Invited lecture/Review

Pathogen-Plant Interactions in Plant Membrane Perforation

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Abstract:
Plants are targets of many pathogens that produce a lot of different effectors to damage plant cells during infection. For plant survival, it is, therefore, crucial to possess an efficient immune system, which in contrast to mammalian immunity, consists only of innate immunity. Traditionally, plant immunity is divided into two branches, i.e. pattern-triggered (PTI) and effector-triggered immunity (ETI), but the accumulating knowledge has shown that the division cannot be strictly maintained. ETI coevolves with pathogen e fector
molecules, which can function in many different ways to escape plant immunity and damage plant cells. Among their targets is the plant plasma membrane, which represents an important cell barrier. There are several different tactics to bypass this barrier, e.g. membrane perforation by proteins or peptides, which is an important and ubiquitously found mechanism of toxicity as well as self-defense in all kingdoms of life. It can be used to get specific molecules from cells, for signaling, or even to deliver effector molecules into the cytoplasm. The exact knowledge on plant membrane perforation, however, is lacking, and the hidden details still await to be unveiled.

Keywords: Plant immune System; Pathogen-Plant Interactions; Plant Membrane Perforation

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1. Introduction

Plants are targets of all pathogen classes, including viruses, bacteria, oomycetes, fungi, nematodes, and feeding insects. During infection and colonization of specific plant species, pathogens produce different effectors, i.e. proteins and small molecules that can facilitate pathogen entry into the host interior, suppress plant immune system, or alter host physiology for their benefit. Effectors can function extra- or intracellularly and target different parts of plant cells. In this review, we will focus on the pathogen-plant interactions that lead to the perforation of the plant plasma membrane. To see and understand a broader picture of the interplay between the pathogen and host-derived molecules, we will begin with a short overview of the plant immune system.

2. Plant Immune System

Besides physical barriers, an efficient immune system is crucial for the survival of any long-lived organism. Jawed vertebrates possess probably the most sophisticated immunity, which consists of a relatively nonspecific innate and a highly specific adaptive branch. Plants have no circulatory system and mobile immune cells to use such circulatory receptors. Therefore, they use much simpler mechanisms, that can be executed by every cell. Nonetheless, they can still respond with high specificity and restricted self-reactivity, and often generate a lifelong 'memory' of the encountered pathogens (Spoel et al., 2012).

All plant immune responses rely solely on innate immunity, which consists of two parts: pattern-triggered immunity (PTI) and effector-triggered immunity (ETI). Each of them uses a variety of receptors that require different early signaling components and activate specific downstream mechanisms. Many final responses of both pathways are nonetheless similar, with a largely overlapping set of genes but with distinct amplitudes and dynamics (Zhang et al., 2010; Yuan et al., 2021).

2.1. PTI

The general components of the plant immune system that pathogens must encounter are pattern recognition receptors (PRRs), which reside in the plasma membrane. They recognize pathogen-associated molecular patterns (PAMPs), which are pathogen-derived molecules, e.g. lipooligosaccharides of gram-negative bacteria, bacterial flagellin, glucans and glycoproteins from oomycetes, or chitin from the fungus cell wall (Zhang et al., 2010).
All currently known PRRs are either receptor-like kinases (RLKs) or receptor-like proteins (RLPs). Both classes conduct signals from the outside to the inside of a cell and are composed of three parts, the first two having identical functions: an extracellular portion of the protein to recognize PAMPs and a transmembrane part to connect the outside with the inside of a cell. The third, intracellular part of PRRs triggers the response with either a kinase domain (RLKs) or through the interaction of a short intracellular tail (RLPs) with adaptor protein bearing a kinase activity (Boutrot et al., 2017; Yuan et al., 2021).
Upon ligand recognition, structural changes of PRRs induce the recruitment of co-receptors to form a receptor complex, in which trans-phosphorylation occurs. Such activated receptor complex further phosphorylates and therefore activates receptor-like cytoplasmic kinases (RLCKs), which activate many downstream proteins. This leads to diverse physiological outputs, e.g. ROS production, Ca^{2+} influx, MAPK activation, production of defense hormones, stomatal closure, and cell wall reinforcement. The responses induce both local as well as systemic immunity (Boutrot et al., 2017; Liang et al., 2018; Yuan et al., 2021).

