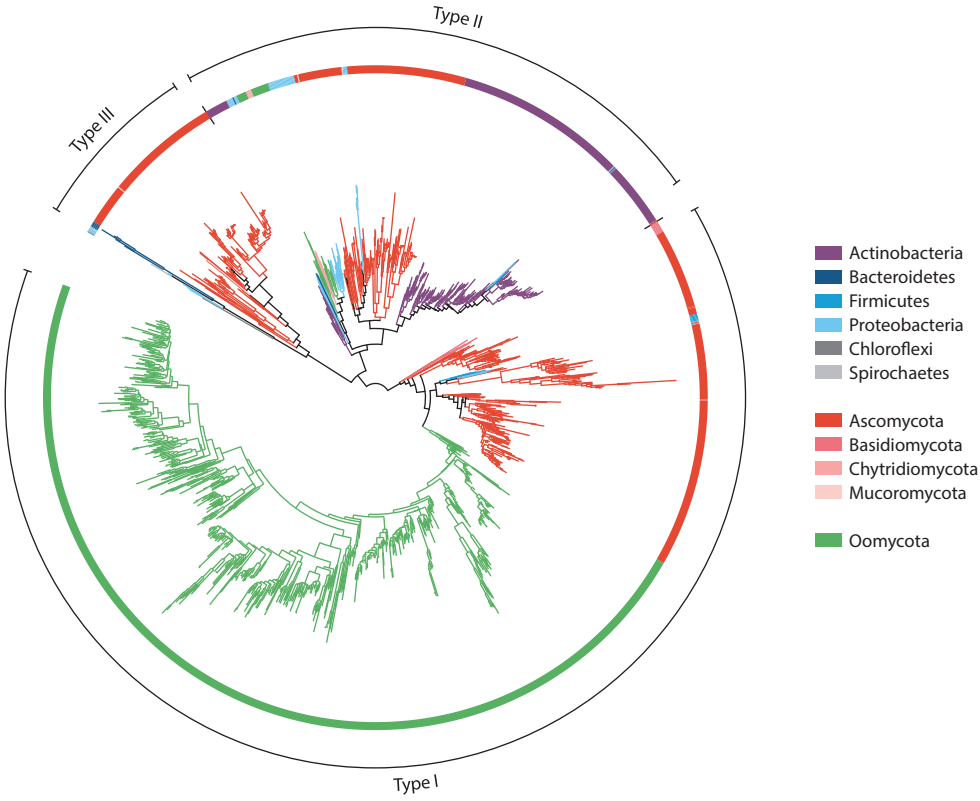

Figure 2

Phylogenetic tree showing diversification of Nep1-like proteins (NLPs). The maximum likelihood phylogeny was based on the previously identified NLPs (**Supplemental Table 1**), for which the match to the NPP1 domain (or type III NLP) was extracted and subsequently aligned using mafft (v7.271; L-INS-i) (46). Poorly aligned regions (alignment columns with a high number of gaps) were removed using trimAl (21). The maximum-likelihood phylogeny was inferred using RAxML [v8.1.15; Whelan and Goldman (WAG) amino acid substitutions model and accounting for site heterogeneity using the gamma distribution] (82). The association of individual sequences to taxonomic groups is indicated by color code (*right*). The three different NLP types are labeled (60).

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the genome average (62). Next to ascomycetes-to-bacteria cross-kingdom transfers, an HGT of NLPs likely occurred from ascomycetes to oomycetes (72). Whole-genome phylogenetic analyses revealed multiple fungus to oomycete HGTs that occurred early in the evolution of oomycetes and largely coincided with the radiation of oomycetes and their emerging capacity to colonize plants (72). One of these HGTs concerns the transfer of an *NLP* to an ancestral oomycete lineage that likely established type I NLPs within this taxonomic group (60, 72). Similarly, it is conceivable that the occurrence of type II NLPs in several oomycete species belonging to the genus *Pythium* is the consequence of an additional, independent HGT (55), likely from bacteria (**Figure 2**).

NLP* gene expansion.** The number of NLPs encoded by individual species differs considerably (**Figure 1*c). Most bacteria encode only one or two NLPs (**Figure 1*c***). Similarly, a considerable number of ascomycetes (~30%), many of which are saprophytes or pathogens of animals or monocotyledonous plant species (**Supplemental Table 2**), contain only a single *NLP* gene. For instance, the wheat pathogen *Zymoseptoria tritici* (previously known as *Mycosphaerella graminicola*) encodes a

single NLP (*Mg*NLP) that is expressed early during infection of susceptible hosts (57). This protein has cell death–inducing activity in leaves of the thale cress *Arabidopsis thaliana* but not in wheat, which is the host species for *Z. tritici*. Although most fungi (75%) only have up to three *NLPs*, some fungal taxa display remarkable expansion of the NLP family (**Figure 1*b***). Saprophytic as well as pathogenic members of the genus *Verticillium* encode an expanded NLP repertoire (75, 76, 100). For example, the vascular wilt pathogen *Verticillium dahliae* encodes eight NLPs (75, 100), of which two NLPs (NLP1 and NLP2) are cytotoxic. The related saprophyte *Verticillium tricorpus* encodes seven NLPs, two of which represent clear orthologs to the cytotoxic NLP1 and NLP2 proteins (76). Gene family expansion has also been observed for members of the genus *Colletotrichum*, in which some species encode up to 14 NLPs (12). Notably, *Colletotrichum* species with broader host ranges compared to their close relatives display increased NLP numbers (12), suggesting that the expansion of NLPs might contribute to the broad host range of these pathogens (12, 49, 75).

