*Tansley insight*

# Disruption of plant plasma membrane by Nep1-like proteins in pathogen–plant interactions

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## Summary

Lipid membrane destruction by microbial pore-forming toxins (PFTs) is a ubiquitous mechanism of damage to animal cells, but is less prominent in plants. Nep1-like proteins (NLPs) secreted by phytopathogens that cause devastating crop diseases, such as potato late blight, represent the only family of microbial PFTs that effectively damage plant cells by disrupting the integrity of the plant plasma membrane. Recent research has elucidated the molecular mechanism of NLP-mediated membrane damage, which is unique among microbial PFTs and highly adapted to the plant membrane environment. In this review, we cover recent insight into how NLP cytolysins damage plant membranes and cause cell death.

## I. Introduction

Microbial plant pathogens use elaborate invasion strategies to successfully colonize plants, as they must overcome numerous physical barriers and inducible plant defenses. To this end, pathogens have evolved a variety of effectors, that is, proteins and small molecules, to manipulate host cellular processes and establish parasitic relationships (Dodds & Rathjen, 2010; Uhse & Djamei, 2018). Research on effector proteins secreted by pathogens during host attack has dominated the field of plant–microbe molecular interactions in the last two decades. In addition to structurally diverse effector proteins, many microbial plant pathogens produce nonproteinaceous cytotoxic compounds to kill their hosts (van't Slot & Knogge, 2002; Horbach *et al.*, 2011), such as fumonisins from various *Fusarium* species (Qu *et al.*, 2022), *Phomopsis amygdali* fusicoccin (Marra *et al.*, 2021), *Alternaria alternata* AAL toxin (Wang *et al.*, 2022), or *Cochliobolus victoriae* victorin (Lorang *et al.*, 2018).

Necrosis- and ethylene-inducing peptide 1 (Nep1)-like proteins (NLPs) are secreted effectors of phytopathogens that have two main roles in plant–pathogen interactions: they act as toxin-like virulence factors that induce tissue necrosis and trigger plant immune responses (Qutob *et al.*, 2006). As described in detail in a recent review by Seidl & Van den Ackerveken (2019), NLPs form one of the largest microbial protein families and are widely distributed among taxonomically unrelated microorganisms such as bacteria, fungi, and oomycetes. These can infect a variety of different crops, including potato, tomato, soybean, grapevine, and tobacco, resulting in enormous economic losses. For example, the oomycete *Phytophthora infestans*, the causal agent of epidemic late blight of potato, caused severe famine in the 19$^{th}$ century (Yoshida *et al.*, 2013) and still poses a major threat to potato and tomato production nearly 200 year later (Cooke *et al.*, 2011).



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Nep1-like proteins are the only known proteinaceous effectors that damage the plasma membrane of plants. In all organisms, the intact plasma membrane forms a barrier between the cell and the extracellular space and serves as a platform for orchestrating signal transduction. Disruption of membrane integrity and function is therefore a mechanism commonly used by toxin-producing organisms and venomous animals, as uncontrolled openings in the plasma membrane can often lead to cell death due to loss of ion gradients and vital nutrients (Peraro & van der Goot, 2016). In contrast to bacterial or mammalian plasma membranes, the composition and structure of plant plasma membranes has only recently begun to be deciphered and exhibits extreme complexity (Gronnier *et al.*, 2018; Mamode Cassim *et al.*, 2019). Glycosylinositol phosphorylceramides (GIPCs), a class of sphingolipids abundant in plants, have been identified as targets for NLP attachment to the plant membrane surface (Lenarčič *et al.*, 2017). Although the cytotoxic activity of NLPs has been known for more than two decades (Bailey, 1995; Veit *et al.*, 2001; Fellbrich *et al.*, 2002), the general molecular mechanism by which NLPs damage the plasma membrane of plants was unknown until recently (Pirc *et al.*, 2022).

