On the front line: structural insights into plant–pathogen interactions

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Abstract | Over the past decade, considerable advances have been made in understanding the molecular mechanisms that underpin the arms race between plant pathogens and their hosts. Alongside genomic, bioinformatic, proteomic, biochemical and cell biological analyses of plant–pathogen interactions, three-dimensional structural studies of virulence proteins deployed by pathogens to promote infection, in some cases complexed with their plant cell targets, have uncovered key insights into the functions of these molecules. Structural information on plant immune receptors, which regulate the response to pathogen attack, is also starting to emerge. Structural studies of bacterial plant pathogen–host systems have been leading the way, but studies of filamentous plant pathogens are gathering pace. In this Review, we summarize the key developments in the structural biology of plant pathogen–host interactions.

Apoplastic effectors
Effectors that are secreted into and act in the apoplast, a tissue-level compartment outside the plant plasma membrane that includes the cell wall.

Cytoplasmic effectors
Effectors that are secreted and translocated across the plant plasma membrane into the host cytoplasm, where they can target different subcellular compartments.

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Modern agricultural practices, including the planting of large areas of crop monocultures that are often selected for consumer-desired traits and not for disease resistance, provide a fertile ground for pathogens. Major diseases of crop plants that are caused by plant pathogens are a considerable threat to global food security[1,2]. The co-evolution of plant pathogens and their hosts has resulted in highly adapted microbial invasion strategies and plant counter-defence mechanisms.

Many bacterial and filamentous (fungal and oomycete) pathogens of plants derive nutrients from living hosts and this necessitates a close parasitic interaction between these organisms. Molecular players from the pathogen and the host compete to affect the outcome of the interaction (BOX 1; FIG. 1). To this end, pathogens secrete effector proteins that modulate host cell physiology to support parasitism[3–6]. The disease-promoting activities of effector proteins typically prompt effector-triggered susceptibility, in which plants become more susceptible to infection in the presence of these proteins. Effector-triggered susceptibility can be a result of the activities of apoplastic (extracellular) or cytoplasmic (intracellular) effectors (BOX 1). In turn, plants have evolved a sophisticated and multilayered surveillance system that triggers signalling cascades resulting in the activation of a defence response against pathogen invasion (BOX 1). Plant cell surface receptors detect conserved microorganism-associated molecular patterns (MAMPs) or damage-associated molecular patterns (DAMPs)[7,8]. These receptors are collectively known as pattern-recognition receptors (PRRs), and sensing of MAMPs and DAMPs by these proteins can initiate PRR-mediated immune responses. The presence of cytoplasmic effectors can be detected by intracellular receptors named after their conserved nucleotide-binding and Leu-rich repeat (NB-LRR) domains. These proteins initiate NB-LRR-mediated immune responses. Adding an additional layer of complexity, many effectors can suppress PRR-mediated immunity, which leads to the idea of an arms race, or molecular battleground, between pathogens and their hosts[5,6,9].

The pace of discovery in assigning roles to the molecular players in plant–pathogen interactions has accelerated with advances in experimental technologies. Genomics, bioinformatics, proteomics, biochemistry and cell biology have all played major parts in identifying, characterizing and exploring the functions of both the pathogen and the plant proteins that are involved. Developments in protein expression technologies and in the instrumentation for biophysical characterization and crystallization of recombinant proteins, as well as access to large multi-user facilities for collection of structural data (synchrotrons for X-ray diffraction from crystals and high-field solution nuclear magnetic resonance (NMR)), have generated new opportunities for structural biology to affect many diverse areas of biology (BOX 2). In this Review, we describe the current developments in the structural biology of plant–pathogen interactions, providing examples from the plant apoplast, the plasma membrane and cytoplasm, and the nucleus. We

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Box 1 | **Plant immunity — lost in acronyms?**

Plants are exploited as nutrient sources by a plethora of different pathogens, including bacteria, fungi, oomycetes, viruses and parasitic plants. Plant immunity is primarily cell-autonomous, as every cell can trigger an immune response[151]. However, following a local infection, systemic signals arise that regulate the spread of the immune response and that can establish a heightened state of immunity throughout the plant[152]. In contrast to vertebrates, plants lack an adaptive immune system and rely entirely on innate immune mechanisms. In nature, this limited repertoire of germline-encoded immune receptors is effective in fending off most pathogens[151]. Pathogens enter plants through natural openings or form specialized structures (for example, appressoria) to penetrate the plant surface[153]. Many plant pathogens secrete glycohydrolases to degrade the plant cell wall, which is a rigid carbohydrate network that surrounds plant cells. The breakdown products of this process can be perceived by the plant via cell surface-resident danger-associated molecular pattern (DAMP) receptors. In addition, plants use microorganism-associated molecular pattern (MAMP) receptors to detect evolutionarily conserved pathogen molecules, such as bacterial flagellin[154].

Ligand binding to the extracellular domains of these pattern-recognition receptors (PRRs) activates their intracellular kinase domains. PRR kinase activity triggers several branching signalling pathways that collectively induce an immune programme termed PRR-mediated immunity or MAMP-triggered immunity. Successful pathogens have evolved effector proteins to prevent induction of PRR-mediated signalling (see main text). Effectors can be either secreted and function extracellularly or they can be secreted and translocated into plant cells. Many host cell-targeted effectors interfere with PRR-mediated immunity at various signalling nodes, a process often referred to as effector-triggered susceptibility. Effectors of plant pathogenic bacteria are primarily translocated via the type III secretion system. Fungal and oomycete pathogens invade plant tissue by protruding hyphae. When in contact with a host cell, these filamentous pathogens establish specialized feeding structures, termed haustoria, which seem to be the main translocation route for effectors[122,124,155]. How effectors from filamentous pathogens are translocated into host cells is still debated[125–127].

Plants have evolved a second class of immune receptors that detect specific pathogen effectors. These intracellular receptors are characterized by a central nucleotide-binding (NB)-ARC domain and carboxy-terminal Leu-rich repeats (LRRs); thus, these proteins bear resemblance to mammalian nucleotide-binding oligomerization domain-containing protein (NOD)-like receptors[144]. Effector-induced activation of NB-LRR receptors induces signal transduction via their amino-terminal Toll and interleukin-1 receptor (TIR) and coiled-coil (CC) domains, which culminates in a defence response that is termed NB-LRR-triggered or effector-triggered immunity. Typically, activation of a single NB-LRR receptor by one pathogen effector (directly or indirectly) is sufficient to establish immunity and prevents further pathogen spread. Therefore, NB-LRR-mediated immunity is the main line of defence against adapted pathogens that effectively block PRR-mediated immunity via effector proteins[6]. NB-LRR-mediated immunity is often, but not always, accompanied by a form of programmed cell death that is termed the hypersensitive response[156]. The hypersensitive response is not strictly required for NB-LRR-mediated immunity and can be genetically separated from plant defence.

Several pathogens have evolved additional effectors that interfere with NB-LRR-mediated immunity[6]. Therefore, the outcome of an attempted infection is determined by both the effector repertoire of the pathogen and the set of immune receptors of the host plant (the 'gene-for-gene' concept). The ensuing molecular arms race can be depicted as a model in which the evolution of effective immune receptors increases the selection for pathogens that can interfere with the corresponding immune receptor-signalling pathway[6].

primarily focus on the molecular players involved in effector-triggered susceptibility (pathogen effectors and host targets) and PRR- and NB-LRR-mediated immunity, highlighting examples in which structural studies have had the biggest effect in the understanding of molecular function (TABLE 1).

Molecular warfare in the apoplast

Having penetrated the outer surface of the plant, bacterial and filamentous plant pathogens reside in the interstitial space between cells, which is known as the apoplast. From here, pathogens deploy proteins in attempts to access individual host cells, and plants fight back by sensing and responding to the presence of pathogens outside their cells.

NLPs — natural killers of plant cells? Necrosis- and ethylene-inducing peptide 1-like proteins (NLPs) are secreted proteins that are widely distributed in bacteria and eukaryotic microorganisms[10,11]. NLPs were identified as elicitors of cell death in dicotyledonous plants and may facilitate infection of hosts by necrotrophic pathogens; they can also stimulate plant innate immunity[12,13]. Interestingly, NLPs are also expressed by hemibiotrophic and obligate biotrophic pathogens and many NLPs from these organisms do not induce cell death when expressed in plant cells[14,15].

Pythium aphanidermatum is an economically important oomycete plant pathogen that causes root and stem rotting and blights in a wide range of hosts, including cucurbits, peppers, cottons and grasses. Structural studies of an NLP from *P. aphanidermatum* were the key to unlocking the mechanism of NLP phytotoxicity[11,16]. The NLP fold comprises a central β-sandwich decorated with α-helices and loops. Database searches showed distant structural homology between NLPs and sea anemone actinoporins, which are pore-forming toxins that transfer their amino-terminal α-helical region into membranes following lipid binding[17]. As this N-terminal region is required for NLP activity[18] it has been hypothesized that phytotoxic NLPs are virulence factors that disrupt plant cell membranes to facilitate cell death during infection[11,16]. Such membrane-damaging activity may result in the production of DAMPs[8], which provides an explanation for how these proteins activate plant immunity. A second NLP structure, from the fungal pathogen *Moniliophthora perniciosa*, which is the causative agent of witches' broom disease of cocoa[19], confirmed all NLPs probably adopt the same fold.