The Expanded Family of Oomycete Nep1-Like Proteins

One of the most striking expansions of NLPs within the tree of life occurred within the oomycetes following the HGTs that independently established type I and type II NLPs within this phylum (55, 72). NLPs have been identified in many oomycete genomes (3, 5, 7, 13, 17, 18, 22, 35, 51, 55, 79, 88), but their presence has been restricted to the Peronosporales and Pythiales, two groups that contain mainly plant-pathogenic species but also a few pathogens of mammals and fungi (within the Pythiales). Notably, *NLPs* are lacking in the obligate biotrophic Albuginales as well as in the marine and freshwater pathogenic Saprolegniales (42, 47, 52, 55, 60). The absence of *NLPs* in Albuginales and Saprolegniales suggests that the HGTs of the initial *NLP* occurred at either the last common ancestor of Peronosporales and Pythiales or the last common ancestor of oomycetes, followed by losses of *NLPs* in some oomycete groups (Albuginales and Saprolegniales).

Genomic organization of oomycete *NLPs*. Oomycetes have highly dynamic genomes that evolve by repeat-driven genome expansions (3, 35, 67, 88, 92), extensive gene duplications and losses (79, 80, 88), gene fusions (43, 56, 79), and HGTs (56, 71, 72, 86). Similar to genomes of many other (plant) pathogens, oomycete genomes are organized in gene-dense regions containing most housekeeping genes and gene-sparse regions (larger intergenic distances) enriched for pathogenicity-related genes (35, 67, 68). This organization is particularly pronounced in the repeat-rich genome of the potato late blight pathogen *Phytophthora infestans* (74% of its genome is composed of repeats), where gene-sparse regions are enriched for structural variations and genes under positive selection (68). Genes belonging to pathogenicity-related gene families such as RXLR effectors or elicitins often colocalize (i.e., they cluster) within these gene-sparse and repeat-rich regions (35, 41, 67), and, consequently, this two-speed genome organization has been proposed to contribute to rapid evolution of pathogenicity-related gene families (26, 68, 77, 78). Notably, comparative genomics between *P. infestans* and four sister species revealed that the NLP family in *P. infestans* is also enriched for rapidly evolving genes and for genes localized in gene-sparse and repeat-rich regions (67).

Duplication as a mechanism of *NLP* gene expansion. *NLPs* form a highly dynamic gene family that is significantly expanded in oomycete genomes, where most species have more than 10 NLP encoding genes (**Figure 1*b***) (3, 5, 7, 13, 17, 22, 35, 51, 55, 88). Additionally, *NLP* genes often cluster in chromosomal segments (7, 17, 22, 25). For instance, the genome of the soybean root rot pathogen *Phytophthora sojae* contains 70 *NLP* genes, many of which are recently duplicated and occur in close proximity, e.g., in two chromosomal segments of 88 kb and 21 kb where eleven

and nine *NLP* genes are located, respectively (25). Gene duplication leads to genetic redundancy, thereby facilitating the emergence of novel or altered functions. It is conceivable that within ascomycetes, duplications and diversification gave rise to the three different NLP types (60). Similarly, oomycete type I NLPs (**Figure 2**) evolved by duplication and subsequent diversification in the lineage of the Peronosporales, leading, for example, to the emergence of type Ia NLPs (60). Additionally, extensive duplications led to many other lineage- and/or species-specific expansions (3, 5, 7, 13, 17, 22, 35, 51, 55, 88). Following duplication, *NLP* genes rapidly diversify. For instance, a subgroup of *P. sojae* and *P. infestans NLP* genes showed evidence of positive selection (25, 67). Gene amplification by duplication in combination with rapid sequence diversification can also lead to the formation of pseudogenes. For example, the expanded NLP repertoire of *P. sojae* (70 *NLPs*) contains 37 pseudogenes (25, 64). Similarly, the obligate biotrophic downy mildew *Hyaloperonospora arabidopsidis* contains 27 NLPs, of which 15 are considered pseudogenes (13, 17). Given the expansion of this family, the high number of pseudogenes, and the positive evolutionary selection of certain NLP genes, it is likely that NLPs play an important role in oomycete biology.

PLANT RESPONSES TO NEP1-LIKE PROTEINS ARE MULTIFACETED

Responses of plants to NLPs are very diverse and cannot be generalized, as they depend on both the kind of NLP and the plant species in which responses are tested. Cytotoxic NLPs can permeabilize the plant membrane in eudicots, leading to cytolysis and associated activation of plant responses, which are similar to those activated during plant immunity. In contrast, noncytotoxic NLPs do not have this toxic effect, even though they are often evolutionarily closely related to cytotoxic NLPs. However, they do have a biological effect: Noncytolytic NLPs trigger strong immune responses in *A. thaliana* and several other plant species. Notably, mutant or truncated cytotoxic NLPs that are no longer able to cause cell death can also trigger these immune responses. The fact that immunity can be activated by noncytotoxic NLPs, i.e., cell death–independent, was not yet known five years ago, and conceivably many of the previously observed responses to NLPs described in literature could be the result of a mix of different NLP activities resulting from (*a*) protein cytotoxicity and (*b*) immunogenicity of NLP patterns. Here, we provide a comprehensive overview of the different biological activities of NLPs (outlined in **Figure 3**) and discuss our current knowledge of the underlying molecular mechanisms.

