Regulation of pattern recognition receptor signalling in plants

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Bibliographies

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Online Summary

- Plants rely on a cell-autonomous innate immune system to detect the presence of microbes and activate immune responses that deter infection. Recognition of conserved microbial features occurs essentially at the cell surface by means of trans-membrane pattern recognition receptors (PRRs).
- PRRs are part of multimeric protein complexes at the plasma membrane, differentially recruiting cytoplasmic kinases that connect PRR complexes to downstream signalling components.
- Ligand binding initiates a series of phosphorylation events within PRR complexes that activates cellular immune signalling, which includes bursts of intracellular reactive oxygen species and calcium, activation of cytoplasmic kinase cascades, and transcriptional reprogramming.
- As in mammals, excessive activation of plant immune responses can have detrimental consequences. Thus, a complex negative regulatory system controls different immune componentes to maintain cellular homeostasis .
- Bacterial pathogens are able to subvert the plant immune system by secreting molecules, such as effectors, that often mimic the mode-of-action of host negative regulators of immune signalling.

Abstract

Recognition of pathogen-derived molecules by pattern recognition receptors (PRRs) is a common feature of both animal and plant innate immune systems. In plants, PRR signalling is initiated at the cell surface by kinase complexes, resulting in the activation of immune responses that ward off microbes. However, the activation and amplitude of innate immune responses must be tightly controlled. In this Review, we summarize our knowledge of the early signalling events that follow PRR activation, and describe the mechanisms that fine-tune immune signalling to maintain immune homeostasis. We also illustrate the mechanisms used by pathogens to inhibit innate immune signalling, and discuss how the innate ability of plant cells to monitor the integrity of key immune components can lead to autoimmune phenotypes upon genetic or pathogen-induced perturbations of these components.

Introduction

Plants do not have a circulating immune system and, as such, they rely on the capacity of each individual cell to initiate innate immune responses against potential pathogenic microbes. To achieve this, plants employ a multi-tier surveillance system that recognizes non-self or modifiedself using plasma membrane-localized and intracellular immune receptors[1,2]. At the cell surface, receptor kinases and receptor-like proteins (RLPs) function as pattern recognition receptors (PRRs) to perceive characteristic microbial molecules — classically known as pathogen-associated molecular patterns (PAMPs) — or host-derived damage-associated molecular patterns (DAMPs)[3,4]. Structurally, plant receptor kinases possess an ectodomain potentially involved in ligand binding, a single trans-membrane domain, and an intracellular kinase domain (**Fig. 1**). RLPs share the same basic conformation, except they lack a kinase domain or any other recognizable intracellular signalling domain. For this reason, RLPs are thought to depend on regulatory receptor kinases to transduce ligand perception into intracellular signalling[5].

Plant PRRs can be distinguished based on the nature of their ligand-binding ectodomain. Leucine-rich repeat (LRR)-containing PRRs preferentially bind proteins or peptides, such as bacterial flagellin or elongation factor Tu (EF-Tu), or endogenous AtPep peptides [3,4]. In turn, PRRs containing lysine motifs (LysM) bind carbohydrate-based ligands, such as fungal chitin or bacterial peptidoglycan[3,4]. Furthermore, lectin-type PRRs bind extracellular ATP or bacterial lipopolysaccharides (LPS), while PRRs with epidermal growth factor (EGF)-like ectodomains recognize plant cell-wall derived oligogalacturonides[3,4,6]. Given the diverse and conserved nature of PAMPs, PRR-triggered immunity (PTI, also known as pattern- or PAMP-triggered immunity) effectively repels most non-adapted pathogens, while contributing to basal immunity during infection.

Intracellular nucleotide-binding domain leucine-rich repeat (NLR, also known as NBS-LRR) proteins represent a second group of immune receptors that is classically associated with the recognition of pathogen-secreted virulence effectors[2,7]. Adapted

pathogens evolved these effectors to suppress host immunity and/or manipulate the host metabolism for virulence. In turn, recognition by NLRs betrays the pathogen in what represents an evolutionary arms race between plants and pathogens[8]. Effector recognition may occur through direct binding or by sensing the perturbing activity of an effector on host components[7]. According to the 'guard model'[9], critical immune components can be guarded by NLRs, which become activated upon effector-triggered modification of their 'guardees' (see **BOX 1**). In an extension of the guard model, plant NLRs can also guard structural mimics (or 'decoys') of key immune components that are normally targeted by effectors[10]. Additionally, integral or partial domains present in immune components targeted by effectors may be fused to NLRs to form 'integrated decoys' or 'integrated sensors' thus directly triggering NLR activation upon effector-mediated modifications[11-14].

An additional intracellular detection system in plants, which is specific for viruses, involves binding and processing of dsRNA by ribonuclease Dicer-like proteins to trigger RNA-based antiviral immunity[15]. Interestingly, NLRs are also involved in anti-viral immunity through recognition of viral proteins or by sensing virus-mediated host manipulation[7]. In addition, recent reports point towards a potential role of receptor kinases during anti-viral immunity[16-18].

Although in mammals PAMPs are perceived both outside and inside the cell[19], PAMP perception occurs essentially at the cell surface in plants. Nevertheless, several parallels can be observed between both innate immune systems[20-24]. In this Review, we will provide an overview of the early signalling events triggered during PTI, while expanding on the negative regulatory mechanisms employed by plant cells to maintain immune homeostasis; as recently reviewed in the case of mammals[25].

Formation and activation of PRR complexes

PAMP recognition by Toll-like receptors (TLRs) plays a crucial role in innate immunity in mammals[26]. TLRs are transmembrane receptors composed of an LRR-containing

ectodomain and a cytoplasmic Toll/Interleukin-1 (IL-1) receptor (TIR) domain. TLRs form multimeric complexes with a variety of co-receptor proteins and use their TIR domain as docking platforms for different TIR-containing adaptors [25,27]. TLRs show selectivity for adaptors, enabling the activation of specific immune responses according to the perceived molecules. MyD88 was the first identified TIR adaptor and is used by all mammalian TLRs (except TLR3). Agglomeration of adaptors into higher-order complexes, such as the 'Myddosome', creates a signalling platform where IRAK/Pelle kinases, or other receptor interacting-protein kinases (RIPKs), are activated to initiate a signalling cascade that leads to transcriptional reprogramming and production of pro-inflammatory cytokines.

