

Releasing hidden MAMPs from precursor proteins in plants

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ABSTRACT

The recognition of pathogens by plants at the cell surface is crucial for activating plant immunity. Plants employ pattern recognition receptors (PRRs) to detect microbe-associated molecular patterns (MAMPs). However, our knowledge of the release of peptide MAMPs from their precursor proteins is very limited. Here, we explore seven protein precursors of well-known MAMP peptides and discuss the likelihood of processing being required for their recognition based on structural models and public knowledge. This analysis indicates the existence of multiple extracellular events that are likely pivotal for pathogen perception but remain to be uncovered.

The recognition of pathogens by plants at the cell surface is mediated by pattern recognition receptors (PRRs), that recognize fragments of conserved polymers of pathogens, known as pathogen-associated molecular patterns (PAMPs) or microbe-associated molecular patterns (MAMPs). MAMP recognition elicits immune responses that include the production of reactive oxygen species (ROS) and ethylene, apoplast alkalinisation, MAP kinase phosphorylation, and transcriptional reprogramming [1]. The release of MAMPs from their precursors appears as an essential step in pathogen recognition, but our knowledge of the process of MAMP release and the involved enzymes is very limited. Here, we discuss seven protein precursors of well-known MAMP peptides including the flagellin-derived FLS2 and FLS3 elicitors; the EF-Tu-derived EFR elicitor; the CSP-derived CORE elicitor; the NLP-derived RLP23 elicitor; the polygalacturonase-derived RLP42 elicitor; and the transglutaminase-derived PERU elicitor. These MAMPs represent a subset of well-studied elicitors that produced knowledge on the minimal bioactive peptide elicitor and relevant residues required for the binding and activation of their respective PRRs. Other peptide elicitors were considered but are less well-defined or their precursor produced unreliable structural models. We discuss the likelihood of processing being required for elicitor release based on structural models and existing public knowledge.

Release of flagellin-derived FLS2 elicitor

Most flowering plants recognize flg22, a highly conserved 22-amino acid peptide sequence present in the N-terminal half of bacterial flagellin [2]. The flg22 sequence is important for bacteria because it comprises the hinge region in the flagellin protein, which is essential for the conformational change in the flagellin polymer when the rotational direction changes [3]. The flg22 peptide is recognized by

flagellin sensing-2 (FLS2), which is a well-studied receptor kinase with extracellular leucine-rich repeats originally identified from *Arabidopsis thaliana* triggering plant immunity [4]. Some FLS2 homologs present in other angiosperms can recognize different sequences of flg22 that escape recognition by Arabidopsis FLS2. Soybean FLS2 (GmFLS2), for instance, is also able to recognise flg22 from *Ralstonia solanacearum* [5], whereas grapevine FLS2 (FLS2^{XL}) also recognizes flg22 from *Agrobacterium tumefaciens* [6]. The originally described flg22 peptide was based on a conserved region of the flagellin of *Pseudomonas aeruginosa* [2]. Six residues in flg22 (underlined in QRLSTGSRINSAKDDAAGLQIA) are essential for recognition by FLS2 [2,5,7–11]. This flg22 peptide can be truncated to flg15 (RINSAKDDAAGLQIA) and still be active as an elicitor in tomato but not in *A. thaliana* or *Nicotiana benthamiana* [2,12–15]. Further truncations of 4 to 7 amino acids residues from the C terminus of flg15 peptides (termed flg15-Δ4 to flg15-Δ7) resulted in competitive antagonists of elicitor-active flagellin peptides and proteins in tomato [2]. Peptides of 18-22 amino acid residues including the flg15 sequence have been detected by mass spectrometry upon incubation of flagellin with apoplastic fluids [16], suggesting the release of the immunogenic peptides by precursor processing in the plant apoplast.

The release of flg22 from its precursor seems necessary because the flg22 peptide is deeply buried in the flagellin polymer, making it entirely inaccessible for FLS2 perception. The flagellin polymer contains ~10⁴ subunits in which each flagellin monomer self-assembled on top of each other in a rod-like structure [17–19]. In this rod-like structure, the exposed surface on each flagellin monomer carries six sites with O-glycosylation, resulting in an O-glycosylated surface of the flagellin polymer [20]. These glycans play a crucial role in delaying flg22 recognition by the plant immune system [21]. Even at the monomeric stage of flagellin, a significant portion of flg22 remains buried within the flagellin structure, with predicted residue exposures falling below 50% (**Figure 1A**). It is therefore evident that recognition of the immunogenic epitope of flagellin by FLS2 requires its exposure, which probably involves deglycosylation, depolymerisation, unfolding, and N- and/or C-terminal processing. The first step of elicitor release from some *P. syringae* strains is probably mediated by a plant-secreted beta-galactosidase (BGAL1), which is thought to remove the terminal glycan residue of specific O-glycans on flagellin [16]. Protease inhibitors can block elicitor release, implicating the need for proteolytic elicitor release by plant proteases [16]. Interestingly, bacteria can avoid recognition by secreting metalloprotease AprA, which is able to inactivate the elicitor peptide [22].

