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Review

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Structural biology of plant defence

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Contents

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Summary

Plants employ the innate immune system to discriminate between self and invaders through two types of immune receptors, one on the plasma membrane and the other in the intracellular space. The immune receptors on the plasma membrane are pattern recognition receptors (PRRs) that can perceive pathogen-associated molecular patterns (PAMPs) or host-derived damage-associated molecular patterns (DAMPs) leading to pattern-triggered immunity (PTI). Particular pathogens are capable of overcoming PTI by secreting specific effectors into plant cells to perturb different components of PTI signalling through various mechanisms. Most of the immune receptors from the intracellular space are the nucleotide-binding leucine-rich repeat receptors (NLRs), which specifically recognize pathogen-secreted effectors to mediate effector-triggered immunity (ETI). In this review, we will summarize recent progress in structural studies of PRRs, NLRs, and effectors, and discuss how these studies shed light on ligand recognition and activation mechanisms of the two types of immune receptors and the diversified mechanisms used by effectors to manipulate plant immune signalling.

I. Introduction

Discriminating between self and non-self is paramount for the survival and fitness of all living organisms to survive and thrive. Animals and plants employ immune systems for self-defence against invading microbes and parasites. Unlike vertebrates that have an adaptive and an innate immune system that is active in the vasculature and designated body cells, plants exclusively rely on their innate immune system that is active in all cells of the organism. While resistance through an adaptive immune system is attainable during the lifespan of the organism, innate immunity is solely heritable. Plants encode immense numbers of such heritable hereditary immune receptor genes, whereas avirulence (AVR) genes (encoding effector proteins) evolved in pathogens to combat plant immunity.

Plants deploy immune receptors on the plasma membrane and intracellular space (Jones & Dangl, 2006; Dangl *et al.*, 2013) (Fig. 1). The front defence line at the plasma membrane is set up by pattern recognition receptors (PRRs) that can recognize pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs), leading to pattern-triggered immunity (PTI) (Bohm *et al.*, 2014; Couto & Zipfel, 2016). The

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pathogen effector proteins manipulate the plant immune response inside the cell in favour of pathogenic fitness. Effectors are functionally diversified as they need to intercept the complex plant immune system, which include branching signalling cascades, transcriptional reprogramming, reactive oxygen species (ROS), nitric oxide (NO) release, calcium influx, phytohormone signalling, and programmed cell death (termed hypersensitive response, HR). In order to detect effector proteins, the intracellular defence line is set up by the nucleotide-binding leucine-rich repeat receptors (NLRs) mediating effector-triggered immunity (ETI) (Jones & Dangl, 2006; Dangl *et al.*, 2013). During the past years, significant progress has been made in our understanding of PTI and ETI signalling and their regulation by pathogen effectors. In this review, we focus on protein structural elucidations of PRRs, NLRs and effectors and the implications of the structures for the mechanistic aspects of the plant innate immune system.

II. Guarding the front defence line – PRRs are the alarm system at the plasma membrane

PRRs are the front line of the immune surveillance on the cell surface. Receptor-like kinases (RLKs) and receptor-like proteins (RLPs) function as PRRs to perceive PAMPs or DAMPs, thereby activating the PTI immune response (Bohm *et al.*, 2014; Couto & Zipfel, 2016; W. L. Wan *et al.*, 2019; Zhou & Zhang, 2020) (Fig. 2). RLKs are composed of an extracellular domain (ECD) that is generally responsible for ligand perception, a single transmembrane region, and an intracellular kinase domain that is important for signal transduction (Han *et al.*, 2014). RLPs share a similar structural organization but lack an intracellular kinase domain. Therefore, RLPs are thought to inherently require a co-receptor kinase for signalling (Gust & Felix, 2014). The ECDs of RLKs vary drastically in size and architecture, enabling them to sense diverse ligands. Based on their ECDs, PRRs can be divided into the leucine-rich repeat (LRR)-, lysin motif (LysM)-, lectin-, wall-associated kinase (WAK) and other subfamilies (Couto & Zipfel, 2016).

Animal PRRs typically are Toll-like receptors (TLRs) that possess an extracellular LRR domain, a single transmembrane domain, and an intracellular Toll/interleukin 1 (IL-1) receptor (TIR) domain (Botos *et al.*, 2011). Ligand perception by extracellular domain of TLRs induces receptor dimerization and triggers intracellular immune signalling cascades (Kawai & Akira, 2010). Similarly, recognition of PAMPs or DAMPs by extracellular domain of plant PRRs induces receptor dimerization and leads to intracellular downstream immune signalling events, such as ROS

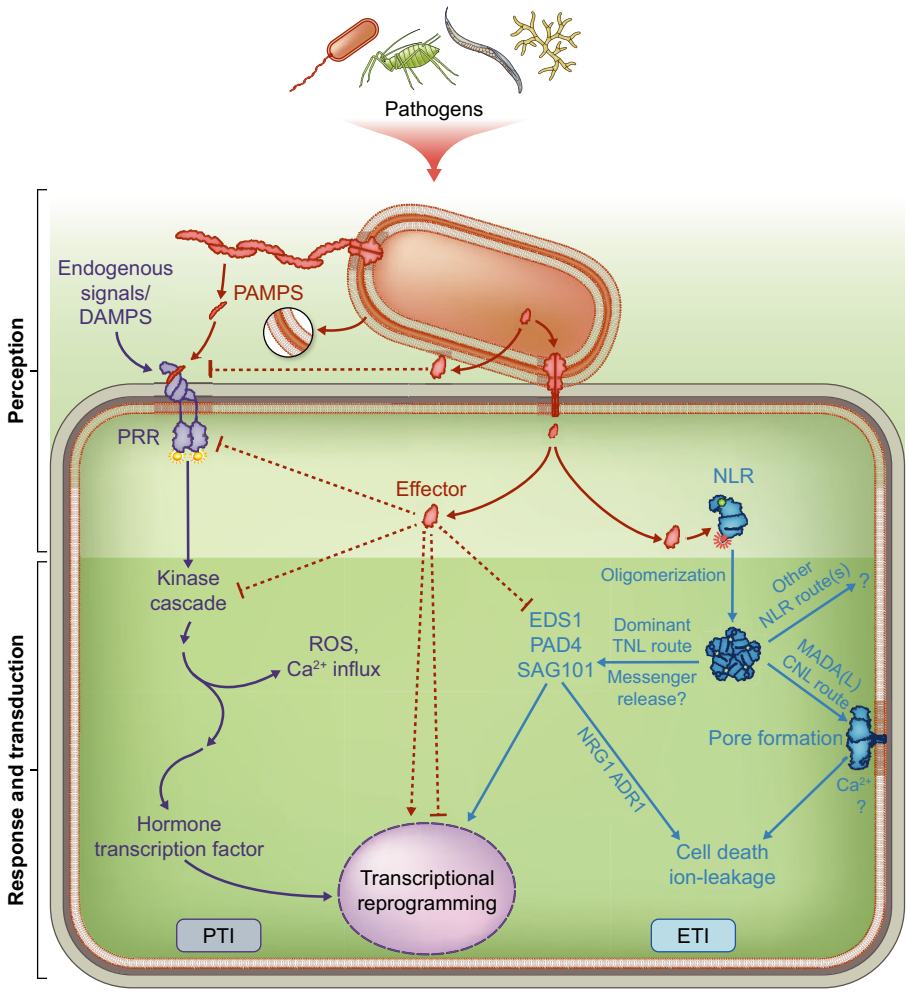

Fig. 1 Schematic diagram of the plant immune system. Plants deploy two tier immune receptors for their immunity. Plants perceive pathogen-associated molecular patterns (PAMPs) via cell surface-localized pattern recognition receptors (PRRs) and initiate pattern-triggered immunity (PTI) (left side). Pathogens deliver effector proteins into plants to manipulate PTI in favour of pathogenic fitness. The intracellular NLR receptors sense the effectors. Direct or indirect effector binding activates NLR oligomerization. NLR oligomerization can form resistosome triggering ion-leakage, EDS1 signalling, transcriptional reprogramming, resulting in NLR-dependent effector-triggered immunity (ETI) (right side).

production, calcium influx, extracellular alkalization, and defence gene expression (Boller & Felix, 2009; Zipfel, 2014; Zhou & Zhang, 2020).

1. Recognition of PAMPs by PRRs

Thus far, many PAMPs have been identified and the best characterized examples are flg22, EF-Tu, chitin, peptidoglycan, and lipopolysaccharides, which are recognized by FLS2 (Gomez-Gomez & Boller, 2000; Zipfel *et al.*, 2004), EFR (Zipfel *et al.*, 2006), CERK1 and LYK3/5 (Miya *et al.*, 2007; Wan *et al.*, 2008), LYM1/LYM3 (Willmann *et al.*, 2011), and LORE (Ranf *et al.*, 2015; Kutschera *et al.*, 2019), respectively (Fig. 2).

As a PAMP molecule, bacterial flagellin can be recognized by plant and animal immune receptors (Gomez-Gomez & Boller, 2000; Hayashi *et al.*, 2001; Zipfel *et al.*, 2006). Flagellin is the monomeric component of the bacterial flagellum. The D1 domain of flagellin is recognized by TLR5 in animals (Fig. 3a) (Donnelly & Steiner, 2002; Yoon *et al.*, 2012), whereas a conserved 22-amino acid fragment flg22 released by multi-step hydrolysis (Buscaill *et al.*, 2019) is recognized by the LRR-RLK FLS2 in Arabidopsis (Zipfel *et al.*, 2004; Sun *et al.*, 2013). In contrast to the planar solenoids of LRR-containing proteins seen in other species (Botos *et al.*, 2011), the structure of the LRR domain of FLS2 ($FLS2^{LRR}$) is superhelical (Fig. 3b) (Sun *et al.*, 2013). Flg22 adopts an extended conformation and binds to the inner surface of the FLS2 solenoid (Fig. 3b). Specific recognition of flg22 by $FLS2^{LRR}$ is through extensive hydrogen bonds and hydrophobic interactions. Flg22-binding induces no conformational change in $FLS2^{LRR}$ but creates a gluing site for interaction with the co-receptor BAK1, explaining why flg22 is required for FLS2 interaction with BAK1 (Sun *et al.*, 2013). In addition to the flg22-mediated interaction, $FLS2^{LRR}$ and $BAK1^{LRR}$ pack against each other through hydrophobic and Van der Waals contacts. These structures offer direct evidence for flg22 recognition by the ECD of FLS2 and demonstrate the sufficiency of ECDs for formation of the flg22-induced FLS2-BAK1 complex. In contrast to animal TLRs that recognize flagellin and induce homo-dimerization, the plant FLS2 receptor recognizes flagellin and heterodimerizes with the co-receptor BAK1. This heterodimeric activation mode is common in plant PRR signalling (Ma *et al.*, 2016; Song *et al.*, 2017).

