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REVIEW PAPER

Deciphering the role of plant plasma membrane lipids in response to invasion patterns: how could biology and biophysics help?

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Abstract

Plants have to constantly face pathogen attacks. To cope with diseases, they have to detect the invading pathogen as early as possible via the sensing of conserved motifs called invasion patterns. The first step of perception occurs at the plasma membrane. While many invasion patterns are perceived by specific proteinaceous immune receptors, several studies have highlighted the influence of the lipid composition and dynamics of the plasma membrane in the sensing of invasion patterns. In this review, we summarize current knowledge on how some microbial invasion patterns could interact with the lipids of the plasma membrane, leading to a plant immune response. Depending on the invasion pattern, different mechanisms are involved. This review outlines the potential of combining biological with biophysical approaches to decipher how plasma membrane lipids are involved in the perception of microbial invasion patterns.

Keywords: Biophysics, innate immunity, invasion patterns, lipids, membrane models, pathogen-associated molecular pattern sensing, plasma membrane.

Introduction

Plants are sessile organisms. The growth and yield of plants are highly influenced by several abiotic and biotic stresses, such as temperature, drought, salinity, and microbial challenge. During their development, plants have evolved a variety of mechanisms to cope with their continuously changing environment. Perception of an invading microbe leads to the activation of early signaling events such as ion fluxes, accumulation of reactive oxygen species (ROS), and phosphorylation cascades (Bigeard *et al.*, 2015). In addition, an intricate network of phytohormone signaling pathways, involving hormones such as salicylic acid, jasmonic acid, and ethylene, regulates appropriate and effective responses encompassing transcription

factors and defense-related gene expression (Aerts *et al.*, 2021). These molecular and cellular responses result in the synthesis of antimicrobial proteins and metabolites (e.g. pathogenesis-related proteins and phytoalexins) and cell wall reinforcement, which together participate in plant resistance or tolerance to pathogens (Zhou and Zhang, 2020).

The recognition of a pathogen by plant cells first occurs at the plasma membrane (PM) level through the sensing of invasion patterns (IPs), also known as apoplastic pathogen-associated molecular patterns (PAMPs), and activating PAMP-triggered immunity (Boutrot and Zipfel, 2017; Schellenberger *et al.*, 2019). Perception of apoplastic IPs classically involves pattern recognition receptors (PRRs), including receptor-like kinases (RLKs) and receptor-like proteins (RLPs) (Macho and Zipfel, 2014; Saijo *et al.*, 2018; Albert *et al.*, 2020; Lu and Tsuda, 2021). Although IP sensing is mostly determined by activation of proteinaceous PM-anchored PRR sentinels, increasing data suggest that some IPs may be perceived by plants through mechanisms that do not directly rely on direct interaction with PRRs but rather involve PM lipids (Montesano *et al.*, 2003; Gust *et al.*, 2010; Henry *et al.*, 2011; Schellenberger *et al.*, 2019).

The PM separates cells from the surrounding environment. It is the platform for intricate orchestration of signal transduction allowing the translation of external signals in a finely tuned response. The plant PM is a complex and highly dynamic structure with a lipid-to-protein ratio of 1:1.3 (Cacas *et al.*, 2016). New extraction methods coupled to technological advances in mass spectrometry and chromatography have improved lipid identification in recent years. The main classes of lipids present in the plant PM are phospholipids, sphingolipids, and sterols. Among the phospholipids, phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are the most abundant in plant PMs. Other phospholipids comprise phosphatidic acid (PA), phosphatidylglycerol (PG), phosphatidylinositol (PI), and phosphatidylserine (PS). The defining component of sphingolipids corresponds to a sphingoid or long-chain base (LCB), which is a carbon amino-alcohol backbone, generally with 18 carbons. This diverse group of compounds includes LCBs, ceramides, glucosylceramides (GlcCers), and glycosyl inositolphosphoceramides (GIPCs) (Michaelson *et al.*, 2016; Huby *et al.*, 2020). Sphingolipids constitute up to 40% of lipids in the plant PM and GIPCs account for 60–80% of lipids in the external PM leaflet (Cacas *et al.*, 2016). In plants, the sterol family consists of more than 250 compounds and could represent 20–40% of PM composition. Most of them are 4-desmethyl sterols. The three main sterols are sitosterol (29 carbons), stigmasterol (29 carbons), and campesterol (28 carbons). In the PM, sterols occur as free sterol or are conjugated to glucose to form steryl glycosides and acyl steryl glycosides (Moreau *et al.*, 2018).

The two leaflets of the plant PM display an asymmetric distribution of lipids (Gronnier *et al.*, 2018). Although only a few studies have described this asymmetry, it is postulated that glycosphingolipids (GlcCers and GIPCs), sterols (free and conjugated), and the phospholipid PC are enriched in the outer membrane leaflet. Other phospholipids such as PE, PI, and its derivatives are enriched in the inner leaflet, whereas PA and PS are located exclusively in the inner leaflet (Cacas *et al.*, 2016; Mamode Cassim *et al.*, 2019). Another aspect of PM heterogeneity consists of a lateral segregation of lipids. Current PM models include highly specialized and dynamic domains (rafts) involved in several biological processes. These rafts are sterol- and sphingolipid-enriched platforms that exhibit self-assembly and can recruit specific proteins (Yu *et al.*, 2020). Lipidomic analyses have shown that almost all structural phospholipids, such as PA, PC, PE, and PS, are largely depleted in rafts, whereas PI-phosphates are enriched (Furt *et al.*, 2010; Mamode Cassim *et al.*, 2019). Whereas GlcCer content fluctuates between different plant species (Mamode Cassim *et al.*, 2019), a high proportion of GIPCs is found in rafts (Cacas *et al.*, 2016). The amounts of free sterols and also steryl glycosides and acyl steryl glycosides are increased in microdomains (Mongrand *et al.*, 2004; Borner *et al.*, 2005, Laloi *et al.*, 2007; Lefebvre *et al.*, 2007). However, no specific sterol species accumulate in lipid rafts (Moreau *et al.*, 2018). The lipid segregation at the PM seems to be critical for protein localization. Rafts mediate protein–protein and protein–lipid interactions that are critical in cell signaling mechanisms. During plant–pathogen interactions, dynamic changes in the distribution of rafts could be observed upon IP sensing (Burkart and Stahl, 2017; Ott, 2017). Compartmentalization of the PM and both the protein and lipid composition of membrane microdomains are of crucial importance for the activation of specific signaling immune responses (Keinath *et al.*, 2010; Nagano *et al.*, 2016; Ott, 2017; Gronnier *et al.*, 2018; Yu *et al.*, 2020).

Currently, the complexity of biological systems and the limitation of analytical technologies limit the understanding of IP perception on living cells. However, biophysical approaches (Box 1) using artificial biomimetic membranes have provided some interesting data (Fig. 1, Table 1) helping to decipher the role of PM lipids in IP-triggered immunity. The main advantage of biomimetic membranes is that they are devoid of the cellular machinery, which means that they are more stable and less dependent on the environment than biological samples. These models have a lipid composition that is adapted both to the biological relevance (requiring the in-depth analysis of the lipid composition of the plant PM under study) and to the interpretability of biophysical data. One of the most widely used artificial biomimetic membranes is the lipid vesicle, also called the liposome (Fig. 1A). Based on its size and curvature, this model closely mimics the cell structure. Nevertheless, the choice of lipid compositions for liposomes is limited by the criterion of stability of the system over time. It is also technically difficult to form vesicles with an asymmetric composition between the outer and inner leaflets. The planar lipid bilayer is an alternative model to study the effect of membrane asymmetry on the kinetics of interactions and on the physicochemical and structural properties of membranes (Fig. 1B) (Clifton *et al.*, 2020). The lipid monolayer, on the other hand, is a model of choice for studying the lipid specificity of the interaction with

Box 1. Basic principles of some biophysical techniques for lipid–bioactive molecule interaction studies.

Only techniques for studying the interaction itself and the impact on membrane lipids are presented. Techniques specific to structural and conformational analysis are not considered. For these, the reader can refer to Munusamy *et al.* (2020) and Huggins *et al.* (2019).

