Opinion piece

Challenges in the identification of microbe-associated molecular patterns in plant and animal innate immunity: a case study with bacterial lipopolysaccharide

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Immunity against pathogen infection depends on a host's ability to sense invading pathogens and to rapidly trigger defence reactions that block pathogen proliferation. Both plants and animals detect conserved structural motifs of microbe-specific compounds, so-called microbe-associated molecular patterns (MAMPs), through germline-encoded immune sensors, which are accordingly termed pattern recognition receptors (PRRs) (Akira *et al.*, 2006; Boller and Felix, 2009). Activated PRRs initiate signal transduction and trigger innate immune responses. MAMPs are generally derived from elements essential for microbial fitness and are conserved across species, thus enabling the host to detect a range of potential pathogens. In mammals, innate immune sensing of MAMPs is not only crucial for basal immune responses but is also tightly connected with and required for a subsequent adaptive, antibody-mediated immunity (Akira *et al.*, 2006; Janeway and Medzhitov, 2002). Plants, lacking an adaptive immune system, have apparently evolved a greater capacity to detect a broader repertoire of MAMPs. Different plant species possess distinct sets of highly specific PRRs, but the downstream signalling pathways are rather conserved and converge on common signalling steps. This allows the transfer of PRRs, even to different plant families, whilst maintaining their functionality and specificity (Zipfel, 2014). This also enables researchers to use well-studied, genetically amenable model systems for the identification of MAMPs and their respective PRRs. Several examples of interfamily PRR transfer have demonstrated that the introduction of novel PRRs into plant species can confer relevant levels of resistance to otherwise susceptible plants (e.g. Afroz *et al.*, 2011; Hao *et al.*, 2015; Lacombe *et al.*, 2010; Mendes *et al.*, 2010; Schoonbeek *et al.*, 2015; Tripathi *et al.*, 2014). Hence, MAMP sensing by PRRs has great potential for the engineering of disease resistance in crop plants. In recent years, it has therefore become a major task to identify and isolate MAMPs from a range of microorganisms, and their respective PRRs, to study their role in innate immunity and their application potential.

IDENTIFICATION OF MAMPS—LOOKING FOR THE RIGHT COMPOUND?

The identification of a novel MAMP usually begins with the detection of the capacity of a microbe-derived molecular fraction to elicit early immune responses in a plant. Such MAMP-like activity is often detected within a crude extract derived from different microbial samples, such as culture filtrates, cell wall components or water-soluble extracts. MAMPs are generally sensed by their cognate PRRs in the picomolar to nanomolar concentration range. Hence, only small amounts of a certain MAMP within such a complex mixture will usually be sufficient to induce the observed immune reactions and it is not necessarily the most obvious and abundant compound within this mixture that is causal for the observed activity. In this regard, it is noteworthy that several MAMPs have been identified serendipitously as a 'contaminating' activity when bacterial culture preparations have been analysed for activities of other MAMPs in plants. Flagellin, for instance, was the actual active component in a harpin preparation from *Pseudomonas syringae* pv. *tabaci*, EF-Tu (elongation factor thermo unstable) in boiled extracts from an *Escherichia coli* mutant lacking flagellin and bacterial cold-shock protein (CSP) in a peptidoglycan (PGN) preparation (Felix and Boller, 2003; Felix *et al.*, 1999; Kunze *et al.*, 2004). A major challenge is to isolate this highly active, but often less abundant, substance in sufficient amounts and purity to facilitate its identification by state-of-the-art analytical methods. Depending on the chemical nature of the putative MAMP, this can be extremely laborious, demanding sophisticated biochemical and analytical skills. In some cases, purified MAMPs have not yet been identified, but the cognate PRRs have nevertheless been identified through genetic screening (Jehle *et al.*, 2013; Zhang *et al.*, 2013).

Following the successful elucidation of the molecular nature of a microbial elicitor, the next step is usually to identify the minimal MAMP-active motif and to characterize its structural features, mostly by the identification of modifications that render the MAMP inactive. Such structure–function relations will already provide a good clue if the compound under investigation is indeed that