Chitin is a polymer of *N*-acetylglucosamine (NAG) (Tang *et al.*, 2015) (Fig. 3c). The chitin oligosaccharides from the degraded fungal cell wall can be recognized as a PAMP by the RLK chitin elicitor receptor kinase 1 (CERK1) and lysin-motif receptor-like kinase 5 (LYK5) in Arabidopsis (Miya *et al.*, 2007), and by OsCERK1 and RLP OsCEBiP in rice (Kaku *et al.*, 2006). All these receptors have the LysM extracellular domain, which is a conserved carbohydrate-binding module found in all kingdoms of life (Buist *et al.*, 2008). The LysM forms a conserved β-α-α-β structure in which the two α-helices pack against the inner antiparallel dyadic β-sheet (Fig. 3d). The LysM domain of AtCERK1 features three LysMs (LysM1, LysM2 and LysM3), which form a trimeric structure through tight packing and interdomain disulphide bonds (Liu *et al.*, 2012). Despite the similarity of the three LysMs, only LysM2 of AtCERK1 binds the

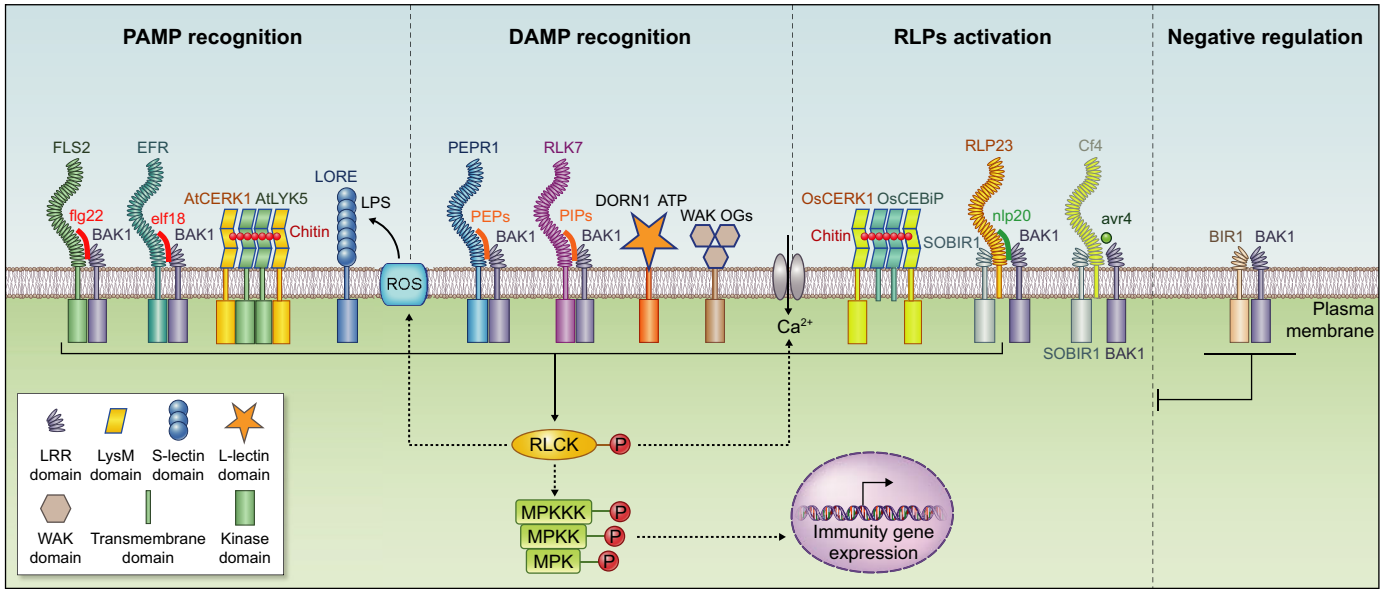

Fig. 2 Overview of signalling mediated by plant pattern recognition receptors (PRRs). PRRs on the cell surface perceive pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs), triggering immune response. PAMPs (like flg22, elf18, chitin and LPS) are perceived by PRRs (such as FLAGELLIN SENSING 2 (FLS2), ELOGATION FACTOR-TU RECEPTOR (EFR), CHITIN ELICITOR RECEPTOR KINASE1 (CERK1), LYSIN MOTIF RECEPTOR KINASE 5 (LYK5), LECTIN S-DOMAIN-1 RECEPTOR-LIKE KINASE (LORE)). DAMPs (such as Peps, PIPs, ATP and OGs) are perceived by PRRs (such as PEP RECEPTORS (PEPRs), RECEPTOR-LIKE KINASE 7 (RLK7), DOES NOT RESPOND TO NUCLEOTIDES 1 (DORN1), WALL-ASSOCIATED KINASES (WAKs)). Ligand-induced homo- or hetero-dimerization of extracellular domains of PRRs results in dimerization of their intracellular kinase domains and trans-phosphorylation, leading to downstream RLCKs and MPKs phosphorylation, production of reactive oxygen species (ROS), Ca^{2+} influx, and transcriptional reprogramming. As receptor-like proteins (RLPs) lack an intracellular kinase domain, regulatory or co-receptor kinases such as BRASSINOSTEROID INSENSITIVE 1-ASSOCIATED RECEPTOR KINASE 1 (BAK1) and SUPPRESSOR OF BIR1-1 (SOBIR1) are required for their activation.

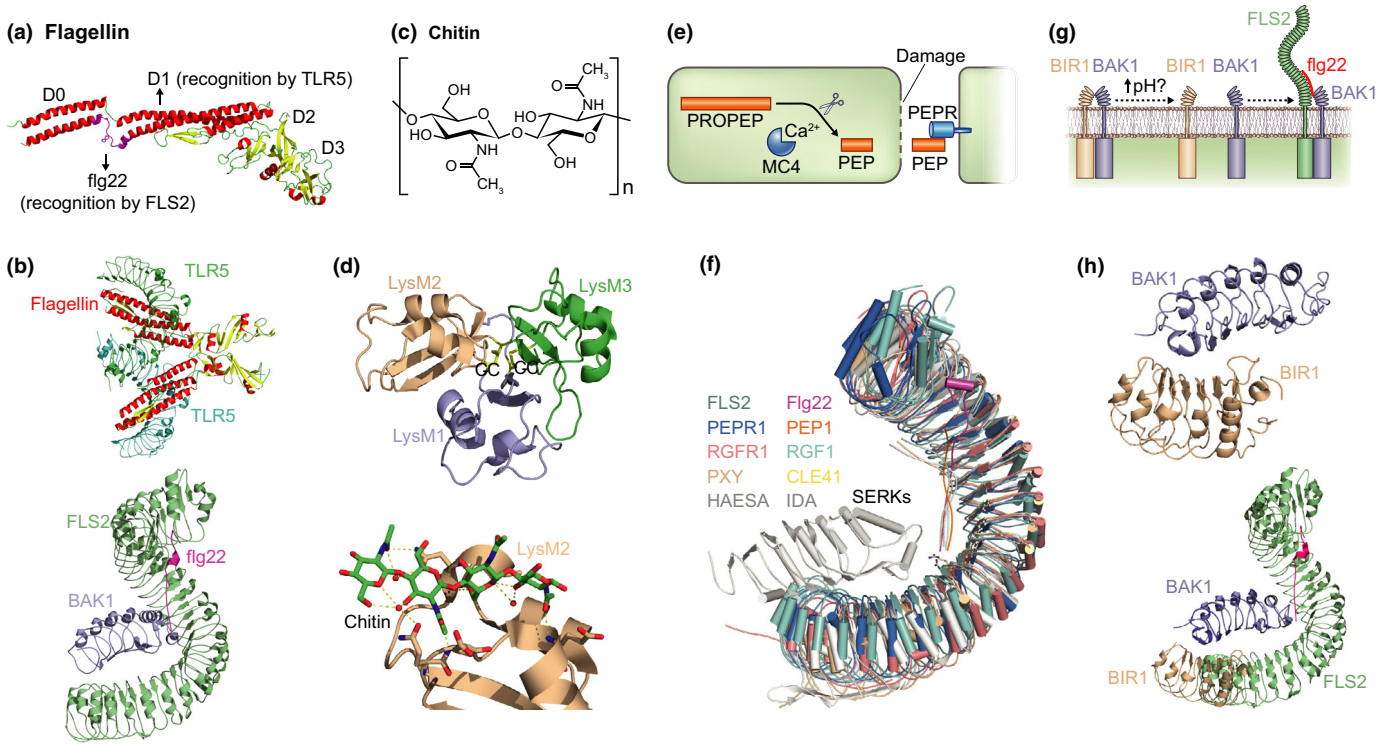

Fig. 3 Ligand recognition, receptor activation and negative regulation of pattern recognition receptors (PRRs) in plant. (a) Structure of flagellin. Different structural domains of flagellin are labelled. The D1 domain and the N-terminal loop region (flg22) recognized by TLR5 in animals and FLS2 in plants, respectively, are indicated. (b) (upper panel) Overall structure of TLR5LRR -flagellin complex; (lower panel) overall structure of the FLS2LRR -flg22-BAK1LRR complex (FLS2LRR in green, BAK1LRR in blue and flg22 in pink). (c) Chemical structure of chitin. (d) Overall structure of the extracellular domain of AtCERK1 (LysM1 in blue; LysM2 in brown; LysM3 in green and the disulfide bonds in yellow). Binding of the chitin pentamer NAG5 to LysM2 domain is highlighted below (NAG5 in green and LysM2 in brown). (e) Carton illustration of the maturation process of Pep peptides. (f) Structural alignment of flg22-FLS2LRR, PEP1-PEPR1LRR, RGF1-RGFR1LRR, CLE41-PXYLRR-SERK1LRR and IDA-HAESALRR). Colour codes for the structures are indicated. (g) Carton illustration of the molecular mechanism for BIR-mediated negative regulation of pattern-triggered immunity (PTI). (h) BIR1 sequesters BAK1 from FLS2. (upper panel) Overall structure of BIR1LRR-BAK1LRR (BAK1LRR in blue and BIR1LRR in brown); (lower panel) structural superposition of the BIR1LRR-BAK1LRR and FLS2LRR-flg22-BAK1LRR complexes.

chitin oligomer $(NAG)_5$ (Fig. 3d). This binding feature is also found in OsCEBiP (Liu *et al.*, 2016). The NAG units adopt an extended conformation closely matching the surface topology of the groove in the loop region of LysM2 and have a rotation along the chain. Specific recognition of the N-acetyl moieties in NAGs allows AtCERK1 to distinguish chitin from glucose. The mechanism of chitin recognition by AtCERK1 is highly consistent with OsCEBiP, revealing a conserved chitin perception by plant LysM-PRRs (Liu *et al.*, 2016). Biochemical data show that $(NAG)_8$ can induce AtCERK1 homo-dimerization, suggesting a ligand-induced homo-dimerization model on activation of AtCERK1 (Liu *et al.*, 2012). Interestingly, AtLYK5, which is required for chitin-induced AtCERK1 activation, is able to bind chitin with a higher affinity (Cao *et al.*, 2014). Thus, it is also possible that chitin binding to AtLYK5 leads to its interaction with AtCERK1 and activating AtCERK1 kinase activity (Cao *et al.*, 2014). Similarly, in rice chitin binding to RLP OsCEBiP induces the association of OsCEBiP with OsCERK1 and initiates the OsCERK1 kinase activity (Shimizu *et al.*, 2010). In addition, AtCERK1 engages with the LysM-type RLPs, LYM1 and LYM3, which enables perception of peptidoglycans (PGNs), triggering immunity to bacterial pathogens (Willmann *et al.*, 2011).