Plant PRRs recruit regulatory receptor kinases upon ligand binding and signal through receptor-like cytoplasmic kinases (RLCKs), which provide a link between extracellular ligand perception and downstream signalling[4,28]. Interestingly, the kinase domains of plant receptor kinases and RLCKs are phylogenetically related to IRAK/Pelle kinases[29]. Thus, plant receptor kinase-type PRRs (at least LRR-type) could be seen as an 'all-in-one' Myddosome complex in which the ligand-binding PRRs are directly fused to intracellular kinase domains; thus bypassing the requirement of TIR-containing adaptors. While different adaptors can provide TLR signalling with flexibility and possibility of activating different downstream pathways[25,30], similar properties may be achieved in plants by differential recruitment of regulatory receptor kinases, and most importantly of distinct RLCKs (**Fig. 1**).

Heteromeric complexes with co-receptors.

Both receptor kinase- and RLP-type PRRs form dynamic complexes with regulatory receptor kinases at the plasma membrane to activate immune signalling. For example, the *Arabidopsis thaliana* (*At*, hereafter *Arabidopsis*) LRR-receptor kinases FLAGELLIN SENSING 2 (FLS2), EF-TU RECEPTOR (EFR), ELICITOR PEPTIDE 1 RECEPTOR 1 (PEPR1) and PEPR2, which recognize bacterial flagellin (or the flagellin epitope flg22), EF-Tu (or the EF-Tu epitopes elf18 or elf26), and the endogenous AtPep1 (and related

peptides), respectively, all associate with the regulatory LRR-receptor kinase BRI1-ASSOCIATED RECEPTOR KINASE 1 (BAK1) (also known as SERK3) and with related SOMATIC EMBRYOGENESIS RECEPTOR KINASES (SERKs) in a ligand-dependent manner[31-36]. BAK1 acts as a co-receptor for flg22 and is critical for activating signalling[35]. Co-crystallization of FLS2 and BAK1 ectodomains together with flg22, revealed that the C-terminus of FLS2-bound flg22 clenches onto BAK1 ectodomain to stabilize the FLS2–BAK1 heterodimer by acting as a 'molecular glue'[35]. Modelling and mutagenic analysis suggested that BAK1 is recruited to the PEPR1–AtPep1 complex in an identical manner[36]. FLS2–BAK1 heterodimerization occurs almost instantly following flg22 perception[31,33,35], suggesting these receptor kinases might be already present in pre-assembled complexes at the plasma membrane. However, a recent study using multiparameter fluorescence imaging spectrometry (MFIS) did not find evidence for FLS2–BAK1 pre-assembled complexes or for FLS2 homodimerization[37], which in the latter case could be detected by co-immunoprecipitation[38]. Intriguingly, FLS2 and BAK1 re-organize in multimeric complexes several minutes after the initial flg22-triggered heterodimerization[37], but the biological relevance of these larger complexes is not yet understood.

Interestingly, SERK proteins form multimeric complexes with a multitude (if not all) LRR-containing receptor kinases and RLPs, whether involved in immunity, growth or development[39-46]. While SERKs may often act as co-receptors whose complex formation with the main ligand-binding receptor is enabled by the ligand itself, other mechanisms of complex formation may also exist. Indeed, crystal structure of the growth-promoting peptide phytosulfokine (PSK) bound to its receptor PSKR1 revealed that SERK1 does not participate in PSK binding, but instead PSK induces allosteric modifications on the surface of PSKR1 that enable subsequent recruitment of SERK1[43].

LRR-RLPs, which lack a signalling kinase domain, constitutively associate with SOBIR1 or SOBIR1-like LRR-receptor kinases to form a bimolecular equivalent of a genuine receptor kinase[5,39]. BAK1 or other SERKs seem to be only recruited to the RLP–SOBIR1

complex upon ligand binding, as recently shown for *Arabidopsis* RLP23 and tomato Cf-4[47,48]. Similarly, BAK1 and SOBIR1 associate with or are required for the function of additional LRR-RLPs involved in immune recognition[49-55].

Importantly, SERK recruitment to PRRs is not always ligand-dependent. For example, the rice (*Oryza sativa*, Os) LRR-receptor kinase XA21 constitutively associates with the BAK1 orthologue OsSERK2[56]. Whether ligand binding could enhance this association however remains to be tested; something enabled by the recent identification of the *Xanthomonas oryzae* pv. *oryzae* (*Xoo*)-derived PAMP RaxX (or derived epitope RaxX21-sY) as an XA21 agonist[57].

Notably, BAK1 together with BAK1-LIKE 1 (BKK1, also known as SERK4) is additionally proposed to negatively regulate cell death based on the autoimmune phenotype (for example, dwarfism) exhibited by *bak1 bkk1* double mutants[58]. The molecular mechanism underlying this regulation is not yet fully understood, but it requires proper protein glycosylation of cell surface receptor kinases[59]. It is also theoretically possible that the autoimmune phenotype of double *bak1 bkk1* mutants is caused by the activation of NLRs that normally 'guard' the integrity of BAK1–BKK1 complexes (see **BOX 1**), as recently proposed for other immune components[60]

Analogous to the role of BAK1 with LRR-type PRRs, the CHITIN ELICITOR RECEPTOR KINASE 1 (CERK1) appears to act as a regulatory receptor kinase that associates with different LysM-containing PRRs to activate immune signalling. In rice, the LysM-RLP CHITIN ELICITOR-BINDING PROTEIN (CEBiP) forms a homodimer upon chitin binding that is followed by heterodimerization with OsCERK1, creating a signalling-active sandwich-type receptor system[61,62]. Two other LysM-RLPs, LYP4 and LYP6, act as dual-specificity receptors for both chitin and peptidoglycan, associating with OsCERK1 in a ligand-dependent manner[63,64]. Although LYP4 associates with LYP6, as well as with CEBiP, these complexes partially dissociate following ligand perception[64]. Further studies, including structural analysis of ligand-bound complexes, will be required to consolidate these data and

improve our understanding of chitin perception in rice. In *Arabidopsis*, AtCERK1 was thought to be the unique chitin receptor, as it homodimerized upon direct chitin binding[65-67]. However, a recent study demonstrated that the LysM-receptor kinase LYK5 displays higher chitin-binding affinity than AtCERK1[68]. Notably, LYK5 (and to a lesser extent its closest homologue LYK4) is genetically required for chitin responsiveness, and forms a chitin-dependent complex with AtCERK1[68,69]. Whether LYK5 and AtCERK1 organize into a sandwich-type receptor system similar to OsCEBiP and OsCERK1 remains to be shown. Furthermore, AtCERK1 is also recruited by the OsLYP4 and OsLYP6 paralogues in *Arabidopsis,* LYM1 and LYM3, during peptidoglycan recognition to mediate anti-bacterial immune responses[70-72]. Intriguingly, LYM1 and LYM3 do not seem to play a role in commonly-measured chitin-induced responses[70], but the paralogous LYM2 protein contributes to chitin-triggered plasmodesmata closure, thus controlling symplastic communication between plant cells and contributing to anti-fungal immunity[73]. Interestingly, this LYM2-dependent role does not involve AtCERK1, raising the possibility that additional co-receptors may function with chitin PRRs in *Arabidopsis*.