Release of flagellin-derived FLS3 elicitor

In addition to recognizing flg22, specific solanaceous plant species also recognize flgII-28, a 28-residue-long fragment located in the middle of the flagellin sequence [23,24]. The flgII-28 peptide is recognised by the flagellin sensing-3 (FLS3), a receptor kinase with extracellular leucine-rich repeats which was originally identified from tomato [25]. The flgII-28 elicitor remains to be further

characterised by further truncations as no minimal elicitor has been reported. Furthermore, the detection of naturally released FLS3 elicitors remains to be identified. Natural variation in the flgII-28 elicitor showed that substitutions A13V or S16F reduce elicitor activity [23,24]. Likewise, the naturally occurring N18K substitution in the flgII-28 epitope of flagellin of *Xanthomonas* evades immunity in tomato [26]. These studies suggest the importance of these three residues for recognition by FLS3 (underlined in ESTNILQRMRELAVQSRNDNSSTDRA).

Although flgII-28 is less deeply buried in the flagellin polymer compared to flg22, the flgII-28 sequence is still embedded, with less than 50% of its residues exposed to the protein surface (**Figure 1B**). In addition, the region containing the flgII-28 sequence folds underneath the flagellin glycans. This analysis indicates that, as with the release of the FLS2 elicitor, the release of the FLS3 elicitor will also require deglycosylation, depolymerisation, unfolding, and N-and/or C-terminal processing.

Release of EF-Tu-derived EFR elicitor

Elongation factor Tu (EF-Tu) is an abundant and conserved bacterial protein recognized by the Brassicaceae-specific elongation factor receptor (EFR), a receptor kinase with extracellular leucine-rich repeats triggering plant immunity [27]. EFR recognizes the elf18 peptide elicitor, which comprises the first 18 N-terminal amino acids of EF-Tu [27,28]. The elf18 was originally identified from *Escherichia coli* and residue substitution analysis revealed that five residues are important for elicitor activity (underlined in SKEKFERTTKPHYNVGTIG) [28–30]. The elf18 sequences from important phytopathogenic bacteria showed strong conservation with a maximum of only five residues being different from the *E. coli*-based elf18 [29]. Synthetic elf18 peptides from these bacteria all induced similar immune responses, except for elf18 from *X. campestris* pv. *campestris* and *P. syringae* pv. *tomato* DC3000 (*Pto*DC3000), which have a strongly reduced elicitor activity, due to key residue substitutions in elf18 of *X. campestris* pv. *campestris* (K2R) and *Pto*DC3000 (V12C) [28–30].

Structural modeling of the EF-Tu protein from *P. syringae* with AlphaFold2 [31] indicates that the N-terminal 12 amino-acid residues of elf18 are exposed on the protein surface (**Figure 1C**), indicating that they can interact with EFR without being released from the precursor [28]. However, the first 12 amino acids N-terminal of elf18 (elf12) are not immunogenic, which indicates the remaining six C-terminal residues are crucial to elf18 bioactivity [28]. Importantly, these last six C-terminal residues of elf18 are buried within the EF-Tu protein and are not exposed (**Figure 1C**). Also, the elf18 to elf26 peptides are equally active [28], suggesting that longer peptides than elf18 might be released and activate immunity *in planta*. This suggests that elicitor detection requires precursor unfolding and/or processing before it can bind EFR. No studies on detection of naturally occurring EFR elicitors have been performed yet.