2. Recognition of DAMPs by PRRs

DAMPs are endogenous danger signals that can be recognized by PPRs triggering PTI (Boller & Felix, 2009; Choi & Klessig, 2016). Unlike PAMPs, DAMPs are host-derived molecular patterns that are released from pathogen-infected cells or wounded tissue (Rubartelli & Lotze, 2007). In plants, many endogenous molecules including peptide signals, nucleotides and oligogalacturonides can serve as DAMPs (Fig. 2). When plants are confronted with biotic or abiotic stress, polypeptide precursor proteins can be proteolytically processed and released to the extracellular space to act as DAMP signals. Examples for this type of DAMP include systemin (Pearce *et al.*, 1991); plant elicitor peptides (Peps), which is recognized by pep receptors (PEPRs) (Huffaker *et al.*, 2006; Yamaguchi *et al.*, 2006; Yamaguchi *et al.*, 2010); and PAMP-induced secreted peptides (PIPs), which is recognized by receptor-like kinase 7 (RLK7) (Hou *et al.*, 2014). Nucleotides can reach the extracellular space, serving as a DAMP signal. One such type of DAMP is the nucleotide ATP that is recognized by the RLK Does Not Respond to Nucleotides 1 (DORN1) (Choi *et al.*, 2014). Degradation of plant cell wall releases oligogalacturonide (OGs) DAMPs, which are recognized by WAKs (Kohorn *et al.*, 2009; Brutus *et al.*, 2010).

Peps are a critical DAMP signal in plants. Immune response and tissue damage induce ProPep maturation through Ca^{2+}-dependent metacaspase processing (Hander *et al.*, 2019) (Fig. 3e). Mature Peps are 23 amino acids long and are released from the intracellular to the extracellular space (Pearce *et al.*, 2008). The LRR-RLKs PEPR1/2 in Arabidopsis are receptors of Peps (Yamaguchi *et al.*, 2006; Yamaguchi *et al.*, 2010). Like $FLS2^{LRR}$, $PEPR1^{LRR}$ assumes a superhelical solenoid structure (Tang *et al.*, 2015) (Fig. 3f). The receptor-bound Pep1 adopts an extended conformation, interacting with the inner surface of $PEPR1^{LRR}$. The C-terminal 15 residues of Pep1 are well-defined in the complex structure, whereas the N-terminal eight residues are largely disordered, supporting an essential role of the C-terminal fragment in immune signalling. Consistently, modelling and biochemical data indicate that the C-terminal region of Pep1 mediates PEPR1 interaction with its co-receptor BAK1 (Tang *et al.*, 2015). Although Pep1 and flg22 are sequence-unrelated, recognition by their cognate receptors is considerably conserved. Notably, a similar recognition mechanism is also demonstrated in some development-related peptide signals such as CLE41, IDA and RGF1 (Santiago *et al.*, 2016; Song *et al.*, 2016, 2017; Zhang *et al.*, 2016), suggesting a conserved peptide recognition mechanism of LRR-RLKs (Fig 3f). Interestingly, the C-terminal side of these peptides have a conserved conformation and interact with the N-terminal loop region of the co-receptor BAK1 or other SOMATIC EMBRYOGENESIS RECEPTOR KINASES (SERKs), offering a possible explanation of how SERKs act as co-receptors in multiple signalling pathways. Furthermore, structural comparison revealed that two arginine residues (termed RxR motif, x represents any amino acid) are conserved in a subfamily of LRR-RLKs and interact with the C-terminal free carboxyl group of Pep1, CLE41 and IDA. By mixing small peptides with purified RxR-containing receptors, followed by size-exclusion chromatography and mass spectrometry identification, the RxR motif led to identification of RGF peptide receptors (Song *et al.*, 2016).

PAMPs and DAMPs recognition by PRRs is crucial for plant innate immunity. Deciphering the molecular mechanism of specific recognition provides a better understanding of plant immune strategies and also offers possibilities to improve the plant immune system. Thus far, only the mechanisms of peptide and chitin-like molecule recognition by PRRs are revealed. It is still unclear how the other PAMPs or DAMPs, such as lipopolysaccharides and nucleotides, are specifically recognized by PRRs. More structural studies for specific recognition of PAMPs and DAMPs by plant PRRs are needed in the future.

3. Activation of PRRs by co-receptor kinases

PRRs depend on their kinase domain for signal transduction (Oh *et al.*, 2011; Han *et al.*, 2014). Dimerization is likely the common activation mode of single transmembrane receptors as demonstrated for receptor tyrosine kinases in animals and further confirmed for receptor-like kinases in plants (Lemmon & Schlessinger, 2010; Han *et al.*, 2014). Ligand-induced homo- or hetero-dimerization of extracellular domains results in dimerization of the intracellular kinase domains of the receptors, leading to their trans-phosphorylation. In contrast with ligand-induced homo-dimerization of receptor tyrosine kinases and TLRs in animals, most of the well-characterized LRR-RLKs are activated through hetero-dimerization with the SERK family members as a co-receptor (Ma *et al.*, 2016) (Fig. 2). As RLP proteins lack an intracellular kinase domain, regulatory or co-receptor kinases are required for their activation (Fig. 2). For example, the LysM-RLP OsCEBiP was suggested to be activated by the LysM-RLK OsCERK1 (Shimizu *et al.*, 2010; Hayafune *et al.*, 2014). Multiple LRR-RLPs have been shown to be associated with the LRR-RLK SUPPRESSOR OF BIR1-1 (SOBIR1), forming a bimolecular equivalent of a genuine RLK (Gust & Felix, 2014; Liebrand *et al.*, 2014). Ligand binding to an RLP–SOBIR1 complex further recruits SERK co-receptors for activation, as shown for many RLPs such as RLP23 in Arabidopsis (Albert *et al.*, 2015) and Cf-4 in tomato (Postma *et al.*, 2016). Hetero-dimerization between two RLKs or an RLK and an RLP is mainly mediated by homotypic ECD interaction, though the reason for this remains unclear. RLPs as membrane receptors not only sense PAMPs but also recognize effectors to trigger immunity (Jamieson *et al.*, 2018). Unfortunately, structural information of ligand recognition by an RLP and activation of the RLP-SOBIR1 complexes is unavailable.

4. Negative regulation of PRR signalling

Excessive activation of immune responses is damaging to hosts. Plants therefore have evolved many strategies to maintain immune homeostasis (Trujillo & Shirasu, 2010; Couto & Zipfel, 2016). Here, we will discuss recent structural studies showing how PRR signalling is negatively regulated (Ma *et al.*, 2017; Hohmann *et al.*, 2018). Arabidopsis BAK1-INTERACTING RECEPTOR-LIKE KINASE 1 (BIR1) is an LRR-RLK that was initially identified as a negative regulator of cell death. Loss-of-function of BIR1 results in activation of cell death and immune responses (Gao *et al.*, 2009). There are four BIRs (BIR1–BIR4) in Arabidopsis and they all interact with BAK1 (Halter *et al.*, 2014a). Both BIR2 and BIR3 were shown to have a critical role in negatively regulating flg22-induced responses by controlling BAK1-FLS2 complex formation in a ligand-dependent manner (Halter *et al.*, 2014b) (Fig. 3g). Biochemical and structural data indicated that the extracellular LRR domains are sufficient for the interaction between BIR1 and BAK1 (Ma *et al.*, 2017). The crystal structure of $BIR1^{LRR}$-$BAK1^{LRR}$ shows that BIR1 and BAK1 form a 1 : 1 heterodimer (Ma *et al.*, 2017) (Fig. 3h). The $BIR1^{LRR}$-$BAK1^{LRR}$ interaction is mediated by both polar and hydrophobic contacts. A lateral side of $BIR1^{LRR}$ packs against the C-terminal inner surface and the C-terminal capping domain of $BAK1^{LRR}$. The N-terminal loop region of $BIR1^{LRR}$ makes extensive contacts with $BAK1^{LRR}$. W71 in the N-terminal loop region of BIR1 forms extensive hydrophobic interactions with BAK1. Mutation of this residue disrupted the $BIR1^{LRR}$-$BAK1^{LRR}$ interaction *in vitro*, whereas BIR1 mutant W71A transgenic plants displayed a seeding lethality, phenocopying the *bir1-1* mutant (Ma *et al.*, 2017). These data indicate that the $BIR1^{LRR}$-$BAK1^{LRR}$ interaction is important for the negative regulation of immunity, suggesting that signals relieving BIR1-mediated inhibition of plant immunity may exist in the apoplast.

Structural comparison between BIR1^{LRR}-BAK1^{LRR} and FLS2^{LRR}-flg22-BAK1^{LRR} shows that the C-terminal portion of FLS2^{LRR}, which interacts with BAK1^{LRR}, completely overlaps with BIR1^{LRR}. This structural observation suggests that BIR1 sequesters BAK1 from FLS2 and negatively impacts flg22-induced immune responses (Fig. 3h). Interestingly, *in vitro* data showed that formation of the BIR1^{LRR}-BAK1^{LRR} complex is pH-dependent with lower pH promoting and higher pH impairing the interaction of the two proteins (Fig. 3g). This suggests that pH might have a role in regulating the BIR1-sequestered BAK1 and consequently affecting PRR-mediated immunity (Ma *et al.*, 2017).

III. Guarding the inner defence line – NLRs detect and defend from inside the cell

1. Domain structural organization of NLRs

The second major class of immune receptors encodes proteins belonging to the NLR family. Unlike PRRs that expose their ligand-recognizing domain into the apoplast, NLR proteins typically perceive pathogen invasion in the cytoplasm (Dangl *et al.*, 2013). Plant NLR proteins have a three-partite domain structure, harbouring a C-terminal LRR domain, a middle nucleotide-binding oligomerization domain (NOD) and either an N-terminal coiled-coil (CC) domain or a TIR homology domain. Many animal NLRs, such as the apoptosome-forming Apaf-1 and the inflammasome-forming NAIP2/NLRC4 share this three-partite domain organization. However, there is a certain degree of variability, for instance, when the LRR domain is replaced by a twin WD40 domain (Apaf-1) or the N-terminal domain by a triple BIR domain (NAIP2) or a CARD domain (Apaf-1 and NLRC4). Also in plants there exists a remarkable degree of variation in the NLR domain-composition of NLR proteins (Jacob *et al.*, 2013). For example, some NLRs harbour additional integrated domains (ID), while others lack the LRR domain or both the LRR and NOD domain (TN and TX, respectively). The TIR or CC domain can also be replaced by other domains in NLRs of bryophytes. Many NLRs oligomerize upon activation but vary in the subunit count. For instance, the animal NAIP2/NLRC4 inflammasome forms an undecamer (stoichiometry 1 : 10 between NAIP2 and NLRC4) (Zhang *et al.*, 2015); the plant ZAR1 resistosome forms a pentamer (Wang *et al.*, 2019a); the animal Apaf-1 apoptosome forms a heptamer (Zhou *et al.*, 2015); and the animal Dark/CED4 apoptosome form octamers (Qi *et al.*, 2010; Cheng *et al.*, 2017).