Necrosis Resulting from Nep1-Like Protein–Induced Cell Death

NLPs were first identified from culture filtrates of different microbes by activity-guided purification, assaying fractionated samples for necrosis induction in plants, followed by protein sequencing (10, 54, 58). The cell death–inducing activity of these purified proteins, as well as of recombinantly produced NLPs, can be tested in different ways. Infiltration of protein into the leaf apoplast using a needleless syringe is an efficient and established method of protein delivery (95). Alternatively, NLPs can be taken up through the xylem and transported by the transpiration stream by placing leaf petioles in protein solution (8). Finally, NLPs have been applied to leaves by spraying a protein solution with a surfactant that allows the proteins to enter plant tissues (40). This latter method has been proposed as a herbicide to specifically kill eudicot plants (39). In all cases, plant tissue collapses within 24 h and initially stays green but will further develop into a necrotic lesion of dried-out tissue in the ensuing days.

An alternative method of testing for necrosis-inducing activity, which does not require first producing and purifying the recombinant protein, is by *Agrobacterium*-mediated transient gene expression (17, 45). This method is based on the efficient transfer of T-DNA from *Agrobacterium*

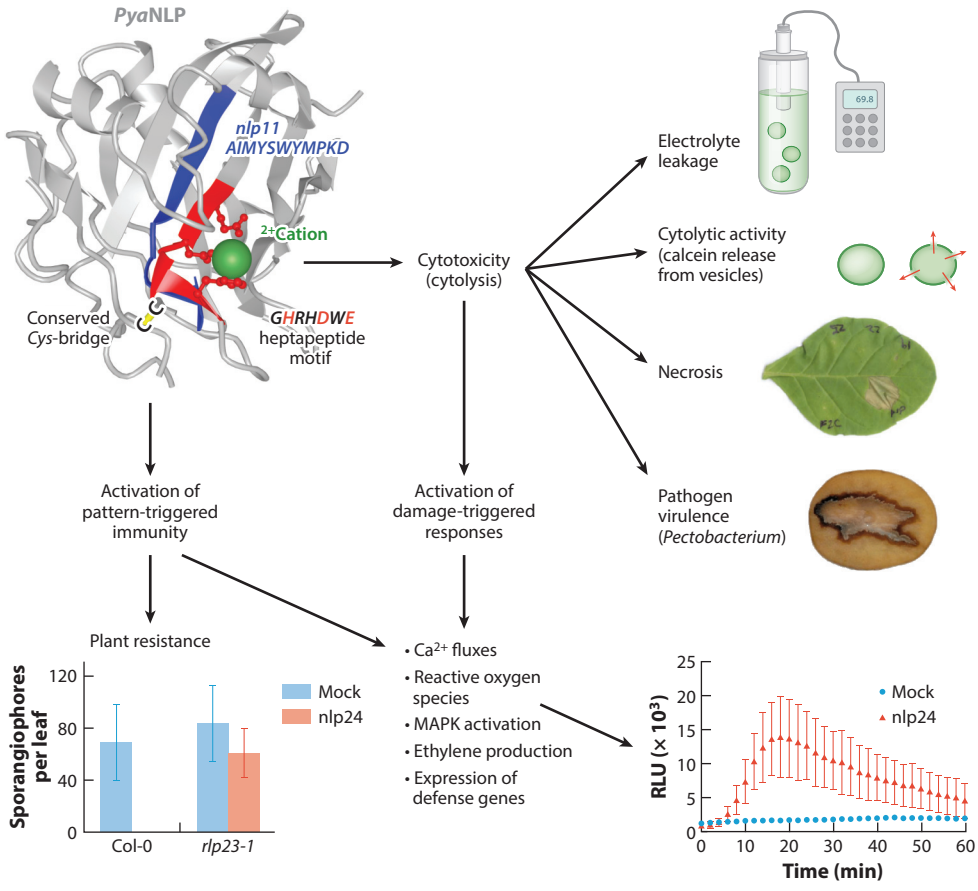

Figure 3

Activities of Nep1-like proteins (NLPs) on plants. Schematic overview of the two main activities of NLPs: cytotoxicity and immunogenicity. Cytolytic activity of NLPs can be assayed by measuring electrolyte leakage of treated leaf tissue (99) or the release of fluorescent calcein from vesicles made from plant-derived plasma membranes (61). The cytolytic activity causes cell death that can be visually scored as leaf necrosis following protein infiltration or *Agrobacterium*-mediated transient expression (8, 17). The cytotoxic activity of NLPs (e.g., *Pya*NLP shown in the top left corner) requires the conserved cysteine bridge (*yellow*), as well as the cation binding site (*red*) that is keeping the Mg^{2+} or Ca^{2+} (*green*) in the cavity (61), to be intact. Cell death also contributes to virulence in a number of pathogen species, e.g., in the potato soft rot bacterium *Pectobacterium carotovorum* (54). An important second activity of NLPs is the induction of plant defense through recognition by the plant immune system, e.g., the detection of peptide fragments of NLPs by the *Arabidopsis thaliana* RLP23 immune receptor (2). Even a small, eleven amino acid internal peptide of *Pya*NLP (nlp11; *blue*) is sufficient to trigger immunity, e.g., as shown in the bar graph for nlp24-induced resistance to downy mildew of *A. thaliana* (59). Interestingly, both the nlp pattern and the damage caused by cytolytic NLPs trigger plant responses that have strong immune signatures [e.g., Ca fluxes; reactive oxygen species, expressed as relative light units (RLUs) from luminol bioluminescence; mitogen-activated protein kinase (MAPK) activation; ethylene production; and defense gene expression] (16, 61, 65). Notably, this response is secondary in the case of cytolytic NLPs but primary for noncytotoxic NLPs.

tumefaciens to plant cells and the subsequent transient expression of cloned genes therein. In the case of transient NLP protein expression, full-length coding sequences are used to encode the SP of the pathogen. In case the SP is not effective in the plant, the mature protein-coding sequence can be fused to an efficient plant SP, e.g., that of the PR-1 protein (64, 65). Following secretion, cytotoxic NLPs cause cell death and tissue necrosis, making them extremely powerful and sensitive tools, in particular in the tobacco species *Nicotiana tabacum* and *Nicotiana benthamiana* (11), in which this method is highly efficient.