Release of the CSP-derived CORE elicitor

Cold shock protein (CSP) is yet another bacterial protein that is recognised by certain solanaceous plants [32]. CSP is recognised by CORE, a receptor kinase with extracellular leucine-rich repeats originally identified from tomato [33]. CORE recognises csp22, a 22-residue N-terminal consensus fragment of CSP from bacteria [32]. Truncation analysis revealed that residues 5-19 of csp22 (csp15) are sufficient to trigger responses. Alanine substitution of a single amino acid residue within this csp15 peptide revealed that five residues (underlined in VKWFNAEKGFGFITP) are essential for recognition, while F10 can be substituted by Y retaining the full activity [31]. The first five and last eight residues in csp15 show high conservation with more than 90% identity among more than 150 bacterial CSP sequences [32]. No studies on naturally occurring variations of CORE elicitors have been performed yet.

CSP proteins are relatively small (7 kDa), and occur as dimers. The N-terminal half of the csp15 peptide is exposed and might be able to interact with CORE directly. However, the C-terminal half of csp15 is mostly buried, and this includes F12, which is essential for recognition (**Figure 1D**). Therefore, unfolding and/or C-terminal processing seem still required for csp15 recognition.

Release of the NLP-derived RLP23 elicitor

Necrosis and ethylene-inducing peptide 1 (Nep1)-like proteins (NLPs) have a broad taxonomic distribution among plant pathogens including bacteria, fungi, and oomycetes. Pathogen-secreted NLPs bind to exposed hexoses on the head group of an eudicot-specific membrane sphingolipid called glycosyl inositol phosphoryl ceramide (GIPC) to cause plant cell lysis [33]. NLPs are recognized by Arabidopsis RLP23, a receptor-like protein with extracellular leucine-rich repeats triggering plant immunity [35,36]. Truncation analysis identified nlp20, a 20-amino acid elicitor peptide from the middle of the NLP protein of the oomycete plant pathogen *Phytophthora parasitica* (*PpNLP*) as the minimum immunogenic peptide recognised by RLP23 [36]. Substitutions of each amino acid by alanine demonstrated that four residues (underlined in AIMYSWYFPKDSPVTGLGHR) are essential for elicitor activity [36]. Synthetic nlp20-like peptides from bacterial, fungal, and oomycete origin exhibit elicitor activity and present high conservation of the four residues important for immune elicitation. No studies to detect naturally occurring RLP23 elicitors have been performed yet.

Structural modelling of *PpNLP* revealed that the N-terminal half of nlp20 is completely buried within the *PpNLP* protein (**Figure 1E**). The C-terminal seven residues of nlp20 are more exposed. Given the fact that the crucial residues are within the buried part of the elicitor, nlp20 perception probably requires *PpNLP* unfolding and/or processing. *PpNLP* also carries a single putative N-glycosylation site and one disulphide bridge that might suppress elicitor exposure and release.

Release of the polygalacturonase-derived RLP42 elicitor

Several fungal pathogens secrete endopolygalacturonases (PGs) to hydrolyse plant cell walls and induce tissue maceration [37–39]. These PGs are recognised by RLP42, which has also been called Responsiveness to Botrytis Polygalacturonaes1 (RBP42) [40]. RLP42 is a receptor-like protein with extracellular leucine-rich repeats in *A. thaliana* that recognises a conserved 9-amino-acid fragment, pg9, located in the middle of the polygalacturonase 6 (PG6) protein from the fungal plant pathogen *Botrytis cinerea* [41]. Substitution analysis revealed that nearly all the residues (underlined in AHNSDGFVDV) are essential for its elicitor activity. Among the tested substitutions, N3A, D5A, and F7A inactivate the peptide, whereas A1G, H2A, D8A, and V9A decreased the immunogenic activity of pg9 [41]. No studies on the role of naturally occurring variation in RLP42 elicitors have been performed yet and the naturally occurring elicitor peptides remain to be detected.

The pg9 elicitor is an essential part of the polygalacturonase structure contributing to a beta-sheet in the core structure of the enzyme with amino acids required for enzymatic activity [42]. Consequently, most of the pg9 peptide is buried in the protein structure with predicted exposures of critical residues below 20% (**Figure 1F**). Perception of the pg9 elicitor, therefore, requires unfolding and/or N- and C-terminal processing. The PG6 protein also contains six *N*-glycans and three disulphide bridges which may hamper elicitor release.

Release of the transglutaminase-derived elicitor pep13

The oomycete cell wall transglutaminase (TGase) of *Phytophthora* species triggers defense responses in various plants including parsley, potato, grapevine, and *N. benthamiana* [43–47]. The 13-residue elicitor fragment pep13, located in the C-terminal half of GP42, a TGase from *P. sojae*, is sufficient to activate plant defence [45,46]. The pep13 immunogenic peptide pattern is conserved within plant pathogenic *Phytophthora* species and are sensed by the recently identified potato surface receptor Pep-13 receptor (PERU), which enhances immunity to *Phytophthora infestans* infection [48]. Substitution analysis revealed that two residues (underlined in VWNQPVRGFKVYE) are indispensable for elicitor activity [44,45]. No studies to detect naturally occurring pep13-containing elicitors have been performed yet.