A prominent feature in the NLP structures is a cavity of net negative surface potential, which may bind a divalent metal cation[11,16,19]. A heptapeptide GHRHDWE motif[10,12,20] that maps to this region is required for the activity of NLPs in *in vitro* and *in planta* assays. Two exposed hydrophobic loop regions that are located adjacent to the negative cavity[16,19] might mediate an initial lipid bilayer interaction, and mutations in one of these loops were sufficient to disrupt NLP cytolytic activity[19].

So far, there are no examples of structures for non-cytotoxic NLPs that could explain how these proteins avoid activation of cell death despite, presumably, having a similar fold to that of cytotoxic NLPs. Such structures may shed light on the range of functions that are encoded by this intriguing protein family.

PGIPs — man the borders! *Endo*-polygalacturonases (*e*PGs) are phytopathogenic fungal virulence factors that contribute to disease by depolymerizing homogalacturonan, which is a component of plant cell wall pectin[21,22]. This activity contributes to the breaching of this physical barrier, which enables the fungus to gain access to the plant cell membrane. In turn, plants secrete

Necrotrophic
An organism that kills host cells before invasion and gains nutrients from the dead plant tissue.

Hemibiotrophic
An organism that feeds on living tissues for a period and then switches to necrotrophic colonization of dead tissues.

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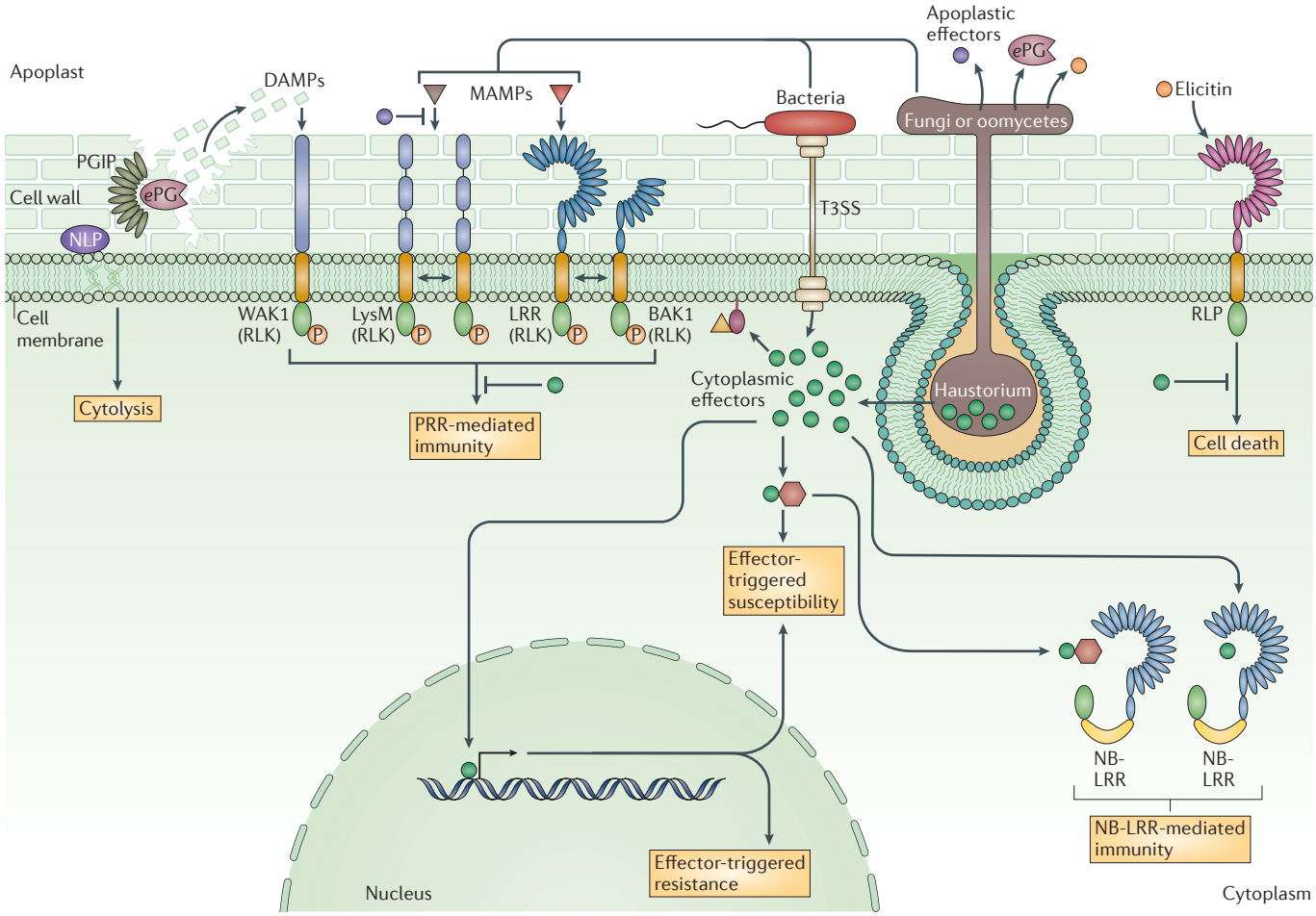

Figure 1 | **Overview of some of the molecular players in plant–pathogen interactions.** Plants are under continuous threat from bacterial and filamentous pathogens, which propagate in the apoplastic space of plant tissues. Some pathogens produce necrosis- and ethylene-inducing peptide 1-like proteins (NLPs), which damage cell membranes, leading to cytolysis. Certain fungi secrete *endo*-polygalacturonases (*e*PGs) to hydrolyse the plant cell wall. In turn, plants produce PG-inhibiting proteins (PGIPs), which interact with fungal *e*PGs and favour the accumulation of cell wall fragments (damage-associated molecular patterns (DAMPs)), which may activate plant defence responses via DAMP receptors (for example, the candidate DAMP receptor wall-associated receptor kinase 1 (WAK1)). Conserved micro-organism-associated molecular patterns (MAMPs) are released from pathogens in the apoplastic space that can be perceived by cell surface pattern-recognition receptors (PRRs; receptor-like kinases (RLKs) or receptor-like proteins (RLPs)) and trigger PRR-mediated immunity. RLKs comprise an extracellular domain that is responsible for the perception of MAMPs (through the Leu-rich repeat (LRR) or LysM domains, which typically detect proteinaceous and oligosaccharide ligands, respectively) and an intracellular kinase domain to activate the downstream signalling process. Some PRRs recruit co-receptors to initiate PRR-mediated immunity (for example, BRI1-ASSOCIATED RECEPTOR KINASE 1 (BAK1)). RLPs can recognize elicitins (small proteins secreted by pathogens) and can induce cell death to restrict pathogen spread. Pathogens secrete effectors to the apoplast (apoplastic effectors) or deliver them inside host cells (cytoplasmic effectors) to perturb plant cell physiology. Apoplastic effectors can interfere with the perception of MAMPs, whereas cytoplasmic effectors migrate to various cellular compartments to carry out their activities (effector-triggered susceptibility or in some cases, effector-triggered resistance). Plants carrying intracellular nucleotide-binding (NB)-LRR receptors can recognize cytoplasmic effectors either directly or indirectly and can initiate NB-LRR-mediated immunity. P, phosphate; T3SS, type III secretion system.

PG-inhibiting proteins (PGIPs), which associate with the plant cell wall. PGIPs interact with *e*PGs to modulate their activity, which results in the accumulation of oligogalacturonides in the apoplast. These oligogalacturonides can function as DAMPs[8], eliciting a host immune response that might be mediated by wall-associated receptor kinase 1 (WAK1), which is a candidate oligogalacturonide receptor-like kinase (RLK)[23].

Despite the importance of LRR-containing proteins in plant immunity, the crystal structure of a PGIP from the common bean *Phaseolus vulgaris* remains the only structure of a plant LRR defence protein that has been determined so far[21,24]. The LRR of PGIP comprises ten repeats, which fold into a right-handed super-helix. The concave face of the structure forms a β-sheet displaying a pocket of negative surface potential. This region could

Obligate biotrophic
An organism that can only complete its life cycle on living plant tissue; such organisms actively prevent host cell death and feed on living plant tissue.

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Box 2 | **Key technological developments**

It seems remarkable that only a few decades ago samples for structural studies of proteins were only available by purifying proteins from their natural sources. In modern laboratories this is extremely rare and, were it not for major innovations at every step in the process of obtaining protein structures, the Protein Data Bank (PDB; the repository for determined protein structures) would not be as rich and diverse as it is today. More importantly, the types of fundamental biological questions that could be addressed with structural biology would be limited. Many technological advances have revolutionized structural biology, some of which were specifically developed for this purpose, whereas others are of more general use. There are too many to cover in detail here but some of the key developments include:

- Bioinformatics: the prediction of domain boundaries and ordered–disordered regions of proteins, which enables design of constructs most suited to structural studies. The availability of example structures for many protein folds that can be used as templates for obtaining similar structures from X-ray diffraction data (molecular replacement) or homology modelling.
- Cloning for protein expression: the design of expression vectors and DNA-cloning strategies to rapidly assemble constructs for protein expression that is suited to structural studies in heterologous hosts.
- Protein expression in multiple hosts: the use of multiple heterologous expression hosts for protein production, including *Escherichia coli* (many different strains with varied benefits), yeast, insect cells, mammalian cell culture and plants. In particular, the increasing use of eukaryotic expression systems to produce samples for proteins that were difficult to produce by other methods.
- Automated protein purification: one-run, multistep systems for rapid protein purification from cell lysates and culture supernatants.
- Miniaturization of protein crystallization: robotics to enable nanolitre-scale crystallization trials, which enables an extended range of crystallization conditions (frequently >1,000) to be investigated for a given sample.
- Large multi-user data collection facilities: the availability and accessibility of modern synchrotron radiation sources (such as high-intensity X-ray and microfocus beams) and high-field nuclear magnetic resonance facilities that enable high-quality data collection.

interact with positively charged residues near the active site in *e*PGs that are involved in substrate binding, which suggests a mechanism for how PGIPs inhibit or modulate *e*PG activity. A second β-sheet, on the convex face of the structure, is absent in other (mammalian) LRR proteins for which structures are available.