Nep1-Like Proteins as Virulence Factors

Cell death caused by cytotoxic NLPs could be beneficial for necrotrophic pathogens, which thrive on dead plant tissue, or for advancing to the necrotrophic stages of plant infection by hemibiotrophic pathogens. This idea was supported by the observation that cytolytic NLPs are not expressed early during infection but rather when the pathogen switches from biotrophy to necrotrophy. For example, NPP1.1 of *P. infestans* is expressed at four days post-inoculation, which is when the pathogen switches from a biotrophic to a necrotrophic lifestyle (44); PsojNIP of *P. sojae* is expressed during the transition from biotrophy to necrotrophy at two days post-inoculation (64). Similarly, the promoter of the cucurbit pathogen *Colletotrichum orbiculare NLP1* gene is activated, as assessed by expression of the *GFP* reporter gene, at late biotrophic and necrotrophic stages of infection but not during preinvasive or early biotrophic growth (37).

To test the importance of NLPs for virulence, mutants have been generated in different pathogen species, which resulted in very different phenotypes. A strong effect on virulence on potato tubers was observed upon inactivation of the single cytotoxic *NLP* genes in *Pectobacterium atroseptica* and *P. carotovorum*. In contrast, on potato stems, only the *P. atroseptica* mutant showed reduced virulence (54, 63). Owing to the clearly reduced virulence of *NLP* mutants, the activity of various cytotoxic NLPs and mutant variants from other species has been tested in the potato tuber assay as a readout of cytotoxicity-mediated virulence (50, 61). Similarly, two of the seven *NLP* genes of the tomato wilt fungus *V. dahliae* are cytotoxic (75). Deletion of *NLP1* or *NLP2* significantly reduced virulence on tomato (*Solanum lycopersicum*) and *A. thaliana* plants. Only the *NLP1* deletion mutant was compromised in virulence on Australian tobacco (*N. benthamiana*) and showed reduced fungal growth in vitro (75). Instead of loss of function, overexpression of a cytotoxic NLP increased fungal virulence on the weed *Abutilon theophrasti*, as was demonstrated for transformants of the fungus *Colletotrichum coccodes* that overexpress the *F. oxysporum Nep1* gene (4). These examples collectively demonstrate that, at least in a selection of pathosystems, NLPs can act as virulence factors.

In contrast, the virulence of *F. oxysporum* on coca plants was not affected by *Nep1* gene inactivation nor by *Nep1* overexpression, which did result in increased production of the protein from in vitro–grown mycelium (9). A similar lack of phenotype was observed in an *NLP* deletion mutant of *Z. tritici* (57). This monocot-infecting fungus encodes a single cytotoxic NLP that is expressed early during infection, but deletion of the gene did not affect fungal virulence nor did it impact asexual reproduction of the fungus (57). Similarly, the simultaneous deletion of four *NLP* genes, of which three encode cytotoxic NLPs, in the rice blast fungus *Magnaporthe oryzae* did not affect virulence on rice nor did it have any effect on the fungus grown in vitro (28). Because oomycete genomes encode an expanded NLP repertoire (**Figure 1*c***), gene deletions of all *NLPs* are not yet possible, and thus the role of cytotoxic NLPs in hemibiotrophic oomycetes remains unclear.

Cytolytic Activity of Nep1-Like Proteins

Determination of the crystal structure of cytotoxic *Pya*NLP (also known as NLP_{Pya} or PaNie) of the oomycete *Pythium aphanidermatum* was a milestone in NLP research (61). A second, highly

similar structure was obtained for the cytotoxic *Mp*NEP2 protein of the basidiomycete fungus *Moniliophthora perniciosa*, the causal agent of Witches' Broom disease of cacao (*Theobroma cacao*) (99). *Pya*NLP and *Mp*NEP2 are proteins with a central β-sandwich that resembles lectins (of fungi) and actinoporins (of sea anemones), two protein classes known to target cell membranes. Whereas lectins merely bind to sugar moieties (94), actinoporins insert into host membranes and form pores (74). Prompted by these observations, Ottmann and colleagues (61) tested the cytolytic activity of *Pya*NLP using small vesicles from isolated plant plasma membranes filled with the fluorescent dye calcein. Treatment of vesicles from the eudicots *A. thaliana* and *N. benthamiana* with *Pya*NLP, *Pcc*NLP, or *Pp*NLP (of *Phytophthora parasitica*) resulted in membrane disintegration and the rapid release of calcein into the medium, revealing that the proteins have cytolytic activity. In contrast, vesicles from the monocotyledonous Asiatic dayflower *Commelina communis* stayed intact, thereby confirming that these NLP proteins are toxic only to eudicot membranes. The rapid cytolytic activity is the most likely cause of toxicity on eudicot plant cells. Equally important, the NLP-induced activation of plant defenses is strictly associated with its cytolytic activity. Mutant NLP proteins with reduced or impaired cytotoxicity also displayed a similarly reduced activation of plant immune responses (61), which strongly argues for a cause and effect relationship in which cytolysis and cytotoxicity cause immune activation.