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causing the observed MAMP activity. Eventually, if possible, a pure synthetic compound should provide the final proof. This is comparatively easy if the MAMP is a simple molecule (e.g. DNA, ATP) that is available synthetically in reasonable amounts and purity, or a protein that can be produced and purified from different sources by commonly available state-of-the-art methods. If correctly folded and native proteins are required, these can, for instance, be transgenically expressed *in planta* or be purified from heterologous expression systems. In other cases, synthetic peptides representing the minimal MAMP motif may be sufficient to trigger immune responses. As a cautionary note, given the recent examples of contaminations with unrelated immune-inducing 'pirate' peptides in peptide preparations, independent batches of synthetic peptides (or receptor mutants of the suspected contaminants) should be used for validation (Mueller *et al.*, 2012). Furthermore, modifications of proteins/peptides occurring under natural conditions may be required for their optimal activity (Kunze *et al.*, 2004; Lee *et al.*, 2012; Matsubayashi, 2011). Although a non-modified protein version can also be active if applied at higher concentrations, it may be preferable to use the physiologically relevant MAMP structure for the most conclusive structure–function relation. For complex structures, such as oligomeric carbohydrates (e.g. β -glucan, chitin), or even mixed conjugates of peptide, lipid and carbohydrate moieties (e.g. PGN) (Felix *et al.*, 1993; Gust *et al.*, 2007; Sharp *et al.*, 1984; Silipo *et al.*, 2010), the identification, purification and characterization of the MAMP-active component are particularly challenging. Chemical synthesis of such complex conjugates is often not feasible by currently accessible methods, forcing investigators to rely exclusively on the purification of these compounds from the source organisms, and thus bearing the risk of the co-purification of contaminants with potent MAMP activity.

One example of such a complex glycolipid conjugate is the Gram-negative bacterial cell surface component lipopolysaccharide (LPS). The common structure of these tripartite amphiphilic macromolecules consists of lipophilic lipid A, which functions as a membrane anchor and is linked to a hydrophilic saccharide part consisting of a rather conserved oligosaccharide core region and a highly diverse O-polysaccharide (OPS) consisting of a variable number of repeat units. Both plants and animals have the ability to sense different parts of LPS (Ranf, 2016). Lipid A is sensed in picomolar concentrations by the mammalian innate immune system through unrelated extra- and intracellular immune receptors, namely the cell surface Toll-like receptor 4 (TLR4)/myeloid differentiation factor-2 (MD-2) complex, the serum glycoprotein CD14 and intracellular inflammatory caspases. By contrast, many of the reported LPS-induced responses in plants require high LPS concentrations in the range 50–100 $\mu\text{g/mL}$ (Ranf, 2016). The high LPS concentrations applied in many cases and contradicting findings have, however, raised concerns about the specificity of the analysed responses for LPS. As a result of the intrinsic chemical and

physical properties of LPS, these issues are, however, quite difficult to resolve unambiguously.

WHY IS LPS PARTICULARLY CHALLENGING TO WORK WITH?

The LPS structure differs substantially between bacterial species and strains, and a single bacterium comprises a mixture of structurally different LPS variants. *Pseudomonas aeruginosa*, for example, contains, in parallel, LPS carrying either linear oligo-rhamnans or complex OPS, as well as LPS lacking OPS, all of which can further differ in their lipid A acylation pattern as well as diverse non-stoichiometric decorations (e.g. phosphate, phosphoethanolamine or aminoarabinose residues) (Knirel *et al.*, 2006). Moreover, LPS structures are dynamically regulated by bacteria for adaptation to different and often hostile environments, e.g. through the modulation of negative charges, acylation patterns or the OPS chain length (Ranf, 2016). Hence, LPS extracted from bacterial cell walls intrinsically comprises a complex mixture of different LPS variants which usually also show different biological activities as MAMPs. The composition of the LPS mixture thereby strongly depends on the cultivation conditions (e.g. temperature, pH, Mg^{2+} , Ca^{2+} and Fe^{3+} concentrations, antibiotics, etc.). In addition, the purification conditions may also affect LPS activity through the loss or modification of moieties critical for biological activity (Knirel *et al.*, 2006). The phosphoramidate group in the *Xanthomonas* core oligosaccharide, for example, is sensitive to hydrolysis and the loss of negative residues impedes LPS activity, as interaction with putative receptors supposedly depends on electrostatic interactions (Silipo *et al.*, 2005). The remarkable size and structural heterogeneity of LPS, which are strongly influenced by the bacterial strain and the culture and purification conditions, complicate comparisons between different studies, even when similar bacteria are used.