The results of mutagenesis, natural variation and protein desolvation energy analyses, when mapped onto the PGIP structure, support the assignment of the concave LRR surface as the *e*PG-interaction surface[25,26]. Direct confirmation of the interaction between PGIP and *e*PG was shown using small-angle X-ray scattering[27]. The low-resolution model may prove useful in engineering PGIPs to improve their use against fungal pathogens in the field.

Insights into MAMP perception — teamwork makes you stronger. The first structural insights into MAMP perception have recently been described for the LysM-containing receptor CHITIN ELICITOR RECEPTOR KINASE 1 (CERK1) from *Arabidopsis thaliana*[28,29], which is a PRR that is essential for the perception of chitin. Chitin is the building block of fungal cell walls and *A. thaliana* CERK1 is required for chitin-triggered immunity[30,31]. The ectodomain of *A. thaliana* CERK1 contains three LysM domains, all of which are required for chitin binding[32,33]. In the structure of the *A. thaliana* CERK1 ectodomain, the three LysM domains form intimate contacts with each other, generating a globular structure. Each LysM domain forms the canonical β–α–α–β fold found in other LysM-containing proteins from bacteria to mammals[28].

A ligand-bound structure of *A. thaliana* CERK1 was obtained by soaking crystals with the chitin fragment *N*-acetylglucosamine 5 (NAG_5; chitin is a homopolymer of *N*-acetylglucosamine). Surprisingly, NAG_5 only bound to the second LysM2 domain, which is sandwiched between the loops connecting β_1–α_1 and α_2–β_2. Comparisons of the grooves between the β_1–α_1 and α_2–β_2 loops in the different *A. thaliana* CERK1 LysM domains reveal differences, and it appears that only LysM2 has evolved to tightly bind chitin. This stoichiometry is supported by *in vitro* binding and mutagenesis data that show a 1:1 binding for *A. thaliana* CERK1–NAG_5 that is dependent on the groove between the LysM2 β_1–α_1 and α_2–β_2 loops.

In the *A. thaliana* CERK1–NAG_5 structure, only four of the chitin monomers are clearly defined. Binding between *A. thaliana* CERK1 and NAG_5 is dominated by hydrogen bonding interactions through the branched groups on one side of the extended carbohydrate that deeply penetrate the LysM2 groove. Interestingly, NAG_5 is not sufficient to elicit a robust immune response in plants, and the most potent chitin fragments are NAG_7 and NAG_8 (REFS 32–34). In the *A. thaliana* CERK1–NAG_5 structure, both ends of the chitin fragment extend into solvent, and longer NAG polymers would neither interfere with binding nor probably contribute additional interactions. Therefore, the structure suggests that chitin fragments longer than those explicitly required for *A. thaliana* CERK1 binding could colocalize or cluster receptors to activate signalling, with the minimum required unit being ligand-induced homodimerization. Indeed, this was subsequently shown to be the case[28].

Ligand-induced heteromerization is well established as being important for the MAMP signalling that is mediated by RLKs in plants[35,36]. Future structural studies will no doubt define the molecular details of how this oligomerization activates PRRs, and this knowledge may highlight new opportunities for engineering PRR-mediated immunity in crops.

Molecular warfare inside host cells

In the apoplast, pathogens attempt to manipulate the host by secreting proteins that can be delivered into plant cells. Following translocation into host cells, these proteins can be trafficked to different subcellular compartments, such as the plasma membrane, cytoplasm and the nucleus[37–39]. So far, the effects these proteins have on plant immunity-related signalling pathways have received the most attention. Plants directly or indirectly sense the presence of these foreign molecules through intracellular immune receptors. This can result in activation of the hypersensitive response, which is a form of programmed cell death (PCD) that can limit the spread of biotrophic pathogens.

Hypersensitive response (HR). A specific form of programmed cell death, often induced by effector-triggered immunity and correlated with accumulation of antimicrobial compounds and systemic acquired resistance.

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Table 1 | **Protein structures of relevance to plant–pathogen interactions described in this Review**

Proteins	Origin	Function	Fold	PDB code	Refs
NLP	*P. aphanidermatum*	Cytolytic toxin	Toxin	3GNU	11
PGIP	*P. vulgaris*	Plant defence protein	LRR	1OGQ	24
A. thaliana CERK1 ectodomain	*A. thaliana*	PRR	LysM	4EBY, 4EBZ	28
$AvrPtoB_{250–359}$–BAK1 kinase domain	*P. syringae* and *A. thaliana*, respectively	Kinase inhibitor–host kinase	4-helix bundle–kinase	3TL8	41
$AvrPtoB_{121–205}$–Pto	*P. syringae* and *S. pimpinellifolium*, respectively	Kinase inhibitor–host kinase	4-helix bundle–kinase	3HGK	48
$AvrPtoB_{436–553}$	*P. syringae*	Ubiquitin ligase	4-helix bundle	2FD4	47
AvrPto	*P. syringae*	Kinase inhibitor	3-helix bundle	1R5E	53
AvrPto–Pto	*P. syringae* and *S. pimpinellifolium*, respectively	Kinase inhibitor–host kinase	3-helix bundle–kinase	2QKW	51
HopU1	*P. syringae*	mADP-RT	Cholera toxin-like	3U0J	57
AvrPphF	*P. syringae*	Unknown (mADP-RT?)	Diphtheria toxin-like	1S28	59
AvrPphB (HopAR1)	*P. syringae*	Protease	Papain-like protease	1UKF	64
AvrB	*P. syringae*	Unknown	Fido	1NH1	88
AvrB–ADP	*P. syringae*	Unknown	Fido	2NUN	89
$AvrRps4^{C}$	*P. syringae*	Unknown	CC	4B6X	104
XopL E3 ligase domain	*X. campestris*	Ubiquitin ligase (*in vitro*)	Novel E3 ligase fold	4FC9	70
XopL LRR domain	*X. campestris*	Unknown (molecular interaction?)	LRR	4FCG	70
XopD SUMO protease domain	*X. campestris*	SUMO protease	SUMO protease	2OIV	74
AVR3a11	*P. capsici*	Unknown	WY	3ZR8	128
PexRD2	*P. infestans*	Unknown	WY	3ZRG	128
AVR3a4	*P. capsici*	Unknown	WY	2LC2	129
ATR1	*H. arabidopsidis*	Unknown	WY	3RMR	133
ATR13	*H. arabidopsidis*	Unknown	α-helical	2LAI	134
AvrL567-D	*M. lini*	Unknown	β-sandwich	2QVT	137
AvrL567-A	*M. lini*	Unknown	β-sandwich	2OPC	137
AvrPiz-t	*M. oryzae*	Unknown	β-sandwich	2LW6	138
MLA10 (CC domain)	*B. graminis*	CC-NB-LRR receptor	CC	3QFL	145
A. thaliana TIR domain	*A. thaliana*	TIR-NB-LRR receptor	TIR	3JRN	147
L6 (TIR domain)	*M. lini*	TIR-NB-LRR receptor	TIR	3OZI	148
PthA	*X. axonopodis*	Modulate gene expression	TPR-like	2KQ5	115
PthXo1	*X. oryzae*	Modulate gene expression	Helical-repeat	3UGM	118
dHax3	Artificial with DNA	NA	Helical-repeat	3V6T	116
dHax3	Artificial without DNA	NA	Helical-repeat	3V6P	116

A. thaliana, *Arabidopsis thaliana*; *B. graminis*, *Blumeria graminis*; BAK1, BRI1-ASSOCIATED RECEPTOR KINASE 1; CC, coiled-coil; CERK1, CHITIN ELICITOR RECEPTOR KINASE 1; Fido, Fic–doc; *H. arabidopsidis*, *Hyaloperonospora arabidopsidis*; LRR, Leu-rich repeat; *M. lini*, *Melampsora lini*; *M. oryzae*, *Magnaporthe oryzae*; mADP-RT, mono-ADP ribosyltransferase; NA, not applicable; NB, nucleotide-binding; NLP, necrosis- and ethylene-inducing peptide 1-like; *P. aphanidermatum*, *Pythium aphanidermatum*; *P. capsici*, *Phytophthora capsici*; *P. infestans*, *Phytophthora infestans*; *P. syringae*, *Pseudomonas syringae*; *P. vulgaris*, *Phaseolus vulgaris*; PDB, Protein Data Bank; PGIP, polygalacturonase-inhibiting protein; PRR, pattern-recognition receptor; *S. pimpinellifolium*, *Solanum pimpinellifolium*; SUMO, small ubiquitin-like modifier; TIR, Toll and interleukin-1 receptor; TPR, tetratricopeptide repeat; *X. axonopodis*, *Xanthomonas axonopodis*; *X. campestris*, *Xanthomonas campestris*; *X. oryzae*, *Xanthomonas oryzae*; Xop, *Xanthomonas* outer protein.