The crystal structure of GP42 [49], shows that parts of the pep13 elicitor are exposed, but the crucial W2 and P5 residues are buried deeply inside the structure (**Figure 1G**). Unfolding and/or N- and C-terminal processing seem essential for elicitor perception. A putative *N*-glycosylation site close to the pep13 sequence, and three disulphide bridges might play an important role in avoiding elicitor exposure.

Other peptide elicitors

Besides these seven elicitor peptides, we also evaluated peptide-based elicitors CD2-1 [50], EFa50 [51], and xup25 [52]. The CD2-1 elicitor of flagellin and the EFa50 elicitor of EF-Tu, both from *Acidovorax avenae*, are 166 and 49 residues long, respectively, and have not been subjected to further

truncation analysis. The xup25 peptide is at the C-terminus of its precursor, a xanthine/uracil permease of *P. syringae*, but we were unable to model this region structurally.

Concluding remarks and open questions

There are several open questions to be addressed in the future to understand the molecular mechanism leading to elicitor perception. For instance, is multimer disassembly required for elicitor release and how is this achieved? Do enzymes catalyse disassembly or are altered conditions (pH, ions, temperature, redox) sufficient? Polymer disassembly seems essential for the release of flagellin elicitors, but perhaps not for presumed monomers, like EF-Tu. Also, heteromultimers can affect elicitor release. Polygalacturonases, for instance, might be stabilised by polygalacturonase-inhibiting proteins (PGIPs) [53], which would implicate PGIPs in reducing pg9 elicitor release. Likewise, the interaction of MAMP precursors with other molecules could affect elicitor release. For instance, subtilomycin, produced by the endophyte *Bacillus subtilis* BSn5, binds to its own flagellin and minimizes flg22-induced immunity [54].

Second, is deglycosylation required for elicitor release? Both *O*- and *N*-glycans of microbial phytopathogens are diverse and consist of different carbohydrates that would require specific glycosidases. Glycosidase BGAL1, for instance, only acts on flagellin that carries *O*-glycans with terminal-modified viosamine [16]. *O*-glycans can be species-specific and although *N*-glycans are more conserved between eukaryotes, the terminal residues can be very different. The apoplast contains dozens of glycosidases with uncharacterised activities [55]. Some of them might facilitate elicitor release. However, not every glycan needs to be removed from every site and even a partial glycan degradation might be sufficient for elicitor access.

Third, is protein unfolding required for elicitor release? The thermodynamic stability of proteins depends on the 3D structure and is influenced by general factors like temperature, pH, and ionic strength [56]. Unfolding may not be a prerequisite for processing by proteases because proteases might also initiate protein unfolding by cleaving in exposed loops. Disulphide bridges drastically stabilise extracellular proteins and an enzymatic reduction might be required to unfold these proteins.

Fourth, is protein processing required for elicitor recognition? MAMP precursor processing is essential for the recognition of nonproteinaceous elicitors. For example, chitinases and beta-glucanases degrade fungal cell wall polysaccharides into fragments [57,58]. This process not only weakens the fungal cell wall [59] but also produces soluble fragments of chitin and beta-glucan that activate plant immunity [60,61]. Similarly, the Arabidopsis metazoan lysozyme-like hydrolase (LYS1) releases soluble peptidoglycan (PGN) fragments from insoluble bacterial cell walls, and the resulting cleavage products trigger plant immunity [62]. Furthermore, the ceramide Pi-Cer D from the plant pathogenic oomycete *P. infestans* is cleaved by the Arabidopsis apoplastic ceramidase, NCER2. This cleavage generates a 9-methyl-branched sphingoid base, which is recognized by the lectin receptor-like kinase (RLK) RDA2, subsequently triggering defence responses [63]. By contrast,

precursor processing might not be essential if the unfolded elicitor precursors might already be able to interact with PRRs. If the processing is required for elicitor release, is it required at both the N- and C-terminal sides of the elicitor peptides? Are both endo- and exo-peptidases involved in elicitor release? Have proteases evolved to mediate elicitor release or is elicitor release an unintended by-product of the hydrolytic environment? And what role do secreted proteases have in the stability of elicitor peptides? It is also anticipated that secreted proteases might be involved in degrading MAMPs to prevent growth retardation caused by over-inducing immune responses [64]. Pathogens are also actively engaged in this process of MAMP removal to avoid triggering an immune response [65]. For instance, the bacterial metalloprotease AprA degrades the flg22 elicitor peptide [22]. Addressing these questions requires the characterisation of naturally occurring elicitors but so far these have only been shown for FLS2 elicitors.

