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Novel structural insights at the extracellular plant-pathogen interface

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Abstract

Plant pathogens represent a critical threat to global agriculture and food security, particularly under the pressures of climate change and reduced agrochemical use. Most plant pathogens initially colonize the extracellular space or apoplast and understanding the host–pathogen interactions that occur here is vital for engineering sustainable disease resistance in crops. Structural biology has played important roles in elucidating molecular mechanisms underpinning plant-pathogen interactions but only few studies have reported structures of extracellular complexes. This article highlights these resolved extracellular complexes by describing the insights gained from the solved structures of complexes consisting of CERK1-chitin, FLS2-flg22-BAK1, RXEG1-XEG1-BAK1 and PGIP2-*Fp*PG. Finally, we discuss the potential of AI-based structure prediction platforms like AlphaFold as an alternative hypothesis generator to rapidly advance our molecular understanding of plant pathology and develop novel strategies to increase crop resilience against disease.

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Apoplast, Extracellular, Structural biology, Crystallography, Cryo-EM, AlphaFold.

Introduction

Diseases caused by plant pathogens pose significant threats to global agriculture and food security [1], particularly in the face of climate change and reduced use of agrochemicals. Plant pathogens typically invade host tissues through wounds, natural openings (*e.g.*, stomata) or by actively penetrating through the epidermis [2]. Following host entry, most plant pathogens colonise the extracellular space or 'apoplast' [3]. Plants detect the presence of pathogens by recognising pathogen-associated molecular patterns (PAMPs) like bacterial flagellin, damage-associated molecular patterns (DAMPs) or effector proteins secreted by the pathogen leading to the onset of immune responses [4]. These responses include the secretion of a cocktail of hydrolytic enzymes into the apoplast to resist infection [5]. Pathogens also release hydrolases into the apoplast, including cell wall degrading enzymes (CWDEs) to promote colonization [6]. In addition, both plants and pathogens secrete inhibitors to suppress the hydrolases secreted by their counterpart [7]. Greater understanding of the molecular mechanisms underlying these dynamic and highly complex apoplastic plant-pathogen interactions could unlock new strategies to combat plant disease.

In recent years, structural biology has provided important novel insights into apoplastic plant–microbe interactions [8–12]. Structural biology typically involves the purification of proteins before using techniques such as X-ray diffraction or cryogenic electron microscopy (cryo-EM) to obtain high-resolution information about their three-dimensional structure [13] (Box 1). These approaches can provide unique insights into biological mechanisms as the structure of proteins is inherently linked to their function [14].

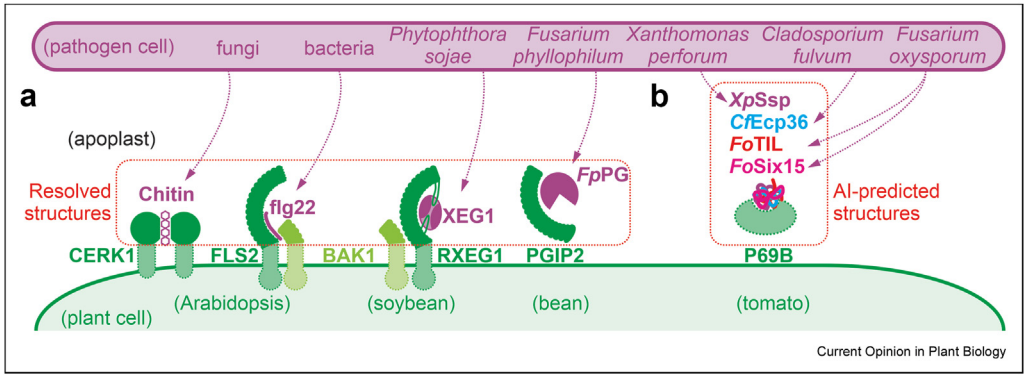
Here, we review recent studies that highlight the capacity of structural biology to shed new light on the molecular mechanisms underpinning apoplastic plant-pathogen interactions by discussing four examples of cross-kingdom protein complexes featuring host and pathogen components: CERK1-chitin, FLS2-flg22, RXEG1-XEG1 and *Pv*PGIP2-*Fp*PG [8–12] (Figure 1A and Table 1). The availability of structural information in each of these cases enabled the elucidation of novel mechanisms of apoplastic pathogen manipulation and host recognition.

Finally, we also discuss the application of artificial intelligence (AI)-guided protein structure prediction

Box 1. Main approaches to study 3D structures of protein complexes.