Fluorescence spectroscopy:

- *Fluorescence quenching*: The lipid domain formation within the lipid bilayer of liposomes and the effect of bioactive molecules on the lateral organization of this membrane can be studied by fluorescence quenching of diphenylhexatriene (DPH) and the use of a fluorescence-quenching phospholipid, 1-palmitoyl-2-(12-doxyl)stearoylphosphatidylcholine (12SLPC) (Xu *et al.*, 2001). In lipid mixtures containing quencher lipid, the occurrence of domain formation results in lower DPH quenching.
- *Calcein or carboxyfluorescein release*: If the membrane is permeabilized/destabilized by a bioactive molecule, the self-quenched fluorescent probe initially encapsulated within the liposomes is released into the external medium and gives rise to an increase in fluorescence emission (Fiedler and Heerklotz, 2015).
- *Probe generalized polarization*: The generalized polarization (GP) measures the fluorescence emission shift of the probe initially partitioned within the lipid bilayer of the liposomes. It depends on the order state of the probe environment. The presence of the bioactive molecule can increase or decrease the GP value, corresponding to a lower or a higher fluidity of the bilayer, respectively (Parasassi *et al.*, 1990).
- *Sterol exchange measurements*: Donor liposomes containing molecules of the fluorescent sterol derivative dehydroergosterol are mixed with acceptor liposomes containing non-fluorescent sterols. In donor liposomes, DHE is self-quenched, resulting in less radiation energy transfer and a decrease in fluorescence polarization. The molecular transfer of sterols, including DHE, by the bioactive molecule can be visualized as the increase in steady-state polarization of DHE in the donor–acceptor mixture (Mikes *et al.*, 1997).

Solid-state NMR (ssNMR): Based on the observation of nuclear spin behaviors in a magnetic field, ssNMR spectroscopy is a non-invasive and non-destructive technique to characterize the behavior of biomolecules in a lipid environment (Furlan *et al.*, 2020). ^{31}P-ssNMR (via chemical shift anisotropy) and ^{2}H-ssNMR (via quadrupolar splitting) are useful to study the lipid dynamics of liposomes, and more specifically the polar head dynamics and the membrane hydrophobic core order, respectively. Other NMR methods are useful for studying biomolecule insertion and location as well as the structural features of biomolecules inside a lipid environment (see Furlan *et al.*, 2020 and Munusamy *et al.*, 2020).

Quartz crystal microbalance with dissipation (QCM-D): QCM-D is an ultra-sensitive weighting device based on the changes in the mechanical resonance of a quartz crystal, excited by an external electric field, which are very sensitive to the mass and mechanical properties of the weighted material, such as a lipid bilayer, deposited on it. The frequency and dissipation signals provide information on the thickness and viscoelastic properties of the bilayer. The monitoring of these parameters when a bioactive molecule is added on the bilayer allows analysis of its adsorption kinetics and of its influence on the bilayer's properties.

Atomic force microscopy (AFM): AFM is a local probe technique that allows visualization of surface structures and lateral organization with a lateral resolution of ~1 nm and vertical resolution of ~0.1 Å. It is a powerful tool to observe phase-separated domains (ordered and disordered phases) of supported lipid bilayers at the micro- and nanoscales, and to monitor membrane remodeling and alteration upon interaction with bioactive molecules (Deleu *et al.*, 2014).

Neutron reflectometry (NR): NR gives data on the density profile of layered samples, such as a lipid bilayer, deposited on a silicon block. The principle is to measure the ratio between the reflected intensity of a neutron beam interacting with the atomic nucleus of the sample and its incident intensity as a function of the momentum transfer, calculated from the angle of incidence and the wavelength of the

Box 1. Continued.

neutron beam. After mathematical fitting, the thickness of the layers of the lipid bilayer is determined to within a few Å resolution (Mattauch *et al.*, 2018). NR allows precise determination of the penetration depth of an incoming molecule into a lipid bilayer and the transverse distribution of the different components, providing that deuterated molecules are available (Rondelli *et al.*, 2016).

Isothermal titration calorimetry (ITC): ITC measures the heat flow (with a sensitivity of 1 µcal) due to the interaction between a bioactive molecule and biomimetic liposomes by titrating one binding partner (which is the calorimeter syringe) into the sample cell of the calorimeter containing the other binding partner (Jelesarov and Bosshard, 1999; Heerklotz and Seelig, 2000). After a mathematical treatment of the thermogram, the complete thermodynamic picture (binding constant, variation of enthalpy, entropy, and free energy) of the interaction is obtained, giving information about the affinity of the bioactive molecule for the liposomes and the nature of their interaction.

Langmuir monolayer technique: This technique allows the characterization of the interaction between a bioactive molecule and the outer leaflet of a membrane. It is also the only currently available technique for studying the lipid specificity of the interaction (Eeman and Deleu, 2010; Deleu *et al.*, 2014). Two modes of measurement exist: (i) the measurement of the surface pressure increases as a function of time in order to determine the insertion power and kinetics of a bioactive molecule into a lipid monolayer, initially deposited at an air–water interface of the device; (ii) the measurement of compression isotherms, that is, the surface pressure as a function of the mean molecular area of the spread of the compounds (i.e. the bioactive molecule and the different representative lipids) at the air–water interface of the device. The thermodynamic analysis of these compression isotherms gives information about the miscibility behavior of the compounds and their possible specific interaction (attraction or repulsion).

Cryo-transmission electron microscopy (Cryo-TEM): By flash freezing samples of liposomes in the absence or presence of bioactive molecules, cryo-TEM provides a direct observation of the structural organization of lipid/bioactive molecule systems in terms of both internal structure and global morphology (Deleu *et al.*, 2008). Cryo-TEM is based on the visualization of the electrons transmitted by the sample bombarded by an electron beam.

a bioactive molecule (Fig. 1B). It allows analysis of single lipids, even those that are not capable of forming liposomes, such as sterols. Other membrane models, for example, bicelles and nanodiscs, have been developed to overcome technical limitations specific to some techniques, such as liquid-state NMR (Munusamy *et al.*, 2020).

In this review, we will focus on the role of plant PM lipids in the sensing of microbial IPs of either polysaccharidic, proteinaceous, or lipidic nature. The key players that are involved in this dynamic network will be discussed to underline different modes of action and the diversity of interactions between IPs and the lipid phase of the PM. This review will show how complementary approaches including molecular biology, biochemistry, and biophysics have greatly increased our knowledge of this challenging and original topic.

Trapping of plasma membrane sterols

Several proteinaceous IPs from pathogens are secreted in the apoplastic compartment of plant cells. Elicitins are extracellular proteins from *Phytophthora* and *Pythium* oomycetes, belonging to a large multigenic family of 10 kDa proteins (Fig. 2) (Ricci *et al.*, 1989; Jiang *et al.*, 2006). The most studied elicitins to date are cryptogein from *Phytophthora cryptogea* and INF1 from *Phytophthora infestans*, which share 79% (93 of 118 amino acids) identity at the amino acid level (Derevnina *et al.*, 2016).

Cryptogein activates canonical signaling events such as ROS production, medium alkalinization, mitogen-activated protein kinase (MAPK) phosphorylation, calcium influx, endocytosis, or the induction of gene expression (Gómez-Gómez and Boller, 2002; Simon-Plas *et al.*, 2002; Garcia-Brugger *et al.*, 2006; Chinchilla *et al.*, 2007; Denoux *et al.*, 2008; Leborgne-Castel *et al.*, 2008). Moreover, cryptogein induces a hypersensitive response (HR) in tobacco leaves (Ricci *et al.*, 1989) or cell suspensions (Hirasawa *et al.*, 2004; Kadota *et al.*, 2004). In contrast, INF1 or cryptogein do not induce an HR in tomato. However, the absence of cell death does not compromise their capacity to induce resistance against pathogens. In tomato, cryptogein and INF1 activate ethylene and jasmonic acid signaling (Kawamura *et al.*, 2009; Starý *et al.*, 2019). In tobacco, a salicylic acid-dependent pathway is stimulated, leading to local plant resistance to pathogens (Keller *et al.*, 1996a, b). Elicitins are also able to mount a systemic acquired resistance against a broad spectrum of pathogens (Kamoun *et al.*, 1993; Keller *et al.*, 1996a; Ricci, 1997).

The ELR (elicitin response) surface RLP, cloned from the wild potato species *Solanum microdontum*, senses a broad spectrum of elicitins through highly conserved domains (Du *et al.*, 2015; Domazakis *et al.*, 2020, Preprint). Expression of ELR in diverse