2.1.1. Resistance to PTI

PAMPs are molecules that are crucial for the fitness of pathogens. Therefore, they are conserved within a class of microbes and are less likely prone to mutations, which makes them a good target for PTI (Medzhitov et al., 1997). Pathogens, however, adapted to overcome PTI with the production of a wide variety of effector molecules, that interfere with PTI and are recognized by ETI.

2.2. ETI

Unlike PAMPs, effectors are species, race, or strain specific. Since not only pathogens but also plants are constantly coevolving, they have responded to these effectors with the development of resistance (R) proteins, which act as the sensing component of ETI (Thomma et al., 2011).

Most R-genes identified to date encode nucleotide-binding domain leucine-rich repeat (NLR) immune receptors (Sánchez-Martín et al., 2021). NLRs can be classified into two functional groups: sensor NLRs that recognize the effector and helper NLRs required by sensor NLRs to trigger ETI (Sun et al., 2020; Cox, 2021). It is important to note that R proteins can detect either the presence of or the activity of their target effector (Jonathan D.G. Jones et al., 2006). Because of selection pressure, pathogen effectors are constantly evolving to escape the R protein recognition, and new effectors are occurring to suppress ETI. But not just effectors, plant R proteins are also continuously coevolving to be able to effectively trigger and execute ETI (Thomma et al., 2011).

It has been postulated for a long time that ETI response is similar to PTI but "accelerated and amplified". Besides ROS production, Ca^{2+} influx, MAPK activation and transcriptional reprogramming, it results in programmed cell death at the infection site, referred to as the hypersensitive response (HR). First, a zig-zag model of plant immune system was proposed (**Figure 1A**) (Jones and Dangl, 2006), but recent experimental evidence calls for its refinement (**Figure 1B**). It was shown that PTI and ETI are functionally linked – many signaling components were found to participate in both of them (Yuan et al., 2021.

2.3. The Interplay Between PTI and ETI

With the accumulating knowledge, it has become evident, that dividing pathogen-derived molecules into PAMPs and effectors, and separating PRRs and R proteins cannot strictly be maintained (Thomma et al., 2011). Certain effectors are so widely spread, that they should be characterized as PAMPs. Such an example are necrosis- and ethylene-inducing peptide 1 (Nep1)-like proteins (NLPs) that are conserved among bacteria, fungi, and oomycetes (Gijzen et al., 2006). On the other hand, some PAMPs have a rather narrow distribution or, despite being widespread, they are not widely recognized among plants. These examples implicate that there is no strict line, but rather a continuum between PTI and ETI (Thomma et al., 2011).

3. Pathogen-Plant Interactions in Plant Membrane Perforation

The plasma membrane represents an important cell barrier, that separates the interior from the outside world and enables the cell to maintain its homeostasis. As pathogens depend on host cell metabolism or intracellular components (e.g. nutrients), they developed different solutions to efficiently bypass this barrier. This can be either through a) endocytosis of effectors, followed by their escape from intracellular vesicles (described in (Kale et al., 2011), b) direct intracellular delivery of effectors through the membrane, or c) with the help of pore-forming effectors, which are able to perforate their target membrane. Besides pathogen-driven permeabilization of the plant membrane, the regulation of its permeability as a self-defense mechanism is also discussed in this chapter.