Targeting kinase active sites — neutralize the scout. The intracellular kinase domains of PRRs transduce the perception of MAMPs and DAMPs on the outside of plant cells to the intracellular signal-transduction pathways that mediate defence-associated responses. Phytopathogenic bacteria deliver type III secreted effectors (T3SEs) that directly interact with these kinase domains to modulate PRR-dependent immunity.

Pseudomonas syringae is a key model pathogen for the study of pathogen–plant interactions[40]. More than 50 *P. syringae* pathovars have been isolated from different hosts. *P. syringae* injects over 30 T3SEs into plant cells during infection, including the two kinase inhibitors, AvrPtoB (also known as HopAB2) and AvrPto. AvrPtoB is a 553 amino acid protein that can interact with the PRR *A. thaliana* CERK1 (REFS 41–43), the *A. thaliana*

Pathovars
Pathogenic variants within a species that are defined by a characteristic host range and/or tissue specificity.

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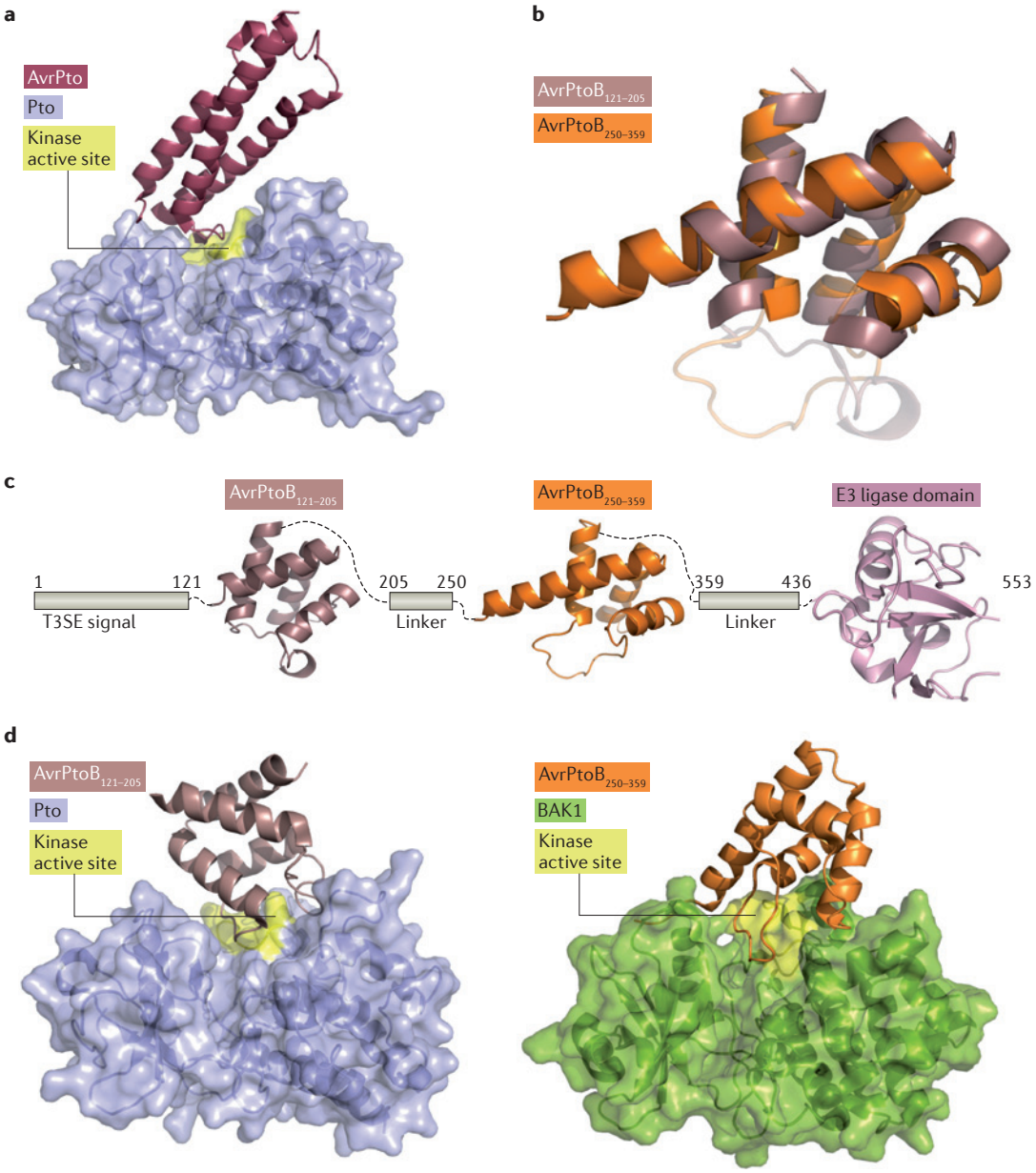

Figure 2 | **Structural basis of the interaction between *Pseudomonas syringae* T3SEs AvrPtoB and AvrPto and immune kinases.** **a** | Crystal structure of AvrPto in complex with the Pto kinase. The kinase active site is highlighted. **b** | Superposition of the two AvrPtoB four-helix bundles ($AvrPtoB_{121–205}$ and $AvrPtoB_{250–359}$) that mediate interaction with immune kinases shows that they adopt a similar structure. The structural overlay was generated using the secondary-structure-matching algorithm in the COOT molecular graphics programme[157]. **c** | Schematic representation of AvrPtoB, showing the regions of the protein for which structures have been determined: the two four-helix bundles and the E3 ligase domain are shown. The type III secreted effector (T3SE) signal and linker regions are indicated. **d** | Complexes of the two AvrPtoB four-helix bundles with immunity-related kinases. Left panel, complex of $AvrPtoB_{121–205}$ with the Pto kinase, which results in defence activation by the Pto–Prf complex. Right panel, interaction of $AvrPtoB_{250–359}$ with the pattern-recognition receptor (PRR) co-receptor BRI1-ASSOCIATED RECEPTOR KINASE 1 (BAK1), which results in suppression of PRR-mediated immunity. The figure was prepared using PyMol.

CERK1-related PRR Bti9 from tomato[44] and the *A. thaliana* co-receptor BRI1-ASSOCIATED RECEPTOR KINASE 1 (BAK1; a co-receptor for FLAGELLIN-SENSING 2 (FLS2) and ELONGATION FACTOR RECEPTOR (EFR)), which interferes with the formation of PRR–BAK1 signalling complexes[45]. AvrPtoB also has E3 ligase activity (see below). Based on AvrPtoB-deletion mutants these different activities are thought to reside in specific regions of the protein[41–43,45–47].

Crystal structures of distinct domains of AvrPtoB have revealed the existence of discrete modules that encode different activities[41,47,48] (FIG. 2). $AvrPtoB_{1–387}$ is

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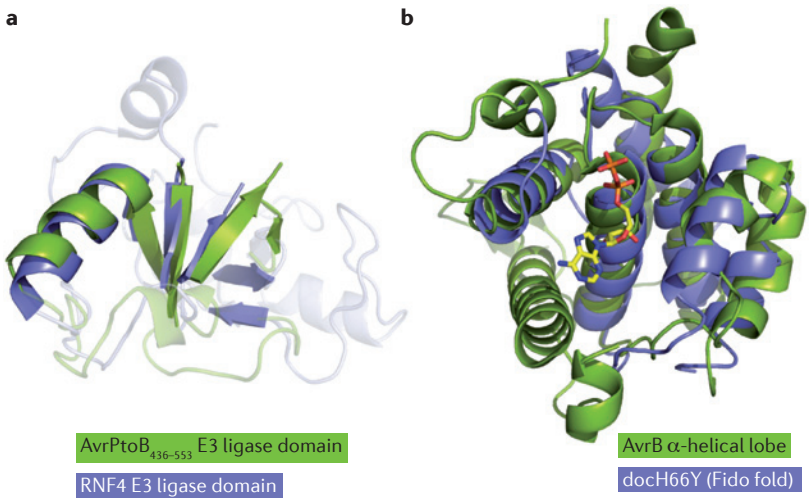

Figure 3 | **Structural homology between bacterial effectors and conserved enzymes suggests protein function. a** | Superposition of the E3 ligase domains of the *Pseudomonas syringae* type III secreted effector (T3SE) $AvrPtoB_{436–553}$ and rat RING-finger protein 4 (RNF4; Protein Data Bank (PDB) ID: 4AP4 (REF. 158)). **b** | Superposition of the α-helical lobe of the *P. syringae* T3SE AvrB with docH66Y from phage P1, an example of the Fic–doc (Fido) fold (PDB ID: 3DD7 (REF. 159)). The ADP molecule bound by AvrB is shown in stick representation. Structural overlays were generated as described for FIG. 2. The figure was prepared using PyMol.

sufficient to interact with the BAK1 kinase domain and to suppress certain PRR-dependent immune responses in *A. thaliana*[45]. The minimal fragment of AvrPtoB that interacts with and inhibits the BAK1 kinase domain was mapped to $AvrPtoB_{250–359}$ (REF. 41). The crystal structure of $AvrPtoB_{250–359}$ in complex with the BAK1 kinase domain revealed that it adopts a four-helix bundle fold, which blocks the kinase active site (FIG. 2). The loop between αC and αE in AvrPtoB makes specific contacts with the crucial BAK1 kinase domain P + 1 loop. Structure-based mutations in the surfaces of both $AvrPtoB_{250–359}$ and BAK1 disrupt the interaction between these proteins *in vitro* and affect virulence activity *in vivo*[41].