Recruitment of regulatory receptor kinases seems to be specified by the type of PRR ectodomain. Accordingly, BAK1 is dispensable for chitin-triggered responses, whereas CERK1 does not participate in flg22-mediated signalling[72,74]. Remarkably, neither BAK1 nor CERK1 are required to mediate signalling by the S-lectin-receptor kinase LORE, which was recently identified as the *Arabidopsis* receptor for bacterial LPS[6], suggesting the latter may interact with yet unknown co-receptors, if any.

RLCKs as direct PRR substrates.

The *Arabidopsis* and rice genomes encode over 160 and 280 RLCKs, respectively[75]. Most remain uncharacterized, but in recent years several RLCKs were reported to play important roles in PTI (**Fig. 1**). BOTRYTIS-INDUCED KINASE 1 (BIK1), a member of *Arabidopsis* RLCK subfamily VII, is the best-studied example. Under resting conditions, BIK1 associates

with FLS2, and likely with BAK1[76,77]. Upon flg22 elicitation, BAK1 associates with FLS2 and phosphorylates BIK1[76,77]. In turn, BIK1 phosphorylates both BAK1 and FLS2 before dissociating from the PRR complex to potentially activate downstream signalling components[76,77]. BIK1 and the closely-related PBS1-LIKE KINASE (PBL) proteins are also required to activate immune responses triggered by elf18, AtPep1 and chitin[76-78], thus representing an early convergence point for distinct PRR-mediated pathways.

Another RLCK from subfamily VII, PCRK1, also mediate BAK1-dependent PTI responses[79]. Furthermore, OsRLCK176 and OsRLCK185, members of rice RLCK family VII, both interact with CERK1 and positively regulate responses to peptidoglycan and chitin[64,80]. Similarly, PBL27, the OsRLCK185 orthologue in *Arabidopsis*, specifically mediates immune responses triggered by chitin, but not by flg22[81]. Interestingly, BSK1, a RLCK from subfamily XII, which was previously associated with growth signalling, dynamically associates with FLS2 to regulate specific subsets of flg22-induced, but not elf18-induced, immune responses[82]. Together, these observations raise the possibility that plants may, in part, owe the robustness and flexibility of their immune system to their large repertoire of RLCKs. In turn, these RLCKs vary in terms of their affinity for different PRRs as well as in their ability to activate distinct branches of PTI signalling (**Fig. 2**), and are possibly subjected to different regulatory constraints.

Activation of cellular immune signalling

Upon ligand binding and subsequent PRR complex activation, a branched signalling cascade is initiated within minutes to promote local and systemic defence responses in the plant that can last up to several days[83]. Rapid ion-flux changes at the plasma membrane, accompanied by the rise of cytosolic Ca^{2+} levels, and production of extracellular reactive oxygen species (ROS) are amongst the first outputs recorded after PAMP or DAMP perception[83]. In turn, activation of Ca^{2+}-dependent protein kinase (CDPK) and mitogen-

activated protein kinase (MAPK) cascades conveys immune signalling to the nucleus, resulting in transcriptional reprograming to establish PTI[83-85] (**Fig. 2**).

A direct link between PRR complex activation and ROS production was recently established whereby RESPIRATORY BURST OXIDASE HOMOLOGUE PROTEIN D (RBOHD), which is the NADPH oxidase responsible for PRR-triggered ROS bursts in *Arabidopsis*, associates with the PRR complex and is directly phosphorylated by BIK1 and related PBLs upon PRR elicitation[86,87]. These findings provided for the first time a mechanism connecting activated PRRs to a cellular immune output. BIK1-mediated AtRBOHD phosphorylation is critical for ROS production, which in turn acts as a key messenger to promote closure of stomata and limit entry of bacterial pathogens into leaf tissues[86,87]. Other RLCKs, such as BSK1 and PCRK1, are genetically required for PAMP-triggered ROS burst and may thus also directly phosphorylate AtRBOHD[79,82], although this remains to be determined experimentally. In contrast, phosphorylation of AtRBOHD by PBL13 was recently proposed to negatively impact ROS production by regulating AtRBOHD[88]. The activity of RBOH enzymes is further regulated through Ca^{2+} binding to conserved EF-hand motifs and CDPK-mediated phosphorylation[89-93]. This is in line with a synergistic model where initial BIK1-mediated phosphorylation primes RBOH activation by enhancing its sensitivity to subsequent Ca^{2+}-dependent regulation[86,94]. This mechanism by which RBOHD needs to be activated by two different types of kinases (namely BIK1 and CDPKs) may help maintaining signalling specificity[94]. Interestingly, it was very recently found that FLS2 and BIK1 associate with heterotrimeric G proteins, which contributes to the regulation of BIK1 steady-state levels and potentially to RBOHD activation [95]. In addition, the rice AtRBOHD orthologue, OsRBOHB, is positively regulated by the small GTPase OsRac1, which is in turn activated by OsCERK1-phosphorylated OsRacGEF1[90,96,97].

Besides controlling RBOHD, BIK1 and PBL1 are also required for the PAMP/DAMP-triggered cytosolic Ca^{2+} burst that precedes ROS production[87,98,99]; however, the identity of

the channel(s) responsible for the Ca^{2+} burst and their activation mechanisms remain elusive. The Ca^{2+} burst activates calcium-dependent protein kinases (CDPKs), which not only regulate RBOHs, but are also important regulators of transcriptional reprogramming during PTI. Multiple knockout of *Arabidopsis CPK4, CPK5, CPK6* and CPK*11* impaired flg22-induced transcription of specific sets of genes[92,93], as well as flg22- and oligogalacturonide-induced ethylene production and resistance to the necrotrophic fungus *Botrytis cinerea*[100]. These CDPKs phosphorylate a group of WRKY transcription factors during NLR-mediated immunity[101]. Whether these or other transcription factors are directly phosphorylated by CDPKs during PTI remains to be shown.