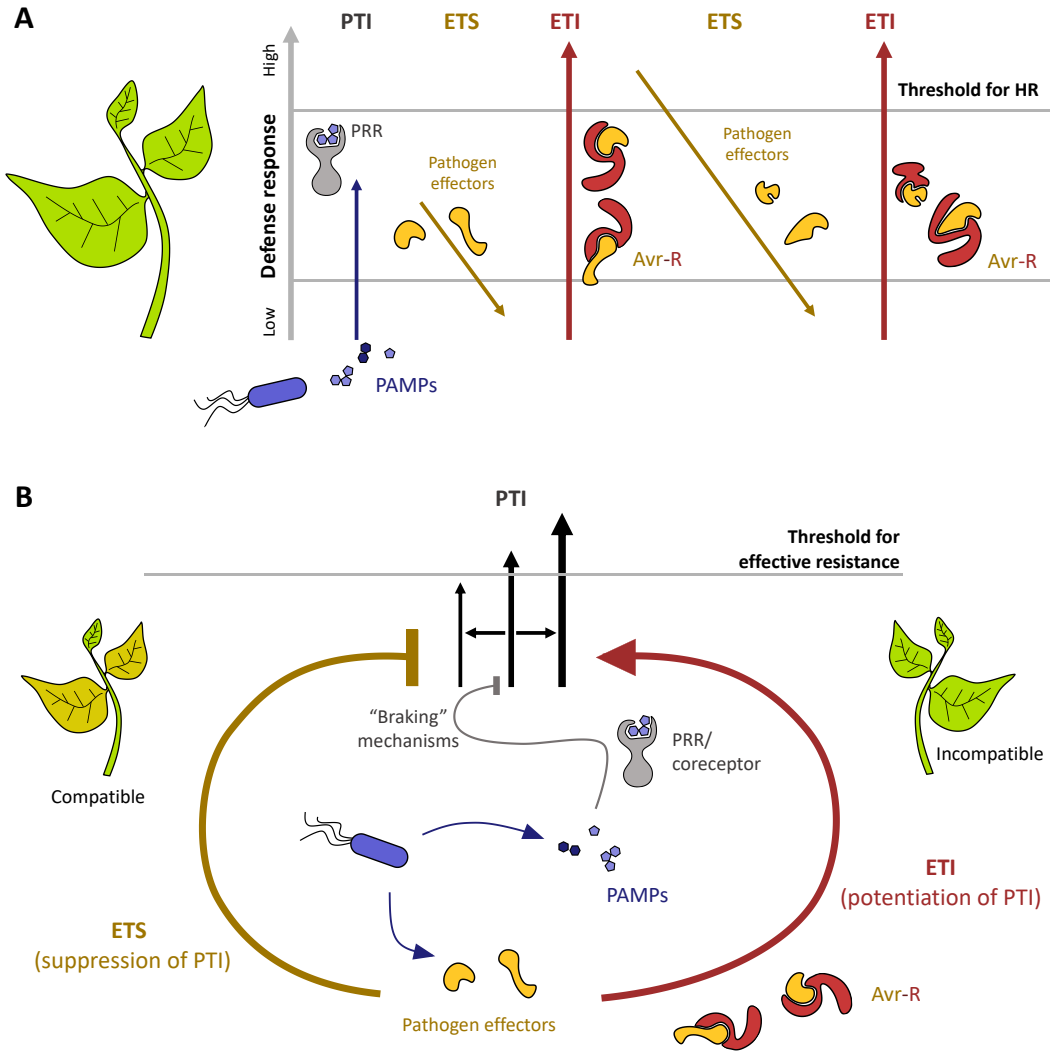

Figure 1: (A) A zig-zag model of plant immune system. First, PAMPs are recognized by PRRs, triggering PTI. Pathogen effectors can interfere with PTI, which results in effector-triggered susceptibility (ETS). Such effectors can be recognized by R proteins that trigger ETI, causing disease resistance and, usually, an HR. Effectors recognized by R proteins are called avirulence factors (Avr). Natural selection leads pathogens to further avoid ETI, evolving new effectors. The same mechanism is present in plants, evolving new R proteins. **(B) An updated model of plant immunity.** PTI acts as the primary defense mechanism. Its components are under negative control by the endogenous »braking« mechanisms of plants to prevent over activation and also by pathogen effectors (grey and yellow blunt arrows). The effectors are recognized by R proteins, which trigger ETI. This leads to upregulation of PTI components, causing potentiation and restoration of PTI (red arrow). "PTI + ETS" is usually associated with compatible interactions (left), while "PTI + ETI" with the incompatible ones (right).