Interestingly, the four-helix bundle adopted by $AvrPtoB_{250–359}$ is structurally homologous to the fold that was previously identified for $AvrPtoB_{121–205}$ (REF. 48), despite these regions only sharing 20% amino acid sequence identity (FIG. 2). $AvrPtoB_{1–307}$, which contains the first helix bundle region, is sufficient for interaction with *A. thaliana* CERK1 and tomato Bti9 (REFS 41–44). Although there is no structural information on this interaction, structure-directed mutagenesis of AvrPtoB revealed that mutations in the αC–αE loop of $AvrPtoB_{121–205}$ do not disrupt the interaction with the Bti9 kinase domain[44]. Therefore, it seems that the separate helical bundles bind host kinases via different interfaces (indeed, $AvrPtoB_{121–205}$ interacts with the Pto kinase in a different orientation from that of $AvrPtoB_{250–359}$–BAK1, see below). BAK1 is dispensable for *A. thaliana* CERK1 function[42], and it is conceivable that acquisition of the first helix bundle of AvrPtoB has extended the function of this effector to suppression of BAK1-independent signalling routes. Notably, a similar arrangement of two structurally related four-helix bundles is found in other effectors of the HopAB family[49].

E3 ligases
Enzymes required to attach the molecular tag ubiquitin to proteins. This tag modifies protein function or targets the protein for proteosomal degradation.

AvrPto is a single-domain, 164 amino acid protein that, like AvrPtoB, forms complexes with the kinase domains of several *A. thaliana* PRRs and suppresses mitogen-activated protein kinase (MAPK) activation downstream of FLS2 and EFR[45,50]. AvrPto functions *in vitro* as a kinase inhibitor[51]. Whether it also binds BAK1 and interferes with formation of PRR–BAK1 signalling complexes remains a matter for debate[45,50,52]. The NMR structure of AvrPto revealed that three tightly packed α-helices constitute the ordered core of the effector[53]. The AvrPto mutation Tyr89Asp maps to a loop connecting the αC and αD helices and abrogates interaction with BAK1, suppression of PRR-mediated immunity and inhibition of kinase activity *in vitro*[45,51,53].

Targeting kinase transcripts — intercept the messenger. The *P. syringae* T3SE HopU1 has mono-ADP ribosyltransferase (mADP-RT) activity[54,55] and targets several RNA-binding proteins, including *A. thaliana* GLY-RICH RNA-BINDING PROTEIN 7 (GRP7), which contributes to PRR-dependent responses and immunity towards *P. syringae* infection[54]. GRP7 binds transcripts of the PRRs *FLS2* and *EFR* via its RNA-recognition motif[56]. The HopU1 crystal structure showed that the effector adopts the fold of mADP-RTs from the cholera toxin group[57]. The structure also revealed two extended loops that are not present in other mADP-RTs, and structure-guided mutagenesis confirmed that these loops form the substrate-binding interface for GRP7. HopU1 specifically targets Arg49 in the GRP7 RNA-recognition motif, which is a region that is required for binding *FLS2* and *EFR* transcripts[54,56–58]. In this way, HopU1 perturbs FLS2 signalling by manipulating translation of *FLS2* transcripts, which reduces the protein levels during infection[56].

The crystal structure of the *P. syringae* T3SE AvrPphF (also known as HopF1) revealed a fold that superimposes well onto that of the mADP-ribosylating diphtheria toxin[59]. Although mADP-RT activity has not been shown for AvrPphF, the sequence-related T3SE HopF2 ADP-ribosylates two *A. thaliana* proteins *in vitro*, MAPK KINASE 5 and RPM1-INTERACTING PROTEIN 4 (RIN4)[60,61]. AvrPphF and HopF2 share 92% sequence similarity, and structural motifs that form the active site of HopF2 are conserved in AvrPphF[55]. Therefore, AvrPphF is likely to be an active mADP-RT.

Targeted degradation of immune components — removal from the battlefield. In addition to the kinase-interacting domains, AvrPtoB contains a third domain for which a structure has been determined[47]. The crystal structure of $AvrPtoB_{436–553}$ showed a fold that was homologous to eukaryotic U-box and RING-finger E3 ligases (FIG. 3). Indeed, AvrPtoB encodes a functional ubiquitin E3 ligase that interacts with host E2 enzymes and leads to targeted degradation of *A. thaliana* CERK1 and other immune kinases[42,47,62]. The remarkable combination of two kinase-inhibiting domains and an E3 ubiquitin ligase makes AvrPtoB a multitasking effector protein that interferes with signalling from several immune-related kinases in host cells.

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AvrPphB (also known as HopAR1) is another *P. syringae* T3SE that eliminates immune regulatory kinases[63]. The crystal structure of AvrPphB showed a fold that is similar to that of papain-like Cys proteases[64], which confirms previous results obtained from secondary structure prediction and mutagenesis of the predicted catalytic Cys-His-Asp triad of Cys proteases (Cys98-His212-Asp227 in AvrPphB)[65]. The structure gave an insight into the preference of AvrPphB to cleave the Gly-Asp-Lys motif in the activation loop of several cytoplasmic kinases that function in signal transduction downstream of activated PRRs[63,64]. A knockout of one of these kinases, BOTRYTIS-INDUCED KINASE 1 (BIK1), results in partially impaired signalling from *A. thaliana* CERK1, FLS2 and EFR[63,66]. In resistant plants, AvrPphB-mediated cleavage of another cytoplasmic kinase, PBS1, results in activation of the NB-LRR receptor RESISTANT TO *P. SYRINGAE* 5 (RPS5) and subsequent PCD[67–69].

Xanthomonas campestris pv. *vesicatoria* is a bacterial pathogen that infects tomatoes and peppers, causing leaf spot. *Xanthomonas* outer protein L (XopL) is a T3SE protein from *X. campestris* pv. *vesicatoria* that, like AvrPtoB, has E3 ubiquitin ligase activity. Structure determination of two ordered XopL fragments showed that the protein consists of an N-terminal LRR domain fused to a carboxy-terminal four-helix bundle[70]. Unlike AvrPtoB, the C-terminal region of XopL shows no structural homology to known ubiquitin E3 ligases. The absence of Cys residues in this region prevents the formation of XopL–ubiquitin thioester intermediates. Despite this, the C-terminal region of XopL specifically interacts with plant E2 enzymes and promotes the elongation of ubiquitin chains *in vitro*, although the exact mechanism involved is unknown. The arrangement of LRR and E3 ligase domains in XopL is reminiscent of two effector proteins from *Shigella* spp. and *Salmonella* spp. that use LRR domains as target-binding modules for polyubiquitylation by an E3 ligase domain[71,72]. Identification of plant proteins that bind to XopL will show whether XopL functions in an analogous manner.

The *X. campestris* pv. *vesicatoria* T3SE XopD comprises a DNA-binding domain, a central domain carrying two EAR-type transcriptional repressor motifs and a C-terminal small ubiquitin-like modifier (SUMO) peptidase domain. XopD SUMO protease activity is required for suppression of salicylic acid- and ethylene-driven immunity[73]. Crystallization and structure determination of the XopD SUMO protease domain identified the XopD residues that determine its specificity for plant SUMO proteins[74]. During infection of tomato, XopD targets the tomato transcription factor ethylene response factor 4 (ERF4), which drives transcription of ethylene biosynthesis genes. XopD catalyses the cleavage of tomato SUMO1 from ERF4 in plant cells and targets ERF4 for degradation by the proteasome[75].

Diverse bacterial effectors that target a single regulator — deploy multiple weapons. RIN4 is a plasma membrane-associated *A. thaliana* protein that is a negative regulator of PRR-mediated defence[76,77]. Although the molecular mechanism involved remains to be fully defined[78,79], RIN4 associates with at least three bacterial T3SEs[76,80,81]. This implicates perturbation of endogenous RIN4 activity as a possible virulence function of bacterial effectors. Furthermore, the status of RIN4 is monitored by two NB-LRR proteins in plant cells[76,80,81].

Following delivery, the *P. syringae* T3SE proteins AvrRpm1 and AvrB are myristoylated[37] and induce the phosphorylation of RIN4 (REF. 82). Delivery of another *P. syringae* T3SE protein, AvrRpt2, results in proteolytic cleavage of RIN4 (REFS 83,84). This activates the NB-LRR protein RPS2, whereas the NB-LRR protein RPM1 induces defence signalling on effector-induced phosphorylation of RIN4 (REFS 82,84–86). The host cytoplasmic kinase RPM1-INDUCED KINASE (RIPK) was shown to interact with and to phosphorylate AvrB and RIN4 (REF. 87), which identifies another molecular player in this effector recognition complex.

