

# How plants differ in toxin-sensitivity

Toxicity to eudicots, but not monocots, is caused by differences in membrane lipids

By Guido Van den Ackerveken

**T**he story of a family of microbial proteins that are toxic to plants started in an unexpected way. In 1995, Bryan Bailey (of the U.S. Department of Agriculture) was studying fungi that could be used to destroy coca plants (which are used to produce cocaine). He studied a strain of the fungus *Fusarium oxysporum* that causes disease in these plants. From culture filtrates of the fungus, he purified a protein that when applied to coca induced necrosis (tissue cell death) and the production of the plant hormone ethylene, which is produced in response to environmental stress (1). The toxin was therefore named necrosis- and ethylene-inducing peptide 1 (Nep1). When tested on different plant species, he showed that Nep1 is toxic to eudicot plant species (such as tomato and bean) but not to monocots (such as cereals and leek) (1). Now, 22 years later, the molecular basis of specificity of members of the Nep1-like protein (NLP) family of toxins is described on page 1431 of this issue by Lenarčič *et al.* (2).

The NLPs have a broad taxonomic distribution, occurring in three kingdoms of life: bacteria, fungi, and oomycetes (3). NLP genes are most common in plant-associated microorganisms and have experienced large gene expansions in the genomes of oomycetes—for example, with 27 NLPs in *Phytophthora infestans*, the Irish potato famine pathogen (4). NLPs have an N-terminal signal peptide for secretion that enables microorganisms to expose plant cells to the proteins. The tissue necrosis that results from NLP activity can aid infection by necrotrophic pathogens that live on dead plant tissues, as exemplified by infection of potato tubers by the soft-rot bacterium *Pectobacterium carotovorum* (5).

The crystal structure of NLP from the oomycete plant pathogen *Pythium*

*aphanidermatum*, NLP<sub>Pya</sub>, revealed a striking structural similarity to lectins and actinoporins (6). These are soluble, single-domain proteins that, through their surface-exposed cavity, target membrane lipids by attaching to their sugar head groups. Whereas lectins merely adhere to carbohydrates, actinoporins, upon sugar head group binding, insert flexible N-terminal domains into the lipid membrane, forming a multimeric pore that causes cell lysis and toxicity (7). Eudicot plant membrane vesicles filled with a fluorescent dye were used to show that NLPs also have strong cytolytic activity caused by membrane disruption. By contrast, monocot vesicles stayed

Because the structure of NLP<sub>Pya</sub> resembles actinoporins, which are toxins of sea anemones that target the lipid sphingomyelin, they next investigated whether NLP membrane binding also occurred through lipids (2). Biochemical analyses revealed that the NLP<sub>Pya</sub> protein binds with high specificity to an abundant plant sphingolipid, glycosylinositol phosphorylceramide (GIPC). In plants, GIPC is a major constituent of membranes, comprising up to 40% of plasma membrane lipids and even up to 60 to 80% of lipids in the outer leaflet of the plasma membrane (8). GIPC consists of an inositol phosphoceramide, which keeps

the molecule in the membrane, and a head group consisting of glucuronic acid and a variable number and form of terminal hexoses (6-carbon sugars, such as glucosamine) (see the figure). The sugar moiety is exposed on the extracellular surface of the membrane and is thus accessible for binding. Different biochemical assays confirmed that NLPs bind GIPC with high affinity, but not any other lipids. Because the free sugars glucosamine and mannosamine can also bind to the NLP, they were used to determine crystal structures of the complex.

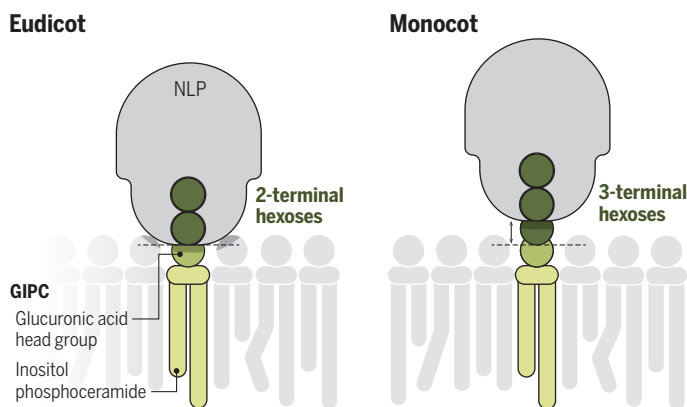
Importantly, sugar binding resulted in structural changes in the NLP<sub>Pya</sub>. An opening in the protein between loop L2 and L3, close to where Mg<sup>2+</sup> binds, changed conformation upon sugar binding. This suggests that NLPs use this opening to fit around the GIPC sugar head

group, allowing them to bind to plant membranes. Several experiments confirmed the requirement of GIPC in the plasma membrane and its exposed sugar groups for NLP binding and cytolytic activity. When sugar groups were enzymatically removed from plant membranes, NLP-induced cell lysis was reduced. Last, *A. thaliana* mutants with lower GIPC levels or altered sphingolipid profiles showed reduced sensitivity to the toxic effect of NLPs.

So, why are NLPs only cytotoxic to eudicots and not monocots? A major difference in the GIPCs between the two plant groups

## Model for NLP toxin specificity

NLPs bind exposed sugars (hexoses) on the head group of the plant membrane sphingolipid GIPC. The three hexoses of monocot GIPCs prevent the NLP from inserting into the membrane, whereas eudicot GIPCs have only two hexoses, allowing the NLP to contact and then insert into the lipid bilayer.



intact in this assay (6). Until now, it was unclear why the membranes of monocots and eudicots differ in NLP sensitivity.