3.1. Pore-Forming Proteins

Membrane perforation by pore-forming proteins (PFPs) is an important and ubiquitously found mechanism in all kingdoms of life. PFPs can serve as toxins or as a part of the immune system. They are produced as soluble monomers, which, upon the target membrane recognition, change their conformation, and form multimeric, active transmembrane pores (reviewed in (Dal Peraro et al., 2016; Mesa-Galloso et al., 2021)) (**Figure 2**). There is a large amount of knowledge about PFPs, however, the information about their roles in plants was missing for a long time. Not long ago, it was shown that effector proteins NLPs can specifically bind to plant plasma membrane and are capable of its perforation (Lenarčič et al., 2017). These findings opened a completely new area in plant research, with many important milestones achieved in the past few years.

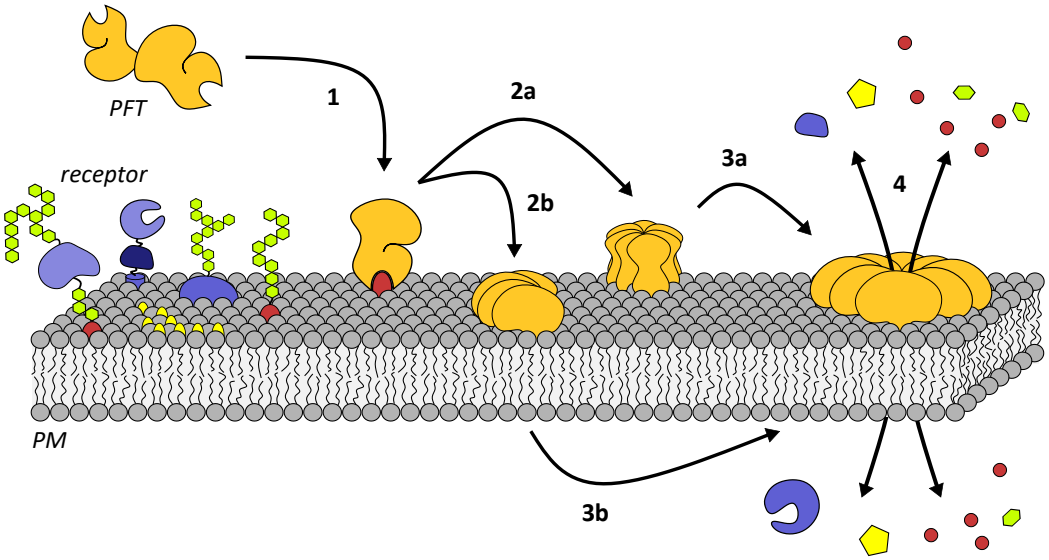

Figure 2: Generalized mechanism of pore formation by PFTs. Soluble PFTs recognize their membrane receptor, which can be either (glyco)protein or (gylco)lipid (1). Oligomerization occurs either through a pre-pore complex on a membrane surface (2a), which eventually undergoes conformational rearrangement to form a pore (3a), or the membrane insertion is concomitant with a sequential oligomerization, leading to partial (2b) or complete pore formation (3b). This results in increased membrane permeability (4).