The crystal structure of AvrB showed a bi-lobal fold[88]. A subsequent study showed that the larger AvrB α-helical lobe binds ADP in a large cavity comprising residues that are highly conserved across the protein family[89]. The same study also determined the structure of AvrB bound to $RIN4_{142–176}$, which is the minimal RIN4 peptide that interacts with AvrB in solution[90]. $RIN4_{142–176}$ predominantly binds to the smaller α–β-lobe of AvrB and to the inter-lobe cleft. Notably, two RIN4 residues that are phosphorylated by RIPK are also located in this cleft (Ser160 and Thr166). When the independent structures are overlaid, the two ligands come into close proximity. Mutations in either the $RIN4_{142–176}$-binding or the ADP-binding sites interfered with AvrB-induced NB-LRR signalling by RPM1 (REF. 89).

Notably, the α-helical lobe of AvrB superimposes well onto the Fic–doc (Fido) fold found in several effector proteins from pathogenic bacteria of mammals and plants[91,92] (FIG. 3). Fic domains catalyse the covalent modification of target proteins with different phosphoderivatives, including AMP and UMP[91,93,94]. AvrB lacks conservation of residues that are essential for catalysing the addition of AMP or UMP by other Fic proteins, so it is currently unclear whether it possesses either of these activities[87,92].

Triggering of NB-LRR signalling cascades — tripping the wire. Above, we discuss how structural biology has contributed to elucidating the virulence functions of the *P. syringae* T3SE proteins AvrPto and AvrPtoB. In this section, we describe how such studies have also provided an understanding of the molecular basis of AvrPto and AvrPtoB recognition by NB-LRR protein complexes. Although AvrPto and AvrPtoB are not recognized in *A. thaliana*, several tomato cultivars have evolved a protein complex that comprises the Ser/Thr kinase Pto and the NB-LRR protein Prf, rendering them immune to infection with *P. syringae* strains that deliver AvrPto or AvrPtoB[95].

Pto specifically interacts with $AvrPtoB_{121–205}$, the first helix bundle of AvrPtoB, which is the same region that binds *A. thaliana* CERK1 and Bti9 kinase domains[42–44,48]. The crystal structure of the $AvrPtoB_{121–205}$–Pto complex

Salicylic acid
Also known as salicylate, this is a central plant hormone signal that induces local and systemic defence responses in plants, collectively known as systemic acquired resistance.

Ethylene
A gaseous, unsaturated hydrocarbon that acts as a plant hormone to promote growth and development and as an inhibiting stress factor.

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showed that the interaction is dominated by two interfaces, with both being required for the AvrPtoB–Pto interaction and for recognition of AvrPtoB (FIG. 2). The first interface involves the $AvrPtoB_{121–205}$ α–β–α loop binding to the P + 1 loop of Pto. Targeted mutagenesis confirmed that this interface is required for the interaction with both Bti9 and Pto[44,48]. The second interface, involving the short $AvrPtoB_{121–205}$ αD-helix binding to a surface groove on Pto, is dispensable for Bti 9 binding. Thus, it seems that Pto evolved an additional interface that enables it to recognize AvrPtoB.

Pto also uses a third distinct interface to specifically recognize AvrPto[51]. The tip of the AvrPto helical bundle and a loop in Pto preceding β1 forms this interface (FIG. 2). An interesting feature of all three AvrPto and AvrPtoB kinase complexes is that the effectors interact with and stabilize the P + 1 loop of the kinases in a conformation that is reminiscent of that of the active form. Autophosphorylation of Pto Ser198-Thr199 in the activation segment of the kinase seems to be a prerequisite for recognition of AvrPto and AvrPtoB[51,96], and the Pto mutation Thr199Ala attenuates the interaction with both effectors[48,51]. In the structures of AvrPto and $AvrPtoB_{121–205}$ bound to Pto, Thr199 is in a phosphorylated form and forms salt-bridge interactions with two residues in the P + 1 loop, which stabilizes this conformation.

Fen, another kinase that signals through Prf, binds the second helix bundle of AvrPtoB and is targeted for proteasomal degradation by the E3 ligase domain[47,97]. Unlike Fen, Pto escapes AvrPtoB-mediated ubiquitylation, possibly by phosphorylating Thr450 in the AvrPtoB E3 ligase domain[98]. As mutations that compromise the interaction between $AvrPtoB_{121–05}$ and Bti9 also prevent interaction with Pto[44], and as Fen and BAK1 are expected to share two of the three interfaces that mediate binding to $AvrPtoB_{250–59}$ (REF. 41), it is probable that the effector domains interact with their respective kinases via the same interfaces. As a role for Pto and Fen in plants, other than defence against pathogens, has yet to be established, it has been suggested that they might function as molecular decoys of the host kinases targeted by AvrPto and AvrPtoB[99,100].

The *P. syringae* T3SE protein AvrRps4 is recognized by a pair of *A. thaliana* NB-LRR proteins, RESISTANT TO *P. SYRINGAE* 4 (RPS4) and RESISTANT TO *RALSTONIA SOLANACEARUM* 1 (RRS1)[101–103]. On delivery into plant cells, AvrRps4 is processed, and a fragment comprising the 88 C-terminal amino acids ($AvrRps4^C$) is sufficient for recognition[104,105]. The crystal structure of $AvrRps4^C$ revealed that it forms an antiparallel α-helical coiled coil (CC)[104]. Structure-guided mutagenesis of surface-exposed residues identified amino acid Glu187 as required for recognition of AvrRps4. It remains to be established if AvrRps4 interacts directly with RPS4, RRS1 or possibly RPS4–RRS1 complexes. Whether AvrRps4 associates with the lipase-like protein ENHANCED DISEASE SUSCEPTIBILITY 1, which is required for RPS4–RRS1 signalling, also remains debated[104,106,107]. The $AvrRps4^C$ structure and the identification of non-recognized AvrRps4 variants provide an important molecular toolkit for validating the physiological relevance of $AvrRps4^C$ interactions with host proteins.

TAL effectors — hijacking host gene transcription. Unlike T3SEs that intercept plant immune signalling pathways by forming protein complexes with host proteins, transcription activator-like (TAL) effectors directly bind to DNA sequences in the promoter regions of host genes. TAL effectors are modular T3SEs from xanthomonads and *Ralstonia* spp. that comprise an N-terminal translocation signal, a C-terminal nuclear localization signal and transcriptional activation domain, and a central repeat region (1.5 to >30 repeats), which binds to DNA[108]. TAL effectors activate expression of individual host genes by binding to target promoter regions in a sequence-specific manner[109,110]. In the natural interaction, activation of host gene expression by TAL effectors can result in either enhanced susceptibility or resistance to the pathogen. Within the central repeat region, two adjacent variable residues, at positions 12 and 13 of the 33–34 repeat (known as the repeat-variable di-residue (RVD)), determine the nucleotide specificity of TAL effectors. The sequence of the RVD recognizes one base pair in the target DNA[111,112]. The use of custom TAL effector DNA-binding domains as a mechanism for achieving sequence-specific DNA modifications has generated considerable interest[113,114].

The first atomic-level structural information on a TAL effector was obtained for a 1.5-repeat unit of PthA from *Xanthomonas axonopodis* pv. *citri*, determined by NMR[115]. This showed a fold similar to that of the protein scaffolding domains of tetratricopeptide repeat (TPR) proteins. However, this structure lacked the context of the repeat units and how they interact with DNA. In 2012, the crystal structures of two TAL effector central repeat regions, bound to DNA, were determined[116–118]. These were from the *Xanthomonas oryzae* protein PthXo1 bound to its natural DNA target sequence and of the artificially engineered protein dHax3 bound to its DNA element. All of the repeats from both proteins adopt highly similar two-helix bundles that pack together to form a left-handed super-helix. In the complexes, the DNA adopts a canonical B-form conformation. The structure of dHax3 was also solved without a ligand[116]. Comparison with the DNA-bound form shows a marked difference in conformation; the unliganded protein was more extended or the super-helix was slightly unwound. This is consistent with dynamic light-scattering analysis of unbound and DNA-bound forms of the PthA central repeat region, which revealed compaction of the protein in the presence of DNA[115].

The crystal structures of PthXo1 and dHax3 bound to DNA showed that the RVD is presented on a loop between the two helices of the repeat. Surprisingly, the first residue of the RVD (usually a His or Asn) does not interact with DNA, but forms a hydrogen bond with a backbone carbonyl oxygen of a residue in the first helix. This anchors the loop between the helices and presents the more variable second residue of the RVD to a position that is suitable to form base-specific interactions

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with the DNA strand[117]. The different combination of repeats in each TAL effector probably provides the overall sequence selectivity of these proteins.

Remarkably, single TAL effectors are not limited to interacting with unmodified double-stranded DNA. Recently, dHax3 was shown to bind a DNA–RNA hybrid[119]. In the structure of the complex, dHax3 forms interactions with only the DNA strand (dHax3 interacts with only bases on one strand in the double-stranded DNA complex (as does PthXo1)), and the protein imposes a distorted B-form conformation on the DNA–RNA hybrid (DNA–RNA hybrids usually adopt A-form-like structures in solution). Structural studies have also showed the molecular basis of how dHax3 accommodates 5-methyl cytosine at specific positions in its DNA element[120]. This suggests that TAL effectors may be useful in studies of epigenetics and cancer if they can be targeted to these specific DNA modifications[120]. It is interesting to speculate that such TAL effectors have evolved in nature to target specific modified DNA elements or nucleotide hybrid structures of relevance to pathogenesis.

Intriguingly, regions outside of the TAL effector central repeat region can also adopt the distinctive two-helix repeat structure[121] and seem to mediate non-specific binding to DNA. Therefore, a model for TAL effector binding to DNA *in vivo* can include both non-specific and specific interactions that enable these proteins to seek out and to bind their specific targets. Structural understanding of how TAL effectors interact with DNA will facilitate improvements in the design of custom proteins that are tailored to specific biotechnological applications.