MAPKs represent a second vehicle to trigger transcriptional changes upon PAMP or DAMP perception. At least two distinct cascades lead to the activation of four MAPKs in *Arabidopsis* within a few minutes of PAMP or DAMP treatment. MPK3 and MPK6 are activated by the MAPK kinases (MKKs, also known as MEKs) MKK4 and MKK5, but their corresponding MAPK kinase kinase (MAP3K, also known as MEKK) remains unknown[102,103]. A second cascade comprising MEKK1, and MKK1 and MKK2 activates MPK4, and its closely related homologue MPK11[103-106]. MPK4 was initially characterized as a negative regulator of plant immune signalling, as mutations associated with this MAPK cascade were accompanied by severe autoimmune phenotypes, including over-accumulation of salicylic acid and spontaneous cell death[103,107]. It was later found that the integrity of the MPK4 cascade is actually guarded by the NLR SUMM2 (BOX 1), in a process that involves MPK4-dependent phosphorylation of MEKK2/SUMM1 and PAT1, a component involved in mRNA decay[108-110]. Although MPK4 is required for flg22-induced gene transcription[111], expression of constitutively-active MPK4 versions negatively impacted *Arabidopsis* immune responses[112], which complicates our views on the exact role of MPK4 in PTI signalling. One cannot exclude that while conveying PAMP-triggered signalling, MPKs may activate downstream substrates that are themselves negative regulators of PTI, and thus part of a feedback loop

maintaining cellular homeostasis (discussed below). Accordingly, a negative role in PTI was also recently proposed for MPK3[111].

The link between PRR complexes and MAPK cascade activation remains an unsolved riddle. None of the RLCKs currently known to play a role in PTI or any of the above-mentioned CDPKs are required for flg22-dependent MAPK activation[93,113]. However, loss of *PBL27* or *OsRLCK185* specifically impaired MAPK activation in response to chitin but not flg22[80,81]. Whether these RLCKs directly activate MAP3Ks, or act themselves as MAP3Ks to directly phosphorylate MPKKs, remains to be shown. Interestingly, neither PBL27 nor OsRLCK185 are required for chitin-triggered ROS burst[80,81], suggesting that RLCKs have pathway- and ligand-specific roles, and that signalling starts to branch at the level of the PRR complex (**FIG. 2**).

Interestingly, a recent study revealed that protease IV (PrpL) secreted by the bacterial pathogen *Pseudomonas aeruginosa*, with homologues in other bacterial genera, triggers PTI responses in *Arabidopsis*[114]. PrpL activates MPK3 and MPK6 via a heterotrimeric G-protein pathway, where RACK1 acts as a scaffold linking G-protein subunits to all tiers of the MAPK cascade[114]. Importantly, activation of MPK3 and MPK6 by flg22 did not follow the same pathway. How PrpL is perceived by plants, and whether RLCKs are involved in activation of the G-protein–RACK1–MAPK complex, remains to be shown.

Downstream of MAPKs and CDPKs, a number of transcription factors are responsible for immune transcriptional reprogramming, resulting in production of antimicrobial compounds or enzymes, reinforcement of extracellular barriers, for example by deposition of callose at the cell wall, and synthesis of hormones that may induce secondary transcriptional waves[85,115]. Collectively, these responses lead to the establishment of PTI.

Negative regulation of PRR-mediated immunity

Excessive or untimely activation of immune responses lead to development of autoimmune and inflammatory diseases in mammals[25,116,117]. Plants must similarly maintain immune homeostasis, and do so via different strategies to adjust the amplitude and duration of PTI responses. These include limiting the ability of PRRs to recruit their cognate regulatory receptor kinases, regulation of signalling initiation and amplitude at the level of PRR complexes, monitoring of cytoplasmic signal transducing pathways, and control of transcriptional reprogramming (**Fig. 3**). In addition, signalling is integrated into a complex network of hormones and endogenous peptides, which act in a cell-autonomous manner, as well as at the tissue and organ levels (**Fig. 3**). These regulatory mechanisms are, in some cases, hijacked by pathogens, for example through the secretion of proteins or compounds, in order to manipulate the host cell and promote virulence (see **BOX 2**). In the next sections, we address in more detail the molecular mechanisms that fine-tune PTI signalling at these different steps.

Regulation of PRR complexes by pseudokinases.

Pseudokinases account for at least 10% of all human and *Arabidopsis* kinases[118,119]. However, their role and mode of action has only recently started to be understood in mammals[120,121], whereas in plants they remain, for the most part, enigmatic. While canonical kinases mostly act as signalling enzymes through ATP hydrolysis and protein phosphorylation, pseudokinases may represent important signalling regulators by acting as allosteric activators of other kinases, or by promoting or preventing protein–protein interactions[122]. IRAK-M (also known as IRAK3) is a prime example of a pseudokinase that negatively regulates mammalian TLR signalling by controlling the dynamics of TLR–adaptor complexes. During stimulation of TLR4 or TLR9, IRAK-M binds to MyD88-IRAK4 complexes, preventing IRAK1 phosphorylation and subsequent interaction with TRAF6[123]. Expression of

IRAK-M is mostly confined to immune cells and is induced during TLR signalling, which is thought to be necessary for restricting inflammation and cytokine production[124].

In *Arabidopsis,* the LRR-receptor kinase BIR2, which is a pseudokinase, dynamically associates with BAK1[125-127]. Notably, BIR2 negatively regulates BAK1–FLS2 complex formation[125,126]. Binding of flg22 by FLS2 is likely to enhance the affinity of BAK1 towards FLS2 in detriment of BIR2. In the absence of BIR2, the threshold required for FLS2–BAK1 interaction is likely to be lowered and facilitate complex formation. BIR2 is phosphorylated by BAK1 kinase domain *in vitro*[126,127]; whether phosphorylation by BAK1, or other kinase, mechanism accounts for BIR2 dissociation from BAK1 remains to be shown.

Regulation of PRR complex phosphorylation status.

Recruitment of TIR-adaptors upon ligand perception by TLRs creates a platform where kinases, such as IRAK1 and IRAK4, are brought into close proximity, allowing their trans-phosphorylation and activation[128,129]. In plants, PRR activation most likely follows a different approach. The kinase domains of receptor kinases or RLP-SOBIR1 bimolecular PRRs function themselves as platforms for interaction and phosphorylation of regulatory receptor kinases and RLCKs. These kinases form complexes even under resting conditions; nevertheless, signalling is generally only initiated upon ligand recognition. This indicates that the activation of these complexes and subsequent immune signalling relies on a combination of activation mechanisms, as well as on the active release of inhibitory mechanisms (**Fig. 4**), especially since kinases like BAK1 and BIK1 possess strong enzymatic activity[77,130,131]. The prominence of kinases within PRR complexes dictates that their phosphorylation status must be kept under tight regulation, especially by protein phosphatases (**Fig. 4**). The reversible nature of this regulation allows plant cells not only to prevent unintended signalling activation, but also to modulate signalling amplitude and fine-tune immune responses.