2. The LRR domain – effector binding and stabilization of the NLR resting state

Since the LRR domain is the most polymorphic part of plant NLR proteins it is very adaptable to evolving novel binding capacity required in effector recognition. The high polymorphism likely enables the LRR domain to acquire novel binding specificities, making it a key feature of the animal and plant innate immune system, and even of the adaptive immune system of jawless vertebrates (Han *et al.*, 2008; Boehm *et al.*, 2012). As observed in other LRR-containing proteins, the LRR domain of NLRs such as Arabidopsis ZAR1 and animal NLRC4 form a curved solenoid consisting of a variable number of parallel β-sheets (Hu *et al.*, 2013; Wang *et al.*, 2019b). The LRR domain of plant NLRs has been implicated in direct and indirect binding of pathogen-derived effectors. Many studies showed that the LRR domain is involved in binding to a host protein (guardee) and that modifications of the guardee by pathogen effectors lead to NLR activation (guard model) (Jones & Dangl, 2006). Structural evidence for indirect effector recognition comes from the study of ZAR1, showing that the LRR domain mediates ZAR1 recognition of effector AvrAC from *Xanthomonas campestris* pv *armoraciae* through the host proteins RKS1 and PBL2 (Wang *et al.*, 2019b). Some studies support direct interaction between the LRR domain of plant NLRs and an effector (Dodds *et al.*, 2006; J. Chen *et al.*, 2017; Saur *et al.*, 2019) but biochemical demonstration of this has proven more difficult. Therefore, direct binding of LRRs to their cognate effectors was speculated to be highly transient and therefore hard to capture *in vitro* (Saur *et al.*, 2019). Aside from ligand perception, the LRR domain of plant NLRs plays an important part in maintaining their resting state by sequestering them in a monomeric state as demonstrated in Arabidopsis ZAR1 (Wang *et al.*, 2019b) and mouse NLRC4 (Hu *et al.*, 2013). This function might not be conserved, because autoinhibition of rabbit NOD2 for example might not rely on the LRR domain (Maekawa *et al.*, 2016).

3. Janus-faced with prion-like character – the NOD module switches between two striking conformations

The NOD module is the signature domain that classifies NLRs as AAA+ ATPases of the signal transduction ATPases within numerous domains (STAND) protein family (Iyer *et al.*, 2004; Leipe *et al.*, 2004). The NOD module can be further divided into the nucleotide-binding domain (NBD), helical domain 1 (HD1) and winged helical domain (WHD) (Fig. 4a). The NOD module binds ADP/dADP in its monomeric inactive state and ATP/dATP in the oligomeric active state (Maekawa *et al.*, 2016; Wang *et al.*, 2019a,b). Mutations in these conserved regions are generally categorized as auto-activating or inactivating based on whether they stabilize ATP-binding or entirely inhibit nucleotide-binding (Tameling *et al.*, 2006; van Ooijen *et al.*, 2008; Williams *et al.*, 2011; Sukarta *et al.*, 2016; Tran *et al.*, 2017). Ligand perception promotes exchange of ADP/dADP with ATP/dATP and thereby activation of NLRs. NLRs are therefore believed to function as a nucleotide switch. Despite being AAA+ ATPases, it remains debatable whether NLRs have ATPase activity in their fully activated state (Tameling *et al.*, 2002; Reubold *et al.*, 2009; Williams *et al.*, 2011). NLRs harbour the catalytic elements of an ATPase, but they differ structurally from a canonical AAA+ ATPase, as their catalytic components are formed by an individual monomer rather than by two neighbouring units of the oligomer seen in canonical AAA+ ATPases (Wendler *et al.*, 2012; Tafoya *et al.*, 2018).

In addition to nucleotide-binding, the NOD module is primarily responsible for oligomerization of an NLR protein in response to ligand perception. As indicated by a number of structures, asymmetric packing of this module results in the formation of a wheel-like structure with a varying number of

protomers (Qi *et al.*, 2010; Hu *et al.*, 2015; Zhang *et al.*, 2015; Zhou *et al.*, 2015; Cheng *et al.*, 2017; Wang *et al.*, 2019a). These studies further show that all three subdomains of the NOD are involved in NLR oligomerization.

4. To kill or not to kill? – α helix 1 of CC domains can work like a jack knife

CC-NLRs (CNLs) are the dominant class of NLRs in monocots but they also exist in most phyla ranging from eudicots to bryophytes (Jacob *et al.*, 2013). The CC domain of CNLs forms a four α-helix bundle in its inactive state (Hao *et al.*, 2013; Casey *et al.*, 2016; Wang *et al.*, 2019b). Fold-plasticity of the CC domain is triggered by its activation and α-helix 1 flips out in a jack-knife-like fashion (Wang *et al.*, 2019a,b) (Fig. 4b). This mechanistic model is mainly based on the structural model of inactive and activated ZAR1 and it could even be argued that the crystal structures of Sr33 and Rx, but also of MLA10 are reminiscent of this inactive and active conformation, respectively. ZAR1 carries a MADA motif, which together with the related MADA-like motif defines a CNL subclass that represents ~20% of all CNLs. The CC domain of MLA10, Sr33, Sr50 (MADA-like) and ZAR1 (MADA)

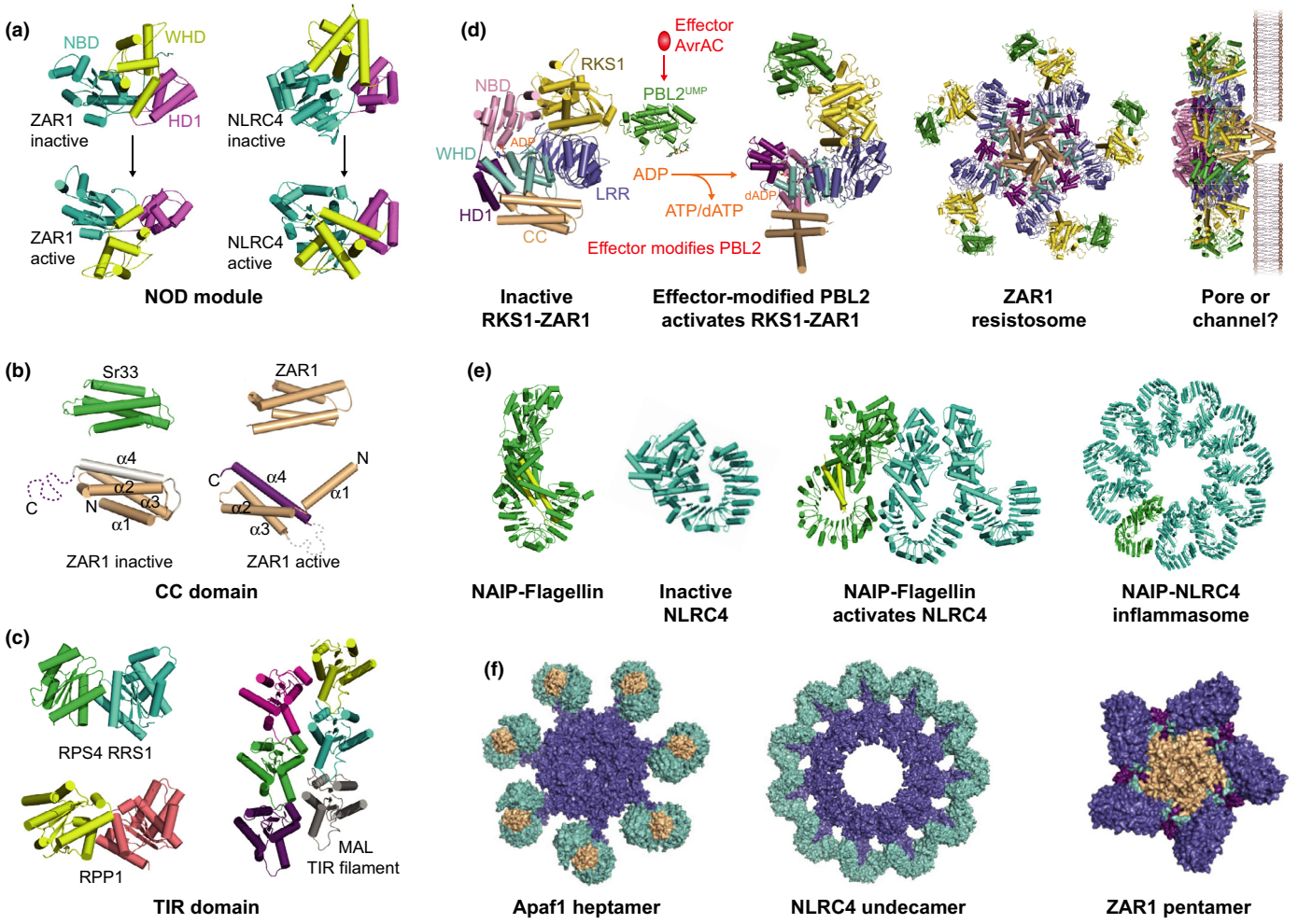

Fig. 4 Structural mechanisms of plant nucleotide-binding leucine-rich repeat receptor (NLR) activation. (a) Nucleotide-binding oligomerization domain (NOD) module conformational change in ZAR1 (left) and NLRC4 (right) upon activation. NOD consists of NBD (cyan), WHD (yellow) and HD1 (magenta). During activation, WHD shows a dramatic flipping in relation to NBD and HD1. (b) (upper panel) CC domain of inactive of Sr33 (green) and ZAR1 (brown) have striking structural similarity. (lower panel) Fold-plasticity of ZAR1 CC domain during activation. α-helix 1 flips out and contributes to funnel-formation in oligomer. During activation, α-helix 4 incorporates additional C-terminal amino acids that were formerly disordered. (c) TIR domains of plant RPS4, RRS1, RPP1 (left), and animal MAL (right). RPS4-RRS1 TIR forms heterodimers in the crystal (green and cyan). Homodimer of RPP1 TIR (yellow and red) and shares interaction interface with RPS4-RRS1 heterodimer. MAL TIR filament highlighting shared interface with RPS4-RRS1 and RPP1-RPP1 (magenta-green). In the filament new interfaces are found (magenta-yellow, green-purple). (d) Molecular mechanism of indirect effector-trigged ZAR1 activation. Different domains are indicated with different colours. RKS1 (yellow) and PBL2-UMP (green) are plant proteins that mediate ZAR1 activation. Going through a transitional complex of ZAR1, RKS1, and PBL2-UMP, five protomers form a wheel-like resistosome. The five α-helices 1 form a funnel-shaped feature that might insert into the membrane forming a pore. (e) Molecular mechanism of flagellin-trigged NAIP-NLRC4 activation. Flagellin (yellow) binds to NAIP5 (green) and induces oligomerization of 10 NLRC4 (cyan) subunits. NAIP5-flagellin functions to induce a nucleated polymerization of NLRC4, a mechanism that might be mirrored by paired plant NLRs. (f) NLR oligomers have different subunit count. Shown are heptameric Apaf1 apoptosome (left), undecameric NLRC4-NAIP inflammasome (middle), and pentameric ZAR1 resistosome (right). Structural model of NLRC4 ignoring NAIP and assuming perfect radial symmetry. All three oligomers also share the continued interaction with their activation-inducing agents, CytC (apoptosome), flagellin (inflammasome), and modified host RKS1-PBL2^{UMP} (resistosome).

(Maekawa *et al.*, 2011; Casey *et al.*, 2016; Wang *et al.*, 2019a) and even a truncated α-helix 1 of NRC4 (MADA) are able to induce cell death (Adachi *et al.*, 2019), indicating that some CC domains are autonomous signalling modules of CNLs. This further shows that the MADA-like motif is a functional equivalent of the MADA motif in inducing cell death when exchanged between full-length CNLs (Adachi *et al.*, 2019). In the ZAR1 pentameric structure, the exposed N-terminal α-helices 1 feature a funnel-shaped structure that has been suggested to form a pore in the plasma membrane (Wang *et al.*, 2019a). Indeed, pore-forming activity has been shown for the CC domains of NLR unrelated proteins including the HeLo domain of fungal Het-S (Seuring *et al.*, 2012) and animal MLKLs (Su *et al.*, 2014). However, whether the CC domain cell death function follows the same mechanism in the remaining 80% of non-MADA CNLs awaits further investigation.