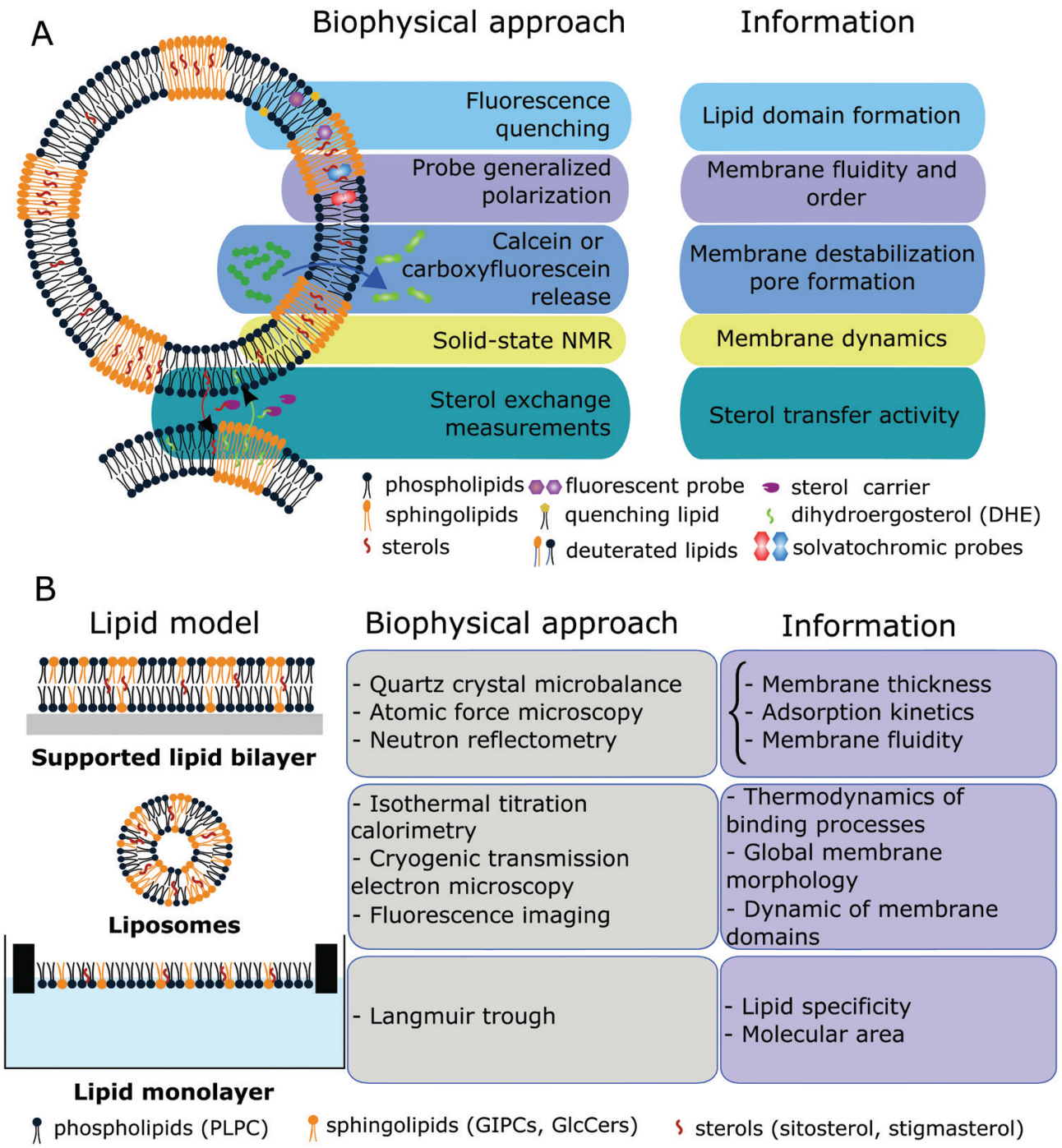

Fig. 1. Overview of biophysical models and associated approaches developed (A) and potential future developments (B) to unravel the interactions between plant plasma membrane lipids and microbial invasion patterns and their impact on membrane properties. The main challenge for the potential future developments is the preparation of artificial membranes with a composition close to that of the plant plasma membrane (left part of B). GIPCs, glycosyl inositolphosphoceramides; GlcCers, glucosylceramides; NMR, nuclear magnetic resonance; PLPC, 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphatidylcholine. See Box 1 for the basic principles of the different approaches. Technologies specific to structural and conformational analysis are not considered. For these, the reader can refer to Munusamy *et al.* (2020) and Huggins *et al.* (2019).

potato cultivars confers enhanced resistance against the mildew agent *P. infestans* (Du *et al.*, 2015). Co-immunoprecipitation experiments showed that downstream signaling activation also involves the transmembrane leucine-rich repeat (LRR) receptor kinases Suppressor of BIR1-1 (SOBIR) and Brassinosteroid insensitive1-associated kinase1/Somatic embryogenesis receptor kinase3 (BAK1/SERK3) to activate the immune response (Fig. 3) (Du *et al.*, 2015; Peng *et al.*, 2015; Domazakis

Table 1. Advantages and limitations of the main biophysical techniques described in Fig. 1 and Box 1 (based on Deleu *et al.*, 2014, Clifton *et al.*, 2020, Furlan *et al.*, 2020, and Benjin and Ling, 2020)

Biophysical techniques	Advantages	Limitations/drawbacks
Fluorescence spectroscopy	Applicable to complex membrane models Provides different kinds of information according to the fluorescence probe used (see Fig. 1A)	Non-disturbing fluorescent probes able to selectively label plant lipids or plant lipid domains and not interact with the bioactive molecule are required Average measurement
Solid-state NMR	Non-invasive and non-destructive No fastidious crystallization step Provides information about lipid dynamics and biomolecule insertion and location	Artificially labeled molecules (lipids and/or bioactive molecules) are often required High complexity of the spectral signal when more than three to four classes of lipids are present High amount of material (mg) needed to obtain an acceptable signal-to-noise level
Quartz crystal microbalance	Label-free Highly sensitive Binding kinetics in real time and physiological conditions Accurate affinity (dissociation constant) value Provides information about the hydrodynamic friction	The sample must be deposited on to a solid support, which can limit the degree of freedom of the molecules Average measurement over the entire surface
Atomic force microscopy	Provides information on the lateral organization with a lateral resolution ~1 nm and vertical resolution ~0.1 Å Real-time imaging is possible in physiological conditions	The sample must be deposited on to a solid support, which can limit the degree of freedom of the molecules Single-lipid mapping requires development of specific probes
Neutron reflectometry	Provides information on the cross-sectional structure at an angstrom scale Neutron beam is not damaging to biological samples	Perdeuterated lipids and/or bioactive molecules are required Samples are supported on a solid substrate, which can limit the degree of freedom of the molecules Average measurement over the entire surface Long time required for data acquisition Complexity of data analysis Requires access to large national or international facilities
Isothermal titration calorimetry	Labeled molecules are not required High sensitivity (1 µcal) Low amount of material (µM) required Quantification of the membrane binding affinity	Average measurement of the interaction Highly sensitive to small changes in experimental conditions, leading to misinterpretation
Langmuir monolayer technique	Single lipid system can be considered Easy control of the experimental design Straightforward and easily deployed technique	Only half of the membrane bilayer is considered, i.e. the mutual influence of the outer and inner leaflets is not considered
Cryo-transmission electron microscopy	Flash freezing maintains the sample in a native state Direct visualization of lipid bilayer Suitable for studying short-term biological events thanks to fast freezing with a time resolution of tens of milliseconds	Electrons can damage the sample Resolution not suitable for visualization of lateral lipid organization Decrease of sample contrast in the presence of organic substances (sugar, DMSO, glycerine)

et al., 2018). Cryptogein treatment induces protein and lipid rearrangements in the PM that are important for a functional immune response. A quantitative proteomic approach in cryptogein-treated tobacco cells demonstrated the delocalization of different proteins including dynamin and 14-3-3 proteins in lipid rafts (Elmayan *et al.*, 2007; Stanislas *et al.*, 2009). Moreover, RbohD, an NADPH oxidase, which is responsible for the ROS production after cryptogein treatment and is a key component of PAMP-triggered immunity, associates exclusively with lipid rafts (Lherminier *et al.*, 2009).

Interestingly, elicitins belong to a family of conserved lipid transfer proteins and can transfer sterols from plant PMs to oomycete membranes (Mikes *et al.*, 1998; Vauthrin *et al.*, 1999). Structural analysis through X-ray crystallography and molecular docking revealed that elicitins possess a hydrophobic cavity located inside the protein core that is able to bind fatty acids and phytosterols (Mikes *et al.*, 1997; Lascombe *et al.*, 2002; Dobeš *et al.*, 2004). Biophysical experiments using a dequenching fluorimetric method revealed that elicitins can trap and transfer sterols between plant membranes, artificial liposomes, or micelles (Fig. 3) (Mikes et al., 1997, 1998; Vauthrin *et al.*, 1999). The scavenging ability of cryptogein is independent of the membrane sterol structure. By the same biophysical approach, the presence of the Lys13 residue in cryptogein was shown to play a key role in the transfer of sterols but not in its binding to the hydrophobic protein cavity (Pleskova *et al.*, 2011). This unique ability of cryptogein to bind PM sterols is important for some immune signaling events in plants (Osman *et al.*, 2001; Hirasawa *et al.*, 2004).