Method	Description	Advantages	Limitations
X-ray crystallography	Purified protein complexes are crystallized from a supersaturated solution using diverse methods, *e.g.* vapour diffusion of microbatch. Structures are determined by monitoring the diffraction pattern of X-rays passed through the crystals.	High-resolution structures.	Crystallisation conditions vary and can be difficult to optimize.
Cryogenic Electron Microscopy (cryo-EM)	Frozen purified protein complexes are bombarded with an electron bean and scattered electrons are detected resulting in a large number of 2D 'projection images' of the protein complex in various orientations. These images are compiled to obtain a 3D structure.	Can elucidate structures of large protein complexes.	Often lower resolution when compared to crystallography.
Structural prediction	Platform trained on resolved structures is used to predict the 3D structure of protein complexes using protein alignment and artificial intelligence or machine learning.	No protein or specialised equipment required.	Structural models still require experimental validation.

Figure 1



Novel structural insights at the extracellular plant-pathogen interface.
Shown are pathogen-secreted molecules (purple) produced by various pathogen species (purple) that directly interact with secreted or apoplast-exposed plant proteins (green). (**a**) Resolved structures (purple box) and their context. (**b**) AI-predicted structures of four P69B inhibitors in their context.

platforms to predict extracellular interactions (Figure 1b). AlphaFold leverages deep learning algorithms to predict protein structures with remarkable accuracy [16]. A recent study demonstrates the potential of *in silico* approaches to rapidly accelerate investigations of plant-pathogen interactions by providing structural predictions that can be used to drive hypotheses and complement experimental approaches.

Table 1

Discussed molecular structures.

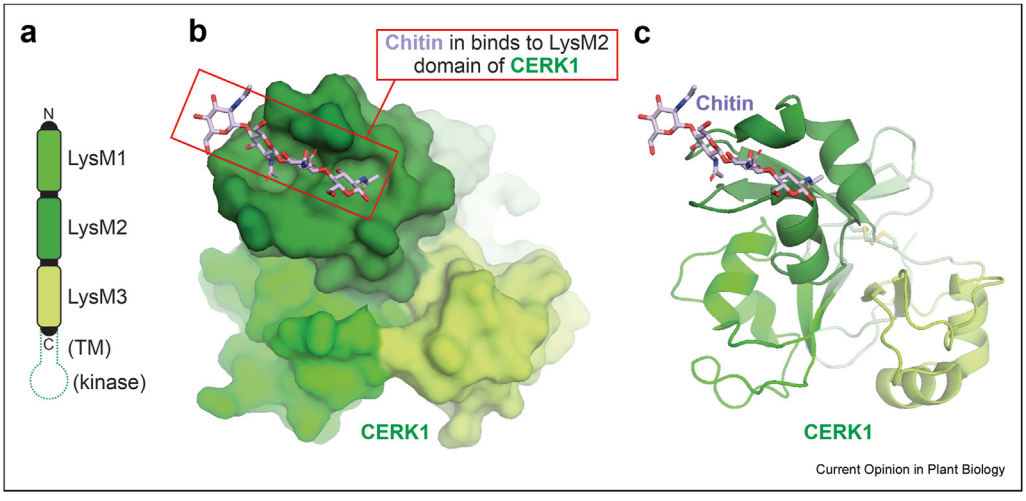
Pathogen component	Plant protein(s)	PDB	Reference
Chitin pentamer (fungal pathogens)	*At*CERK1 (Arabidopsis)	4ebz	[8]
Chitin hexamer (fungal pathogens)	*Os*CERK1 (rice)	7vs7	[9]
Chitin tetramer (fungal pathogens)	*Os*CEBIP (rice)	5jce	[15]
flg22 (bacterial pathogens)	FLS2, BAK1 (Arabidopsis)	4mn8	[12]
XEG1 (*Phytophthora sojae*)	RXEG1 (*N. benthamiana*)	7w3v 7drb	[10]
XEG1 (*Phytophthora sojae*)	RXEG1, BAK1 (*N. benthamiana*)	7drc	[10]
*Fp*PG (*Fusarium phyllophillum*)	*Pv*PGIP2 (*P. vulgaris*)	8ikw	[11]