It should be further noted that LPS is not water soluble, but immediately forms aggregates and micelles in hydrophilic solutions. Together with the large size and structural heterogeneity, it is therefore difficult to assess the actual bioactive concentration of isolated LPS. LPS aggregation might further hinder its access through the cell wall matrix and binding to its receptor(s) (Zähringer *et al.*, 2008). Indeed, in the mammalian immune system, diverse LPS-binding proteins facilitate the disaggregation of such LPS aggregates into LPS monomers and promote receptor binding (Ranf, 2016). In plants, no such LPS-binding proteins have been identified to date. The reduced bioavailability may be one reason why the LPS concentrations required in plants are quite high compared with the situation in mammalian systems and with other MAMPs in plants, most of which are active in the nanomolar range. Alternatively, it has been suggested that plants may possess only a low-affinity perception system for LPS (Zeidler *et al.*, 2004). Recently, however, we have identified the first LPS receptor

component, the receptor-like kinase LORE (LipoOligosaccharide-specific Reduced Elicitation), which mediates sensitive perception of *Pseudomonas* and *Xanthomonas* LPS at concentrations of about 0.2–0.5 µg/mL (approximately corresponding to 50–100 nM) in *Arabidopsis thaliana* and triggers typical pattern-triggered immunity (PTI) responses (Ranf *et al.*, 2015). The low-affinity NO-inducing (Zeidler *et al.*, 2004) and sensitive $[Ca^{2+}]_{cyt}$ elevation-inducing perception might, however, originate from the recognition of different LPS moieties through distinct receptors.

In addition, isolated LPS might cause non-specific stress to plants with increasing concentrations because of its amphiphilic 'detergent-like' nature. LPS has been reported to integrate into biological membranes via its lipophilic lipid A moiety (Nomura *et al.*, 2008), which may disturb membrane integrity and activate stress responses. Moreover, it cannot be excluded that the observed activities are not caused by LPS itself, but might potentially be a result of minor but potent contaminants in the LPS preparations. In this regard, several reports from the animal field have raised the suspicion of the misinterpretation of results because of contamination of MAMP preparations. In mammals, highly active lipoprotein/lipopeptide contaminants in PGN or LPS preparations are probably the actual TLR2 activators, as re-purification of such preparations abolished TLR2 activation, whereas PGN or LPS itself most probably do not activate TLR2 (Zähringer *et al.*, 2008). Similarly, in *Drosophila*, immune responses to commercial LPS preparations were caused by PGN contaminations. As purified LPS is inactive, *Drosophila* most probably does not detect LPS, but distinguishes between Gram-negative and Gram-positive bacteria through the sensing of specific PGN forms (Leulier *et al.*, 2003). Analyses based on knockout mutants of the established plant PRR EFR (EF-Tu receptor) also pointed to significant contaminations with bioactive EF-Tu proteins/peptides in some commercially available LPS and PGN preparations. Plant defence responses to these LPS and PGN preparations were also dependent on the co-receptor BAK1, as reported previously (Shan *et al.*, 2008), whereas in-house prepared, protein-free LPS does not require BAK1 (Ranf *et al.*, 2015). Correspondingly, proteinase K treatment of the former preparations completely abolished EFR- and BAK1-dependent responses (S. Ranf *et al.*, unpublished results). Along this line, it should be noted that the core oligosaccharide of *Pseudomonas* LPS is extensively phosphorylated (Knirel *et al.*, 2006) with an increased propensity to interact with undesirable contaminants through negative charges. Indeed, we identified a polyamine co-purifying with our *Pseudomonas aeruginosa* LPS. This polyamine was so strongly associated with the negative core residues that it was only removed on complete chemical deacylation and dephosphorylation of the LPS (Ranf *et al.*, 2015). The isolated polyamine led to a shift in the calcium resting level, potentially by interacting with membrane

phospholipids or inducing a pH shift in the apoplast (S. Ranf and N. Gisch, unpublished results).

The cell surface receptor-like kinase LORE specifically mediates the perception of *Pseudomonas* LPS-type lipid A structures in cruciferous plant species (Ranf *et al.*, 2015). Accordingly, defence responses induced by LPS in other plant species, e.g. rice or tobacco, or by enterobacterial *Escherichia coli* or *Burkholderia cepacia* LPS, as reported in *Arabidopsis*, are not mediated by LORE. Further structure–function analyses, as well as genetic studies, are required to resolve whether these LPS perception mechanisms follow a classical PRR-mediated pathway, possibly a non-canonical PTI mechanism, or are caused by non-specific stress reactions to LPS. Only the identification of the exact patterns within the LPS causing the different activities and their respective receptors will clarify the role of LPS from different bacterial species in plant–microbe interactions. Future studies will thus reveal whether single plant species also possess several LPS immune sensors in parallel, like mammals, and whether the plant kingdom has evolved different LPS sensors in different plant lineages.

PERCEPTION OF MOLECULAR PATTERNS—ONE LIGAND FOR ONE RECEPTOR?