Alongside initiating downstream signalling, the CC domains of RPS5, Rx and LOV1 are also physically involved in indirect effector recognition (Ade *et al.*, 2007; Sacco *et al.*, 2007; Lorang *et al.*, 2012). Whether such guardee-binding stabilizes the inactive conformation of these CNLs, as was shown for RPS5 (Qi *et al.*, 2012) and the exact conformational changes that occur at the CC-guardee module during activation, needs to be addressed in future.

5. To kill or not to kill? – the TIR domain deadly mechanism remains elusive

Like the CC domain, expression of truncated TIR in plants leads to cell death possibly through a different mechanism (Swiderski *et al.*, 2009; Bernoux *et al.*, 2011; Nishimura *et al.*, 2017). The TIR domain has a flavodoxin-like fold and consists of a central five-stranded parallel β-sheet (Nanson *et al.*, 2019), as shown by single plant TIR structures of TNLs (see Table 1; Fig. 4c). Two recent studies showed that the TIR domain of TNLs possesses NADase activity, which is required for TNL-mediated cell death and immune responses (Horsefield *et al.*, 2019; L. Wan *et al.*, 2019). NADase activity is only displayed at high protein concentration, which argues in favour of oligomerization as an activator of catalytic activity (Horsefield *et al.*, 2019). The animal TIR domain protein from MAL was found to oligomerize into filament–like structures (Ve *et al.*, 2017) (Fig. 4c). Interestingly, MAL TIR domains can also form cofilaments with TIR domains of other proteins like TLR4 and MyD88 (Ve *et al.*, 2017). Filament formation is a common theme in the innate immune system of animals and can be found among non-NLR TIR-containing proteins (Nanson *et al.*, 2019). However, there has been no evidence for filament-forming activity of the TIR domain of TNLs. Nonetheless, structural studies suggest that homo-dimerization (Bernoux *et al.*, 2011) or even hetero-dimerization (Williams *et al.*, 2014) mediated by TIR domains is important for TNL activation. The dimerization interfaces vary in the solved structures, suggesting that different interfaces may exist in oligomeric TNLs.

Like CC domains, also TIR domains might be physically involved in pathogen recognition (Burch-Smith & Dinesh-Kumar, 2007). It will be interesting to investigate whether and how TIR domain oligomerization and NADase activity can be maintained after activation despite partial occupancy of such TIR domains by (indirect) effector binding.

6. Fused with their guard – atypical NLR proteins have integrated additional domains

Besides the canonical three-partite domain organization, some plant NLRs harbour additional or alternative domains. NLRs carrying additional domains are generally called ID-NLRs, in which the ID typically follows the LRR domain but can also occur at the very N-terminus or before the NOD. According to the integrated decoy model, IDs present another method of effector recognition (Grund *et al.*, 2019). However, indirect effector binding is also possible, where an ID binds to a host guardee (Mucyn *et al.*, 2006). Direct binding of an ID to an effector is supported by a crystal structure of the HMA domain of Pikm-1 in complex with AVR-PikA (Maqbool *et al.*, 2015; De la Concepcion *et al.*, 2018). Thus, most IDs confer sensor capacity to an NLR and can have diverse identity including HMA, protein kinase and WRKY (Le Roux *et al.*, 2015; Sarris *et al.*, 2016). Interestingly, many CC-type ID-NLRs lost the cell death inducing MADA motif in their CC domains, suggesting that they may need other partners for cell death (Adachi *et al.*, 2019). Indeed, these NLRs work in pairs with an ID-less executor NLR containing a conserved MADA/MADA-like motif (Adachi *et al.*, 2019). Alongside 'monogamous' NLR pairing, 'polygamous' pairing also exists, here diverged sensor NLRs converge on a single executor NLR (e.g. NRC4) (Adachi *et al.*, 2019). Current models on the activation of paired NLRs hypothesize that effector-induced conformational change in the sensor NLR is perceived by the genetically and physically linked executor NLR, forming a signalling-competent oligomer of the two NLRs (Cesari *et al.*, 2013, 2014; Huh *et al.*, 2017)

Another class of atypical NLRs carry a CC domain homologous to RPW8/HR family and are called CC_R-NLRs (Barragan *et al.*, 2019). NRG1s and ADR1-Ls are the most comprehensively researched CC_R-NLRs (Collier *et al.*, 2011). Although NRG1s and ADR1-Ls lack a MADA/MADA-like motif, overexpression of their CC domains is sufficient to induce cell death in tobacco (Moffett *et al.*, 2002; Adachi *et al.*, 2019).

7. Truncated NLRs – lack LRR domains or LRR-NOD modules

There is another class of atypical TIR-NLRs that lack either the LRR domain (TN) or both the NOD and LRR domain (TX). TN and TX proteins are widely present in plants, accounting for more than 25% of the NLRs in Arabidopsis (Meyers *et al.*, 2002, 2003). TN and TX may have evolved independently from canonical TNLs (Meyers *et al.*, 2002, 2003; Nandety *et al.*, 2013). Nonetheless, the structure of AtTIR1 is strikingly similar to those of TIR domains of TNLs (Chan *et al.*, 2010). Furthermore, like the TIR domain of TNLs, overexpression of TX proteins in plants induces cell death and activates defence responses (Nandety *et al.*, 2013), suggesting that TX and TNL signalling may be convergent for induction of plant immunity. TNs and TXs were proposed to function as

Table 1 PDB codes.

Protein	Species	PDB code	Method	Comment
NLR full length				
ZAR1, RKS1, PBL2	Thale cress	6J5T (Wang *et al*., 2019a)	CryoEM	First plant NLR resistosome structure
ZAR1, RKS1, PBL2	Thale cress	6J5U, 6J5W (Wang *et al*., 2019b)	Cryo EM	Plant NLR in inhibition state
NLR single domains				
NRC1 NOD domain	Tomato	6S2P (Steele *et al*., 2019)	X-ray crystallography	Only plant NOD domain structure besides ZAR1 NOD
Sr33 CC domain	Wheat	2NCG (Casey *et al*., 2016)	X-ray crystallography	Helix bundles of CC domains in inactive conformations
MLA10 CC domain	Barley	5T1Y, 3QFL (Maekawa *et al*., 2011)	X-ray crystallography	
Rx0 CC domain	Potato	4M70 (Hao *et al*., 2013)	X-ray crystallography	
L6-TIR	Flax	3OZI (Bernoux *et al*., 2011)	X-ray crystallography	Suggest two interfaces for TIR-TIR interaction
RPS4-TIR	Thale cress	4C6R (Williams *et al*., 2014)	X-ray crystallography	NLR TIR structure
RRS1-TIR	Thale cress	4C6S (Williams *et al*., 2014)	X-ray crystallography	
RPP1-TIR	Thale cress	5TEB (Zhang *et al*., 2017)	X-ray crystallography	
TIR domain of SNC1	Thale cress	5TEC (Zhang *et al*., 2017)	X-ray crystallography	
TIR domain of RPV1	Grapevine	5KU7 (Williams *et al*., 2016)	X-ray crystallography	
AtTIR TN10	Thale cress	3JRN (Chan *et al*., 2010)	X-ray crystallography	
RUN1-TIR in complex with NADP	Grapevine	6O0W (Horsefield *et al*., 2019)	X-ray crystallography	Structural evidence for TIR as enzyme binding substrate
RPS4-TIR and RRS1-TIR	Thale cress	4C6T (Williams *et al*., 2014)	X-ray crystallography	Functional TIR dimer
Heavy metal domain of Pikp1	Rice	5A6P (Maqbool *et al*., 2015)	X-ray crystallography	NLR ID domain
RGA5A-HMA	Rice	5ZNE (Guo *et al*., 2018)	X-ray crystallography	
AVR-PikD and HMA of Pikp1	Rice	6FU9 (De la Concepcion *et al*., 2018)	X-ray crystallography	Direct interaction of effector and NLR ID domain
AVR-PikE and HMA of Pikp1	Rice	6FUB (De la Concepcion *et al*., 2018)	X-ray crystallography	
AVR-PikA and HMA of Pikp1	Rice	6FUD (De la Concepcion *et al*., 2018)	X-ray crystallography	
Animal structural models for NLRs				
NLRC4	Mouse	4KXF (Hu *et al*., 2013)	X-ray crystallography	Autoinhibition mechanism of NLR proteins
NAIP5-Flagellin	Mouse	5YUD (Yang *et al*., 2018)	X-ray crystallography	Ligand recognition by NLR proteins
NLRC4-NAIP2	Mouse	3JBL (Zhang *et al*., 2015)	Cryo EM	Heteromeric oligomer of NLR proteins
NLRC4-NAIP5 inflammasome	Mouse	6B5B (Tenthorey *et al*., 2017)	Cryo EM	Heteromeric oligomer of NLR proteins
NLRC4-NAIP5	Mouse	5AJ2 (Diebolder *et al*., 2015)	Cryo EM	Helical arrangement of an NLR polymer
NOD2	Rabbit	5IRM, 5IRN (Maekawa *et al*., 2016)	X-ray crystallography	Autoinhibition mechanism of NLR proteins
ASC inflammasomes	Human	3J63 (Lu *et al*., 2014)	Cryo EM	Immune adaptors forming a filament structure
ASC-CARD filament	Human	6N1H (Li *et al*., 2018)	Cryo EM	
Mal filament	Human	5UZB (Ve *et al*., 2017)	Cryo EM	TIR domains forming a filament structure
PRRs				
FLS2-flg22-BAK1	Thale cress	4MN8 (Sun *et al*., 2013)	X-ray crystallography	PAMP-induced heterdimeric PRR complex
CERK1-Chitin	Thale cress	4EBY, 4EBZ (Liu *et al*., 2012)	X-ray crystallography	First structural model for PAMP recognition by plant PRRs
OsCEBiP-Chitin	Rice	5JCD (Liu *et al*., 2016)	X-ray crystallography	Conserved chitin recognition mechanism of LysM-PRR

Table 1 (Continued)

Protein	Species	PDB code	Method	Comment
PEPR1-pep1	Thale cress	5GR8 (Tang *et al*., 2015)	X-ray crystallography	First structural model for DAMP recognition by plant PRRs
TLR5-flagellin	Salmonella	3V47 (Yoon *et al*., 2012)	X-ray crystallography	Flagellin recognition by animal PRR
Effectors				
AvrPtoB-Bak1	*Pseudomonas syringae* pv tomato	3TL8 (Cheng *et al*., 2011)	X-ray crystallography	Multi-functional effector that inhibits kinase domains of plant PRRs
AvrPtoB-Pto	*Pseudomonas syringae* pv tomato	3HGK (Dong *et al*., 2009)	X-ray crystallography	
AvrPtoB	*Pseudomonas syringae* pv tomato	3HGL, 2FD4 (Janjusevic *et al*., 2006; Dong *et al*., 2009)	X-ray crystallography	
Ecp6-chitin	*Passalora fulva*	4B8V, 4B9H (Sanchez-Vallet *et al*., 2013)	X-ray crystallography	Effectors bound to chitin
CfAvr4	*Cladosporium fulvum*	6BN0 (Hurlburt *et al*., 2018)	X-ray crystallography	
GlcN-NLPPya	*Phytium aphanidermatum*	5NNW, 5NO9 (Lenarcic *et al*., 2017)	X-ray crystallography	Possibly pore-forming effector bound to PM-localized GlcN
PthXo1-dsDNA	*Xanthomonas oryzae* pv *oryzae*	3UGM (Mak *et al*., 2012)	X-ray crystallography	TALEN-effector bound to DNA
dHax3-dsDNA	*Xanthomonas campestris* pv *armoraciae*	3V6T, 4GJR (Deng *et al*., 2012)	X-ray crystallography	TALEN-effector bound to DNA

PDB, protein data bank; PRRs, pattern recognition receptor; NLR, nucleotide-binding leucine-rich repeat receptor.

adaptor proteins (Meyers *et al*., 2002), but evidence for this is still lacking. Much less is known about the upstream and downstream signalling mediated by TXs and TNs. For example, the TX protein RBA1 likely oligomerizes and can cause cell death in response to the effector HopBA1, but direct binding of RBA1 to HopBA1 could not be demonstrated (Nishimura *et al*., 2017). Also truncated forms of CNLs exist as exemplified by Arabidopsis RPW8 that might be able to directly induce cell death (Li *et al*., 2020).