3.1.1. Nep1-like Proteins

NLPs are widely spread apoplastic effectors produced by several plant pathogens from bacteria, fungi, and oomycetes, including *Phytophthora infestans*, which caused the Great Irish Famine (Oome et al., 2014 a). They trigger leaf necrosis and immunity-associated responses in several plants (Jennings et al., 2000; Veit et al., 2001; Fellbrich et al., 2002; Bae et al., 2006; Garcia et al., 2007; Feng et al., 2014; Xiang et al., 2022). For the necrotic activity, an N-terminal secretion signal peptide and a conserved heptapeptide motif are needed (Oome et al., 2014 a; Seidl et al., 2019), while PTI is triggered through a highly conserved region of 20 or 24 amino acids (Böhm et al., 2014; Oome et al., 2014 b). According to the number of the cysteine residues they contain, they can be divided into three classes: type I, which is the most abundant and contains two cysteines, type II, which contains four cysteines, and type III, which contains six cysteines. They might be toxic or non-toxic (Seidl et al., 2019), and structurally resemble cytolytic PFTs actinoporins (Küfner et al., 2009; Ottmann et al., 2009). While actinoporins target glycolipid sphingomyelin (Birck et al., 2004; Kristan et al., 2009), NLPs were found to bind the sugar headgroup of plant-specific sphingolipids glycosylinositol phosphorylceramides (GIPCs) (Lenarčič et al., 2017) (**Figure 3A**). Plant sterols and low salt concentration were also shown to promote the membrane binding of NLPs (Pirc et al., 2022).

Distinguishing Between the Monocot and Dicot Membrane Components?

It was soon noticed that in general, NLPs affect dicot, but not monocot plants, which can be explained by the difference in their GIPC-composition: dicot plants contain mostly GIPCs with two terminal hexoses, while in monocots the predominant species are GIPCs with three terminal hexoses (Gronnier et al., 2016). Although NLPs can bind both kinds of GIPCs, a membrane disrupting activity was restricted to plasma membrane vesicles from dicot plants. One possible explanation is that the longer sugar headgroup in monocots creates a distance too large for the bound NLP to reach the membrane (Lenarčič et al., 2017) (**Figure 3B**). However, cytolytic NLPs are produced by pathogens of monocots as well. Recently, their cytotoxic activity on monocots was shown Steentjes et al., 2022), but the mechanism of membrane binding and pore formation was not assessed.

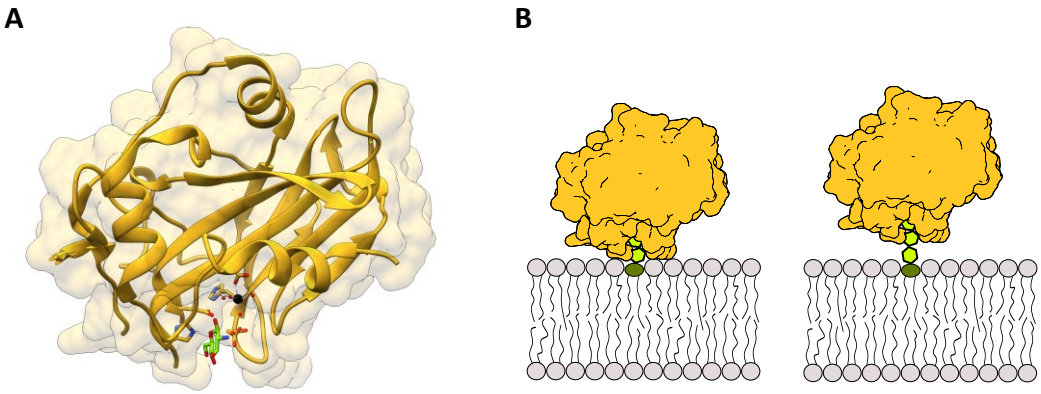

Figure 3: (A) NLP$_{Pya}$ crystal structure (PDB 5NNW) with bound glucosamine. Glucosamine is one of the possible terminal hexoses of GIPCs. NLP$_{Pya}$ is shown in yellow, glucosamine in green, and Mg^{2+} in black**. (B) A model of NLP binding to GIPC with two (left) or three (right) terminal hexoses.** In the first case, NLP can interact with the membrane- surface, while in the latter it cannot.

Mechanism of Pore Formation

According to the current knowledge, a unique mechanism of pore-formation by NLP$_{Pya}$, the cytotoxic model NLP, was recently proposed (**Figure 4**). As a soluble monomer, NLP$_{Pya}$ recognizes its target, i.e. the polar headgroup of GIPCs through electrostatic interactions, and its interaction is strengthened in the presence of sterols. One molecule of NLP binds multiple GIPCs, which leads to its aggregation and small pore formation. The mechanism is unique among currently known PFPs due to the shallow membrane binding of NLP$_{Pya}$, and the transient nature of the pores (Pirc et al., 2022).