The structural basis of chitin perception

Plants perceive PAMPs in the apoplast via the ectodomains of receptor proteins embedded in the cell surface [17,18]. Chitin is a polymer of *N*-acetyl-D-glucosamine (NAG) found in fungal cell walls and is a potent elicitor of plant immune responses [19]. The receptor-like protein chitin oligosaccharide elicitor binding protein (CEBiP) from rice (*Oryza sativa*) was the first chitin receptor identified in plants [20]. The extracellular domain of *Os*CEBiP contains lysine motifs (LysMs), structural modules involved in the recognition of polysaccharides containing NAG [21]. In the model plant *Arabidopsis thaliana*, chitin is perceived by CERK1 (chitin elicitor receptor kinase 1) [22]. The ability of CERK1 to recognise chitin is dependent on its LysM ectodomain which contains three individual LysM modules [23,24].

The crystal structure of the CERK1 ectodomain in complex with the chitin pentamer $(NAG)_5$ was determined at 1.79 Å resolution using X-ray crystallography (Figure 2) [8]. The interaction is mediated specifically by LysM2 which binds to the three central NAG residues of the pentamer, while the terminal NAG rings extend beyond LysM2 and are exposed to solvent on either side. This structural observation is consistent with previous observations that longer chitin fragments elicit stronger immune responses than chitin pentamers [23,25] and aligns with the hypothesis that longer-chain chitins could simultaneously bind to two CERK1 molecules [8]. Subsequent binding assays confirmed that chitin octamers induce dimerization of CERK1 and revealed that this mechanism is important for the full activation of chitin-triggered immune signaling [8].

Figure 2



The chitin tetramer binds to the second LysM domain in the ectodomain of Arabidopsis CERK1.
(**a**) The crystalized ectodomain of CERK1 contains three LysM domains. (**b**) The chitin tetramer (sticks) binds to a shallow groove in the second LysM domain of the CERK1 ectodomain (surface representation). (**c**) Cartoon model of the CERK ectdomain showing the three LysM domains. Based on crystal structure 4ebz [8].

Following this, the structure of the OsCEBiP ectodomain in complex with the chitin tetramer $(NAG)_4$ was determined using X-ray crystallography at 2.51 Å resolution [15]. The structure revealed that *Os*CEBiP also has three LysM domains, but only LysM2 binds to chitin [15]. The LysM2 residues directly involved in chitin binding are conserved among CEBiP homologs in diverse plant species but are highly variable in LysM1 and LysM3, likely explaining their low affinity for chitin [15]. Structure-based modelling indicates that longer-chain chitin hexamers could simultaneously bind to two *Os*CEBiPs, similar to the homodimerization mechanism described for AtCERK1 [8,15].

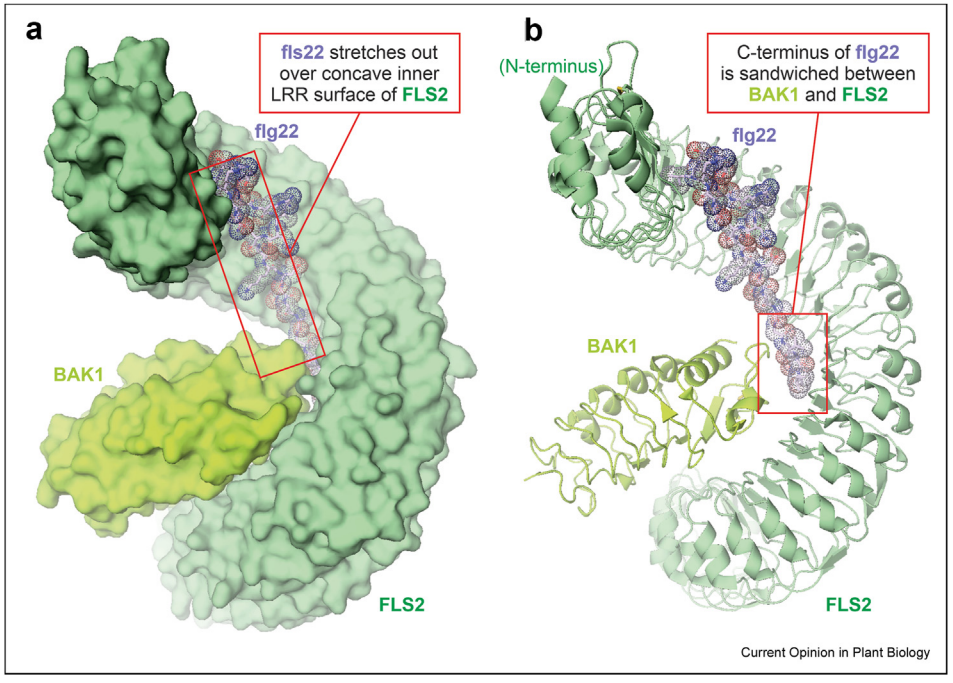
Rice also contains a homolog of Arabidopsis CERK1 known as *Os*CERK1 which functions co-operatively alongside CEBiP [26]. Although *Os*CERK1 is essential for chitin-induced immune signaling [27], it does not exhibit a strong binding affinity for chitin [26]. Sensitive isothermal titration calorimetry (ITC) assays later revealed that *Os*CERK1 can bind weakly to chitin [9]. The structure of *Os*CERK1 in complex with the chitin hexamer $(NAG)_6$ was solved at a 2.02 Å resolution [9]. Similar to Arabidopsis CERK1 and rice CEBiP, the chitin binding ability of *Os*CERK1 is dependent on its LysM2 motif and mutations in this region disrupt chitin binding [9]. Interestingly, certain subspecies of *O. sativa* have naturally acquired substitutions in the LysM2 region of *Os*CERK1 producing a receptor with a shallow binding groove that is better suited for binding chitin [9]. Rice plants expressing this $OsCERK1^{TT}$ variant exhibit greater resistance against fungal pathogens [28]. This $OsCERK1^{TT}$ variant binds to chitin with a similar binding affinity as Arabidopsis CERK1.