The answer lies in sphingolipid differences in membranes between monocots and eudicots (2). Lenarčič *et al.* exposed individual plant cells of the eudicot *Arabidopsis thaliana* to labeled extracellular NLP and observed that the plant cell membranes quickly accumulated the toxin and cells lysed within minutes. Even protease-treated cells remained sensitive to the toxin, indicating that the NLP receptor on the plant cell membranes is not a protein.

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is in the number of sugar residues on the glucuronic acid head groups. Whereas eudicots mainly have A series GIPCs, which have two sugars linked to the glucuronic acid-inositol phosphoceramide, monocots predominantly have series B GIPCs, which have three sugars (9). One group of monocots—orchid species belonging to the genus *Phalaenopsis*—do contain both series A and B GIPCs and were found to be sensitive to NLPs. Strikingly, NLPs are able to bind to monocot GIPCs just as well as those of eudicots (2). It is proposed that the longer sugar head groups on monocot GIPCs preclude the NLP from interacting with the exposed membrane surface. This could prevent the NLP from inserting into the membrane and thereby safeguard the plant (see the figure). It remains unclear how the NLPs lyse the cell membrane. One can envision that a region of the protein inserts into the host membrane and that NLPs oligomerize to form a pore, similar to actinoporins (7). Future structural data on the NLP pore will hopefully clarify this issue.

Two main questions remain concerning the binding of NLPs to GIPCs. Why do microbial pathogens of monocots produce NLPs that are cytolytic when tested on eudicots but do not have a toxic effect on their monocot hosts? For several monocot pathogens, it was shown that evolutionary conserved NLP genes are not needed for virulence—for example, for the wheat fungus *Mycosphaerella graminicola* (which has a single NLP gene) or the rice blast fungus *Magnaporthe oryzae* (which has four NLP genes) (10, 11). The other big question relates to the many noncytotoxic NLP family members that have been identified (3, 12). Do they also bind to GIPCs, and in what way would that aid the pathogens that express them during plant infection? The structural knowledge on the NLP-GIPC interaction provided by Lenarčič *et al.* can now help to answer these questions. ■

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## ENGINEERING

# Rethinking digital manufacturing with polymers

## Data-driven approaches and improved resin materials can expand applications

By Justin Poelma and Jason Rolland

**A**dditive manufacturing (AM) is poised to radically change the way objects are manufactured, ranging from critical applications such as aircraft components and medical devices to more commonplace, yet highly engineered, products such as running shoes. The ability to produce three-dimensional (3D) objects from a digital template can have advantages over traditional manufacturing techniques (such as machining, injection molding, and thermoforming), including mass customization, formation of complex part geometries that are not readily molded or cast, on-demand

**“..liquid photopolymer resins present a rich opportunity to tune final materials properties by introducing various monomers, oligomers, additives, and additional reactive functionalities.”**

inventory, elimination of tooling costs, and reduced lead time. To realize these advantages, digital manufacturing requires materials that not only achieve the requisite mechanical properties and economic targets but are also designed to work in software-controlled, data-centric, fabrication technologies. We focus here on this challenge in the realm of polymeric materials.

Despite the initial excitement about AM dating back to the 1980s, a lack of suitable materials that can be printed economically and with sufficient quality for many production applications has prevented 3D printing from reaching its potential (1). One major limitation for polymers is that 3D-printed

parts often behave differently than their injection-molded counterparts, which greatly limits their use in manufacturing applications. This limitation is especially problematic for AM techniques, such as fused deposition modeling (FDM) and powder bed fusion (PBF), that use heat to process industrially relevant thermoplastics such as acrylonitrile butadiene styrene (ABS), polylactic acid, and polyamides. Although the inherent material properties are suitable for a wide range of applications, the layer-by-layer process by which the starting materials are deposited or sintered results in anisotropic mechanical properties resulting from poor adhesion between deposited layers of powder or filament (2–4).

However, not all manufacturing applications require fully isotropic properties or defect-free parts, and the ability of FDM and PBF to process high-performance thermoplastic materials, such as Ultem polyimide and polyether ether ketone (PEEK), is attractive for low-volume applications in aerospace and medical devices. For example, Airbus, in conjunction with Stratasys, announced in 2015 that the A350 aircraft contained more than 1000 polymeric 3D-printed parts, developed using FDM, that meet U.S. Federal Aviation Administration regulations for flame and smoke toxicity. Compared to traditional methods, up to 90% less energy and raw materials were used to produce the parts, and the reduced weight of the parts led to operational savings (5). With inventory-on-demand enabled by AM, Airbus can manage an inventory exceeding 3.5 million replacement parts for their airliners (6).

Light-based AM technologies, such as stereolithography, use digital projection or lasers to cure a photopolymer resin and produce parts with resolutions of 10 to 100  $\mu\text{m}$ . Light has advantages over heat in that it offers excellent spatial and temporal resolution and allows for direct synthesis of polymers from monomers contained in the photopolymer resin. Despite being the largest category of AM materials by sales (\$350 million in 2016) (7), photopolymer resins have poor mechanical properties and machinability (for example, they cannot easily have a hole drilled into them) compared to injection-molded thermo-

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