It has long been suspected that protein phosphatases were important regulators of plant immunity, as treatment of cell cultures with phosphatase inhibitors was sufficient to initiate responses similar to those triggered by PAMPs[132,133]. Several studies have now revealed that PRRs are negatively regulated by protein phosphatases type 2C (PP2Cs). For example, the rice PP2C XA21-BINDING PROTEIN 15 (XB15) dephosphorylates XA21 *in vitro* and negatively regulates XA21-mediated immune responses[134]. XA21 phosphorylates XB15 *in vitro*[134], but whether this represents a regulatory mechanism remains to be tested. XA21 is further regulated by the ATPase XB24, which is thought to promote auto-phosphorylation of specific XA21 phosphorylation sites to inhibit its kinase activity[135]. The XB15 orthologues in *Arabidopsis* POLTERGEIST-LIKE 4 PLL4 and PLL5 associate with EFR and play a negative role in EFR-mediated responses, demonstrating that PRR regulatory mechanisms are conserved between distantly-related plant species. Another *Arabidopsis* PP2C, KINASE-ASSOCIATED PROTEIN PHOSPHATASE (KAPP), interacts with the FLS2 cytoplasmic domain in yeast two-hybrid assays and its over-expression inhibits flg22 responsiveness[136]. However, the specificity of this action is unclear since KAPP can interact with a number of unrelated receptor kinases[137].

A recent study identified a specific *Arabidopsis* protein phosphatase type 2A (PP2A) holoenzyme, composed of subunits A1, C4, and B'η, that constitutively associates with and negatively regulates BAK1 activity[138]. The activity of the BAK1-associated PP2A was reduced following PAMP perception[138], suggesting that PP2A itself is negatively regulated via a yet-unknown mechanism to allow PRR complex activation. Importantly, treatment with cantharidin, a PP2A-specific inhibitor, was sufficient to induce BIK1 hyper-phosphorylation[138]. This is consistent with previous reports of phosphatase inhibitors spontaneously triggering ROS bursts, and demonstrates that a tight regulation of BAK1 is crucial to prevent unintended activation of downstream RLCKs in the absence of PAMPs.

Interestingly, we could recently reveal that BIK1 phosphorylation is similarly under dynamic regulation. Indeed, the *Arabidopsis* protein phosphatase PP2C38 dynamically associates with BIK1, controls its phosphorylation, and negatively regulates BIK1-mediated responses.

Notably, PP2C38 is phosphorylated upon PAMP perception, presumably by BIK1, which is required for dissociation of the PP2C38–BIK1 complex, and likely to enable full BIK1 activation (D. C., R. Niebergall and C. Z., unpublished observations).

Recently, a 'shotgun' proteomics study identified the MAP3K MKKK7 as part of the FLS2 complex[139]. MKKK7 becomes rapidly phosphorylated in response to flg22 to attenuate MPK6 activation, as well as ROS production, suggesting that it acts at the level of FLS2 complex[139]. Whether MKKK7 controls the phosphorylation status or recruitment of FLS2 interaction partners remains to be addressed.

Regulation of the PRR complex by protein turnover.

Attachment of K48-linked poly-ubiquitin chains is a universally conserved mechanism amongst eukaryotes to selectively mark proteins for proteasomal degradation, and an effective way to control the levels of signalling components in the cell[140,141]. A number of E3 ubiquitin ligases mediate ubiquitination and degradation of TLR signalling components in order to attenuate or shut-down immune signalling[141].

Members of the *Arabidopsis* Plant U-box (PUB) family of ubiquitin E3 ligases are known to negatively regulate PTI responses. Successive disruption of *PUB22*, *PUB23* and *PUB24* in higher-order mutants results in a gradual increase of PTI responses, such as ROS production and immune marker gene expression[142]. PUB22 is stabilized upon flg22 perception and mediates proteasomal degradation of Exo70B2, a subunit of the exocyst complex that is required for PTI responses[143]. How the exocyst complex affects early immune signalling, and whether these ligases have additional substrates required for PTI remains to be addressed. Two other partially redundant members of the same E3 ligase family, PUB12 and PUB13, have been implicated in the degradation of FLS2. Upon flg22 treatment, BAK1 phosphorylates PUB12 and PUB13 promoting their transfer to FLS2, which is then ubiquitinated[144].

Degradation of integral plasma membrane proteins typically follows the endocytic route, which can also be regulated in an ubiquitin-dependent manner. FLS2 and other PRRs undergo ligand-dependent endocytosis, but whether this process is required for sustaining or terminate PRR-mediated signalling, or to allow replenishment of the plasma membrane with newly-synthesized PRRs is still a matter of debate[145]. Mutation of *DRP2b*, a dynamin required for scission and release of clathrin-coated vesicles during endocytosis, partially compromises flg22-induced FLS2 endocytosis[146]. In addition, it enhances flg22-induced ROS production, while rendering plants more susceptible to bacterial infection[146]. Mutants on other components of the endocytic machinery produced similar bacterial susceptibility phenotypes[145]. However, the conclusions taken from these experiments must be carefully considered, as interference with the endocytic routes may affect cargoes other than PRRs themselves involved in PTI signalling and plant immunity[145].

Similarly, it was recently reported that modulation of PTI signalling amplitude in *Arabidopsis* can be achieved by fine-tuning BIK1 protein levels. The *Arabidopsis* Ca^{2+}-dependent protein kinase CPK28 constitutively associates with BIK1 to control its proteasome-dependent turnover[147]. *CPK28* mutants exhibit increased BIK1 levels and PAMP responsiveness, while *CPK28* over-expression results in decreased BIK1 levels and PTI responses[147], suggesting that BIK1 is a rate-limiting factor during PTI signalling. The mechanism by which CPK28 regulates BIK1 turn-over is not yet understood, but it is likely to involve CPK28-dependent phosphorylation of specific BIK1 residues that would facilitate the recruitment of a yet unknown ubiquitin E3 ligase. Intriguingly, the XLG2–AGB1–AGG1(or AGG2) heterotrimeric G protein complex was recently shown to attenuate BIK1 proteasomal degradation and hence modulate PTI activation[95]. Whether the XLG2 complex acts by limiting the access of CPK28 to BIK1, or via a CPK28-independent mechanism, remains to be tested.

Regulation of MAPK signalling cascades.

MAPKs are instrumental for transcriptional reprogramming by directly or indirectly controlling the activity of transcription factors following PAMP perception[85,115,148,149]. Thus, the actions of MAPKs must be also controlled. Phosphorylation of both Tyr and Thr residues in their activation loop is critical for MAPK activation; consequently, dephosphorylation of any of these residues renders them inactive[150]. Dual-specificity protein phosphatases (DUSPs, also known as MAPK phosphatases (MKPs)) dephosphorylate both these residues and are important modulators of MAPK activity during innate immunity in mammals[149,150]. In *Arabidopsis*, DUSPs, as well as protein Tyr phosphatases (PTPs) and protein Ser/Thr phosphatases (in particular PP2Cs) also target PRR-activated MAPKs.