The structural basis of flagellin perception

The 22-amino acid peptide flg22 derived from bacterial flagellin is a PAMP perceived by the plasma-membrane resident receptor FLS2 (flagellin-sensitive 2) in Arabidopsis [29]. FLS2 consists of an extracellular leucine-rich repeat (LRR) domain, a transmembrane domain and an intracellular kinase domain [30]. Binding of flg22 to the FLS2 ectodomain promotes its association with the co-receptor BAK1 (BRI1-associated receptor kinase 1), a plasma membrane-resident LRR-containing receptor-like kinase (RLK), triggering reciprocal phosphorylation of intracellular kinase domains that initiate immune signalling [31].

To understand the molecular mechanisms of flagellin perception in greater detail, the crystal structure of the FLS2 and BAK1 LRR ectodomains in complex with flg22 was resolved at 3.06 Å resolution (Figure 3) [12]. Flg22 binds to the inner concave surface of the FLS2 ectodomain spanning the length of 14 LRRs, with the

Figure 3



Receptor FLS2 interacting with both elicitor flg22 and coreceptor BAK1.
(**a**) Peptide elicitor flg22 (shown as sticks and dots) interacts with the inner concave surface of the leucine-rich repeats (LRRs) of the FLS2 ectodomain (green surface representation) of *Arabidopsis thaliana* and the ectodomain of BAK1 (light green surface representation) of *Arabidopsis thaliana*. (**b**) The C-terminus of flg22 (sticks and dots) interacts with both FLS2 (green cartoon) and BAK1 (light green cartoon). Based on crystal structure 4mn8 [12].

N-terminus of the peptide at LRR3 and the C-terminus at LRR16. Interestingly, the C-terminal region of flg22 appears to function as a molecular glue that facilitates the association between FLS2 and BAK1. Consistent with this, substitution of bulky residues at the C-terminus of flg22 abrogates the FLS2-BAK1 interaction [12]. BAK1 also interacts directly with the C-terminal portion of FLS2 in the presence of flg22 [12]. Mutagenesis of contact points at the flg22-FLS2 interface led to the identification of several amino acids in flg22 that are critical for recognition by FLS2, including Ala17 and Ile21 [12]. These residues are conserved among bacteria that trigger FLS2-mediated immune responses [12].

Earlier work had demonstrated that N-terminally truncated variants of flg22 such as flg15 can still elicit immune responses in diverse plant species, although it is far less potent than flg22 in Arabidopsis [32]. C-terminal truncations of flg15 do not trigger immunity in plants but instead serve as competitive antagonists for flg15 perception in tomato cells [32]. Similarly, the flg22-AYA variant, with three substitutions close to the C-terminus, antagonizes flg22 binding in Arabidopsis [33]. These findings are consistent with the fact that in the structure the N-terminal portion of flg22 interacts with FLS2 but the C-terminal region is crucial for assembly of the FLS2-BAK1 signaling complex [12].