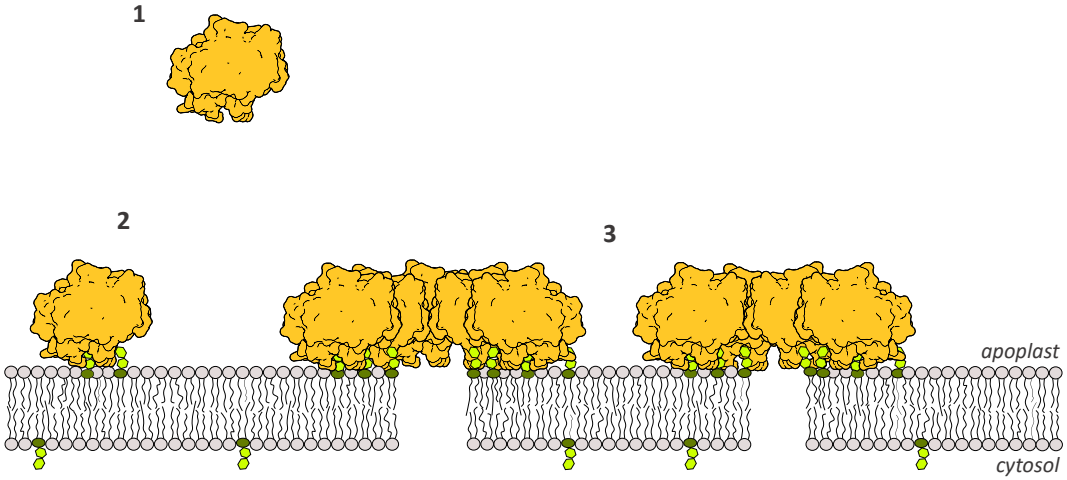

Figure 4: Mechanism of pore formation by NLPs. Soluble NLP (1) binds multiple membrane receptors, i.e. GIPCs (2). This leads to the aggregation of NLP molecules and the formation of small transient pores (3).

Facilitated Oligomerization

Recently, the analysis of *A. thaliana* accessions with different sensitivities to NLP toxicity showed that there are also other factors contributing to NLP-pore formation. A gene encoding a leucine-rich repeat (LRR)-only protein NTCD4 (NLP-triggered cell death on chromosome 4) promotes the toxicity of NLP if secreted into the apoplast. Although its mode of action is not yet well understood, NTCD4 was shown to physically interact with NLP, facilitating its oligomerization (Chen et al., 2021).

3.2. Pore-Forming Peptides

Besides PFPs, pore formation can be also caused by peptides. Usually, peptides are used as host defense to damage pathogens, such as bacteria. In plants, it was shown that alamethicin causes apoptosis-like death of plant cells (Rippa et al., 2010). Alamethicin is a membrane-active peptide produced by the beneficial plant root-colonizing fungus *Trichoderma viride*. It kills pathogenic fungi and bacteria around the root, but can also form pores in the plant membranes, i.e. plasma membrane, inner mitochondrial membrane, and plastid inner envelope (Matic et al., 2005; Aidemark et al., 2010).

3.3. Type III Secretion System

Type III secretion system (T3SS, **Figure 5**) is an organelle present in the most plant as well as animal pathogenic or symbiotic Gram-negative bacteria. It is complex machinery composed of more than 20 different components. Spanning both bacterial membranes, it also contains a filamentous part called an Hrp pilus in plant pathogens or a needle in animal pathogens (He et al., 2004). The filamentous part of plant pathogens reaches up to 2 μm in length, which is much longer than the needle of animal pathogens, which reaches up to 80 nm. A longer filamentous part is needed to overcome the plant cell wall, which is at least 100 nm thick (Ji et al., 2015).