Eudicot-Specific Membrane Sugars Enable Cytolysis by Nep1-Like Proteins

Since the finding of differential necrosis-inducing activity of Nep1 in 1995 (8), the main question has been why eudicots and monocots differ in their sensitivity to cytotoxic NLPs. Recently, the mechanisms underlying this specificity have been unraveled in a ground-breaking study by Lenarčič and colleagues (50) and illustrated in an accompanying commentary (90). Cyanine3-labeled *Pp*NLP was used to study the binding of NLPs to plant membranes, showing that the protein quickly accumulated on the plasma membrane of *A. thaliana* protoplasts (within a minute), resulting in the collapse of cells within ten minutes. Because NLPs display structural similarity to sphingomyelin-binding actinoporins (61), *Pya*NLP binding to plant-specific sphingolipids was tested, revealing specific binding to glycosyl inositol phosphoryl ceramide (GIPC) (50). GIPC is the most abundant class of sphingolipids in plants, yet GIPCs also occur in fungi and protozoa (19). GIPC is composed of inositol phosphoceramide, by which it is anchored in the membrane, and a head group that consists of glucuronic acid and terminal hexoses (six-carbon sugars, such as glucosamine). Because *Pya*NLP was co-crystalized with free sugars, protein structures with sugar bound in the elongated crevice between the two loops L2 and L3 were obtained. *Pya*NLP can bind glucosamine or mannose, sugars that also occur in the headgroup of GIPCs (20). Amino acid residues of the highly conserved heptapeptide motif (**Figure 1*a***) are located in the crevice, of which the fifth aspartic acid and seventh glutamic acid residues bind Mg^{2+} (**Figure 4**, indicated in red). Strikingly, the second histidine in the heptapeptide motif, which normally binds Mg^{2+} via H_2O, is crucial for interacting with the sugar moiety. These observations led to a model in which, upon sugar binding by the L2–L3 loop, the crevice widens (50). This then allows Mg^{2+} to move more to the inside of the protein, resulting in a shift in protein conformation, making the L3 loop protrude and bringing an exposed tryptophan residue closer to the membrane. One can speculate that, similar to actinoporins (74), the L3 loop can then insert into the membrane and form a multimeric NLP structure that could act as a pore. Notably, the number and chemical structure of terminal hexoses differ between taxonomic groups, e.g., two hexoses in eudicots and three in monocots. Even though NLP can bind both eudicot and monocot GIPCs, the protein is proposed to interact only with the membrane in eudicot plants to exert its cytolytic activity. Thus, the reason pathogens of monocots produce cytotoxic NLPs that do not affect the monocot hosts remains

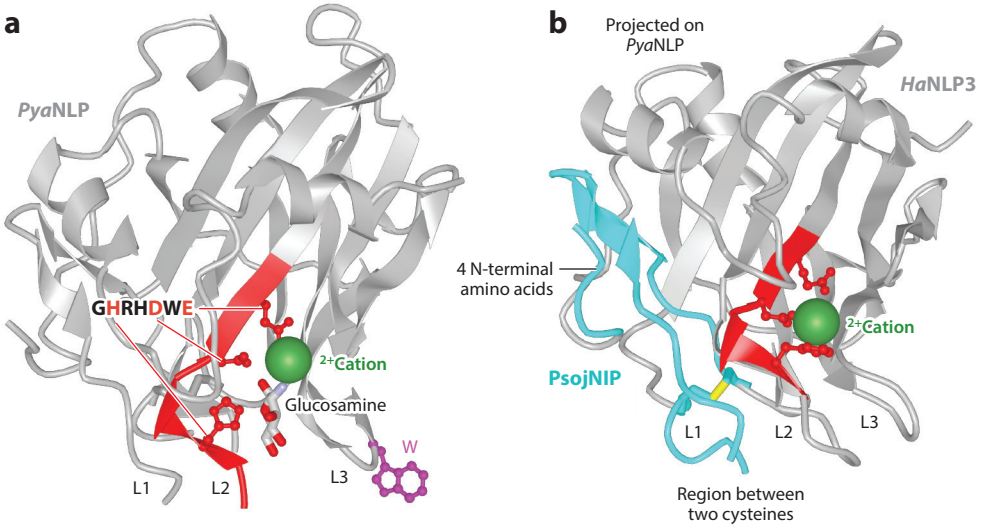

Figure 4

Nep1-like protein (NLP) structures. (*a*) Sugar binding changes conformation of *Pya*NLP, as observed when glucosamine was co-crystalized with the NLP protein (50). Structure determination revealed that the cation (*green*) in the elongated crevice between loops L2 and L3 has moved into the protein (compare to situation without sugar in panel *b*), and the histidine at position 2 of the heptapeptide motif (*red*) now interacts with the glucosamine molecule. The L3 loop, and its terminal tryptophan (W) residue, is predicted to move closer to the membrane and could thereby contribute to the cytolytic activity of *Pya*NLP. It should be noted that in vivo, *Pya*NLP would interact with the sugar head group of membrane-bound glycosyl inositol phosphoryl ceramide (GIPC) sphingolipids and not with free sugar monomers. (*b*) Projection of the noncytotoxic *Ha*NLP3 (*gray*) on the *Pya*NLP structure to show that the exchange of a minor portion of *Ha*NLP3 with two parts of the N-terminal regions of PsojNIP (*blue*) renders the protein cytotoxic (fusion 11) (17). This demonstrates that most of the *Ha*NLP3 structure, including loops L2 and L3, support a cytotoxic function.

unknown. NLPs of several monocot-infecting pathogens such as *Z. tritici* and *M. oryzae* are not required for virulence (28, 57). It could be that cytotoxicity is an evolutionary relic in these NLPs or their biological activity is not targeted to the host plant and unrelated to cytolysis of eudicot plant cells. Therefore, these could be functionally more similar to noncytotoxic NLPs.