RXEG1 receptor protein also inhibits its ligand, XEG1

Some plant pathogens deploy cell-wall degrading enzymes (CWDEs) to compromise cell wall integrity [6]. In some cases, CWDEs or the hydrolyzed cell wall fragments they produce can be perceived by the plant [34]. The oomycete pathogen of soybean *Phytophthora sojae* secretes the glycoside hydrolase family 12 (GH12) protein XEG1 at the early stages of colonisation to degrade xyloglucan and β-glucan in the plant cell wall [35]. XEG1 can be perceived by the PM-localized *Nicotiana benthamiana* receptor-like protein (RLP) RXEG1 (Response to XEG1), triggering immune responses [36]. RXEG1 contains an extracellular LRR domain to associate with XEG1 in the apoplast [36]. XEG1 binding triggers the association of RXEG1 with BAK1, triggering further immune signalling by protein phosphorylations [36].

The crystal structure of the complex of XEG1 with the ectodomains of RXEG1 was recently solved by X-ray crystallography at 3.30 Å resolution and with cryogenic electron microscopy (cryo-EM) at 3.11 Å resolution [10]. RXEG1 arches around XEG1 and fixes it in place with two RXEG1 loopout regions occupying the substrate-binding groove of XEG1 from both sides (Figure 4) [10]. Deletion of the N-terminal loop-1 of RXEG1 strongly disrupted the interaction with XEG1, supporting this proposed model [10]. The structure of the complex of XEG1 with the ectodomains of both RXEG1 and BAK1 was solved in the same study using cryo-EM at 3.30 Å resolution [10]. This ternary complex revealed that XEG1 binding induces conformational changes in loop-2 of RXEG1 that enhance the interactions with BAK1 [10].

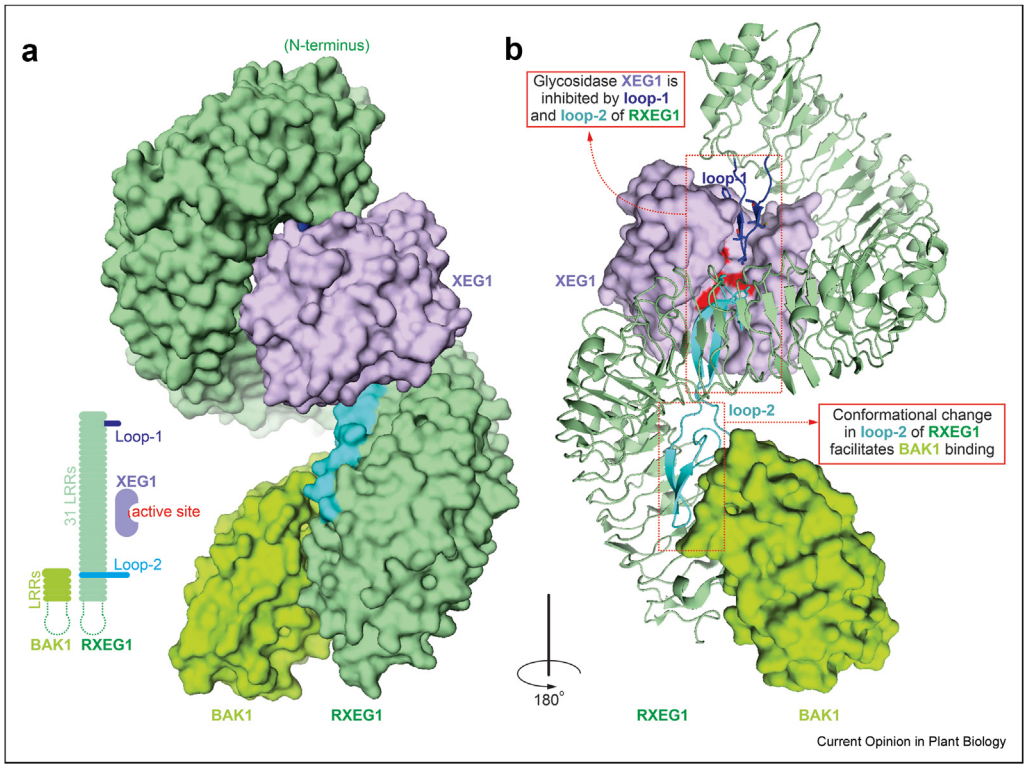
The XEG1-RXEG1(-BAK1) structures elucidated the mechanism and revealed the wider biological relevance of this interaction. Firstly, the presence of the XEG1 active site at the interface of the RXEG1 interaction prompted enzymatic assays, which confirmed that RXEG1 inhibits the endoglucanase activity of XEG1, thereby suppressing its virulence function [10]. Secondly, amino acid sequence alignment of XEG1 homologues from *P. sojae* and *Phytophthora parasitica* showed that the residues involved in the RXEG1 interaction are conserved, and cell death assays confirmed that these XEG1 homologues are indeed also recognized by RXEG1 [10]. Thirdly, the structure of the ternary complex revealed that RXEG1 interacts with BAK1 via four C-terminal LRRs conserved in other RLPs, explaining the role of BAK1 as a co-receptor for many other LRR-RLPs [10]. Finally, the ternary complex also revealed that XEG1 binding is distal from the RXEG1-BAK1 interface, indicating that XEG1 promotes BAK1 recruitment not directly, as in flg22 perception, but indirectly, through a conformational change in loop-2 [10].