8. NLR-mediated immunity

NLRs in slumber – an intracellular minefield for effectors Plant NLRs differ greatly in their subcellular localization. Specific NLRs localize in the cytoplasm or the nucleus, or they attach to guarded host proteins (which themselves have a specific localization), to the plasma membrane or to membranes of the endomembrane system. For example, the tomato CNL Tm-2^2, the Arabidopsis CNLs RPS5, RPS2 and RPM1 all localize as peripheral proteins to the plasma membrane (T. Chen *et al*., 2017; Noman *et al*., 2019). While RPS2 and RPS5 carry N-terminal palmitoylation and acylation signals, respectively (Qi *et al*., 2012), RPM1 is held at the membrane by its prenylated or palmitoylated guardee RIN4 (Kim *et al*., 2005; Gao *et al*., 2011). Not only in CNLs, but also in TNLs membrane localization often seems to be encoded in the most extreme N-terminus. For example, GFP fusions of the first 30 amino acids of L6 and M are sufficient for targeting the Golgi apparatus membrane and the tonoplast, respectively (Takemoto *et al*., 2012). Other NLRs like MLA10 or RPS4 are imported into the nucleus as well as into the cytoplasm and their activation leads to separable downstream effects (Heidrich *et al*., 2011; Bai *et al*., 2012). For example, activation of nuclear RPS4 leads to transcriptional reprogramming through EDS1, while activation of the cytoplasmic RPS4/RRS1-paired NLR complex leads to cell death (Heidrich *et al*., 2011; Huh *et al*., 2017). ZAR1 and the potato CNL R3a are in the cytoplasm before activation and change localization upon activation to the plasma membrane or endosomes, respectively (Engelhardt *et al*., 2012; Wang *et al*., 2019a). How activated NLRs are delivered to new locations in the cell and the function they execute there will be exciting topics of future structural and functional studies.

NLRs waking up – conformational changes and oligomerization A striking similarity between NLRs is that they switch from an ADP-bound 'off' state to an oligomerized ATP-bound 'on' state. Structural studies of ZAR1 provided insight into the switching mechanism of NLRs (Fig. 4d). Binding of the AvrAC-uridylated PBL2 stabilizes a loop region of RKS1, which in turn allosterically induces a conformational change in the NBD of ZAR1, ADP release and subsequent priming of ZAR1. Owing to much higher concentrations of ATP than ADP in cells (Chandra *et al*., 2006), the primed ZAR1 preferentially binds ATP, triggering further conformational changes in WHD together with the C-terminal LRR domain. ATP in this process functions to stabilize the active conformation of ZAR1. The sequential conformational changes lead to pentamerization of the ZAR1-RKS1-PBL2 complex,

forming the ZAR1 resistosome. Assembly of the heptameric Apaf-1 apoptosome likely follows a similar mechanism. Here, the binding of cytochrome c to the WD40 domains of Apaf-1 triggers a conformational change in the NBD domain (Zhou *et al.*, 2015). Similar to ZAR1 and Apaf-1, activation of NLRC4 also involves structural re-organization of its C-terminal segment (Hu *et al.*, 2015; Zhang *et al.*, 2015; Wang *et al.*, 2019a,b). Notably, the positioning of the three subdomains of WHD of these three NLRs is highly conserved (Fig. 4a). However, ATP/dATP-binding appears dispensable for the assembly of the NLRC4 inflammasome as evident in structural and functional data (Hu *et al.*, 2015; Zhang *et al.*, 2015; Jubic *et al.*, 2019). One reason for this can be that NLRC4 is self-activated, an activity induced by its paired NLRs NAIP2/5 (Fig. 4d). Interestingly, mutations of the P-loop have no effect on the functionality of sensor NLRs such as RGA5 and RRS1 (Cesari *et al.*, 2014; Williams *et al.*, 2014), but only on the corresponding executor NLRs RGA4 and RPS4. Thus, the activation mechanism of some plant sensor and helper NLRs may be analogous to mammal NLRC4 and NAIP2/5.

Sticks and stones – an assortment of NLR oligomer shapes Most NLR oligomers studied so far have circular shapes, for example the ZAR1 resistosome, the Apaf-1/CED4/DARK apoptosomes (Qi *et al.*, 2010; Zhou *et al.*, 2015; Cheng *et al.*, 2017) and the NAIP2/5-NLRC4 inflammasome (Hu *et al.*, 2015; Zhang *et al.*, 2015; Yang *et al.*, 2018) (Fig. 4f). Although varying in the number of protomers, these oligomers have their perceptive parts (WD40, LRR) facing outwards. Formation of the ring-like structures is predominantly mediated by the NOD module of these NLRs. Located at the centre of the circular structures are their N-terminal signalling domains (CARD, CC). While the CARDs of apoptosomes and inflammasomes act as adaptors to recruit downstream components, the oligomerized CC domain of the ZAR1 resistosome functions differently as discussed earlier. Although oligomerization is likely a general mechanism of NLR activation, whether other plant NLRs can form resistosome-like structures is an interesting question for future research. The NAIP2/5-NLRC4 inflammasomes appear to be an attractive model for plant paired NLRs like RRS1/RPS4 and RGA4/RGA5 or even sensor NLRs together with the helper NLRs NRCs. However, there has been no evidence that they can form hetero-oligomers similar to the inflammasomes.

Oligomeric NLRs can also assume other shapes than ring-like structures. For example, the prototype NLR protein MalT from bacteria forms curved oligomers with linear symmetry upon ligand-binding (Larquet *et al.*, 2004). After activation, MalT functions as a transcription factor directly binding to DNA (Larquet *et al.*, 2004). It could be speculated that the curved oligomers of MalT are structurally more suitable for this function. Given that DNA-binding activity was reported for some plant NLRs (Fenyk *et al.*, 2015; Fenyk *et al.*, 2016), it will be interesting to explore whether these NLRs can form similar structures during transcriptional reprogramming. In addition to the ring-like structures, helical polymers were also shown for NLRC4 inflammasomes (Diebolder *et al.*, 2015; Li *et al.*, 2018). However, the biological significance of these structures remains unclear.

NLR signalling Compared with NLR signalling in animals, signalling mediated by plant NLRs is much less well understood (Fig. 5). Generally, NLR activation leads to HR cell death at the infection site and is correlated with events like calcium influx and the generation of ROS and NO in different cellular compartments (Hammond-Kosack & Jones, 1996; Torres *et al.*, 2006). Distal parts of the plant are informed through mobile signals and react with systemic acquired resistance (SAR), which is associated with massive transcriptional reprogramming (Fu & Dong, 2013; Shine *et al.*, 2019). Although HR cell death is a hallmark output of NLR signalling, RLP signalling can also lead to HR (Peng *et al.*, 2018). Generally, HR signalling trough CNLs follows a different route than that of TNLs.

The TNL route of signalling predominantly converges on ENHANCED DISEASE SUSCEPTIBILITY 1 (EDS1). The EDS1-SAG101-NRG1 axis is a signalling module promoting TNL-dependent local cell death, while the EDS1-PAD4-ADR1 axis promotes distal SAR (Peart *et al.*, 2005; Qi *et al.*, 2018; Castel *et al.*, 2019; Lapin *et al.*, 2019) (Fig. 5). Although the link between local TNL signalling, and distal activation of EDS1-PAD4-ADR1 modules is yet to be identified, it is likely that small molecules created by TNL NADase activity are of importance.

The CNL route of signalling does not involve EDS1 and PAD4. Instead, some CNLs might trigger cell death more directly by forming a pore in the membrane (Wang *et al.*, 2019a,b). It is not yet clear whether the ion leakage of CNL oligomers of the ZAR1-type (MADA/MADA-like CNLs) is strong enough to directly cause cell death. It seems more plausible that ion traffic through the ZAR1 pore is somehow amplified to reach a certain signalling threshold, for example by calcium channels. Alternatively, calcium-dependent procaspases like AtMC4 might be activated in response (Pitsili *et al.*, 2020). The involvement of AtMC4 is an attractive hypothesis. It makes a link to SAR signalling by the cleavage of the precursor protein PROPEP1, which releases the danger peptide Pep1 to trigger defence signalling similar to SAR (Huang *et al.*, 2018; Hander *et al.*, 2019). It still remains a challenge to know whether other CNLs can form ZAR1 resistosome-like structures for signalling, and how they induce local HR and distal SAR.

The identification of the ZAR1 resistosome as a potential channel or a pore also illustrates the convergence of plant and animal NLR signalling. However, multiple adapter proteins are involved in the recruitment of the pore-forming protein Gasdermin D by the NLRC4 inflammasomes (Shi *et al.*, 2017; Ruan *et al.*, 2018). Thus, the spatial separation between oligomerization of NLRC4 and pore-formation allows branching of the signalling cascade, likely rendering it possible to uncouple NLRC4-mediated cell death from immune responses (Shi *et al.*, 2017). Like in animals, it is possible that the non-MADA CNLs of plants rely on the recruitment of CNLs or other proteins with pore-forming capacity.

IV. Invaders and their armoury – negative regulation of plant immunity by pathogen effectors

Pathogens secrete effectors to perturb the plant immune system through various mechanisms. One common strategy is to impede