What Is the Function of Noncytotoxic Nep1-Like Proteins?

The first noncytotoxic NLPs were observed in *P. infestans* when three cDNA NLP sequences were identified (44). When tested by transient expression in *N. benthamiana*, only PiNPP1.1, and not PiNPP1.2 or PiNPP1.3, induced necrosis. Genome analysis of different microbial species confirmed the presence of both cytotoxic and noncytotoxic NLP-encoding genes in each of the genomes (**Table 1**). For instance, 11 of the 18 tested NLPs of *P. sojae* are noncytotoxic (25). In contrast, all NLPs of the downy mildew *H. arabidopsidis* are noncytotoxic, as tested by transient expression in tobacco (13, 17). This was not surprising, as *H. arabidopsidis* depends on living host cells for growth and reproduction, and killing its host using cytolytic NLPs would be obstructive for its biotrophic lifestyle.

Noncytotoxic NLPs are not restricted to oomycetes; fungi also encode noncytotoxic NLPs. For instance, the genome of the phytopathogenic fungus *Colletotrichum higginsianum* encodes six NLPs, of which two were tested in *N. benthamiana* (48); ChNLP1 caused severe necrosis, whereas ChNLP3 did not. The expression of *ChNLP1* and *ChNLP2* peaks when the fungus switches from

Table 1 Number of cytotoxic versus noncytotoxic Nep1-like proteins (NLPs) in a broad range of microorganisms

Organism	Taxon	Lifestyle	Number of NLP genes[a]	Cytotoxic	Noncytotoxic	Reference
Phytophthora infestans	Oomycete	Hemibiotrophic	27	1	2	35, 44
Phytophthora sojae	Oomycete	Hemibiotrophic	33 (70)	7	11	25
Phytophthora capsici	Oomycete	Hemibiotrophic	39 (65)	4	6	22
Hyaloperonospora arabidopsidis	Oomycete	Obligate biotrophic	12 (27)	0	10	13, 17
Pythium aphanidermatum	Oomycete	Necrotrophic	5	1	ND	1, 95
Colletotrichum higginsianum	Fungus	Hemibiotrophic	6	1	1	48
Colletotrichum orbiculare	Fungus	Hemibiotrophic	7	1	ND	6, 31
Verticillium dahliae (*JR2*)	Fungus	Hemibiotrophic	8	2	6	75
V. dahliae (*V592*)	Fungus	Hemibiotrophic	9	2	7	100
Magnaporthe oryzae	Fungus	Hemibiotrophic	4	3	1	28
Botrytis cinerea	Fungus	Necrotrophic	2	2	0	23
Botrytis elliptica	Fungus	Necrotrophic	2	2	0	81
Sclerotinia sclerotiorum	Fungus	Necrotrophic	2	2	0	24
Zymoseptoria tritici	Fungus	Hemibiotrophic	1	1	0	57
Moniliophthora perniciosa	Fungus	Hemibiotrophic	3	2	ND	32
Pectobacterium carotovorum	Bacterium	Necrotrophic	1	1	0	54, 63

[a]Total number of genes, including pseudogenes, in parentheses (taken from cited references but may differ from the numbers presented in **Supplemental Table 1**, as these are based on our independent analyses, as described in **Figure 1**).
Abbreviation: ND, not determined.

Supplemental Material >

biotrophic to necrotrophic growth. In contrast, *ChNLP3* is mainly expressed during the appressorial stage, just like *ChNLP5* (48). Similarly, noncytotoxic NLPs also occur in the fungi *V. dahliae* (six out of eight) and *M. oryzae* (one out of four) (28, 75).

The noncytotoxic HaNLP3 protein of the oomycete *H. arabidopsidis* was studied in more detail (17) by making chimeras with the cytotoxic NLP PsojNIP of *P. sojae* (64). A chimeric protein, named fusion 11, was particularly informative: By exchanging a relatively small part in HaNLP3, namely the first four amino acids of the mature PsojNIP and its 25-amino-acid region between the two conserved cysteines, a cytotoxic chimera could be engineered in a noncytotoxic backbone (17). The exchanged region represents an exposed area on the protein that contains loop L1 but not the region encompassing the heptapeptide motif involved in cation binding and sphingolipid interaction (**Figure 4**), as was shown by comparison of the structure of *Pya*NLP (61). Surprisingly, the region corresponding to the GIPC-binding domain in the cytotoxic *Pya*NLP, loops L2 and L3 (50), appears to be functional in HaNLP3, as it contributes to the cytotoxic activity of fusion 11 (17). However, many other noncytotoxic NLPs have substitutions in the heptapeptide motif that are likely disturbing the cation binding pocket and GIPC binding site. Therefore, further biochemical and structural studies are needed to determine the molecular activities of noncytotoxic NLPs.