Bean PGIP2 alters the activity of fungal glycosidase to produce DAMPs

Polygalacturonases (PGs) are a class of CWDEs that target pectins by hydrolyzing polygalacturonic acids (PGAs) [37]. Plants secrete polygalacturanose-inhibiting proteins (PGIPs) into the apoplast to suppress PG activity [38]. Although PGIP blocks the degradation of PGA into monomers by PG, certain oligogalacturonides accumulate that elicit an immune response [39]. These PGA fragments are damage-associated molecular patterns (DAMPs) that are recognised by wall-associated kinases (WAKs) [40].

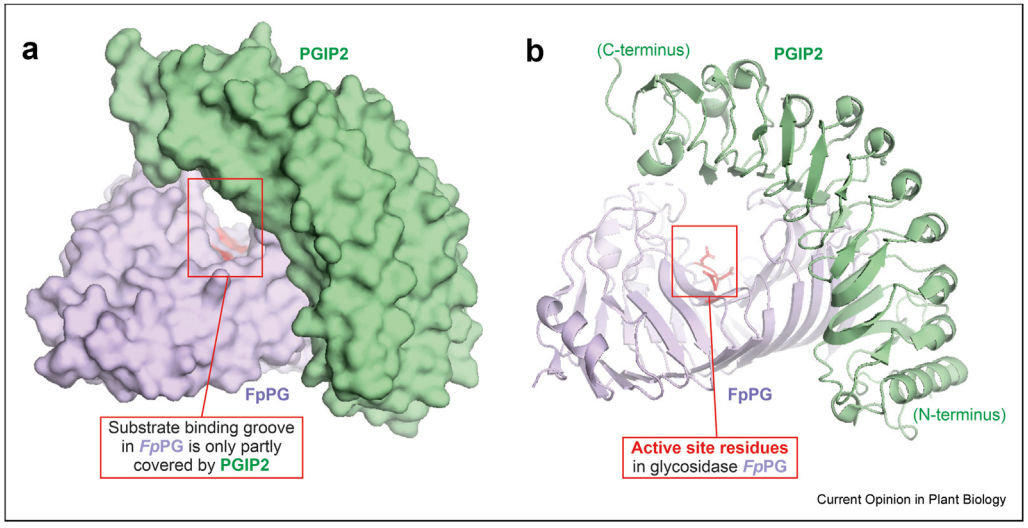
The well-characterised *Fp*PG effector from the fungal pathogen *Fusarium phyllophilum* is inhibited by the secreted protein *Pv*PGIP2 of the common bean plant *Phaseolus vulgaris* [41]. The crystal structure of *Pv*PGIP2 was first reported in 2003 [42], and its complex with *Fp*PG was recently solved at 1.93 Å resolution using X-ray crystallography (Figure 5) [11]. Notably, this complex could only be obtained using a variant of *Pv*PGIP2 containing an N274D substitution at a predicted glycosylation site [11]. Glycosylation is a common feature of secreted proteins [43] and can hinder protein crystallization [44]. The structure confirmed that *Pv*PGIP2 does not obstruct the active site of *Fp*PG, and PGA-binding assays showed that the interaction

Figure 4



Receptor RXEG1 interacts with both xyloglucanase XEG1 and coreceptor BAK1.
(**a**) Xyloglucanase XEG1 of *Phytophthora sojae* (purple surface representation) interacts with the concave inner surface of the leucine-rich repeats (LRR) of the ectodomain of receptor RXEG1 of *Nicotiana benthamiana* (green surface representation) whilst the ectodomain of coreceptor BAK1 of *Nicotiana benthamiana* (light green surface representation) interacts with the C-terminal LRRs of RXEG1. (**b**) Loop-1 (dark blue) and loop-2 (cyan) of RXEG1 occupy the substrate binding groove of XEG1 (purple surface representation) and inhibit XEG1 activity. XEG1 binding causes a conformational change in loop-2 of RXEG1 that creates a binding site for coreceptor BAK1 (light green surface representation). Based on cryo-EM structure 7drc [10].