Based on the lock-and-key principle designed by Emil Fischer, it was initially thought that a certain receptor (the lock) would only be activated by highly specifically binding a single ligand (the key). However, it is evident that a receptor may actually bind several ligands. Mostly, however, these ligands belong to a family of related ligands that share a common core structure. Examples of such ligand families are the CLE peptides involved in plant development or the AtPep peptides involved in plant defence responses (Bartels *et al.*, 2013; Kucukoglu and Nilsson, 2015). In the case of PRRs, the conserved core structure would represent the 'molecular pattern', the part of the ligand common to different species. Different hetero-complexes formed by combinations of different receptors may perceive structurally different ligands, e.g. mammalian TLRs (O'Neill *et al.*, 2013). In other cases, however, even structurally completely unrelated ligands have been proposed to bind and/or activate the same receptor. This has raised substantial concerns as to whether these proposed ligands are indeed genuine ligands of these receptors or whether contaminations with traces of the established and mostly very potent ligands of these receptors cause the observed responses. For example, several endogenous mammalian compounds, which share no structural similarity to lipid A at all, have been reported to bind or activate TLR4 (Erridge, 2010). However, in several cases, subsequent studies using candidate ligands in a highly purified form, as well as LPS-free preparatory and test reagents, could not confirm these previous observations,

suggesting that the TLR4 activation was instead caused by traces of LPS in either the ligand preparation or other experimental reagents. Similarly, the bacterial Ax21-derived peptide and the plant CLV3-related peptide, which regulates the shoot apical meristem during development, have been proposed to bind to the immune PRR FLS2 (Flagellin Sensing2) (Danna *et al.*, 2011; Lee *et al.*, 2011). Although FLS2 is activated by its genuine ligand, the bacterial flagellum-related peptide MAMP flg22, already at subnanomolar concentrations, significantly higher concentrations were required for both Ax21- and CLV3-related peptides. As such concentrations are probably not physiologically relevant and subsequent studies could not confirm these results, it was supposed that trace contaminations with the highly potent flg22 peptide caused the observed phenotypes (Mueller *et al.*, 2012; Segonzac *et al.*, 2012). However, both studies used slightly different versions of the peptides and different experimental conditions that possibly might also account for the different results (Danna *et al.*, 2012; Lee *et al.*, 2012). Later studies showed that the Ax21 peptide does not act as a MAMP recognized by the rice immune receptor XA21 (Bahar *et al.*, 2014), raising further doubts about the proposed activation of FLS2 immune signaling by Ax21-derived peptides in Arabidopsis.

GENERAL CRITERIA FOR MAMP IDENTIFICATION—WHAT CAN WE LEARN FROM PAST EXPERIENCES?

Ultimately, there are no universal rules that can be applied to distinguish a 'real' MAMP from a 'contamination', but we can propose some 'logical' guidelines from former experiences that may aid in the purification and characterization of candidate MAMPs.

1. Always use the most purified form of your candidate MAMP.
2. Perform dose–response analyses to assess the activity of the candidate MAMP in a physiologically relevant concentration range. Use a robust and quantitative read-out method. Make sure that the response is reproducible, also between different batches of MAMP preparations.
3. Determine an unequivocal structure–function relationship by identifying the minimal motif, i.e. the 'molecular pattern', sufficient for the activation of immune responses, and by using MAMP preparations from different source organisms.
4. Eventually, obtain genetic evidence, either on the microbial side, e.g. by alteration of the proposed MAMP, or on the plant receptor side, e.g. by the use of established receptor mutants to exclude contaminations or by the identification of mutants insensitive to the candidate MAMP.
5. Whenever possible, demonstrate direct binding of the MAMP to the proposed PRR, e.g. by receptor binding assays or receptor–ligand co-crystallization, and determine the binding affinity, e.g. by thermodynamic interaction studies.

CONCLUSIONS

The identification of a MAMP is not a trivial task and not all the above criteria are feasible in each case, possibly because of technical constraints. Thus, it remains the researcher's responsibility to carefully evaluate each case with regard to the specific conditions. Generally, however, a host must be able to sense a MAMP at very low concentrations to ensure the early detection of and rapid defence against invading pathogens. Accordingly, well-established MAMPs, either in mammals or plants, are active at very low concentrations. Thus, if high concentrations of a putative MAMP are required to induce immune responses, careful consideration is required to determine whether the compound under investigation is indeed the true MAMP-active component, or whether the observed responses are rather caused by a non-specific stress response induced by high sample concentration instead of a sensitive, receptor-mediated sensing mechanism. Notably, even commercially available substances often do not possess the required purity. Even at a purity of 99%, they still contain 1% of other, possibly highly active, substances. Thus, commercial, non-synthetic MAMP preparations should be carefully analysed for their purity and may require additional purification steps before being applied to a sensitive biological system. Therefore, in future studies of candidate MAMPs, special care must be taken to ensure that elicitor preparations of microbial origin are free from potent undesirable contaminants through stringent purification procedures, and preferably such studies should be combined with a clear structure–function relationship analysis.

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