Nep1-Like Proteins as Immunity-Triggering Patterns

The activation of immune responses by cytolytic NLPs has been a common observation (29, 30, 44, 61, 65, 69, 95, 96). However, it has been unclear whether activation by cytolytic NLPs is indirectly the result of cell death responses or it was triggered directly by NLPs (16). Work on the noncytotoxic *Ha*NLPs of the downy mildew *H. arabidopsidis* revealed that these NLPs are potent triggers of *A. thaliana* immunity, thereby allowing the uncoupling of induction of immunity and cytotoxicity (59). It was a serendipitous discovery when *A. thaliana* transformants

expressing secreted *Ha*NLPs showed a strong growth reduction caused by hyperactivation of the plant immune system. Interestingly, truncations of the NLP transgene still led to reduced growth, indicating that an internal peptide of *Ha*NLP3 is sufficient to trigger the immune response. A synthetic peptide of only 24 amino acids (nlp24) is sufficient to induce *PR-1* expression, ethylene accumulation, and resistance to the downy mildew in *A. thaliana*. Notably, similar peptide fragments from a bacterial (*Bs*NPP1 of *Bacillus subtilis*) and fungal NLP (*Bc*Nep2 of *Botrytis cinerea*) also induced a strong immune response, showing that a conserved amino acid sequence in type I NLPs, which occur in three distinct kingdoms of life (**Figure 2**), can act as a pattern to trigger immunity. In contrast, a fragment of a type II NLP of *P. carotovorum* (*Pcc*NLP) did not activate immunity, suggesting that recognition in *A. thaliana* might be restricted to type I NLPs.

The uncoupling of cytolytic activity and immune responses were further corroborated by the independent observation that mutated and heat-inactivated *Pp*NLP (a cytotoxic type I NLP of the oomycete *P. parasitica*) lost its cytotoxic activity but remained a potent inducer of immunity in *A. thaliana*, as measured by the activation of the defense-associated genes *PR-1* and *PAD3*. In contrast, mutated and heat-inactivated *Pcc*NLP (a bacterial type II NLP) did not induce immunity in *A. thaliana* (16). Analysis of a set of synthetic peptide fragments of *Pp*NLP clearly showed that only two overlapping fragments are able to induce immune responses; an overlapping region of 14 amino acids was active as an inducer. However, a peptide of 20 amino acids (nlp20) containing most of the 14-amino-acid peptide and additional C-terminal amino acids was more potent. Similar nlp20 peptides from other oomycetes, fungi, and bacteria were equally potent inducers of immunity, confirming that *A. thaliana* can recognize a conserved pattern derived from NLPs occurring in three kingdoms of life.

Nep1-like protein detection by plant receptor-like protein RLP23. The plant immune system detects most known (pathogen-derived) patterns through perception by receptor-like proteins (RLPs) or receptor-like kinases (RLKs) (53). A breakthrough in revealing the perception of NLP patterns by the plant was the identification of the receptor complex mediating NLP-triggered immunity in *A. thaliana* (2). By screening a collection of *A. thaliana rlp/rlk* mutants for the loss of nlp20-induced responses, Albert and colleagues identified two T-DNA insertion lines that are both mutated in the *RLP23* gene (2). Interestingly, screening of 135 *A. thaliana* accessions revealed that three were unresponsive to the nlp20 peptide but not to the unrelated flg22 peptide derived from bacterial flagellin that is recognized through the RLK FLS2 (34). These three accessions all carry the same frame-shift mutation, leading to a premature stop codon and rendering RLP23 nonfunctional. RLP23 encodes for a receptor with an extracellular domain consisting of 27 leucine-rich repeats (LRRs), a transmembrane domain, and a small cytoplasmic tail of 17 amino acids (2). Genetic complementation in *A. thaliana* and the nonresponsive species tobacco, tomato, and potato confirmed the requirement of *RLP23* for nlp20 pattern recognition. Similarly, nlp20 peptides derived from the oomycete HaNLP3, the fungal BcNep2, and the bacterial BsNPP1 proteins are no longer detected by the *rlp23* mutant plants, indicating that this single receptor is sensing patterns from three different kingdoms of life. Subsequently, detailed biochemical studies revealed that the nlp20 peptide binds to the LRR ectodomain of RLP23 and that the receptor interacts with other RLKs to initiate signaling (2). In unchallenged plant tissue, RLP23 is associated with the RLK SOBIR1 (SUPPRESSOR OF BIR1-1), and upon nlp20 stimulation, a third partner becomes associated, the RLK BAK1 (BRASSINOSTEROID INSENSITIVE 1–ASSOCIATED RECEPTOR KINASE 1). The downstream immune responses triggered by nlp20 overlap partially with those triggered by the flg22 pattern, but there are also major differences (97). Treatment of *A. thaliana* leaf tissue with flg22 leads to earlier and stronger MAP kinase activation and production of reactive oxygen species (ROS) compared with nlp20. Similarly, transcriptional changes

are more extensive in flg22-treated tissue, with the nlp20-regulated genes representing only a fraction of the flg22-regulated genes. Whereas most downstream regulators appear to function in both immune signaling pathways, BIK1 (BOTRYTIS-INDUCED KINASE 1) acted as a positive regulator in response to flg22 but as a negative regulator in nlp20-induced immune responses (97).

The immune responses triggered by different NLPs are also effective in inducing systemic resistance to subsequent pathogen infection. Pretreatment of a few *A. thaliana* leaves one day before pathogen inoculation renders the whole plant resistant to infection by, for example, the oomycete downy mildew *H. arabidopsidis*, the bacterium *Pseudomonas syringae*, and the fungus *B. cinerea*. The systemic effect is absent in the *A. thaliana ald1* mutant, indicating that ALD1 activity is required (66). ALD1 is an enzyme needed for the production of the signaling molecule pipecolic acid that plays a key role in systemic-acquired resistance (14).