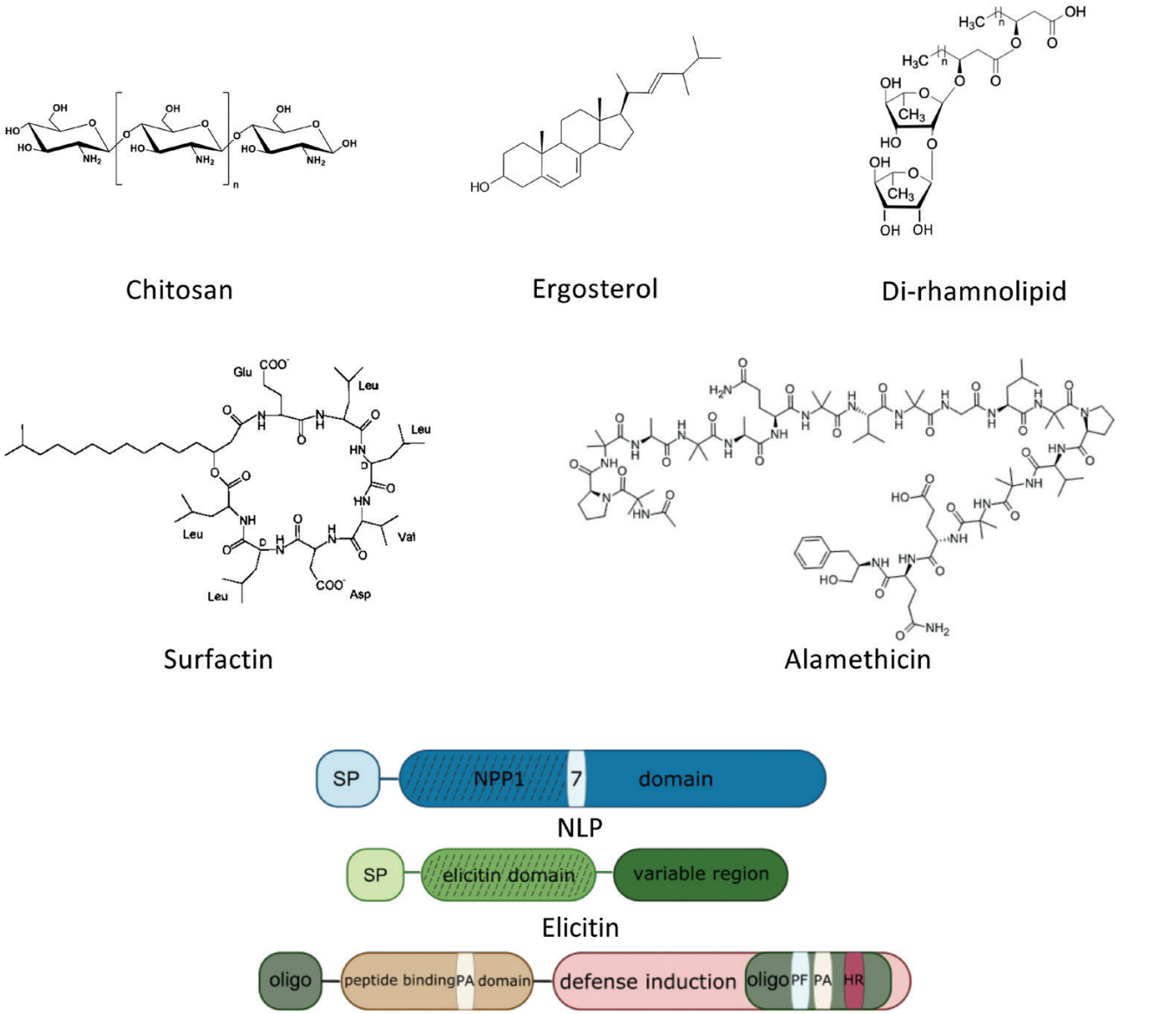

Fig. 2. Chemical structures and schematic overviews of some invasion patterns interacting with plasma membrane lipids. Di-rhamnolipid, surfactin, alamethicin, and HrpZ1 represent examples of rhamnolipids, cyclic lipopeptides, peptaibols, and harpins, respectively. 7, Heptapeptide motif; HR, hypersensitive response; NLP, necrosis- and ethylene-inducing peptide 1-like proteins; NPP1, Pfam domain PF05630; oligo, oligomerization domain; PA, phosphatidic acid binding domain; PF, pore formation domain; SP, signal peptide. Dashed boxes represent domains with conserved cysteine residues.

Like other IPs, cryptogein treatment triggers an increase in the PM order within the first minutes in tobacco or Arabidopsis cell suspensions. Moreover, unlike other IPs, cryptogein specifically induces an increase in PM fluidity, as shown by di-4-ANEPPDHQ mobility during fluorescence recovery after photobleaching. These data suggest a reorganization of the PM through processes such as lateral movements and/or membrane fusion (Gerbeau-Pissot *et al.*, 2014). The ability of sterols to maintain the microfluidity state of the PM is very important for biological processes (Halling and Slotte, 2004; Roche *et al.*, 2008; Grosjean *et al.*, 2018). Accordingly, the use of cryptogein variants with an alteration in their sterol-binding properties confirms a strong correlation between the removal of sterols from the PM and the increased fluidity of the PM (Sandor *et al.*, 2016). These data therefore suggest a model providing a dual cooperative role for cryptogein: the modification of the PM order inducing an IP-common signaling cascade and the binding of PM sterols, leading to the modification of PM fluidity and the induction of ROS production. Interestingly, elicitin mutants that fail to bind sterols still elicit a cell death response, suggesting that sterol binding and the HR remain independent activities (Osman *et al.*, 2001; Dokládal *et al.*, 2012; Derevnina *et al.*, 2016; Norman *et al.*, 2020).

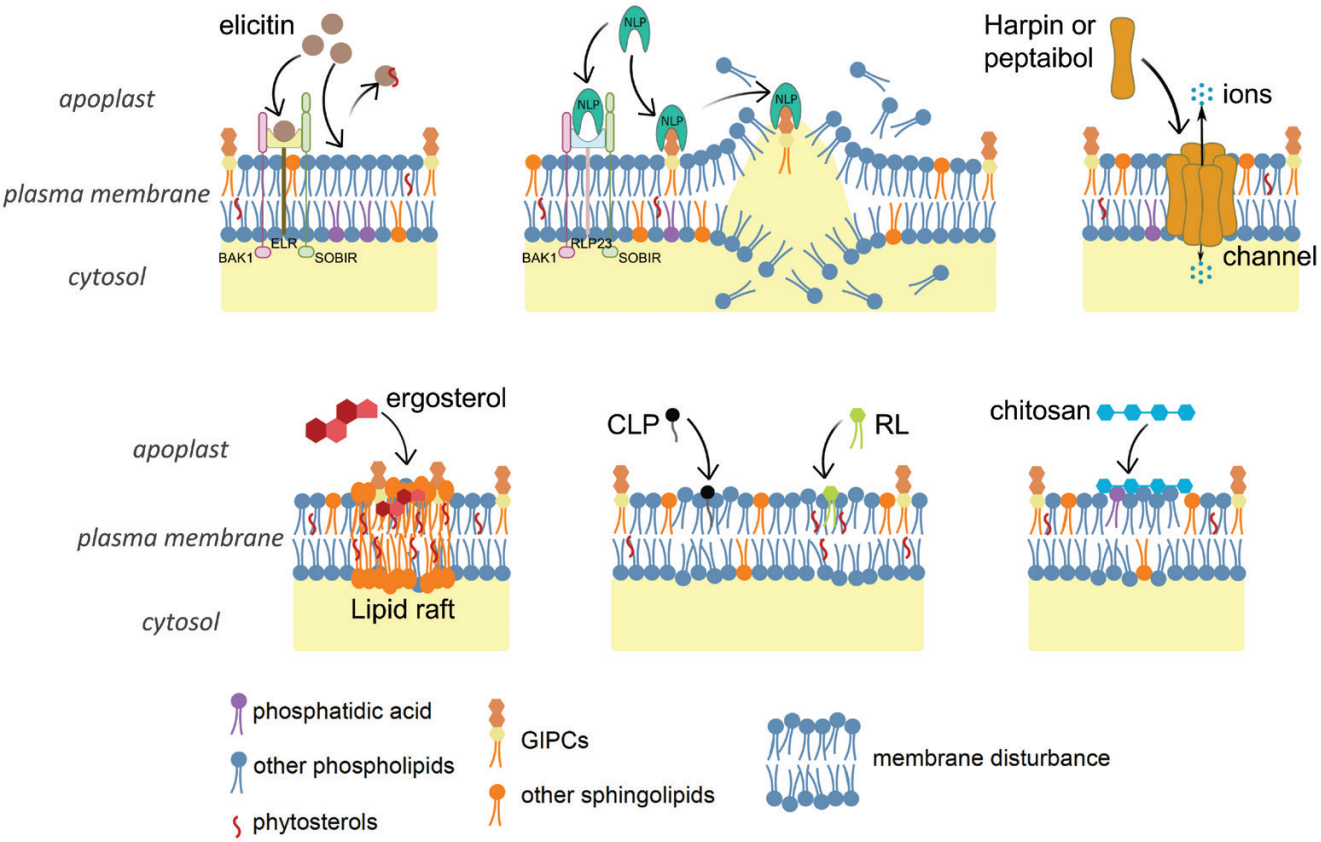

Fig. 3. Representation of the main mechanisms involved in the sensing of invasion patterns by the plasma membrane (PM) and involving lipids. Elicitins are perceived by RLP/RLKs (ELR, BAK1, and SOBIR) but could also act as sterol carrier proteins. NLPs are either perceived by RLP/RLK complexes (RLP23, BAK1, and SOBIR) or interact with GIPCs, leading to the formation of a pore. Harpins or peptaibols induce the formation of channels inside the lipid bilayer of the PM after oligomerization. Ergosterol and chitosan, by interacting with PM lipids (phospholipids and rafts, respectively), and CLP and RL, by inserting their hydrophobic tail inside the PM, could induce a membrane disturbance. All these membrane interactions trigger plant immune responses.