Figure 5



Bean PGIP2 alters the activity of fungal glycosidase to produce DAMPs
(**a**) Polygalacturonase *Fp*PG of *Fusarium phyllophilum* (purple surface representation) interacting with polygalacturonase inhibiting protein PGIP2 of *Phaseolus vulgaris* (green surface representation). The active site of *Fp*PG is highlighted in red. (**b**) Cartoon presentations of the *Fp*PG-PGIP2 interaction with the active site residues shown as sticks (red).

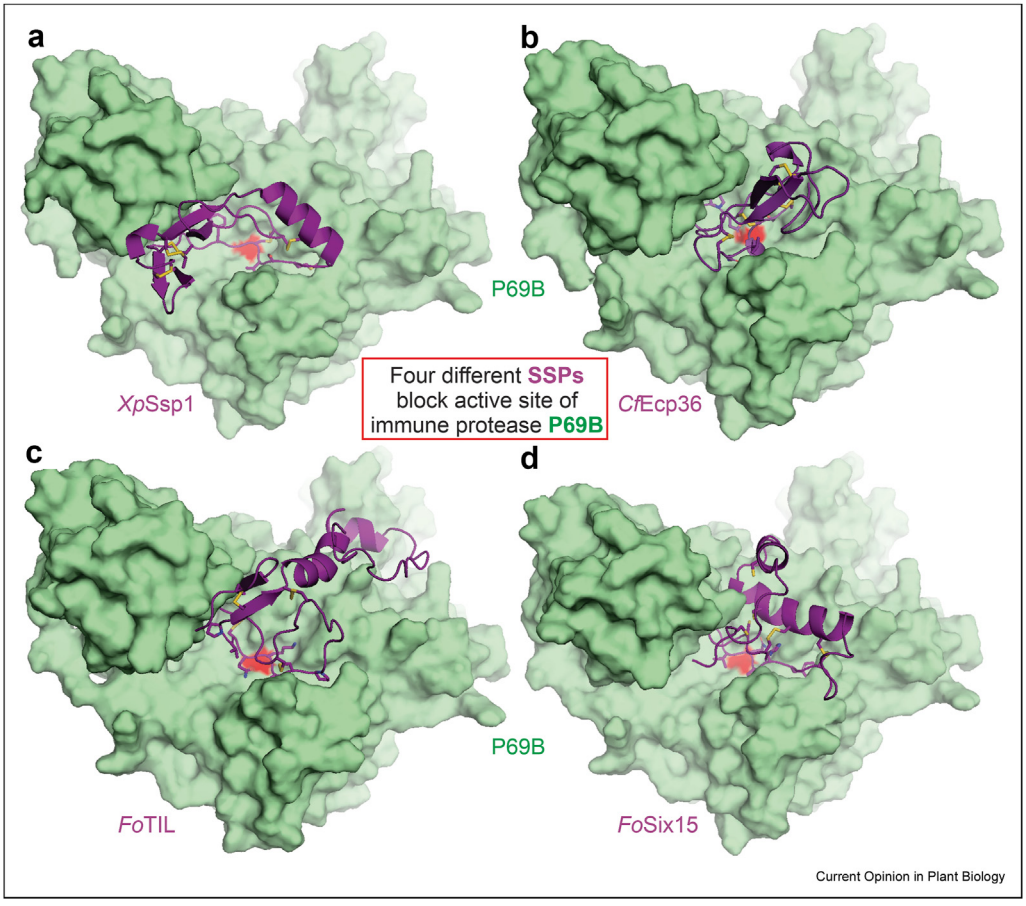
promotes the substrate binding ability of *Fp*PG [11]. Additional assays confirmed that *Pv*PGIP2 binding directs *Fp*PG to preferentially catalyse the production of long-chain immunogenic oligogalacturonides versus shorter oligogalacturonides [11]. Remarkably, these shorter oligogalacturonides were shown to suppress immune signalling, also triggered by other elicitors including flg22 through an unresolved mechanism [11]. Thus, structural biology resolved how PGIP2 converts the virulence activity of *Fp*PG into a trigger for plant immune responses.