The closely related PP2Cs AP2C1 and PP2C5 regulate PRR-dependent MPK3 and MPK6 activation. Single or double mutations of *AP2C1* and *PP2C5* enhanced MPK3 and MPK6 phosphorylation in response to elf26[151], while *AP2C1* over-expression abolished their activation in response to flg22 and oligogalacturonides, compromising MPK3- or MPK6-dependent gene induction and induced resistance to the necrotrophic fungus *B. cinerea*[152]. In addition to its effects on MPK3 and MPK6, AP2C1 was shown to inactivate MPK4 *in vivo*[153].

The DUSPs MKP1 and PTP1 regulate MPK3 and MPK6 in a partially redundant manner. Mutation of *MPK1* increased elf26-dependent responses and decreased bacterial susceptibility, which correlated with enhanced MPK3 and MPK6 activation[154]. Intriguingly, *MKP1* mutation in *Arabidopsis* ecotypes possessing the NLR SCN1 produces an autoimmune phenotype, which is further aggravated by mutation of *PTP1*[155]. This phenotype can be partially rescued by mutating *MPK3*, *MPK6* or *SCN1*, suggesting that the effects of MAPK activation and/or the integrity of the MKP1 pathway may be monitored by a SCN1-dependent pathway[155]. In addition, MPK2 could dephosphorylate both MPK3 and MPK6 *in vitro*; however, *MKP2* over-expression only strongly affected activation of MPK3, but not of MPK6, during the early stages of *B. cinerea* infection[156]. Together, this demonstrates the importance of protein phosphatases in the regulation of MAPKs and immune responses;

however, a more systematic biochemical and functional characterization is required to fully address their role in PTI signalling.

Regulation at the transcriptional level.

Several mechanisms are in place that negatively regulate adequate activation of immune-related genes. Plant-specific WRKY transcription factors have been particularly associated with plant immunity. For example, *Arabidopsis* WRKY33 is responsible for PAMP-induced production of the antimicrobial phytoalexin camalexin[148]. WRKY33 is maintained in an inhibitory complex by MPK4 and the VQ motif-containing protein (VQP) MKS1[157]. Upon flg22 perception, MPK4 phosphorylates MKS1 and releases the MKS1–WRKY33 complex[157], allowing WRKY33 to be phosphorylated and activated by MPK3 and MPK6[158,159]. Interestingly, several other VQPs interact with different WRKYs and are substrates of MPK3 and MPK6, suggesting these proteins are a widespread mechanism that regulates WRKY-dependent gene transcription[85,160-162]. Consistently, over-expression of MPK3/MPK6-targeted VQP1 (MVQ1) inhibits PAMP-induced and WRKY-dependent expression of the immune marker gene *NHL10*, and abolishes PAMP-induced resistance to *P. syringae*[162]. Importantly, phosphorylation by MPK3 and MPK6 upon flg22 treatment destabilizes MVQ1, thus releasing WRKYs from MVQ1-imposed inhibition. Interestingly, other VQPs, such as SIGMA FACTOR BINDING PROTEIN 1 (SIB1) and SIB2, can stimulate the DNA-binding affinity of WRKY33[163]. How different combinations of VQPs and WRKYs interact with MAPKs to regulate transcription during PTI is a challenge to be addressed in the future.

Arabidopsis ASR3 is a plant-specific trihelix transcription factor that acts as a transcriptional repressor during PTI[164]. Accordingly, *asr3* mutants showed enhanced flg22-induced gene expression and increased resistance to *P. syringae*, while early PTI outputs, such as ROS production or MAPK activation were unaffected. Remarkably, phosphorylation of ASR3 by MPK4 upon flg22 elicitation enhances its DNA affinity. With this action, MPK4 promotes

binding of ASR3 to the promoter regions of flg22-upregulated genes, such as *FRK1*, initiating a negative feedback mechanism to fine-tune immune gene expression.

Transcriptional regulation during PTI may also be achieved by direct regulation of the C-terminal domain (CTD) of the largest RNA polymerase II subunit. The CTD is composed of several repeats and is subject to post-translational modifications that ultimately determine its activity[165]. The CTD is phosphorylated in response to different PAMPs by cyclin-dependent kinases C (CDKCs), which are activated by MAPK cascades[166]. In turn, the CTD phosphatase-like protein CPL3, which was identified in a mutant screen as a negative regulator of early PAMP-induced gene expression, dephosphorylates the CDKC-activated CTD to repress transcription[166]. How CPL3 activity is regulated in the context of PTI signalling remains to be addressed; nonetheless this study elegantly demonstrated that coordination between the MAPK–CDKC module and CPL3 dictates the CTD phosphorylation status, and underpins gene activation during PTI.

Attachment of poly(ADP-ribose) (PAR) chains to target proteins is a common post-translational modification catalysed by PAR polymerases (PARPs) in eukaryotes that regulate important cellular processes, such as DNA repair, gene transcription and chromatin remodelling, particularly during stress, including inflammatory responses in mammals[167]. PARP2 accounts for most of *Arabidopsis* PARylation activity in response to DNA damage-inducing agents[168], and its activity is enhanced following flg22 treatment[169]. Consistent with a positive role of PARylation in PTI signalling, *parp1 parp2* double mutants are compromised in flg22-induced gene induction and immunity against *P. syringae*, but not in early PTI responses[168,169]. PARylation can be reverted by the action of PAR glycohydrolases (PARGs). PARG1 was found to negatively regulate PAMP-induced gene transcription in the same mutant screen that identified CPL3[169]. Although their targets remain elusive, it is now evident that the combination of PARP and PARG activities determines the outcome of transcriptional reprograming during PTI.

Regulation by hormones and endogenous peptides.

The plant immune system is highly regulated by a complex network of hormones that integrates both external and internal cues to maintain homeostasis and coordinate immune responses at the spatial and temporal levels[170]. Hormones may act downstream of immune-recognition events and/or modulate immune signalling by controlling the basal levels of signalling components in the cell. Yet, the events leading to up- or down-regulation of hormone biosynthesis following PAMP recognition remain largely unknown.

Salicylic acid and jasmonic acid represent the two major immune-related hormones, and often act antagonistically[170]. Salicylic acid positively regulates basal FLS2 levels and consequent flg22-triggered responses[171,172]. Conversely, jasmonic acid has a negative impact on FLS2-mediated responses, such as ROS burst and callose deposition[171]. Whether this effect is due to perturbation of FLS2 accumulation and/or a reflection of the jasmonic acid–salicylic acid antagonism remains to be shown. Remarkably, several pathogenic *P. syringae* strains produce the phytotoxin coronatine (COR), a structural mimic of a bioactive jasmonic acid conjugate, as well as effector proteins that directly activate jasmonic acid signalling[173]. Consequently, this suppresses salicylic acid signalling and inhibits typical PTI responses, such as stomatal closure and cell wall reinforcement[173].