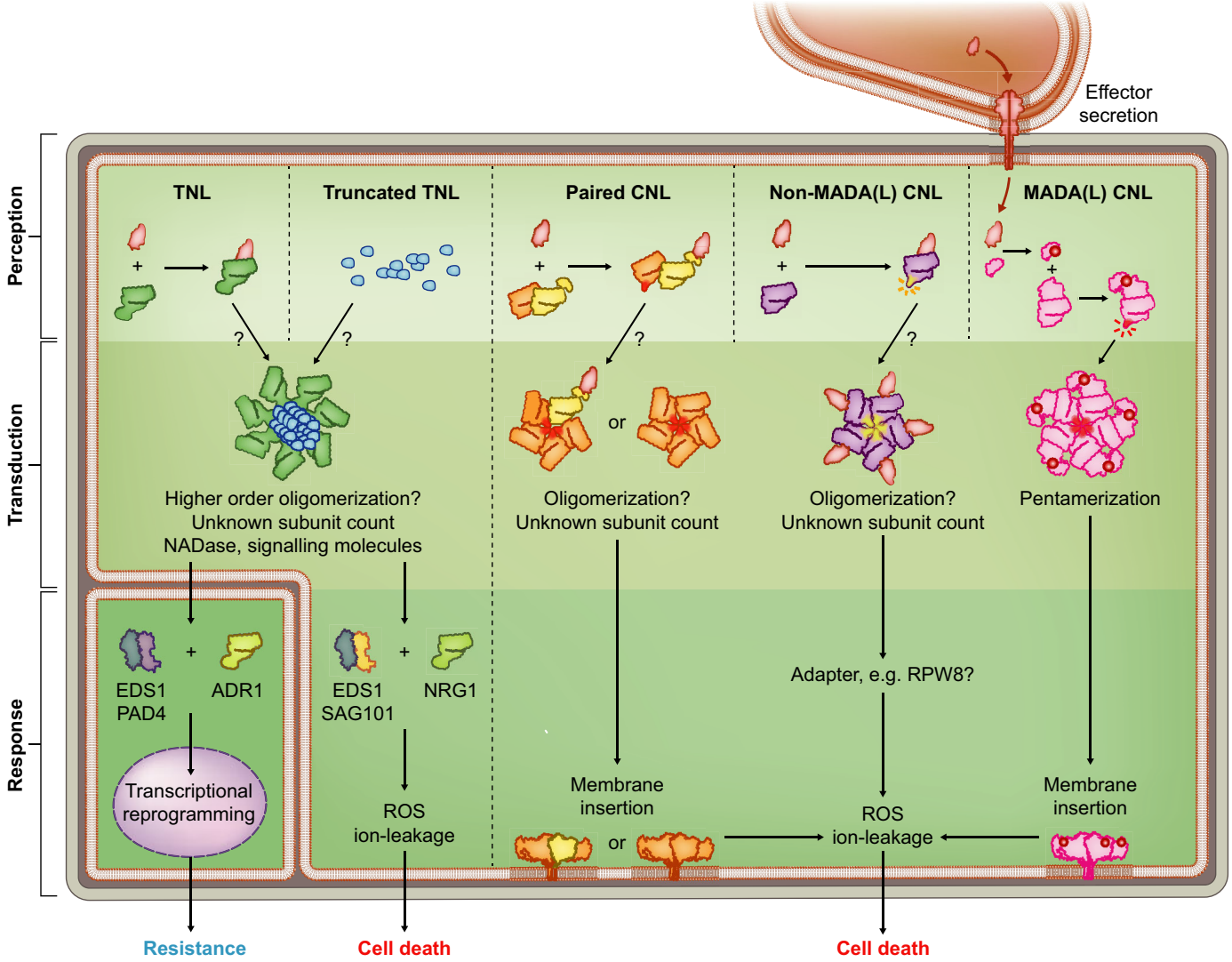

Fig. 5 Cartoon showing the classes of plant nucleotide-binding leucine-rich repeat receptors (NLRs). Mechanisms are suggested for activation of TNLs, truncated TNLs, paired CNLs, non-MADA(L) CNLs and MADA(L) CNLs (left to right). Effectors (red) accumulate inside the cell. Effector binding might be indirect, but is mostly shown here as direct for simplification. TNLs (dark green) are either directly or indirectly activated by an effector. Whether TNLs and truncated TNLs (light blue) oligomerize in response to ligand perception remains unknown. Presumably ligand-induced oligomerization of TNLs or truncated TNLs promotes the NADase activity of the TIR domain, but how the enzymatic activity is translated into the activation of EDS1-PAD4 and EDS1-SAG101 and leads to cell death of the endogenous cell and resistance response in neighbouring cells need to be addressed in future. Paired CNLs (executer in orange and sensor in yellow) associate with each other before activation. Effector binding to the sensor might induce oligomerization of the executor that either includes the sensor or excludes it. Nevertheless, executor CNLs with a MADA(L) motif may directly induce pore formation. Non-MADA(L) CNLs (purple) might be able to oligomerize, but due to the lack of a pore-forming MADA(L) motif, they might either have other pore-forming motifs or rely on associated proteins with pore-forming ability (e.g. RPW8). ZAR1, belonging to the MADA(L) CNL class (purple), forms a pentamer (resistosome) upon indirect activation through an effector. ZAR1 MADA(L) motif containing alpha-helix 1 form a funnel shaped pore in the oligomer. The funnel inserts into the membrane and might be directly associated with ion-leakage leading indirectly to reactive oxygen species (ROS) production and cell death. Cartoon illustration of activation of different CNLs is based on ZAR1, but it remains unclear whether they form a structure similar to that of the ZAR1 resistosome for signalling.

PTI, as exemplified by AvrPto and AvrPtoB from *Pseudomonas syringae*, which directly target and inhibit PRRs and their co-receptors. AvrPtoB can also function as an E3 ligase to mediate PRR degradation (Janjusevic *et al.*, 2006; Gohre *et al.*, 2008; Shan *et al.*, 2008; Zeng *et al.*, 2012). As a hub of PTI signalling, MAPKs are targeted by the *P. syringae* effectors HopAI1 and HopF2 (Zhang *et al.*, 2007; Wang *et al.*, 2010). Similarly, AvrAC interferes with PTI by acting as a uridylyl transferase on the activation loop of Arabidopsis BIK1 and RIPK (Feng *et al.*, 2012). Interestingly, BIK1 is proteolytically cleaved by *P. syringae* effector AvrPphB, demonstrating that these PTI hubs are targeted by various mechanisms (Zhang *et al.*, 2010). Some pathogens modify host

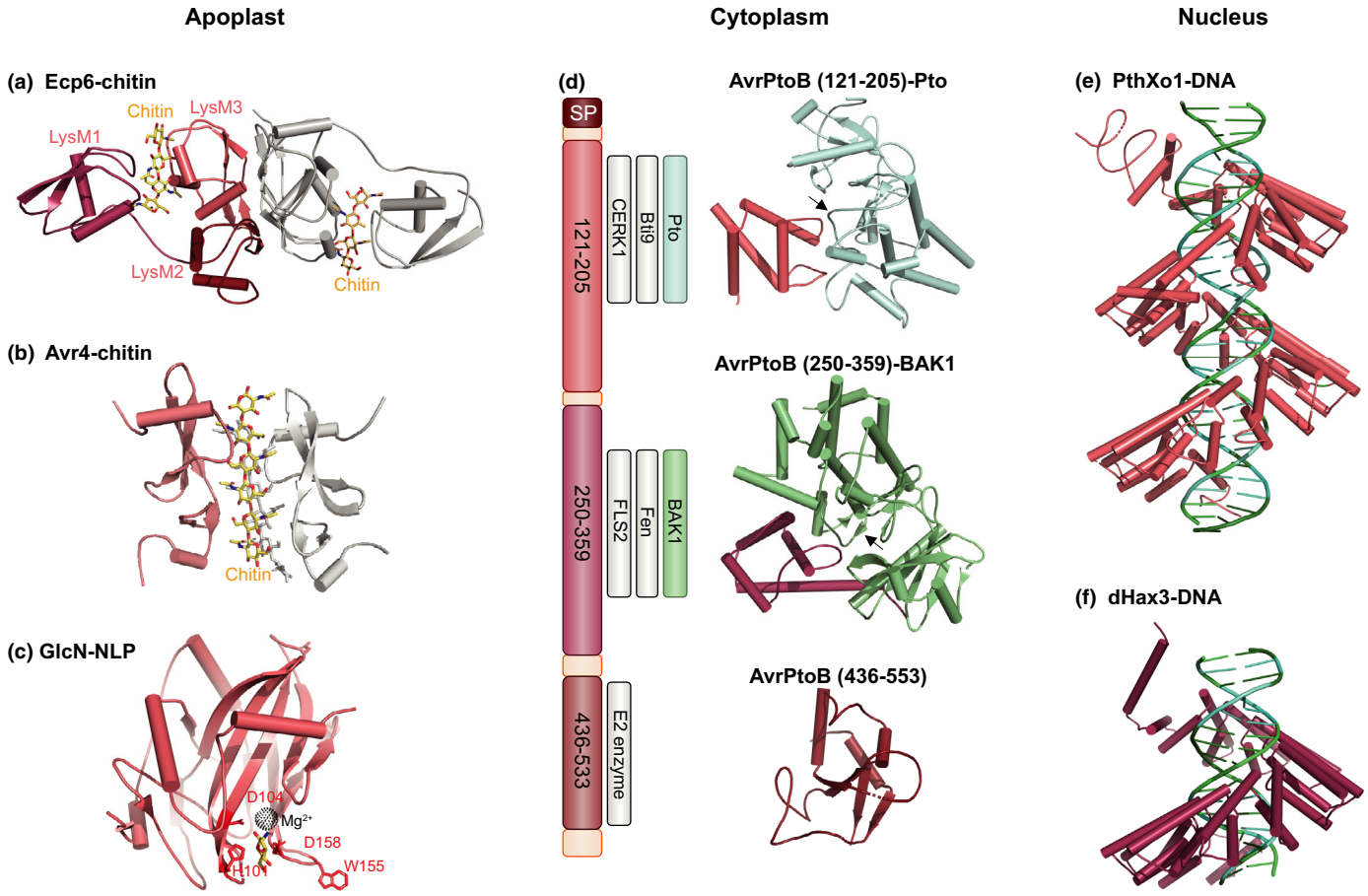

Fig. 6 Structures of pathogen effectors bound to their substrates. (a) Crystal structure of the complex between an Ecp6 dimer and chitin. LysM1–3 in the two monomers are labelled and the two chitin tetramers are indicated by yellow sticks. (b) Crystal structure of the CfAvr4 dimer in complex with chitin. The left monomer is coloured in light red and the chitin hexamers are indicated by yellow sticks. (c) Crystal structure the GlcN-NLPPya complex. GlcN is displayed in yellow stick. Mg^{2+} ions are shown in black spheres. (d) (left) Genomic architecture of AvrPtoB. Different regions and their target proteins are indicated. (right) Crystal structure of AvrPtoB (250–359) in complex with BAK1-kinase, AvrPto (B121–205) in complex with Pto and core domain of AvrPtoB (436–553). The activation sites of BAK1 and Pto are indicated with arrows. (e) Crystal structure of the PthXo1-dsDNA complex. (f) Crystal structure of dHax3 bound by dsDNA.

components and suppress PTI. For instance, AvrRpt2 from *P. syringae* proteolytically cleaves the host protein RIN4 and the cleavage product is a hyperactive suppressor of PTI signalling (Afzal *et al.*, 2011). Another strategy used by pathogens to compete with the host immune system is to sequester their PAMPs from host PRRs as demonstrated for the chitin scavenger ECP6 from *Cladosporium fulvum* (de Jonge *et al.*, 2010).

1. Warfare in the apoplast – shielding PAMPs and piercing membranes

The early competition between plant and pathogen occurs in the apoplast. To neutralize chitin-induced immunity, pathogenic fungi secrete effectors, for example ECP6 of fungal tomato pathogen *C. fulvum*, to sequester the fungal cell-wall-derived PAMP from being perceived by the PRRs. The effector ECP6 has three LysM domains as observed in the PRRs AtCERK1 and OsCEBIP. However, ECP6 binds chitin with higher affinity than these two PRRs (de Jonge *et al.*, 2010; Sanchez-Vallet *et al.*, 2013), allowing ECP6 to outcompete PRRs for chitin-binding. The overall structure of ECP6 is tightly packed, with LysM1 separated by a long and flexible linker from the compact dimer formed by LysM2 and LysM3 (Fig. 6a) (Sanchez-Vallet *et al.*, 2013). In contrast with AtCERK1 and OsCEBiP (Fig. 3d), LysM1 and LysM3 of Ecp6 cooperate to compose a single chitin-binding groove (Fig. 6a). Unlike the largely exposed AtCERK1- and OsCEBiP-bound chitin oligomer, the four GlcNAc units of a chitin oligomer are nearly completely buried in the chitin-binding groove, explaining why ECP6 has an ultra-high affinity for chitin. The remaining singular LysM2 also possesses a lower chitin binding activity and is able to perturb the chitin-triggered immunity, but the precise mechanism remains elusive.