AlphaFold multimer predicts P69B inhibitors

Despite the evident power of structural biology to dissect molecular mechanisms, the technical challenges involved and the need for specialized equipment mean that these approaches are limited in most cases. Crystallisation of secreted proteins is particularly challenging due to the frequency of glycosylation in the apoplast [43,44]. In recent years, *in silico* protein structure predictions have served as a powerful complement to traditional structural biology methods. AlphaFold is an artificial intelligence (AI) platform released in 2021 that predicts protein structures from amino acid sequences with unprecedented accuracy [16,45]. Predicted structures for the entire proteomes of several plant model organisms are available from the AlphaFold database, including Arabidopsis, rice (*O. sativa*), soybean (*Glycine max*) and maize (*Zea mays*) (alphafold.ebi.ac.uk/download#proteomes-section). AlphaFold-Multimer (AFM) is an extension of the 'AlphaFold 2' update with the added capability of predicting protein complexes [46].

AI-guided protein structure prediction models can now be used as a discovery tool to generate hypotheses that can be experimentally tested. For instance, AFM was used to screen 1879 pathogen-produced small secreted proteins (SSPs) for being inhibitors of the P69B immune subtilase of tomato [47]. Four SSPs were predicted by

Figure 6



AI-predicted structural models of four novel inhibitors of immune protease P69B.
AFM-predicted models of P69B in complex with (**a**) *Xp*Ssp1 of *Xanthomonas perforans,* (**b**) *Cf*Ecp36 of *Cladosporium fulvum* (syn *Passalora fulva*), (**c**) *Fo*TIL (*Fusarium oxysporum*) and (**d**) *Fo*Six15 (*Fusarium oxysporum*). P69B is shown in a green surface representation with the active site Ser residue in red, in the substrate binding groove. The inhibitors are shown as purple cartoons with sticks for Cys residues and the residues occupying the substrate binding pockets in P69B. P69B inhibition was confirmed experimentally [47].

AFM to possess an intrinsic fold that may bind the substrate binding groove of P69B. These proteins were produced in a heterologous system and suppressed activity-based labeling of P69B, confirming that they are novel P69B inhibitors (Figure 6). Although this approach did not resolve the structure of these complexes, it did accurately predict the function of the SSP, in a similar manner to how *e.g.*, XEG1 inhibition by RXEG1 was predicted by the resolved structure. Notably, the screen on P69B also uncovered SSPs that seem to bind elsewhere on the surface of P69B. These interactions could result in an allosteric regulation, reminiscent of how PGIP binding alters the function of *Fp*PG.

Such studies illustrate that the advent of AlphaFold enables new possibilities for the study of apoplastic plant-pathogen interactions. For example, AlphaFold 2-predicted structural models of cell surface receptors perform well when compared with experimentally determined structures in library docking screens for ligand identification [48]. The latest AlphaFold release - AlphaFold 3 — is also capable of predicting protein complexes with nucleic acids, glycans and other small molecules [49]. Considering the presence of nucleic acids (*e.g.*, small RNAs) [50], prevalence of glycosylation [43]) and the abundance of small molecules [51] in the apoplast, this added functionality will also prove very useful to unravel additional extracellular plant-pathogen interactions.

Future perspectives

The understanding of extracellular plant-pathogen protein complexes is remarkably shallow since only few complexes have been resolved experimentally. By comparison, many more structures have been resolved for effectors in complex with their targets that reside inside the plant cell, partly because these are easier to produce and resolve. Nevertheless, the few protein complexes resolved by X-ray crystallography and cryo-EM, have provided important molecular insights, *e.g.*, into glycosidase inhibition or manipulation, and in co-receptor recruitment. To some extent, these structures explained phenomena that were described previously, but these structures also generated new hypotheses that were experimentally confirmed. We will see a lot more use of structural models in the near future with the increased use of AI-based structural prediction programs. It is important to note that these predictions produce structural models that remain to be experimentally validated and are dependent on the training set and the depth of the multiple sequence alignment. Current prediction programs can also not yet handle the vast variety of small molecules in the apoplast and predict conformational changes. However, these models overcome a bottleneck in producing purified extracellular proteins, and will generate an increasing amount of intriguing hypothesis on extracellular molecular mechanisms to test experimentally.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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