Oomycete and fungal effectors — new weapons in the armoury. In the sections above, we describe examples in which crucial insights into the activities of proteins involved in plant pathogen–host interactions have been gained through knowledge of their structures either in isolation or in complex with a target molecule. Many of these examples were of phytopathogenic bacterial effectors. Like bacterial pathogens, fungal and oomycete pathogens of plants translocate effector proteins into host cells during infection[122–124].

A major class of oomycete effector proteins contain an RXLR signature motif that is thought to mediate delivery into host cells, although the mechanism by which this occurs remains controversial[125–127]. All current bioinformatic and structural data concerning the RXLR region are consistent with it adopting a disordered conformation, which may be relevant for its function[128–130]. In this section, we focus on the C-terminal domains of these proteins, which are responsible for their biochemical functions inside plant cells[131,132].

So far, the structures of five oomycete RXLR effector proteins have been published: AVR3a4 (REF. 129) and AVR3a11 (REF. 128) from *Phytophthora capsici*, PexRD2 from *Phytophthora infestans*[128] and ATR1 and ATR13 from *Hyaloperonospora arabidopsidis*[133,134] (FIG. 4). With the exception of that of ATR13, these structures comprise a three-α-helix fold, termed the 'WY domain' after the conserved Trp and Tyr residues, which interact to form a stable hydrophobic core[135] (FIG. 4). These proteins typically share <20% sequence identity and their conserved fold was only apparent after the structures were determined[135]. The WY fold has been observed in monomeric forms (AVR3a4, AVR3a11, and ATR1 as a four-helix bundle) and as a homodimer (PexRD2)[128]. In ATR1, two tandem WY repeats are observed and are linked by an additional helix[133] (FIG. 4). The ATR1 structure suggests how tandem WY domains might be arranged in other repeat-containing effectors. The WY fold may provide a flexible, stable scaffold that supports surface diversification of RXLR effectors. It is unlikely that all oomycete RXLR effectors adopt the WY fold, and one current challenge is to obtain the structures of proteins that do not adopt this fold, with the aim of understanding the structural diversity of effectors produced by these pathogens.

Like oomycetes, fungal plant pathogens rely on translocated effector proteins to promote infection[136]. Three-dimensional structures of two alleles of the AvrL567 effector from the flax rust fungal pathogen *Melampsora lini* (AvrL567-A and AvrL567-D) revealed a β-sandwich fold with limited structural similarity to the host-selective toxin ToxA from the fungal pathogen *Pyrenophora tritici-repentis*[137]. The structures also identified two patches of positive surface charge that could represent DNA-binding sites, and the proteins were subsequently shown to bind DNA *in vitro*. However, the biological significance of the structural homology to ToxA and the DNA binding is unknown. Interestingly, the NMR structure of AvrPiz-t, which is an effector from *Magnaporthe oryzae*, a fungal pathogen and the causative agent of rice blast disease, also adopts a β-sandwich fold[138]. In the future, it will be interesting to see whether this fold is found in other effectors from fungal plant pathogens.

One major unanswered question in the study of both fungal and oomycete effectors is how these proteins interact with host cell targets at the molecular level. Structural studies of such complexes will be a major step forward in understanding how these proteins promote pathogenesis.

NB-LRR recognition of oomycete and fungal effectors — know your enemy. Direct recognition of fungal and oomycete effectors by host NB-LRR receptors has been shown for *M. lini* effectors AvrL567 and AvrM, and for the *H. arabidopsidis* effector ATR1 (REFS 139–141). Each of these effectors and the NB-LRRs that recognize them is polymorphic in different pathogen strains and host ecotypes, and 'gene-for-gene' relationships between different alleles are the basis of recognition. In each case, it is the LRR domain of the NB-LRRs that seems to be the relevant region for interaction.

The crystal structures of ATR1, AvrL567-A and AvrL567-D have been used to map residues involved in their interactions with cognate NB-LRRs. These studies have shown that polymorphic residues mapping to the surface of the effectors mediate recognition. For ATR1 and AvrL567, a combination of mapping polymorphic residues and structure-guided mutagenesis showed that

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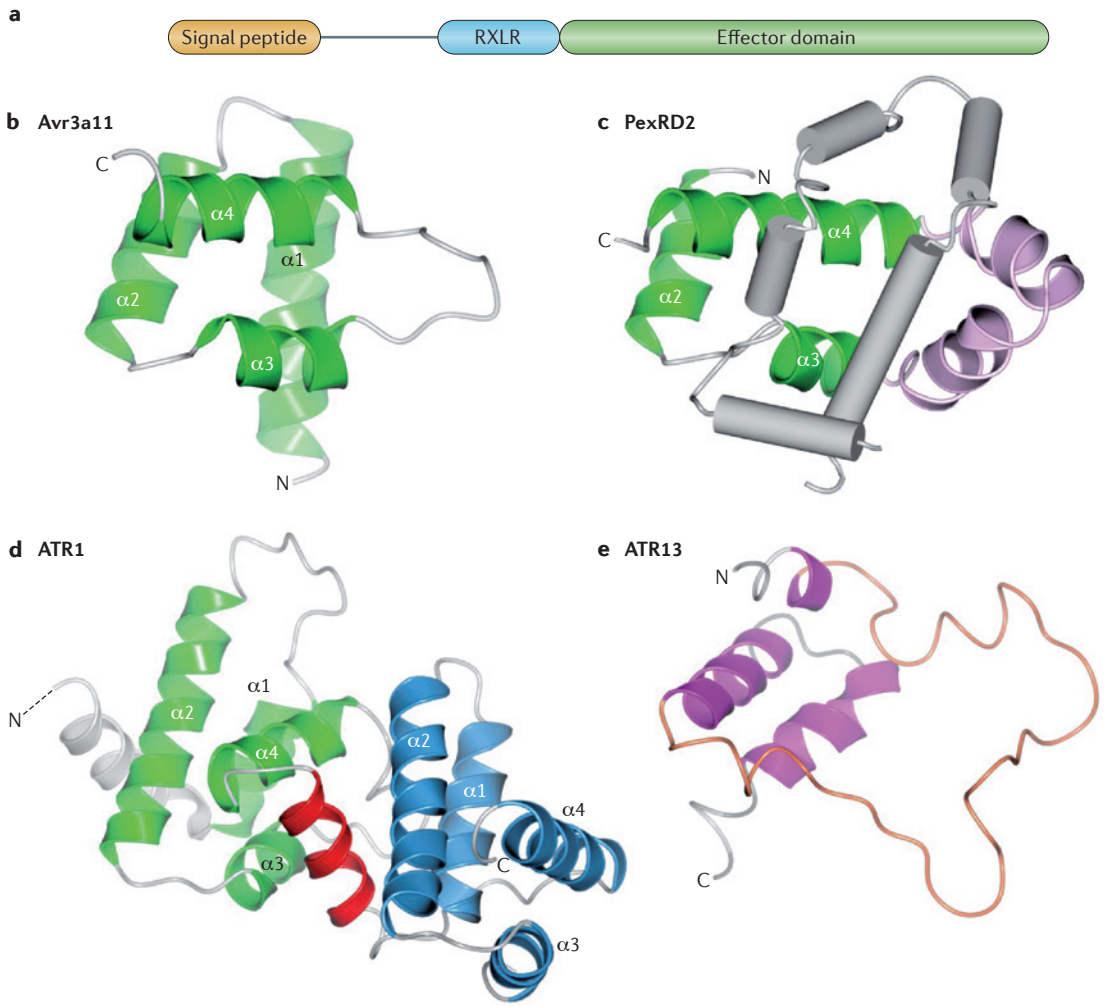

Figure 4 | **Crystal structures of RXLR effector proteins.** **a** | A schematic representation of an RXLR effector, showing the signal peptide linker region, the RXLR motif and the effector domain. **b**–**e** | Ribbon diagrams showing the overall structures of the effector domain of Avr3a11 (part **b**), PexRD2 (part **c**), ATR1 (part **d**) and ATR13 (part **e**). The amino and carboxyl termini are labelled in individual structures. The α-helices of the conserved WY domains are coloured green and labelled α2, α3 and α4. Avr3a11 and ATR1 carry an additional N-terminal helix, labelled α1. In PexRD2, helices that are not part of the core WY domain are coloured pink, and the second PexRD2 monomer of the homodimer is represented as grey helix tubes. ATR1 has two tandem WY domains (green and blue helices) separated by a linker helix, which is coloured red. The non-WY region at the N terminus of ATR1 is not shown. The structure of ATR13 lacks a WY domain and is characterized by three helices (magenta) and a disordered loop (orange) between the first two helices. The figure was prepared using PyMol.

multiple regions distributed across the effector surfaces were important for recognition. Furthermore, the contributions of each of the sites to activation and/or to specificity were shown to be additive.