## II. The toxicity and defense activation by NLPs

Nep1-like proteins have been shown to function as cytolytic toxins that disrupt the integrity of the plasma membrane of many eudicots, making them cytotoxic (Ottmann *et al.*, 2009). Non-cytotoxic members of the NLP family have also been reported, suggesting that NLPs have undergone functional diversification, including functions beyond host infection (Cabral *et al.*, 2012; Lenarčič *et al.*, 2019; Seidl & Van den Ackerveken, 2019). Similarities in three-dimensional structures between the NLP family fold and fungal lectins (Ottmann *et al.*, 2009; Lenarčič *et al.*, 2019) suggest lectin-like roles of noncytolytic NLPs in host adhesion. This view is also supported by the production of such proteins at very early time points during host infection by biotrophic or hemibiotrophic pathogens (Cabral *et al.*, 2012; Dong *et al.*, 2012; Santhanam *et al.*, 2013; Chen *et al.*, 2018). By contrast, cytolytic NLPs are expressed by necrotrophic plant pathogens at the onset of host infection or by hemibiotrophic plant pathogens during the transition from biotrophic to necrotrophic growth (Qutob *et al.*, 2006; Boevink *et al.*, 2020).

The deleterious effects of toxic NLPs on host membranes lead to the activation of defenses in many eudicot plants. This is likely caused by the damage-induced release of damage-associated molecular patterns that trigger defenses in cells adjacent to necrotic lesions (Ottmann *et al.*, 2009; Albert *et al.*, 2015). In addition, in Arabidopsis and related Brassicaceae species, NLPs trigger pathogen-associated molecular pattern (PAMP)-triggered immunity through AtRLP23 receptor-mediated perception of a small NLP-derived 20 amino acid-peptide, nlp20 (Böhm *et al.*, 2014; Oome *et al.*, 2014; Albert *et al.*, 2015).

The number of NLPs encoded in the genomes of individual microbial species varies considerably, with significantly higher numbers in the genomes of oomycetes such as *Phytophthora*, which implies important roles for these proteins in the life cycle of these pathogens (Seidl & Van den Ackerveken, 2019). Limited data are available on host infection-related synergistic functions of structurally different NLPs or of NLPs with other effectors (Boevink *et al.*, 2020). Moreover, pathogens may secrete a cocktail of effector proteins with redundant functions, all of which together contribute to necrosis (Leisen *et al.*, 2022).

Only a few NLPs have been characterized at the protein level, hampering our understanding of NLP dynamics and their role in pathogen life cycle and virulence. Of the > 1700 NLP homologs (Seidl & Van den Ackerveken, 2019), the structures of three NLPs have been published to date (Ottmann *et al.*, 2009; Zaparoli *et al.*, 2011; Lenarčič *et al.*, 2019). Nep1-like proteins are single-domain proteins with a central β-sandwich flanked by α-helices (Fig. 1a). A strongly negatively-charged cavity is formed by three wide loops on one side of the molecule (Fig. 1b), in which a divalent cation ($Mg^{2+}$ or $Ca^{2+}$) is bound (Fig. 1a). The amino acids involved in cation binding are highly conserved, and mutational analyses revealed that this region is critical for virulence and necrotic activities of the protein (Ottmann *et al.*, 2009). Recent advances in our understanding of how NLPs damage plant plasma membranes were made by demonstrating interactions of these proteins with plant-specific sphingolipids, GIPCs (Lenarčič *et al.*, 2017).

## III. GIPCs as receptors for NLP attachment to the membrane

Glycosylinositol phosphorylceramides are found almost exclusively in the outer leaflets of plant plasma membranes (Cacas *et al.*, 2016), in which they are crucial, abundant components that, together with plant-specific sterols, form scaffolds for the formation of lipid domains (Gronnier *et al.*, 2018; Mamode Cassim *et al.*, 2019). The structure of GIPCs is poorly understood, particularly with respect to the polar glycan heads, which contain a variable number of monosaccharides, from two to several (Fig. 1c). Interestingly, the GIPCs of monocots and eudicots can bear the same type of terminal sugars but in different numbers: predominantly two in eudicots (series A GIPC) and three (series B GIPC) or more in monocots (Cacas *et al.*, 2013; Fig. 1c).