Further studies revealed that the lipid composition of the PM is important in the activation of plant defenses by elicitins (Mamode Cassim *et al.*, 2019). Accordingly, an optimal ROS response induced by cryptogein requires the presence of PA produced by diacylglycerol kinases (Cacas *et al.*, 2017). It is now well known that PA can regulate the activity of defense-associated proteins (Pokotylo *et al.*, 2018). In addition, LCBs and their phosphorylated derivatives (LCB-Ps) differentially regulate ROS production induced in tobacco cells by cryptogein (Coursol *et al.*, 2015). Cryptogein-induced ROS production is attenuated by LCBK inhibitors and increased by the overexpression of Arabidopsis SPHINGOSINE KINASE 1 (SPHK1), an enzyme responsible for the synthesis of LCB-Ps. An exogenous supply of LCB-Ps increases the production of ROS induced by cryptogein, while LCBs have the opposite effect (Coursol *et al.*, 2015). These data therefore support a model in which a dynamic balance between LCBs and LCB-Ps regulates early ROS production localized at the membrane level in tobacco cells. These results are also in agreement with a model in which cryptogein activates PM-associated LCBKs, acting upstream of NtRbohD to up-regulate ROS production in tobacco cells. Altogether, these data suggest that the ability of elicitins to induce plant defense responses could rely at least in part on direct interaction of the IP with PM lipids, and that PM dynamics is an element of early signaling processes.

Plasma membrane GIPCs as receptors

Necrosis- and ethylene-inducing peptide 1 (Nep1)-like (NLP) proteins form a large family of proteins that are produced and secreted by plant-pathogenic microorganisms such as bacteria, fungi, and oomycetes (Pemberton and Salmond, 2004; Gijzen and Nürnberger, 2006; Oome and Van den Ackerveken, 2014; Seidl and Van den Ackerveken, 2019). The first NLP family member characterized was the Nep1 protein from *Fusarium oxysporum* causing vascular wilt disease (Bailey, 1995). These proteins, which display strong similarities to cytotoxic actiporins or fungal lectins (Ottmann *et al.*, 2009), have effector-like characteristics and trigger plant defense responses, sometimes associated with necrotic symptoms (Bailey, 1995, Qutob *et al.*,

2006). NLP proteins are able to trigger an oxidative burst, the activation of MAPKs, and the accumulation of phytoalexins (Fellbrich et al. 2000, 2002; Qutob *et al.* 2006). Several studies have shown that the NLPs contribute strongly to the virulence of pathogens (Amsellem *et al.*, 2002; Santhanam *et al.*, 2013; Ono *et al.*, 2020; Schumacher *et al.*, 2020).

NLPs are characterized by conserved structural elements such as an N-terminal 24-amino-acid signal peptide, a domain called NPP1 containing conserved peptide domains such as cysteine residues, and a heptapeptide motif in the central position (Fig. 2) (Van den Ackerveken *et al.*, 1993; Fellbrich *et al.*, 2002). Despite many similarities, NLPs are divided into two groups (Alkan *et al.*, 2015). The group of non-cytotoxic NLPs, identified in hemibiotrophic and biotrophic oomycetes and fungi, elicits a strong immune response without cell death in various plant species (Bailey, 1995; Veit *et al.* 2001; Fellbrich *et al.* 2002; Qutob *et al.* 2006; Dong *et al.*, 2012; Kleemann *et al.*, 2012; Schouten *et al.* 2008; Oome and Van den Ackerveken, 2014). However, the function of these non-cytotoxic proteins for the pathogens is still unclear. The group of cytotoxic NLPs, identified in many necrotrophic or hemibiotrophic plant pathogens, consists of proteins with a strong ability to induce plant immunity but also cell death (Gijzen and Nürnberger, 2006). So far, it is not clear whether plant immunity induced by cytolytic NLPs is indirectly the result of cell death responses or is directly triggered by the NLPs following their recognition by plants (Böhm *et al.*, 2014).

In recent years, various studies have reported that conserved 20- and 24-amino-acid motifs (called nlp20 and nlp24, respectively) in the central part of NLPs act as IPs, since the corresponding synthetic peptides induce an immune response in Arabidopsis (Böhm *et al.* 2014; Oome and Van den Ackerveken, 2014; Oome *et al.* 2014). RLP23, an LRR-RLP, has been identified as the receptor for nlp20 and nlp24 in many plants, including Arabidopsis (Azmi *et al.*, 2018). It has also been reported that, after nlp20 perception, RLP23 forms a complex with SOBIR1 and BAK1 that activates plant immunity (Fig. 3) (Albert *et al.*, 2015, 2019).

In addition to being perceived via an RLP receptor, binding to the PM GIPCs was also suggested to be involved in the recognition of NLPs by plant cells (Fig. 3) (Lenarčič *et al.*, 2017). Accordingly, sedimentation assays using artificial vesicles showed that NLP bound to GIPC-containing vesicles but not to vesicles containing PC only (Lenarčič *et al.*, 2017). Preincubation of NLP with GIPCs also reduced the release of calcein from calcein-loaded vesicles formed from purified Arabidopsis PM, suggesting that prior saturation of the protein with its GIPC receptor hindered its interaction with the vesicles (Lenarčič *et al.*, 2017). X-ray crystallography on NLPs revealed their specific binding to the terminal monomeric hexose moieties of GIPCs, resulting in conformational changes within the protein. It also showed the importance of specific NLP residues for the binding process, explaining the impairment of cytotoxicity of NLPs mutated in those residues (Ottmann *et al.*, 2009; Lenarčič *et al.*, 2017). As for cytolytic actinoporins (Rojko *et al.*, 2016), it is hypothesized that conformational changes in GIPC-NLP complexes induce pore formation in the membrane, leading to cell death (Fig. 3) (Lenarčič *et al.*, 2017; Van den Ackerveken, 2017).

Cytotoxic NLPs have been reported to induce necrosis in eudicots but not in monocots. Biophysical experiments based on calcein release assays with PM vesicles from either tobacco, Arabidopsis, or *Commelina communis* showed a permeabilization effect of NLPs on the first two but not on the latter (Ottmann *et al.*, 2009). The basis for host selectivity was suggested to be related to the structure of the PM GIPCs, more particularly, the length of the GIPC head group (Lenarčič *et al.*, 2017). It was shown by computer simulation that the presence of a third terminal hexose (instead of only two as in eudicots) prevents NLP interaction with the PM (Lenarčič *et al.*, 2017; Van den Ackerveken, 2017). It is still not clear if this interaction leads to the oligomerization of NLPs and thus the formation of a pore inside the PM. NLPs are known as toxins or virulence factors, therefore sphingolipids described as 'receptors' could also be considered 'targets' for NLPs. Finally, it can be hypothesized that the activation of a strong innate immune response leading to an active plant cell death process could be related not only to NLP recognition but also to toxin-like effects mediated through lipid binding (Qutob *et al.*, 2006).

Pore formation

Besides being able to trap sterols from the PM, other proteinaceous IPs, such as harpins or peptaibols, and to some extent NLPs (see above), have the ability to oligomerize and create pores in the PM. Harpins belong to a class of proteins encoded by the *hrp* genes of Gram-negative bacteria and secreted by the type III secretion system during host–pathogen interaction (Fig. 2) (Choi *et al.*, 2013). Harpins are generally characterized as rich in glycine and cysteine free (Liu *et al.*, 2020). They act as virulence factors (or effector helper proteins) for pathogenic bacteria at the host PM level (Medina *et al.*, 2018; Wang *et al.*, 2018). They are able to trigger an HR (Kim *et al.*, 2003; Xie *et al.*, 2017; Liu *et al.*, 2018) and systemic acquired resistance (Dong *et al.*, 1999).