Chitinase plays an important role in plant defence by cleaving chitin and releasing chitin fragments as a PAMP to induce innate immunity (Pusztahelyi, 2018). Accordingly, some fungal effectors protect the cell wall component from being degraded as demonstrated for the effector *Cf*Avr4 from *C. fulvum* (van Esse *et al.*, 2007). The crystal structure of *Cf*Avr4 bound to chitin revealed that two *Cf*Avr4 monomers form a three-dimensional molecular sandwich that laminates two $(GlcNAc)_6$ molecules within the dimeric assembly (Fig. 6b) (Hurlburt *et al.*, 2018). Thus, the effector may inhibit chitinase-mediated hydrolysis of chitin by

shielding the substrate. However, whether the chitin microfibril in the fungal cell wall is shielded by *Cf*Avr4, as soluble chitin is, remains unknown.

Necrosis and ethylene-inducing peptide 1-like proteins (NLPs) function as specific cytolytic toxins for dicots plants but not monocots. The crystal structure of NLPPya complexed with the glucosamine group (GlcN) of plant plasma membrane glycosylinositol phosphorylceramides (GIPCs), which specifically binds NLPs to prevent NLP mediated cytolysis (Lenarcic *et al.*, 2017), provides an elegant explanation to the toxic specificity. The crystal structure of NLP from the oomycete *Pythium aphanidermatum* (NLPPya) revealed that it exhibits a central β-sandwich decorated with α-helices and loops (Lenarcic *et al.*, 2017). Remarkably, NLPs have structural homology with the pore-forming actinoporins of sea anemone (Ottmann *et al.*, 2009; Rojko *et al.*, 2016). In the NLPPya-GlcN complex, a GlcN hexose moiety binds to the elongated crevice between loop 2 and loop 3 of the NLP (Fig. 6c). Interestingly, most of monocot GIPCs carry three-hexose units in their sugar heads rather than two as in eudicot GIPCs. Binding of NLPs to three-hexose head GIPC likely leads to a more distant positioning of the loop 3 to the plant membrane, thus preventing an NLP from inserting into the plasma membrane for cytolysis (Lenarcic *et al.*, 2017).

2. Warfare in the cytoplasm – from Swiss knives to killing the messenger

AvrPtoB from *P. syringae* is a modular protein containing three discrete domains that confer different activities. On the one hand, it directly targets PRRs or their co-receptors, inhibiting their kinase activity and thereby suppressing PTI (Shan *et al.*, 2008; Zeng *et al.*, 2012). One of the PRRs targeted by AvrPtoB is BAK1 (Shan *et al.*, 2008). The central region of AvrPtoB (residues 250–359, $AvrPtoB_{250\text{-}359}$) forms a four-helix bundle primarily interacting with the activation segment of BAK1 (Cheng *et al.*, 2011) (Fig. 6d). This structural observation suggests that $AvrPtoB_{250\text{-}359}$ inhibits BAK1 kinase activity, as further confirmed by *in vitro* biochemical and cell-based assays. On the other hand, $AvrPtoB_{250\text{-}359}$ also interacts with the FEN kinase in tomato but functions to activate ETI mediated by the CNL Prf (Janjusevic *et al.*, 2006). Fen was therefore proposed to be a molecular mimic, or decoy, of host virulence targets for activation of ETI (Rosebrock *et al.*, 2007). Thus, AvrPtoB appears to be a living fossil unveiling the arms race between plants and pathogens, an idea further strengthened by the data discussed later.

The N-terminal region of AvrPtoB (residues 1–307) target the kinase domains of AtCERK1 and the CERK1-like PRR Bti9 from tomato (Zeng *et al.*, 2012). The fragment of AvrPtoB containing residues 121–205 ($AvrPtoB_{121\text{-}205}$) was mapped to directly inhibit these two PRRs. Unexpectedly, this fragment adopts a fold similar to that of $AvrPtoB_{250\text{-}359}$ despite their limited sequence homology (Dong *et al.*, 2009; Cheng *et al.*, 2011), suggesting that these two domains might have arisen by tandem duplication of a progenitor kinase-interacting domain. $AvrPtoB_{121\text{-}205}$ also interacts with Pto to activate Prf-mediated ETI (Xiao *et al.*, 2007). Like FEN, Pto may also function as a decoy of virulence targets of AvrPtoB to activate ETI (Rosebrock *et al.*, 2007; Mathieu *et al.*, 2014). Despite their conserved structures, $AvrPtoB_{121\text{-}205}$ and $AvrPtoB_{250\text{-}359}$ assume different orientations when interacting with Pto and BAK1, respectively (Fig. 6d). Nonetheless, both regions interact with the conserved activation segment to inhibit the kinase activity of Pto and BAK1 (Dong *et al.*, 2009; Cheng *et al.*, 2011). AtCERK1 and Bti9 may be similarly targeted by $AvrPtoB_{121\text{-}205}$ despite the lack of structural information. Interestingly, FEN does not recognize $AvrPtoB_{121\text{-}205}$, though it shares 80% sequence identity with Pto (Jia *et al.*, 1997; Dong *et al.*, 2009). Structural comparison between Pto-$AvrPtoB_{121\text{-}205}$ and BAK1-$AvrPtoB_{250\text{-}359}$ suggests that the specificity determinants for interaction with the effectors are located in the non-conserved regions flanking the activation segment.

Structural study revealed the biochemical function of the C-terminal region of AvrPtoB ($AvrPtoB_{436\text{-}553}$). The structure of $AvrPtoB_{436\text{-}553}$ remarkably resembles those of eukaryotic U-box and RING-finger E3 ligases (Janjusevic *et al.*, 2006) (Fig. 6d). Indeed, biochemical and functional data support the C-terminal region as an E3 ligase. Later on, multiple targets of the E3 ligase were identified. In addition to inhibiting the kinase activity of FLS2 and AtCERK1, AvrPtoB also degrades these two PRRs through its E3 ligase domain, abolishing flagellin- and chitin-induced immunity (Gohre *et al.*, 2008; Gimenez-Ibanez *et al.*, 2009). Similarly, the tomato kinase Fen (Ntoukakis *et al.*, 2009), Arabidopsis EXO70B1 (W. Wang *et al.*, 2019) and NPR1 (H. Chen *et al.*, 2017) were also shown to be degraded by AvrPtoB to suppress immune signalling.

Salicylic acid (SA) is a master phytohormone in plant immunity. It is not surprising that some pathogens have evolved effectors to impede SA signalling. One such pathogen effector is CHORISMATE MUTASE 1 (CMU1) from the fungus *Ustilago maydis*, the causative agent of corn smut. Cmu1 catalyses conversion of chorismate into prephenate instead of phenylpropanoid (Djamei *et al.*, 2011), a key intermediate in SA biosynthesis pathway. Thus, CMU1 blocks SA-induced immune responses by depleting chorismate and consequently lowering the concentrations of SA in plant cells. CMU1 forms a homodimer with each monomer harbouring nine α-helices and an active site (Bange & Altegoer, 2019; Han *et al.*, 2019). In contrast with the housekeeping chorismate mutases in maize (Denance *et al.*, 2013), Cmu1 is insensitive to regulation by the amino acid tryptophan or tyrosine. Cmu1 has an additional α-helix and an extended loop after the second α-helix, blocking the site where these two amino acids bind. To counter against Cmu1 inhibition of SA signalling, maize evolved a kiwellin protein termed ZmKWL1, which specifically interacts with Cmu1 and thereby inhibit its metabolic activity (Han *et al.*, 2019). *Zm*KWL1 is positioned in close proximity to the active site of Cmu1, thus blocking substrates from accessing the active site of Cmu1 to inhibit its chorismate mutase activity.

3. Warfare in the nucleus – I TALE you a different story

Another group of pathogen effector is imported into plant nuclei after injection, suggesting that these effectors may modulate the

normal cellular activities via transcriptional activation and interference with host nuclear processes. One group of such effectors are transcription activator-like effector (TALE), which usually contains an N-terminal translocation signal, a central DNA-binding domain that specifies the TALE target sequence, a C-terminal nuclear localization signal, and a C-terminal acidic activation domain responsible for gene modulation (Boch & Bonas, 2010). The DNA-binding domain is usually composed of repeats of 33 to 35 hyper-conserved amino acids, while residues at position 12 and 13 (repeat variable diresidue, RVD) are hyper-variable and responsible for matching with specific double-stranded DNA (dsDNA) bases. The structures of the *Xanthomonas oryzae* TAL effector PthXo1 and an artificially engineered TALE protein dHax3 bound by dsDNA are nearly identical, with two helical bundles packing together to wrap around the bound dsDNA (Fig. 6g,h) (Deng *et al.*, 2012; Mak *et al.*, 2012; Denance *et al.*, 2013). In the protein-dsDNA complexes, each TAL repeat is connected by the two RVD containing loop, with the first one stabilizing the backbone through hydrogen bonds and the second one making base-specific contacts with the dsDNA. These interactions allow the TALE proteins to adopt a super-helical structure tracking along the dsDNA. The selective activation of individual gene expression not only extend the understanding of effector pathogenicity, but also made TALEs one of the gene editing tools.

V. Future directions

During the past years, structural and biochemical studies provided significant insight into the signalling mechanisms of plant immunity. However, structural biologists are still faced with many challenging yet important questions. One of these is to understand how RLPs as a PRR and their shared co-receptor BAK1 are activated in response to ligand recognition. It is still not well understood why RLP-PRRs with varied sizes and sequences have the same co-receptor for signalling. Structures of full-length PRRs in signalling-competent complexes will be instrumental in understanding how ligands induce activation of the kinase domains of PRRs. Studies of NLPPya and the E3 domain of AvrPtoB highlight the fact that structural biology can be used as a tool for specifying the biochemical function of a protein. The biochemical activities of several pathogen effectors were similarly demonstrated (Chosed *et al.*, 2007; Zhang *et al.*, 2010). Structural elucidations of pathogen effectors can therefore facilitate our understanding of how they interact with plants by revealing their biochemical identities. Compared to PTI, less is known about the biochemical mechanisms of NLR-mediated ETI signalling. Oligomerization of plant NLRs is believed to be important for their function, as supported by studies of ZAR1 and some other NLRs. However, structural and biochemical studies are necessary to demonstrate whether this generally holds true with plant NLRs. If so, do they form structures similar to that of the ZAR1 resistosome? Given the high diversity of NLR activation in plants, it would not be surprising that variations exist in their oligomerized status. Another question concerning oligomerization is why ATP/dATP is required for some but not for other NLRs. Is it possible for some plant NLRs to utilize other nucleotides rather than ATP/dATP to stabilize their active conformation for oligomerization? Defining the biochemical activity of the ZAR1 resistosome and other oligomerized plant NLRs will be crucial for the dissection of ETI signalling. In this aspect, oligomerized TNLs can function as a NADase as demonstrated for the TIR domain proteins, but it remains unknown whether and why oligomerization is important for this activity. More importantly, it will be of interest to understand how the enzymatic activity is associated with the activation of EDS1-SAG101/EDS1-PAD4 and the downstream helper NLRs ADR1-Ls and NRGs. Although the ligand-receptor model on NLR recognition of effectors was proposed many years ago, reconstitution of a direct NLR-effector interaction is still technically challenging, partially due to the difficulty in purification of NLR proteins, which is a critical step toward structural determination of them. With the advances in cryo-EM (Bai *et al.*, 2015), it can be anticipated that more structures of NLR-containing complexes will be resolved, which would reveal more exciting information on the acting mechanisms of NLR proteins.

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