Binding studies and X-ray crystallography revealed that the cytotoxic $NLP_{Pya}$ from the oomycete *Pythium aphanidermatum* (PDB: 3GNZ; Ottmann *et al.*, 2009), forms complexes with the terminal monomeric hexose unit of GIPCs (Lenarčič *et al.*, 2017; Fig. 1a). Binding of glucosamine or its epimer mannosamine near the divalent cation ($Mg^{2+}$) leads to several conformational changes, such as a widening of the $Mg^{2+}$-binding crevice and a $Mg^{2+}$ shift to the interior of the protein, suggesting that NLPs use this opening to bind to the GIPC head group. The insensitivity of monocot plants to NLP cytolysins could be explained by the length of the series B GIPC head groups, which results in insufficient proximity of the NLP molecule to the membrane surface for efficient membrane damage, as well as by the architecture of the NLP sugar-binding site (Lenarčič *et al.*, 2017). Notably, some monocot plants also produce substantial amounts of series A GIPCs in addition to series B GIPCs, making these plants sensitive to NLP cytotoxins (Lenarčič *et al.*, 2017; Steentjes *et al.*, 2022). From a recent study (Steentjes *et al.*, 2022), it can be concluded that a portion of > 20% of series A

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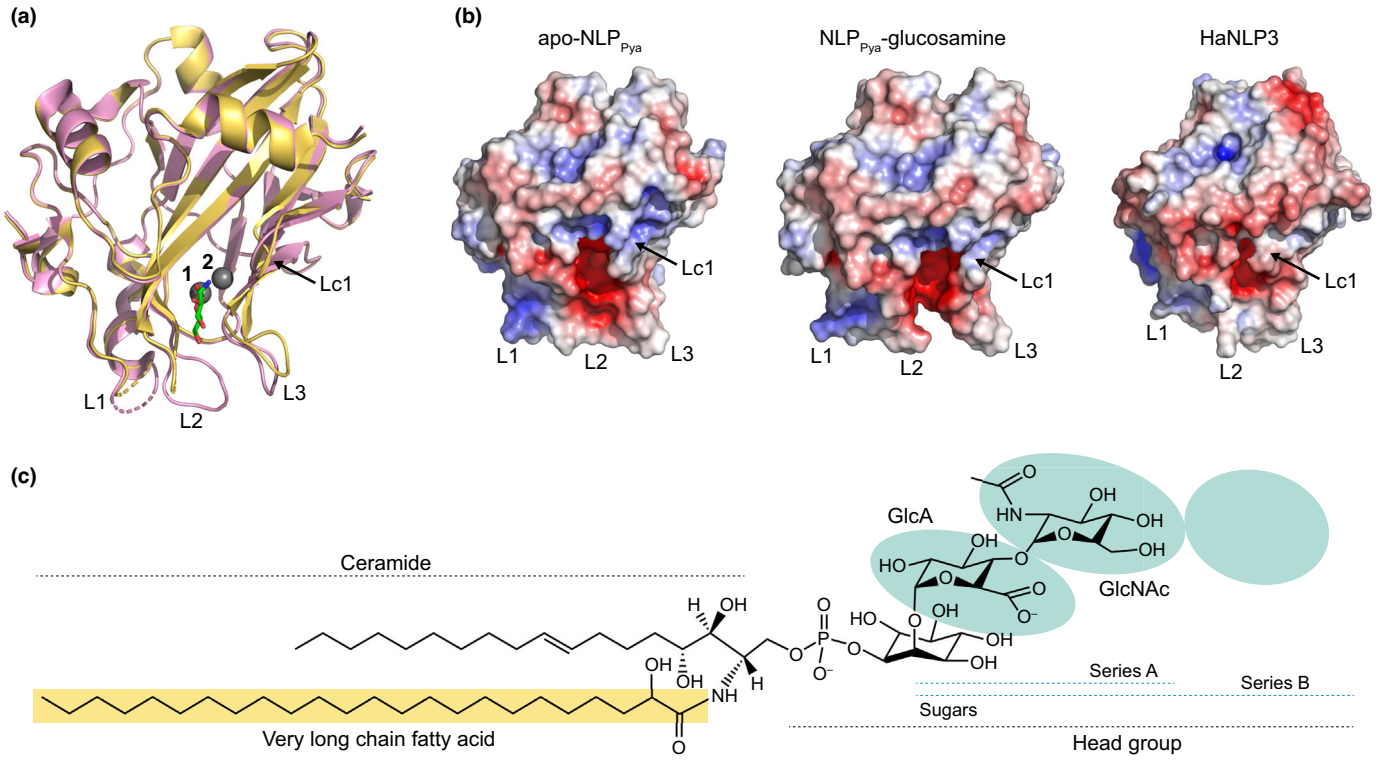