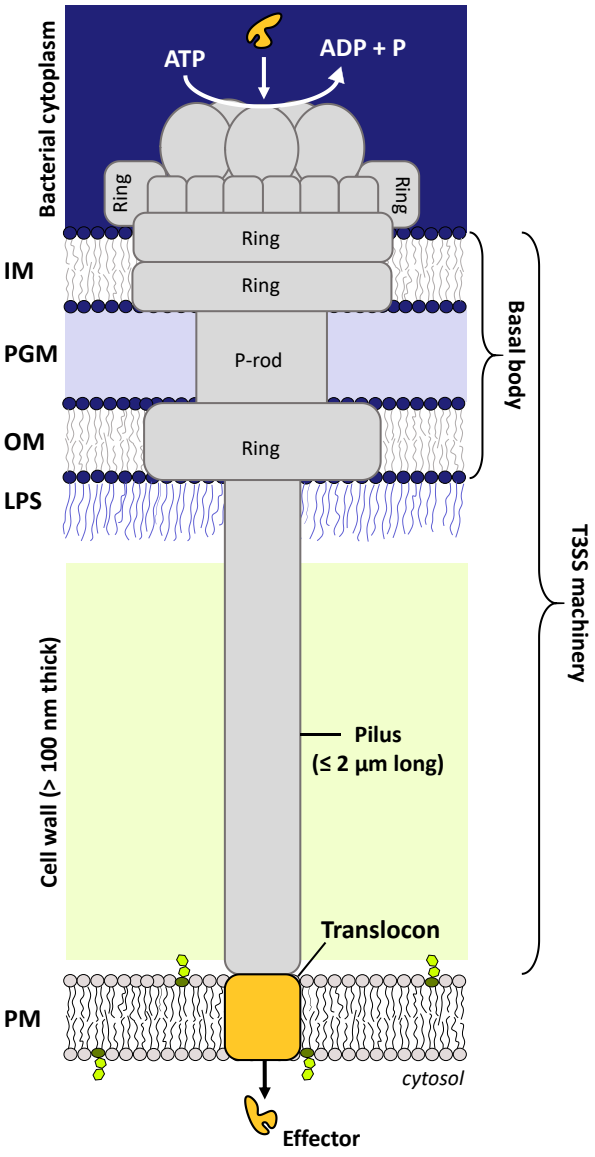

Figure 5: A schematic representation of T3SS machinery. The basal body spans bacterial inner (IM) and outer (OM) membranes and is composed of several rings that are presumably connected by a periplasmic rod (P-rod) across the peptidoglycan mesh (PGM). The filamentous part reaches the host membrane, where it forms a transmembrane translocon to access the host cytosol. In the case of attacking a plant cell, Hrp pilus has to pass the cell wall as well. For the delivery of effector proteins into the host, ATP hydrolysis is needed. LPS – lipopolysaccharide, PM – plant plasma membrane.

3.3.1. Translocon

T3SS can deliver many structurally different effectors directly into the host cell cytoplasm. This is done through translocon, which is a pore, formed at the tip of the filamentous complex (He et al., 2004). T3SS from *Shigella* was shown to be important in animal pathogenesis as well as in the proliferation of *Shigella* in plants (Jo et al., 2019). The pore-forming component of T3SS from *Shigella* is homologous to coiled-coil regions of colicin Ia, a PFP from Gram-negative bacteria that targets bacterial cells. This suggests that colicin Ia and the T3SS pore-forming component could evolve from a common ancestor (Barta et al., 2012). However, T3SS is mechanistically and functionally different from PFPs – its primary role is not to permeabilize and lyse the cell but to deliver effector molecules. Upon reaching the plasma membrane, the pore-forming components recognize the sensors, i.e. plant membrane lipids or proteins. Only then the translocon is formed, and the effectors are injected to the cytoplasm (Ji et al., 2015). After delivering the effectors, the bacterium leaves the site of contact. It was recently observed, that *Pseudomonas aeruginosa* translocon remains anchored into the host cytoplasmic membrane even after that, making the membrane permeable for ions.