Fifth, does natural variation in elicitor protein sequences affect disassembly, unfolding, or processing? Little is known about the role of regions beyond the MAMP epitope. Intriguingly, the S16F substitution in flgII-28 affects the growth of *P. syringae* in Arabidopsis leaves in an FLS2-dependent manner, even though Arabidopsis does not have an FLS3 homolog perceiving flgII-28 [24,25], which indicates that polymorphism within non-immunogenic regions can affect elicitor exposure e.g. through conformational changes. Likewise, a modified *A. tumefaciens* strain carrying the full-length EF-Tu from *PtoDC3000* induced reduced immune responses when compared to *A. tumefaciens* carrying its own EF-Tu with the elf18 sequence from *PtoDC3000* [30], suggesting the involvement of additional residues beyond the elf18 motif contributing to the recognition by EFR. These observations suggest that polymorphisms within immunogenic as well as non-immunogenic sequences might impair protein processing and subsequently the exposure of immunogenic fragments.

Finally, how are nonsecreted proteins recognised extracellularly by cell surface receptors? In contrast to extracellular precursor proteins (flagellin, NLP, and PG), EF-Tu and CSP are cytoplasmic proteins, and GP42 is associated with the cell wall. During microbial growth or division, there is a constant balance between cell wall degradation and biosynthesis. For example, during the remodeling and biogenesis of the bacterial cell wall, there is an imperfect recycling of cell wall materials, leading to the release of components into the extracellular space [66,67]. This suggests that when microbes grow or die in the apoplast, microbial constituents could be exposed and become susceptible to apoplastic hydrolases. Consequently, it is generally assumed that a sufficient quantity of elicitors is released from dying cells, enabling their recognition by cell surface receptors. It is also possible that the release of non-secreted elicitor molecules is facilitated by plant-secreted molecules that disrupt the microbial cell wall. For example, potato Snakin-1 (SN1) is a cysteine-rich antimicrobial peptide that can disrupt the membranes of *E. coli* and confers protection against bacterial and fungal pathogens [66]. Likewise, saponins are present in the cell membranes of many plant species that act by disrupting the cell membranes of invading bacterial and fungal pathogens [67].

Given the importance of PTI in plant immunity, it seems likely that all factors that contribute to elicitor release will be under strong antagonistic selection, and this is likely to involve unexpected mechanisms that remain to be uncovered in future research. This analysis indicates that it will be interesting to determine the contributions of host-secreted proteases and pathogen-secreted protease inhibitors in the control of elicitor release.

Supplemental information: PSE and PDB files of the models used to generate **Figure 1**.

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Declaration of interests

The authors declare no competing interests.

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421

422 **Table 1** Peptide elicitors and their precursors

Elicitor	Precursor	kDa	Microbe	Receptor	Plant
flg22	Flagellin	29*	bacteria	FLS2	Angiosperms
flgII-28	Flagellin	29*	bacteria	FLS3	Solanum
elf18	EF-Tu	43	bacteria	EFR	Brassicaceae
csp15	Cold shock protein CspD	7	bacteria	CORE	Solanaceae
nlp20	Necrosis and ethylene-inducing peptide 1 (Nep1)- like protein (NLP)	23*	bacteria, oomycetes and fungi	RLP23	Brassicaceae
pg9	Polygalacturonase PG6	36*	fungi	RLP42/ RBPG1	<i>Arabidopsis thaliana</i>
pep13	Transglutaminase GP42	55*	<i>Phytophthora</i>	Unidentified	Parsley