Some reports have pointed out that harpins can function both inside and outside plant cells. A harpin-triggered HR in tobacco required foliar infiltration (Choi *et al.*, 2013). Various in-depth studies with several harpins revealed that specific regions of the protein, composed of specific amino acids, are sufficient to induce an HR (Haapalainen *et al.*, 2011). However, when constitutively expressed in the plant, harpins confer resistance to various plant pathogens without activating an HR (Dong *et al.*, 1999; Fontanilla *et al.*, 2005a; Jang *et al.*, 2006; Niu *et al.*, 2019). This suggests that there are potentially target proteins or receptors located inside the plant cell. In addition, spraying harpin at low concentrations on to plant leaves

induces HR-free defense responses associated with disease resistance (Fontanilla *et al.*, 2005b). This indicates that harpins are also active outside the plant cell and that the level of plant response may be correlated with the concentration of harpin, whether or not it induces an HR. These data also suggest that the potential target interactors of harpins are most likely to be present in the plant PM.

Despite knowledge of the different activities of harpins in plants, their mode of action remains poorly understood. Using a yeast two-hybrid system, the HrpN protein has been shown to interact with HrpN-interacting protein from *Malus* (HIPM) from Arabidopsis and rice, which are also localized to the PM (Oh and Beer, 2007). However, no direct interaction was determined *in vivo*. It was shown that $HrpZ_{Psph}$ is able to bind to the tobacco PM and its PM binding correlates with the induction of defense gene expression (Lee *et al.*, 2001a). Interestingly, a protease treatment does not abolish the binding of $HrpZ_{Psph}$ to tobacco microsomal membranes (Lee *et al.* 2001a). Moreover, it is known that effector helper proteins in pathogenic bacteria can form a pore structure in the host PM through oligomerization (Montagner *et al.*, 2011). Accordingly, HrpZ1 has the ability to form oligomers, with up to 16 monomers, possibly contributing to pore formation in the host PM (Haapalainen *et al.*, 2011). In addition, the association of harpin with plant PM lipids has been suggested through the characterization of the ability of harpin to form pores in artificial lipid vesicles (Fig. 3). *In vitro*, HrpZ1 is able to insert into artificial lipid bilayers of purified membranes and to form cation-permeable pores (Lee *et al.*, 2001b). Full HrpZ1 protein is required for the process of pore formation (Engelhardt *et al.*, 2009). Using a binding test with 15 different membrane lipid components, it was shown that HrpZ1 binds specifically to a membrane PA. By using calcein release (Box 1) from artificial lipid vesicles composed of a mixture of PE, PC, PG, and/or PA, it was shown that HrpZ1 pore-forming activity depends on the presence of PA (Haapalainen *et al.*, 2011). In the case of PopA1 harpin, a stronger *in vitro* affinity for sterols and sphingolipids was observed and the presence of calcium was required for the lipid binding and the PopA1 oligomerization (Racapé *et al.*, 2005). Among the phospholipids, PopA1 displays a higher affinity for PC, then PI, PS, and PE. Carboxyfluorescein leakage assays with artificial liposomes and patch clamp measurements on *Xenopus* oocytes have confirmed the ability of this harpin to form pores within the membrane (Racapé *et al.*, 2005). These data therefore show that several harpins can bind to lipids and can form pores in plant PMs.

The formation of pores is correlated with the oligomerization of harpins in the PM of the host and could be linked to ion leakage (in particular K^+ cation efflux) and to PM depolarization (Popham *et al.*, 1995; El-Maarouf *et al.*, 2001; Haapalainen *et al.*, 2012). However, the modulation of anionic fluxes could be specific to host–pathogen interactions, depending on both the harpin sequence and the allelic variation of the plant host. In Arabidopsis, two purified HrpZ harpins from the compatible pathogen *Pseudomonas syringae* pv. *tomato* DC3000 ($HrpZ_{Pto}$) and the incompatible *P. syringae* pv. *phaseolicola* ($HrpZ_{Pph}$) both induce an HR (Haapalainen *et al.*, 2012). Both HrpZ variants trigger an efflux of K^+ cations. In contrast, $HrpZ_{Pph}$ has no effect on anionic fluxes while $HrpZ_{Pto}$ does. This observation suggests that the HrpZ proteins of different pathovars of *P. syringae* may have insertion specificity into the host PM related to genetic variations among bacteria.

In order to understand the mode of action of harpins, the identification of their interactors is therefore essential. They can be either proteinaceous in nature, like the HIPM-type proteins (Oh and Beer, 2007), or lipidic in nature, like PA or sphingolipids (Racapé *et al.*, 2005; Haapalainen *et al.*, 2011).

Peptaibols represent a family of secondary metabolites with antimicrobial activities produced mainly by soil fungi of the genus *Trichoderma* but also by other fungi. They are composed of 5 to 20 amino acids. They are characterized by a non-ribosomal synthesis and the abundance of α-amino-isobutyric acid (Leitgeb *et al.*, 2007; Ramirez-Valdespino *et al.*, 2019). Peptaibols are able to activate plant defenses and systemic resistance in cucumber (Viterbo *et al.*, 2007), Arabidopsis (Chen *et al.*, 2003), lima bean (Engelberth *et al.*, 2001), tobacco (Kim *et al.*, 2000), and moth orchid (Zhao *et al.*, 2018). Alamethicin (ALM), a 20-mer peptaibol, is the most studied because of its ability to elicit defense responses in plants (Fig. 2). ALM induces an HR-like response in Arabidopsis associated with callose deposition, the accumulation of phenolic compounds and the activation of defense gene expression (Rippa *et al.*, 2010). Its capacity to induce programmed cell death depends on the presence of the α-amino-isobutyric acid residue. In lima bean, ALM also triggers the biosynthesis of volatile compounds via the octadecanoic pathway and salicylate accumulation (Engelberth *et al.*, 2001), leading to a crosstalk between these two hormonal pathways to regulate volatile emissions.

Like harpins, peptaibols can form pores in the PM. Thanks to their amphipathic nature, they are able to bind to the surface and to insert into the PM. Their oligomerization within the membrane leads to the formation of voltage-dependent ion channels and membrane permeabilization (Fig. 3) (Cafiso *et al.*, 1994; Duclohier and Wroblewski, 2001). Different biophysical studies have shown that the pore-formation process is dependent on different parameters, such as the molar ratio between the peptide and lipid, the peptide concentration, and the physical state and surface charge of the membrane (Thippeswamy *et al.*, 2009). The presence of PE impedes ALM binding to the lipid bilayer, while sterols such as cholesterol are suggested to enhance ALM adsorption to the membrane (Duclohier and Wroblewski, 2001). Low concentrations of ALM induce a membrane permeabilization in tobacco cells that is non-lethal, but higher concentrations lead to cell death (Matic *et al.*, 2005; Rippa *et al.*, 2010). Interestingly, no permeabilization could be observed after exposure to ALM in membranes displaying low contents of PS and PI and a low sterol to fatty acid ratio (Aidemark *et al.*, 2010). Although not applied

on membrane models relevant for plant PMs, structural analysis of ALM by X-ray crystallography (Fox and Richards, 1982) and different NMR methods (Bechinger *et al.*, 2001; Salnikov *et al.*, 2009) have suggested that the peptide adopts mixed α-/3_{10}-helical structures into a PC bilayer and is oriented perpendicularly to the membrane surface. This transmembrane orientation can explain the pore formation by ALM according to the helical bundle model (Bechinger, 1999).

Recently, the structural organization of some pore-forming proteins in oligomers and pore entities in lipid membranes have been elucidated for several animal toxins. For example, the virulence factor of *Streptococcus pneumoniae*, pneumolysin, oligomerizes to trigger the formation of pores in cholesterol-rich membranes (Tilley *et al.*, 2005). Cryo-transmission electron microscopy (cryo-TEM) and atomic force microscopy (AFM) (Box 1) demonstrated how pneumolysin docks to cholesterol-rich PM owing to an expulsion of lipids from the hydrophilic inner rim of the pore, explaining its cytolytic activity (Vögele *et al.*, 2019). Some pore-forming proteins have adapted some domains that are able to target the lipid part of the PM. For example, the resolution of the pore structure of the lysenin from the earthworm *Eisenia fetida* by AFM and cryo-TEM at the angstrom level in liposomes revealed the position of a binding pocket for interaction with PC or sphingomyelin (Bokori-Brown *et al.*, 2016; Yilmaz *et al.*, 2018). The powerful techniques of cryo-TEM and AFM could be undoubtedly applied to plant pore-forming toxins. Interestingly, thanks to the use of cryo-TEM, the structure of the complex between an effector and its receptor from *Xanthomonas campestris* pv. *campestris*, ZAR1 (HopZ-activated resistance1), was elucidated. These data could explain its activation and further association to the PM to trigger a plant defense response (Wang *et al.*, 2019, 2020).