Direct interaction between other oomycete effectors, such as *P. infestans* AVR3a and *H. arabidopsidis* ATR13, and their cognate resistance proteins have not been established. However, the NMR structure of ATR13 showed that, in contrast to in ATR1, polymorphic residues map to only two discrete exposed surface patches in ATR13, and only one of these is responsible for recognition of ATR13 by the NB-LRR protein RECOGNITION OF *PERONOSPORA PARASITICA* 13 (RPP13). Homology modelling of *P. infestans* AVR3a (on the basis of the crystal structure of AVR3a11) shows that the polymorphic residues $AVR3a_{K80/I103}$ and $AVR3a_{E80/M103}$ map to the same surface of the four-helix bundle. This highlights this region of the AVR3a surface as being important for recognition by the NB-LRR protein R3a. A structural understanding of how NB-LRRs directly interact with effectors will be a crucial step towards enabling the rational design of new recognition specificities.

NB-LRR signalling — joining forces. Intracellular NB-LRR receptors function as molecular traps, either directly detecting effectors or detecting effector-mediated manipulation of target proteins that are 'guarded' by the receptor[142]. N-terminal to the NB domain, most plant NB-LRR receptors carry either a Toll and interleukin-1 receptor (TIR) domain or a CC domain, although some groups of immune receptors are exceptions[143]. Owing to

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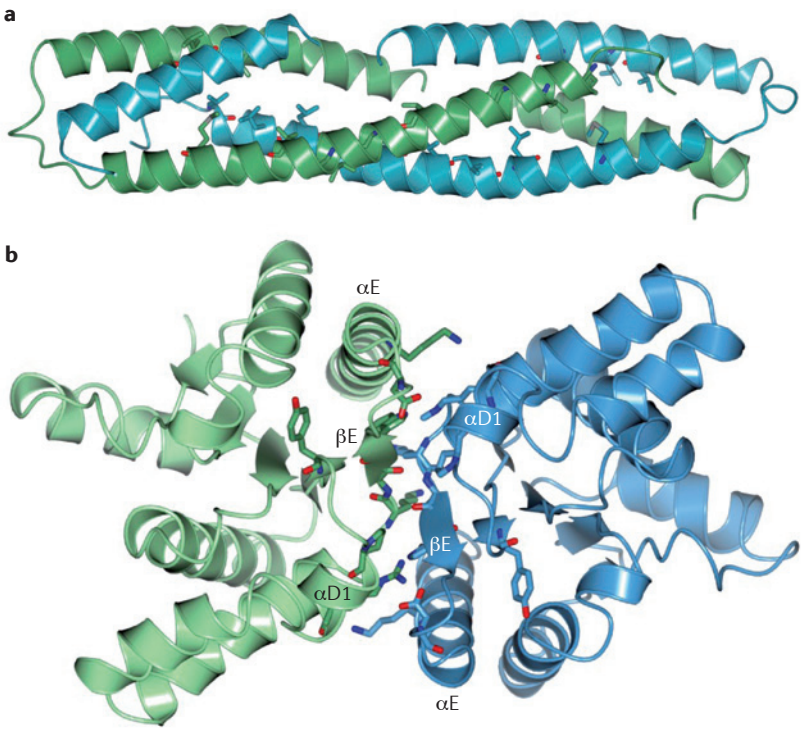

Figure 5 | **Dimerization in the amino-terminal domains of NB-LRR proteins.** Cartoon representations of the dimeric structures of the coiled-coil domain of the MLA10 receptor (part **a**) and the Toll and interleukin-1 receptor domain of L6 receptor (part **b**). The two chains of the homodimers are coloured green and blue. The residues in a stick representation have been shown to be important for stability and/or dimer formation in each protein[145,148]. NB-LRR, nucleotide-binding and Leu-rich repeat. The figure was prepared using PyMol.

the importance of NB-LRRs in plant disease-resistance breeding, many studies and reviews have investigated, hypothesized about and modelled the molecular details of how NB-LRRs might sense effectors or their activities, and how these proteins initiate signal transduction cascades, which lead to disease-resistance responses (see BOX 2 and REFS 143,144). An in-depth understanding of the early events that trigger signalling from NB-LRR proteins is hampered by a lack of structural information about the receptors themselves and about their intramolecular and intermolecular interactions. However, recent reports associating structures of a CC domain and TIR domains with their PCD-inducing activities in plant cells have shed light on the molecular features that are essential for signalling.

The N-terminal CC domain (residues 5–120) from the barley CC-NB-LRR receptor MLA10 forms a domain-swapped homodimer[145] (FIG. 5). This homodimer was stable in solution and in yeast. Several mutations made in the CC-dimerization interface resulted in the protein being unstable when expressed in *Escherichia coli* and abolished dimer formation in yeast. Importantly, the corresponding full-length MLA10 mutants fail to recognize the *Blumeria graminis* effector AVR_{A10} in a bombardment single-cell assay. Interestingly, most CC mutations that attenuate dimerization also abrogate binding of MLA10 CC domain to the barley transcription factor WRKY1, which represses MLA10-triggered defence genes[146]. This suggests that dimerization of the MLA10 CC domain creates the molecular interface for WRKY1 binding and therefore MLA10 signalling.

Two crystal structures of plant TIR domains have been determined, from *A. thaliana*[147] and flax[148]. Despite sharing less than 20% sequence identity with the TIR domains from mammalian Toll-like receptors (TLRs), the plant TIR domains share the same overall α–β-fold as their mammalian homologues. Plant TIR domains contain an insertion in the αD region, and the structure of the *A. thaliana* TIR domain showed that αD is replaced by three short α-helices. Several amino acids are known to be required for initiation of PCD cluster in this region, which suggests that it has an important role in signalling[147].

The structure of the flax L6 TIR domain showed a distinct interface, involving residues from the αD1 and αE helices, the βE strand and the DE and EE loops, which mediate homodimerization of this domain (FIG. 5). Although stable homodimers of the isolated L6 TIR domain were not observed in plant cells, structure-guided mutagenesis identified several amino acids that are not only required for dimer formation *in vitro* and in yeast but also that are essential to trigger effector-independent cell death *in planta*. Notably, dimerization of longer L6 constructs including the NB and NB-LRR regions were not observed, which suggests that the TIR–TIR interaction may occur only on effector recognition. In analogy to the action of mammalian TLRs and nucleotide-binding oligomerization domain-containing (NOD) proteins, dimerization of the N-terminal TIR domains might be promoted by effector-induced oligomerization of full-length NB-LRR receptors[148,149].

So far, there is only limited evidence for effector-induced oligomerization of NB-LRR proteins[150] and, conversely, there is evidence that some NB-LRRs form oligomers in the absence of cognate effectors[67,144]. Therefore, whether effector-induced oligomerization is a conserved feature of NB-LRR proteins remains to be tested. In the near future, structural approaches are expected to determine crucial interfaces for intramolecular and intermolecular interactions between NB-LRR protein domains to help elucidate the early events of receptor activation.

Outlook

Structural biology has provided key advances in our understanding of plant–pathogen interactions in recent years, including: the identification of protein functions that were not apparent from sequences alone (for example, for NLPs and the E3 ligase domain of AvrPtoB); the visualization of molecular interfaces of relevance to pathogen virulence (for example, the interaction $AvrPtoB_{250–359}$ with the BAK1 kinase domain) and to plant immunity (for example, the interaction of Pto with AvrPto and $AvrPtoB_{121–205}$, and the oligomerization of CC and TIR domains of NB-LRRs); and the identification of structural homology in effectors that was unrecognizable in the sequence (for example, helix bundle domains of oomycete effectors and AvrPtoB). The availability of these protein structures not only provides

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direct insights into molecular function but also enables new experiments to test functions that could not otherwise be envisioned. In cases in which structures do not provide a direct link to functions, it is crucial to investigate the biological context of protein activity. For example, knowledge of host targets may be essential to understand effector function based on structure. Finally, protein structures can provide templates for the design of novel activities, such as engineering PRR or NB-LRR proteins to have greater use in agriculture.

Despite the advances made, the impact of structural biology on plant–pathogen interactions is only just beginning to be felt. Looking forward, there are considerable challenges ahead, especially related to the increasing complexity of samples under study, as the greatest insights will come from the structures of multi-domain proteins or multi-protein complexes, some of which will be ligand-bound. For example, key challenges include: visualizing PRRs (in addition to *A. thaliana* CERK1) and NB-LRRs in pre-activation and active states, and defining a role for homo- and hetero-oligomerization; understanding how fungal, oomycete and additional bacterial effectors interact with plant proteins to promote effector-triggered susceptibility; and (re-)defining the route (or routes) of oomycete and fungal effector delivery into host cells, including developing a molecular picture of their interaction with cell surface components.

Overcoming these challenges will require the integration of genomics, bioinformatics, cell biology and biochemical approaches in both model plant species and crops. Large-scale screens for protein–protein interactions are building the foundations of protein interactomes in plant cells. Biochemical approaches that include structural biology are expected to substantiate the biological relevance of these interactions and to highlight protein interfaces and activities that are crucial for function. It is hoped that translation of these findings into crop species will have a meaningful effect on achieving more durable resistance to pathogens in the field. Exciting times must surely lie ahead.

Note added in proof

Since this Review was prepared, the structure of an NB-LRR protein from mouse NOD-like receptor family CARD domain-containing 4 (NLRC4) was determined in the absence of its N-terminal caspase activation and recruitment domain[160]. Readers are referred to this publication, as it may enhance our knowledge of how plant NB-LRRs function. Furthermore, readers are referred to a publication describing the crystal structure of the fungal apoplastic effector extracellular protein 6 (Ecp6)[161]. The structure of Ecp6 was crucial in defining how this effector sequesters chitin fragments to prevent perception by PRRs such as *A. thaliana* CERK1.

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