Breeding for resistance relies to a large extent on the transfer of immune receptors between plant genotypes via classical genetics or by using genetic engineering to allow transfer between species (73). Similar to other pattern-recognition receptors, RLP23 could therefore be used to enhance disease resistance and provide crops with new detection capabilities. Indeed, transformation of NLP-nonresponsive potato plants with a constitutively expressed *RLP23* construct resulted in transgenic lines with reduced disease susceptibility to the late blight pathogen *P. infestans* and to the white mold fungus *Sclerotinia sclerotiorum* (2). These two pathogens are known to secrete NLPs, which activate immune responses in *RLP23*-expressing potato lines (2). The broad occurrence of NLPs and the ability of RLP23 to sense patterns from three kingdoms of life suggest that transgenic expression of RLP23 provides a powerful approach to engineer broad-spectrum resistance to many destructive plant pathogens.

Perception of Nep1-like proteins in other plant species. Along with *A. thaliana*, nlp20 peptide recognition is also observed in a number of other related *Brassicaceae* species (2). For instance, the alpine rock-cress *Arabis alpina*, the field pennycress *Thlaspi arvense*, and the whitlow grass *Draba rigida* respond to nlp20 treatment. Notably, *Arabidopsis lyrata*, the sister species of *A. thaliana*, as well as Solanaceae plants, wheat, and parsley, did not respond to nlp20. However, lettuce (*Lactuca sativa*) shows a strong immune response to the nlp20 peptide and triggers immunity to the downy mildew pathogen *Bremia lactucae* (2, 66). Notably, NLP perception in lettuce differs from that in *A. thaliana*, as lettuce also responds to the type II *Pcc*NLP (66). No *RLP23* ortholog could be identified in the lettuce genome sequence (70), suggesting that NLP perception is mediated by another receptor as a result of convergent evolution.

A third NLP recognition specificity was recently discovered in cucumber (6) through the unexpected behavior of transformants of the fungus *C. orbiculare* constitutively expressing *CoNLP1*. In wild-type *C. orbiculare*, *NLP1* is expressed during late biotrophic and necrotrophic infection phases (37). Constitutive expression of *NLP1* in spores caused complete loss of virulence. Microscopic analysis revealed that the infection was arrested at early stages of infection and was accompanied by callose formation and the production of ROS. An overexpression line producing a noncytotoxic mutant NLP1 protein, with a substitution in the conserved histidine in the heptapeptide motif, was also unable to cause disease on cucumber plants, indicating that early abortion of infection is not caused by the cytotoxicity. This suggested that the NLP acts as a pattern triggering plant immunity, resembling recognition of nlp peptides by RLP23 in *A. thaliana*. CoNLP1 has a conserved region matching the nlp24 peptide of HaNLP3, and a synthetic peptide, Conlp24, was able to trigger a ROS burst in *A. thaliana*, whereas a mutant version (Conlp24Mut) was not. However, cucumber plants still respond to the CoNLP1Mut protein in which the same pattern was mutated. Deletion analysis showed that cucumber is able to respond

to the C-terminal part of CoNLP1, indicating cucumber perceives the protein through a different pattern. Expression of a fusion protein of the C-terminal 32 amino acids of CoNLP1 to a secreted mCherry (a red fluorescent protein) in *C. orbiculare* resulted in loss of virulence, indicating that the 32-amino-acid pattern is sufficient for activation of cucumber immunity. A synthetic peptide corresponding to the CoNLP1 C-terminal region, however, was not effective as an immunogenic pattern.

These observations suggest that at least three different plant perception systems for NLPs have evolved. Given the convergent evolution of plants to detect NLPs as microbe-associated molecular patterns, it is conceivable that NLPs are critical for the biology of a wide range of microbes. It can be anticipated that, along with cytotoxicity on eudicot plants, other NLP activities will be revealed in the near future, possibly on other organisms that associate with plants.

SUMMARY POINTS

1. Named after its founding member, the necrosis- and ethylene-inducing peptide 1 (Nep1), the family of Nep1-like proteins (NLPs) constitutes a large family of microbial secreted proteins occurring in bacteria, fungi, and oomycetes.
2. NLP genes are present in approximately 5% of analyzed species, which often have a plant-associated lifestyle.
3. Phytopathogenic oomycetes encode a large NLP repertoire that is often expanded through evolutionarily recent duplication.
4. Cytotoxic NLPs bind to plant cells via membrane sphingolipids and cause cytolysis and thereby toxicity.
5. In some necrotrophic or hemibiotrophic pathogens, cytotoxic NLPs can contribute to virulence, whereas in others NLP mutation has no effect.
6. Many noncytotoxic NLPs have been identified, but none is as of yet linked to virulence or microbial growth phenotypes.
7. A number of plant species (e.g., *A. thaliana*, lettuce, and cucumber) are able to detect NLPs as patterns and mount successful immune responses.

FUTURE ISSUES

1. To understand the mechanism by which cytotoxic NLPs lyse the membrane of eudicot plant cells, advanced biochemical and structural analysis methods are required to appreciate the protein changes and potential multimerization that are needed for membrane disruption.
2. The role of noncytolytic NLPs for microbial success in plant-associated environments is unknown but might be solved by studying their effect on other microbes occupying the same niche.
3. NLPs can be used to probe different plant species or accessions for pattern-recognition receptors able to respond to them; these novel receptors might be deployed to engineer resistance in crops.

DISCLOSURE STATEMENT

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