3.4. Perforation as a Mechanism of Defense

The immune system of vertebrates uses pore-formation to control infection as well as tumor formation and can kill both pathogen and host cells (Liu et al., 2020). Similarly, it was recently shown that pore-formation is present also in plant defense, as a part of the second layer of plant immunity (ETI) (Burdett et al., 2019).

3.4.1. HOPZ-ACTIVATED RESISTANCE 1 Resistosome

HOPZ-ACTIVATED RESISTANCE 1 (ZAR1) is a sensor NLR with a coiled-coil domain that can sense several effectors. At the same time, it acts like the executor, which can trigger ETI without the need for a helper NLR. It interacts with pseudokinases, including ZED1 and RKS1, and receptor-like cytoplasmic kinases, such as PBL2, to form a pentameric resistosome (**Figure 6A**). Resistosome forms a pore through the membrane, which allows the influx of calcium ions (**Figure 6B**). This triggers a defense response and causes cell death (Burdett et al., 2019; van Wersch et al., 2020; Bi et al., 2021).

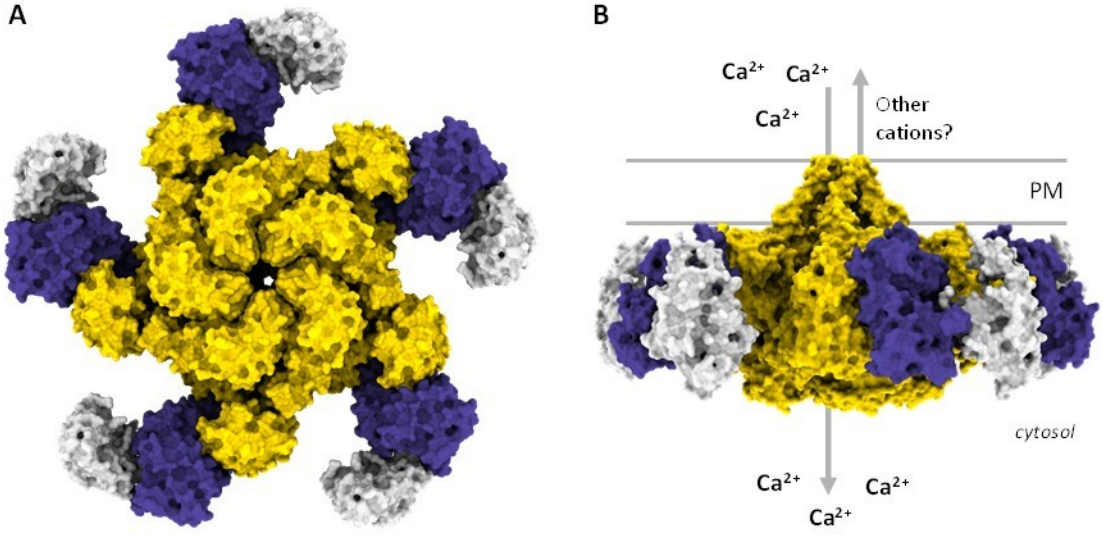

Figure 6: Structure of pentameric ZAR1 resistosome, (A) top view and (B) side view (PDB 6J5T). Resistosome perforates the plant plasma membrane (PM), which allows the influx of calcium ions. Yellow – ZAR1, violet – pseudokinases, grey – receptor-like cytoplasmic kinases.

4. Conclusion

Pathogen-host interactions rely on different mechanisms, one of which is membrane perforation. Such a mechanism can be used either as an attack or a defense mechanism and is relatively well understood among vertebrates and bacteria. The exact knowledge of plant-membrane perforation is still lacking, although it has been stably improving in the past years. It is important, that not only the mechanistic but also structural data is becoming available, which will allow us to understand the pathogen-plant membrane interactions in detail on the molecular level. This will open the possibility to develop greener and more effective strategies for plant pathogen management, which, for our growing population, are of unprecedented value.

Conflicts of Interest: The authors declare no conflict of interest.

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