**Fig. 1** Structural features of Nep1-like proteins (NLPs) and its binding partner glycosylinositol phosphorylceramide (GIPC). (a) Structural comparison of NLP$_{Pya}$ in the absence (apo-NLP$_{Pya}$; magenta; PDB: 3GNZ) and presence of glucosamine (gold; PDB: 5NNW). Glucosamine is shown in green sticks. The $Mg^{2+}$ ion in the absence (position 1) and presence of glucosamine (position 2) is shown as a gray sphere. The loops Lc1, L1, L2, and L3 are indicated. (b) Comparison of protein surfaces of apo-NLP$_{Pya}$ (PDB: 3GNZ), glucosamine-NLP$_{Pya}$ complex (PDB: 5NNW), and HaNLP3 (PDB: 6QBE). Blue and red represent positive and negative charge, respectively. Loops are indicated. (c) Structure of GIPC. Glycosylinositol phosphorylceramides consist of a ceramide embedded in the membrane and an exposed head group containing inositol phosphoryl bound to glucuronic acid (GlcA) and terminal sugar(s), for example, hexose(R1), where R1 is a hydroxyl, amine, or *N*-acetylamine group (GlcNAc).

GIPCs relative to the total GIPC content may render monocot plants sensitive to NLP cytolysins.

Structural characterization of a GIPC-binding site in a noncytotoxic HaNLP3 from the oomycete *Hyaloperonospora arabidopsidis* revealed that the conformations of the loops surrounding the GIPC-binding site differ substantially from those of NLP$_{Pya}$ (Lenarčič *et al.*, 2019; Fig. 1b). Likewise, molecular dynamics (MD) simulations showed limited conformational plasticity of these loops (Lc1 in Fig. 1b) when compared to those of cytotoxic NLP$_{Pya}$ or MpNEP2, thus preventing GIPC binding by this noncytotoxic protein. Overall, these results suggest that the flexibility of the protein and the conformation of the loops surrounding the GIPC-binding site are important structural determinants for the specific recognition of a variety of saccharides present in GIPC head groups.

## IV. Mechanism of pore formation by NLPs

Pore formation in lipid membranes is a multistep process that usually begins with the binding of a soluble monomeric protein to the membrane via a specific lipid or protein receptor, followed by protein oligomerization and structural rearrangements leading to the formation of transmembrane pores (Anderluh & Lakey, 2008; Peraro & van der Goot, 2016). The NLP structure is similar to that of actinoporins, pore-forming toxins (PFTs) from sea anemones (Ottmann *et al.*, 2009). Actinoporins are among the best-studied PFTs that form pores in lipid membranes containing sphingomyelin, which serves as a lipid receptor (Rojko *et al.*, 2016; Fig. 2). In addition to sphingomyelin, cholesterol can also serve as a common receptor for the binding of various PFTs to animal plasma membranes (Peraro & van der Goot, 2016). However, none of these lipids are present in the plasma membrane of plants, and GIPCs are not found in animals.

Recent studies using GIPC-containing lipid model systems and a plethora of biophysical and computational approaches provided insights into NLP pore formation (Pirc *et al.*, 2022). The mechanism underlying NLP$_{Pya}$-induced membrane damage proceeds in several sequential steps, similar to actinoporins (Rojko *et al.*, 2013; Fig. 2), but differs from actinoporins in the final pore-forming step, as NLP pores do not insert deeply into the membrane (Fig. 2). During plant infection, NLPs are secreted into the extracellular space of host plants, the apoplast, and target the GIPC-rich surface of plant cells (Cacas *et al.*, 2016). The low ionic strength in the apoplast enables a predominantly electrostatically driven initial NLP interaction with anionic GIPCs. NLP$_{Pya}$ binds to the GIPC head group and remains bound to the membrane surface throughout the pore formation process. Nep1-like proteins associate into aggregates that are heterogeneous in size and shape



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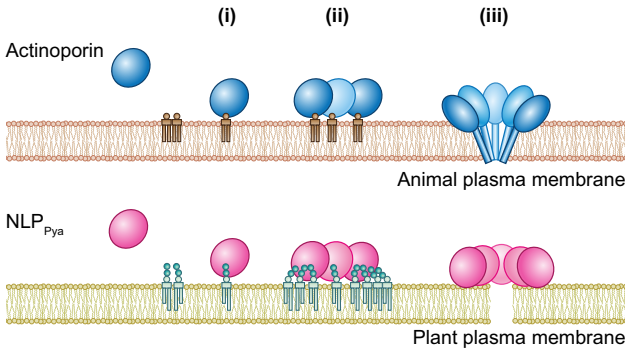