423 *, not including O/N-glycans

424

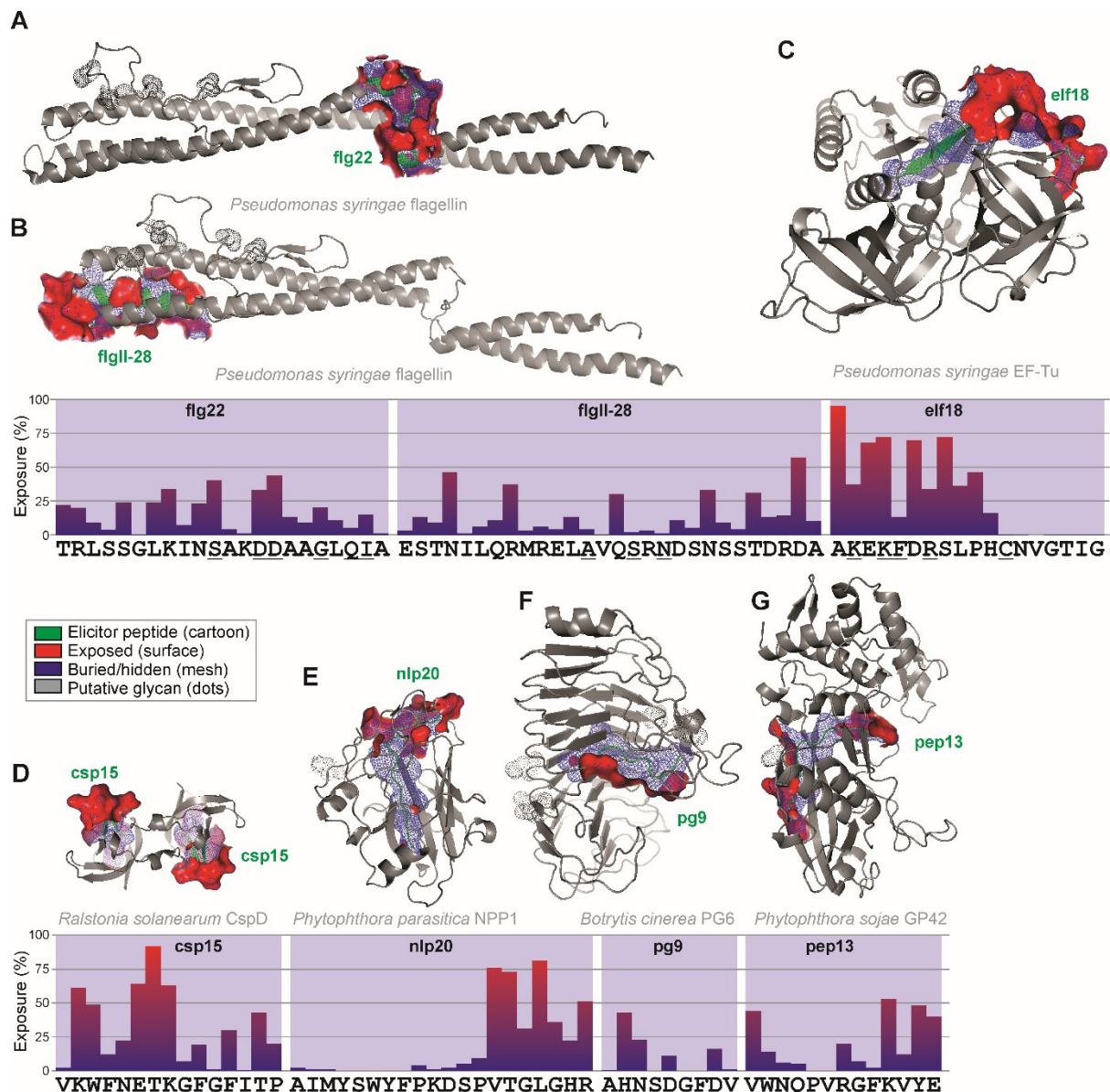


Figure 1 Elicitor peptides hiding in their precursor structures.

Structural models were generated with Swiss Model (A and B on 5wk6; D on 3cam; E on 6qbe; F on 1hg8; G on 3tw5) or AlphaFold2 (C) and rendered with PyMol, showing the elicitor peptide (green cartoon) in the precursor (grey cartoon), with the exposed residues (red surface) and buried or hidden surface (blue mesh). Putative N- or O-glycosylation sites are indicated with grey dots/spheres. The models of flagellin show one monomer within a model of a flagellin polymer and the model of CspD is a dimer. The relative exposure of each residue of the elicitor peptide within the structure was measured from the structural models using the 'get_sasa_relative' command in PyMol. Residues in the elicitor peptides that are important for recognition are underlined. The original PSE and PDB files used to generate this figure can be accessed in **Supplemental File S1**.