A third hormone produced by plants during pathogen attack, ethylene, is important for *FLS2* transcription by controlling the activation of its promoter by the ethylene-responsive transcription factor EIN3[174]. Ethylene plays both antagonistic and synergistic roles in its relationship with salicylic acid, while mostly being synergistic to jasmonic acid[170].

Surprisingly, biosynthesis of all three hormones is increased following flg22 perception[175-177]. Jasmonic acid production seems to be required for flg22-dependent induction of the AtPep1–PEPR1/PEPR2 pathway[177], which further strengthens PTI responses. In turn, this pathway is synergistically activated by ethylene and salicylic acid during elf18-triggered responses[178].

Several growth-promoting hormones have been associated with plant immunity. For example, auxin is known to antagonize salicylic acid signalling, and some plant pathogens have evolved to hijack and use auxin signalling to their advantage[179]. Although concrete data is still missing, such an effect on salicylic acid signalling is likely to negatively influence the levels of PTI signalling components. Accordingly, the microRNA *miR393* is induced upon flg22 perception and targets the auxin receptors to inhibit auxin signalling and alleviate its antagonism on salicylic acid signalling[180,181].

In turn, cytokinins may stimulate salicylic acid signalling and boost immunity[179]; however, many pathogens are known to tamper with cytokinin signalling and to produce cytokinins in order to induce susceptibility[182]. The most remarkable example is perhaps *Agrobacterium*, which manipulates cytokinin and auxin signalling to induce nutrient re-allocation and tumour formation[183]. Moreover, it was recently shown that activation of cytokinin signalling by the *P. syringae* effector HopQ1, or by exogenous cytokinin application, inhibits PTI via repression of *FLS2* transcription[184]. This however contradicts a previous report showing that cytokinin treatment enhanced resistance against *P. syringae*[185], a conflict that may lie on the hormone dosage.

Importantly, brassinosteroids can inhibit PTI responses[186,187], in a process that is mainly mediated by the transcription factor BRASSINAZOLE-RESISTANT 1 (BZR1)[188]. Furthermore, the transcription factor HBI1, which is itself a transcriptional target of BZR1, also negatively regulate PTI signalling, while being a positive regulator of brassinosteroid signalling[189,190]. A model has been proposed where BZR1 integrates brassinosteroid and gibberellin signalling, as well as environmental cues, such as light or darkness, to inhibit PTI via activation of a set of WRKY transcription factors that negatively regulate immunity[188,191]. Interestingly, the expression of brassinosteroid biosynthetic genes is rapidly inhibited following PAMP perception[192], revealing a complex bi-directional negative crosstalk between PTI and brassinosteroid signalling.

An additional layer of complexity is brought about by the growth-promoting endogenous tyrosine-sulphated PSKα and PSY1 peptides, which negatively regulate several PTI responses[193,194]. Perception of PSKα and PSY1 is mainly attributed to the LRR-receptor kinases PSKR1 and PSY1R, respectively, which are both transcriptionally up-regulated upon PAMP perception[193,194], generating a feedback loop that opposes immunity and promotes growth.

Plant hormones make up a flexible and robust system that feedbacks, either positively or negatively, on immune signalling, and is capable of responding against pathogenic threats, while maintaining homeostasis. A parallel could be drawn between plant hormones and pro- and anti-inflammatory cytokines that regulate inflammatory responses during mammalian innate immunity, and are critical to avoid autoimmunity. In particular, IL-10 negatively regulates TLR signalling primarily by controlling transcription of TLR-induced genes[195]. In plants, such a role could be attributed to brassinosteroids and to the endogenous peptides PSKα and PSY1.

Perspectives and future challenges

PRR-triggered immunity is emerging as a highly complex and tightly regulated process. PRRs dynamically associate with different co-receptors, regulatory receptor kinases and RLCKs to initiate immune signalling. Increasing evidence suggests that immune signalling already branches at the PRR complex level, leading to the activation of distinct responses. The underlying molecular mechanisms are not yet fully comprehended, but the diversity of PRR-associated RLCKs is likely to play an important role. Understanding how immune signalling is generated at the cell surface will most likely require multi-disciplinary approaches to help deciphering the macromolecular composition and dynamics of functional PRR complexes, how they are organized at the plasma membrane, and how phosphorylation events, as well as other post-translational modifications, are employed to

activate/regulate PRR-associated signalling components. Moreover, it will be interesting to investigate how the different regulatory mechanisms described in this review work together to integrate immune signalling generated by distinct PRRs. Together with the identification of novel ligand-PRR pairs, such knowledge will provide the foundation to engineer PRR-based broad-spectrum disease resistance into important crops and help with developing a more sustainable agriculture.

BOX 1: Guarding the goods

The activation of plant NLRs occur either as a result of direct recognition of pathogen-secreted effectors, or by detecting effector-mediated manipulation of host components involved in immune signalling or mimic thereof (including ‘sensor’ or ‘decoy’ domains integrated within NLRs)[2,7,9-12]. Consequently, genetically induced loss or alteration of these ‘guardees’ can inadvertently cause NLR activation and autoimmunity. As such, genes whose mutation caused these autoimmune phenotypes could be classified a negative regulators of immunity, *e.g. MPK4*[107]. However, with the identification of NLR mutants (eg. *summ2*[110]) that suppress these autoimmune phenotypes, it is now postulated that many important immune components are actually ‘guarded’, leading to misinterpretation of results solely based on loss-of-function genetic eveidence[60]. Accordingly, while clearly involved in PTI as positive regulators, loss of BAK1, BIK1 and RBOHD, for example, in addition to MPK4, leads to autoimmune phenotypes. Interestingly, many effectors secreted by plant pathogenic bacteria redundantly target PTI components including BAK1, BIK1 and MAPKs[196]. Thus, the identification of the NLRs that guard these important immune components is of great interest, not only for academic reasons, but also as they may enable us to engineer disease resistance against the important pathogens that secrete these effectors.

BOX 2: Manipulation of plant pattern-triggered immunity by bacterial effectors

A common feature of Gram-negative pathogenic bacteria is the use of the type III secretion system (T3SS) to inject effector proteins (virulence factors) directly into host cells. These effectors manipulate host cells to the pathogen advantage, and can suppress plant immunity by targeting key signalling components[196].