Lipid raft modulation

The 5,7-diene oxysterol ergosterol is the main sterol of the fungal PM (Fig. 2) and participates in its stabilization. Ergosterol is able to activate plant immunity (Nürnberger *et al.*, 2004). Due to its similarity to phytosterols located in the PM, ergosterol could target the lipids of the PM, triggering plant immunity. Treatment of plants with ergosterol stimulates early signaling such as calcium fluxes (Vatsa *et al.*, 2011) and a wide range of plant defense responses (Kasparovsky *et al.*, 2004; Laquitaine *et al.*, 2006; Lochman and Mikes, 2006; Rossard *et al.*, 2010; Dadakova *et al.*, 2013; Tugizimana *et al.*, 2014). It was speculated that ergosterol could mediate the inactivation of the jasmonic acid signaling pathway in tobacco since no change in the expression of proteinase inhibitor genes could be detected after ergosterol application (Lochman and Mikes, 2006). In contrast to other sterols, ergosterol stimulates defense markers related to the PM, such as ROS production and alkalinization of the external medium via a transient H^+ influx, in several plant species (Granado *et al.*, 1995; Kauss and Jeblick 1996; Amborabé *et al.*, 2003; Rossard *et al.*, 2006, 2010). In *Beta vulgaris*, this modification of H^+ flux is due to the inhibition of a H^+-ATPase activity (Rossard *et al.*, 2010). The phospholipase A_2 activity inhibitor AACOCF3 prevents ergosterol-induced defense reactions, suggesting that ergosterol elicits defense responses via this enzyme (Kasparovsky *et al.*, 2004).

In *B. vulgaris*, successive applications of ergosterol resulted in a refractory state in the alkalinization response, which was not observed with other sterols (Rossard *et al.*, 2010). These data pointed out the existence of a specific receptor involved in ergosterol sensing. It was also hypothesized that an oxysterol-binding protein (OSBP) could be involved in ergosterol sensing, as in mammalian models, in which it plays a role in vesicle trafficking (Klemptner *et al.*, 2014). Interestingly, proteomics analyses of PM-associated fractions identified several changes in the accumulation of PM proteins, such as chitin elicitor receptor kinase (CERK), aquaporins, or GPI-anchored proteins, in response to ergosterol treatment. However, no specific proteinaceous receptor has been identified so far (Khoza *et al.*, 2019) and ergosterol sensing is still matter of debate. Using membrane models and fluorescence quenching, it was shown that ergosterol can tightly pack to saturated lipids promoting the formation of specialized lipid microdomains (Xu *et al.*, 2001). This suggests that ergosterol can induce perturbations of lipid raft structures (Fig. 3), modulating the function of specific proteins recruited at these sites. Interestingly, and as mentioned above, some RLKs are overexpressed in plant lipid rafts (Cacas *et al.*, 2012), and the NADPH oxidase responsible for the ROS production induced by ergosterol, but not by cholesterol, is located in lipid rafts. Such local perturbations of the PM could thus lead to the induction of a defense signaling cascade and changes in PM protein accumulation.

Intercalation into the plasma membrane bilayer

Amphiphilic compounds are able to interact preferentially with hydrophobic/hydrophilic interfaces such as the two lipid monolayers of the PM. Among bacterial IPs, rhamnolipids (RLs) and cyclic lipopeptides (CLPs) have the characteristic of being amphiphilic (Fig. 2). RLs are glycolipids secreted by *Burkholderia* and *Pseudomonas* species. For these bacteria, RLs are required for virulence, motility, and biofilm formation. They also facilitate the solubilization of some nutrients (Abdel-Mawgoud *et al.*, 2010; Vatsa *et al.*, 2010; Kumar and Das, 2018). RLs are composed of a polar head, composed of one or two rhamnoses, and a lipid tail, consisting of one, two, or three 3-hydroxy fatty acids chains of 6 to 16 carbons in length (Abdel-Mawgoud *et al.*, 2010).

Natural RLs induce plant defense responses in Arabidopsis, which are effective against various pathogens (Sanchez *et al.*, 2012). RLs also trigger an immune response in other plants,

such as grapevine, rapeseed, and wheat (Varnier *et al.*, 2009; Monnier *et al.*, 2018, 2020; Platel *et al.*, 2021).

Interestingly, synthetic bioinspired RLs are also known to induce plant immunity, such as RL bolaforms (Obounou Akong and Bouquillon, 2015; Luzuriaga-Loaiza *et al.*, 2018) or Ac- and Alk-RLs (Nasir *et al.*, 2017), with a carboxyl or a methyl group, respectively, at the end of the carbon chain. Synthetic mono-RLs with a simplified lipid tail also trigger early and late immunity-related plant defense responses and protection against *Botrytis cinerea* in tomato (Robineau *et al.*, 2020) and against *Zymoseptoria tritici* in wheat (Platel *et al.*, 2021). In these studies, structure–function analysis showed that fatty acid chain length is critical for the efficacy of the immune response.

How RLs are perceived by plant cells remains unknown. Interestingly, whereas no proteinaceous receptor for RL perception has been identified so far, the mc-3-OH-acyl building block of RLs is sensed by the lectin S-domain-1 receptor-like kinase LORE (Kutschera *et al.*, 2019). It is postulated that due to their amphiphilic nature, RLs could directly interact with plant membrane lipids (Fig. 3) (Schellenberger *et al.*, 2019). Accordingly, biophysical studies have shown that natural RLs are able to fit into plant lipid-based membrane models (Monnier *et al.*, 2019). By using Fourier transform infrared spectroscopy on 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine liposomes, it was demonstrated that RLs are located near the lipid phosphate groups. It seems that RLs have the same behavior whatever the saturation degree of the lipids (Sánchez *et al.*, 2009; Abbasi *et al.*, 2012). While their insertion inside the lipid bilayer does not strongly affect lipid dynamics according to NMR experiments (Box 1) on PC liposomes, the introduction of stigmasterol, or to a lower extent β-sitosterol, triggers an enhancement in lipid dynamics. This suggests that the nature of the phytosterols could influence the effect of RLs on plant PM destabilization (Monnier *et al.*, 2019). For the synthetic RLs Alk-RL and Ac-RL, it was shown that the presence of sterols tends to increase their interaction with lipid bilayers, giving rise to a fluidizing effect on the lipid alkyl chains (Nasir *et al.*, 2017). On the contrary, for the synthetic RL bolaform, sitosterol rather limits its insertion into the membrane without impairing it (Luzuriaga-Loaiza *et al.*, 2018). RLs are capable of modulating the membrane structure; they have dehydration and tightening effects on the lipid polar heads (Monnier *et al.*, 2019). In the case of the synthetic RL bolaform, a transient perturbation of the bilayer was suggested to play a role in its eliciting activity (Luzuriaga-Loaiza *et al.*, 2018).

The structure of the RLs has also an influence on their membrane properties. The main difference between the mono-RL and the di-RL, which are the two main RLs produced by *Pseudomonas aeruginosa*, is their location in the transverse plane of the membrane, the mono-RL being inserted more deeply. A more favorable insertion of Alk-RL than Ac-RL into the lipid membrane was also observed (Nasir *et al.*, 2017). Interestingly, Alk-RL was shown to be more potent in inducing defense responses than Ac-RL. This suggests that differences in the biological activity of these molecules could be linked to their amphiphilic nature and their capacity to interact with the membrane.

CLPs are multifunctional secondary metabolites secreted by various microorganisms (Raaijmakers *et al.*, 2010). Some CLPs secreted by beneficial bacilli and pseudomonads are effective IPs as they are able to stimulate the plant immune system (Crouzet *et al.*, 2020; Pršic and Ongena, 2020). CLPs from *Bacillus* species including surfactin, mycosubtilins, and fengycins activate early immune-related events and plant defense responses in tobacco, grapevine, strawberry, tomato, and Arabidopsis (Jourdan *et al.* 2009; Debois *et al.* 2015; Farace *et al.* 2015; Han *et al.* 2015; Kawagoe *et al.* 2015; Yamamoto *et al.* 2015; Farzand *et al.* 2019; Li *et al.* 2019). Similarly, orfamides, which originate from *Pseudomonas*, are able to trigger defense responses in rice (Ma *et al.* 2017).

How lipopeptides are perceived by plant cells remains puzzling. Several indications suggest that these compounds are not perceived by high-affinity receptors. Purified lipopeptides activate plant immune responses at relatively high concentrations compared with canonical IPs, which are usually active at nanomolar levels. In addition, immune responses triggered by surfactin in tobacco are not compromised in protease-treated cells (Jourdan *et al.*, 2009; Henry *et al.*, 2011), and successive applications of this IP do not display a refractory state usually linked to IP perception by high-affinity receptors (Jourdan *et al.*, 2009). Some evidence suggests that, instead, lipopeptide perception involves a direct binding to lipids that could be indirectly sensed by plants as a stress response (Fig. 3) (Henry *et al.*, 2011). Lipopeptides are active at different concentrations depending on the plant species (Pršic and Ongena, 2020). This may be explained by the variability of the lipid composition of the PM depending on the plant species/organ. NMR studies (Box 1) showed a deep intercalation of surfactin into the lipid bilayer of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine vesicles and a tilt of the acyl chains. Its strong anchoring in the outer leaflet creates an imbalance in lateral pressure between the outer and the inner leaflets, leading to a curvature strain (Heerklotz *et al.*, 2004). In the case of surfactin, its interaction is dependent on the physical state of the membrane model and is improved in the presence of solid-like domains. Structure–function experiments revealed that longer homologs bind more efficiently to plant biomimetic membranes, which is correlated with their eliciting activities on tobacco cells (Jourdan *et al.*, 2009; Henry *et al.*, 2011). In this case, no destructive action was observed, unlike the detergent effect or pore formation observed on microorganism-derived biomimetic membranes that could explain their antimicrobial activities (Balleza *et al.*, 2019). The lipid phase of membranes could act as an anchoring platform for some of these lipopeptides, and experimental biophysics and also *in silico* dynamic modeling could help to elucidate lipopeptide–lipid interactions (Deleu *et al.*, 2014; Balleza *et al.*, 2019; Nishimura and Matsumori, 2020).