**Fig. 2** Pore formation by an actinoporin and Nep1-like protein (NLP). The mechanism of pore formation by an actinoporin and NLP$_{Pya}$ involves binding to a specific membrane lipid (i), protein oligomerization in the membrane plane (ii), and pore formation (iii). Note that actinoporin forms transmembrane pores (through α-helices formed from up to eight monomers), whereas NLP$_{Pya}$ does not cross the membrane but remains on the membrane surface. The current model suggests that NLP$_{Pya}$ forms small pores due to reorganization of GIPCs triggered by numerous contacts between NLP$_{Pya}$ and glycosylinositol phosphorylceramides.

and give rise to small, transient membrane openings (Pirc *et al.*, 2022).

Glycosylinositol phosphorylceramides are thought to play a structural role due to their long fatty acyl chains (Fig. 1c; Gronnier *et al.*, 2018). It remains unclear how NLP clusters induce small membrane ruptures. However, MD simulations suggest that single NLP molecules interact strongly with multiple GIPCs (Pirc *et al.*, 2022), which may lead to membrane restructuring and pore formation (Pirc *et al.*, 2022). However, because GIPCs also function as sensors of extracellular $Na^+$ and are part of a signaling cascade involving $Ca^{2+}$ influx (Jiang *et al.*, 2019), NLP-mediated perturbation of GIPC homeostasis may also affect cell integrity.

Nep1-like protein oligomerization and cell death have been reported to be promoted by an apoplastic leucine-rich repeat (LRR)-only protein in Arabidopsis (Chen *et al.*, 2021). The molecular mode of action of this protein in NLP-mediated host cell lysis remains elusive. However, NLP aggregates of varying sizes have been observed in plant membranes (Chen *et al.*, 2021), which may be similar to those observed in *in vitro* membrane systems that did not contain plant proteins (Pirc *et al.*, 2022).

## V. Conclusions

Membrane damage induced by NLPs follows the steps of a typical pore-forming process, that is, accumulation of protein at the membrane surface by binding to a specific receptor, protein oligomerization, and finally pore formation. However, NLP$_{Pya}$ does not penetrate deeply into the lipid bilayer, and the loss of membrane integrity could be due to the reorganization of GIPC lipids, leading to the appearance of transient small ruptures in the plasma membrane (Pirc *et al.*, 2022).

New insights into the interaction of toxic NLPs from devastating microbial pathogens with the lipid membrane of plants are pivotal for the development of better strategies for crop protection (Pirc *et al.*, 2021). NLPs represent an important and understudied protein superfamily that is of great importance for phytopharmaceutical use for the following reasons: (1) they play a crucial role in the pathogenesis of major microbial plant pathogens; (2) they have a wide taxonomic distribution and occur in three microbial lineages; (3) they act at the plant cell membrane and are therefore more amenable to phytochemical intervention strategies than intracellular effectors; and (4) they target an extremely conserved part of the plant cell, the GIPC sphingolipids, which may prevent pathogens from readily developing resistance to inhibitor molecules mimicking chemical structures of GIPC head groups.

Major open questions in our mechanistic understanding of NLP functions comprise of whether individual GIPC sugar head groups are targeted by structurally different NLPs produced by the same pathogen species, whereas certain GIPC head groups are not targeted at all. Alternatively, it remains to be investigated whether structurally different NLPs may have evolved to target different GIPC head groups that are found in the same or in different host plants. In addition, the precise molecular mechanisms underlying NLP-induced membrane damage associated with pore formation remain to be elucidated. For example, it is not yet fully understood which parts of the NLP molecule are involved in the interaction with the plant cell surface and which parts are responsible for the toxic effect. It is also not clear whether the molecular mechanism of membrane damage is common to all NLP cytolysins or whether structurally different NLPs may differ in their cytotoxic modes of action. The effects of membrane biophysical properties, such as the role of sterols in increasing the binding affinity of NLPs to GIPC-containing membranes (Pirc *et al.*, 2022) and the formation of lipid domains on the NLP–membrane interaction, require further research. To address these challenging questions, new lipid-based probes, model membrane, and plant plasma membrane systems need to be developed to gain more detailed insight into mechanistic functions of these interesting microbial effectors.

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