Plasma membrane phospholipid disturbance

Chitosan is a β1,4-linked glucosamine polymer and thus a chitin derivative found in the cell walls of many fungi (Fig. 2). The term 'chitosan' refers not to only one molecule but to a family of compounds that differ in, for example, their degree of deacetylation or polymerization and their viscosity (Badawy and Rabea, 2011; Iriti and Varoni, 2015). Chitosan displays some direct antibacterial (Kong *et al.*, 2010) and antifungal activity by inhibiting mycelium growth or spore germination (Hadwiger and Beckman, 1980; Benhamou, 1992; El Hassni *et al.*, 2004; Badawy and Rabea, 2011). Chitosan is more potent against fungi than bacteria and has a greater antibacterial effect on Gram-positive than Gram-negative bacteria (No *et al.*, 2002). By inducing plant defense mechanisms, chitosan protects several plants against fungal, bacterial, or viral pathogens (Badawy and Rabea, 2011), including grapevine against *B. cinerea* and *Plasmopara viticola* (Reglinski *et al.*, 2010; Brulé *et al.*, 2019) or barley against *Blumeria graminis* f sp. *hordei* (Faoro *et al.*, 2008). The efficiency of chitosan depends on its physicochemical characteristics, such as the degree of deacetylation, viscosity, and molecular weight (Iriti and Faoro, 2009).

Chitosan induces typical defense responses (Walker-Simmons *et al.*, 1984; Köhle *et al.*, 1985; Kauss and Jeblick 1996; Zuppini *et al.*, 2004; Aziz *et al.*, 2006; Rossard *et al.*, 2006; Raho *et al.*, 2011). It stimulates the octadecanoid pathway leading to the production of jasmonates in rice (Rakwal *et al.*, 2002). Chitosan-induced resistance against tobacco mosaic virus is mediated by abscisic acid (Iriti and Faoro, 2008). Interestingly, chitosan induces plant immune reactions that are directly connected to sensing and signal transduction taking place at the plant PM level. For instance, chitosan stimulates PA production via the activation of both phospholipase D and phospholipase C/diacylglycerol kinase (Raho *et al.*, 2011) and, like ergosterol, chitosan inhibits the PM H^+-ATPase (Amborabé *et al.*, 2008).

As with ergosterol, a refractory state following a second application of chitosan has been observed (Amborabé *et al.*, 2003), suggesting the existence of a receptor for chitosan. It is plausible that ergosterol and chitosan have different PM targets because after a first hyperpolarization induced by ergosterol, cells remained responsive to chitosan. In wheat, several candidates for the chitosan receptor have been proposed (Liu *et al.*, 2018). These include a potential wall-associated kinase 1 (WAK1) receptor protein and G-type lectin S-receptor-like serine/threonine-protein kinases. In grapevine, two LysM receptor kinases are important for chitosan-triggered immunity (Brulé *et al.*, 2019). However, direct proof that these proteins directly interact with chitosan is still lacking. It was also speculated that CERK1 bound chitin and chitosan (Petutschnig *et al.*, 2010), but another study demonstrated that chitosan was perceived by a CERK1-independent pathway (Povero *et al.*, 2011). Thus, similarly to other IPs, chitosan could generate PM disturbance by interacting with PM lipids, leading to the induction of plant immune responses (Fig. 3). Biophysical studies performed on different membrane models mimicking mammalian or bacterial membranes, but not plant membranes, have suggested that the mechanism of interaction of chitosan with the membrane lipids is dependent on its concentration. At low concentration, chitosan chains form a core-shell structure around liposomes by electrostatic interaction (Tan *et al.*, 2015). At higher concentration, chitosan embeds into the lipid bilayer and increases the membrane fluidity, as shown by fluorescence polarization (Tan *et al.*, 2013), which may lead to membrane disruption. Recently, it was shown with pure lipid Langmuir monolayers that chitosan induces more significant expansion in anionic lipid films than zwitterionic ones (de Oliveira *et al.*, 2020). Moreover, the presence of raft-mimicking lipids in the monolayer increases the effect of chitosan (Pereira *et al.*, 2020). All these perturbations could be at the origin of its eliciting activity.

Conclusions

Biological control is a promising alternative to conventional chemical control of plant pathogens. Deciphering the mechanism of IP recognition by plant cells at a molecular scale is a prerequisite to control its use in agroecological strategies. Moreover, the knowledge of the relationship between the chemical structure of an IP and its mechanism of perception by the plant cell is essential to design new eliciting molecules and predict their efficacy.

Whereas the majority of microbial IPs are known to be perceived by specific proteinaceous PRRs, an increasing number of studies are reporting that the sensing of some IPs involves the lipid phase of the PM as a first target. Depending on the IPs, different mechanisms could be involved, such as sterol trapping or membrane disturbances. Moreover, it was recently demonstrated that GIPCs could act as PM receptors for NLPs. Because of their amphiphilic nature, some IPs are likely to be sensed by a direct intercalation between PM lipids. In this respect, the case of sensing of lipopolysaccharides (LPS) is intriguing. The LORE receptor recognizes the LPS precursor but not the LPS itself (Kutschera *et al.*, 2019). Moreover, a higher degree of complexity is added as some IPs are perceived by several mechanisms. For example, elicitins and NLPs are perceived by PRRs, but they are also able to bind lipids functioning as receptors. This could ensure a rapid and/or strong activation of the plant immune system, as well as their capacity to target a large spectrum of plants. Looking at these data in a new light, this suggests that the organization of the PM may be involved at different levels in the pathways of defense signaling. Although some changes in PM organization are known to correlate with signal initiation, the functional implications and molecular basis of these membrane changes remain to be elucidated.

Thus, it is now evident that PM lipids play central roles in the sensing of IPs, either directly or indirectly. The in-depth study of the localization of an IP and its influence on the organization of membrane lipids is of particular importance for understanding their mechanism of action. However, the identity of the lipids involved in that sensing is far from being solved. For some IPs, only some hypothesis could be formulated. Besides biological studies, biophysical approaches can also be very useful in addressing these questions by combining information obtained from several types of artificial biomimetic membranes and biophysical tools. The main biophysical approaches are summarized in Fig. 1B and Box 1. However, other biophysical techniques, commonly applied to mammalian biomimetic membranes, could also be considered (Deleu *et al.*, 2014; Furlan *et al.*, 2020; Munusamy *et al.* 2020). The combination of several biophysical techniques, with their respective advantages and drawbacks (Table 1), will give a precise picture of the molecular mechanism. Likewise, structural and conformational analysis by X-ray crystallography, NMR, infrared spectroscopy, and circular dichroism, particularly suitable for peptides and proteins, but also by molecular simulation, are of crucial importance to decipher their mode of action. The reader can refer to Munusamy *et al.* (2020) and Huggins *et al.* (2019) for an in-depth description of these techniques. Biophysical approaches help us understand how a molecule could interact with lipids arranged in a membrane. To date, most of the data originate from experiments done on simplified membrane models with a composition far from that of the plant PM, and biophysics approaches on purified plant membranes with appropriate lipid markers will be the next important step. Functional biology as well as *in vivo* binding experiments are necessary to confirm the role of lipids as direct interactors or receptors for IPs. Unfortunately, mutants affected in lipid composition are often non-viable or display pleiotropic effects. Therefore, there is still a serious lack of available methodologies, and the generation of molecular or microscopic tools is essential to better understand the role of lipids in the perception of IPs. In contrast to the mammalian PM, one limitation is also a better understanding of the structure and dynamics of the plant PM, and also the effects of the cell wall on these PM properties. Combining data generated by biophysics with molecular biology, biochemistry, and cell biology should help us better understand the role of PM lipids in the process of IP perception, which is crucial to activate the plant immune system.

Author contributions

SC, JC, SD, MD, and SD-C contributed to the manuscript conceptualization, writing, review and editing; MD, GG, and SD-C prepared the figures.

Conflict of interest

The authors declare no conflict of interest.

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