Similar to host phosphatases that negatively regulate PRR complexes, bacterial effectors interfere with the phosphorylation status of PRR complexes to block the early steps of PTI signalling. The *P. syringae* effector AvrPto acts as general kinase inhibitor, targeting receptor kinases, such as FLS2 and EFR, to inhibit PTI responses triggered by multiple PAMPs[197,198]. Another *P. syringae* effector, HopAO1, displays tyrosine phosphatase activity and inhibits elf18-triggered immunity by dephosphorylating EFR tyrosine residues[199]. The *Xanthomonas campestris* effector AvrAC possesses a previously uncharacterized uridylyl transferase activity that modifies key phosphorylation sites of several RLCKs, including BIK1, to block their kinase activities and thus PTI signalling[113]. Remarkably, *Arabidopsis* detects AvrAC virulence by using the decoy substrate PBL2, which is guarded by the NLR ZAR1[200]. Additionally, the *X. oryzae* effector Xoo1418, of unknown enzymatic function, interacts with several rice RLCKs and prevents CERK1-dependent phosphorylation of OsRLCK185, suppressing both PGN- and chitin-triggered immune responses[80].

HopAI1 from *P. syringae* permanently inactivates MAPKs by removing the phosphate group of phospho-threonines[201]; however, its action on *Arabidopsis* MPK4 is recognized by the NLR SUMM2[110]. In addition, HopF2, also from *P. syringae*, ADP-ribosylates and inactivates MKK5 to prevent downstream activation of MPKs[202].

Some bacterial effectors target immune signalling components for degradation: the *P. syringae* cysteine protease AvrPphB cleaves BIK1 and other PBLs[76], which effect can be recognized by the NLR RPS5[203,204]; whereas AvrPtoB functions as an ubiquitin E3 ligase to promote degradation of FLS2, EFR and CERK1[72,205,206]. Additionally, AvrPtoB can also act as a kinase inhibitor to inactivate BAK1 and the tomato orthologue of AtCERK1, SlBti9[207,208].

Several bacterial effectors subvert jasmonic acid signalling in their favour. RIN4 is an intrinsically disordered protein conserved across plants and was recently found to play an

important role in jasmonic acid signalling and stomatal opening by regulating the H^+-ATPase AHA1[209,210]. Interestingly, a number of effectors have been found to target RIN4, but *Arabidopsis* RIN4 is guarded by two NLRs, RPS2 and RPM1[211-213]. In addition, the *P. syringae* effectors HopZ1a and HopX1 promote degradation of JAZ proteins, the key repressors of jasmonic acid signalling[214,215].

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Figure Legends

Figure 1. Recruitment of regulatory receptor kinases and RLCKs by PRRs in *Arabidopsis* and rice.
PRRs recruit different regulatory receptor kinases according to their ectodomain. In addition, RLCKs are specifically recruited to different PRR complexes. (**A**) In *Arabidopsis*, BAK1 (also known as SERK3), related SERKs and AtCERK1 are recruited upon ligand perception by LRR-receptor kinases and LysM-receptor kinases/RLPs, respectively. Constitutive bi-molecular LRR–RLP–SOBIR1 complexes recruit BAK1 and SERKs upon ligand binding. No regulatory receptor kinases interacting with the LPS-perceiving LORE S-Lectin-receptor kinase have yet been identified. BIK1 is a convergent point for multiple PRR pathways. (**B**) In rice, OsCERK1 is recruited by the LysM-RLPs CEBiP and LYP4/LYP6 upon ligand perception. XA21 constitutively associates with the BAK1 orthologue OsSERK2.

Figure 2. Early branching of PTI signalling.
PAMP perception by PRRs induces immune signalling that branches immediately downstream of the PRR complex, such as ROS production and MAPK cascades. PRRs rely on distinct mechanisms to activate such pathways. For example, the RLCK PBL27 is required for MAPK cascade activation during AtCERK1-dependant responses. The PBL21 orthologue in rice, OsRLCK176, together with OsRLCK185, are required for chitin-mediated MAPK activation. Perception of bacterial Protease IV (by a yet unknown mechanism) triggers MAPK activation via a heterotrimeric G-protein complex composed by GPA1-AGB1-AGG1/AGG2. FLS2-mediated MAPK activation does not follow any of these routes, and remains an unsolved riddle. The RLCK BIK1 (and related PBL1) phosphorylates the NADPH oxidase AtRBOHD on specific sites to activate ROS production after flg22 and chitin perception. In addition, BIK1 and PBL1 are required to initiate a cytoplasmic Ca^{2+} burst; however the source of Ca^{2+} and the identity of the channels involved remain elusive. The FLS2-associated heterotrimeric G-protein complex composed by XGL2-AGB1-AGG1/AGG2 also contributes to ROS production, by controlling BIK1 protein levels and possibly through direct activation of RBOHD by XLG2, which is phosphorylated by BIK1. The RLCKs BSK1 and PCRK1 are also required for flg22-dependent ROS production. In rice, chitin-triggered ROS production requires the small GTPase OsRac1, which is activated by the guanine nucleotide exchange factor OsRacGEF1.

Figure 3. Negative regulation of PTI signalling by a multi-layered system.
The *Arabidopsis* FLS2-dependent pathway is used here to illustrate PTI signalling. At the cell surface, formation of the FLS2-BAK1 heterodimer can be inhibited by the action of LRR-receptor kinases that are pseudokinases, such as BIR2. In the cytoplasm, the signalling output of the PRR complex is modulated through regulation of its phosphorylation status and by protein turnover. Downstream signalling transducers, such as MAPKs have their activity modulated by several phosphatases; mechanisms negatively regulating CDPKs are currently unknown. Transcriptional reprograming is mediated by transcription factors (TFs). WRKYs may be kept in inhibitory complexes, for example by VQPs. In turn, negatively-acting TFs are activated by MAPKs to repress transcription of defence-related genes, in a negative feedback that fine-tunes signalling. The CTD domain of RNA polymerase II (Pol II) is phosphorylated upon PAMP recognition, an action that can be reversed by phosphatases to

modulate the polymerase activity. PTI signalling is integrated in a network of plant hormones that regulates the transcription of defence-related genes and of key PTI signalling components (for example *FLS2*). The biosynthesis of these hormones is repressed or enhanced by the PTI signalling pathway. BRs – brassinosteroids; ET – ethylene; JA – jasmonic acid; SA – salicylic acid.

Figure 4. Negative regulation at the PRR complex level.
The Arabidopsis FLS2-flg22 and rice XA21-RaxX systems are used here as representative models for plant PRR regulation. (**A**) The pseudokinase BIR2 inhibits BAK1 interaction with FLS2; upon flg22 perception BIR2 dissociates from BAK1. In the absence of stimuli, the phosphorylation status of PRR complex components is regulated by different phosphatases: the PP2C KAPP negatively regulates FLS2; PP2A controls BAK1. Following flg22 perception, PP2A is transiently inactivated by an unknown mechanism. Basal BIK1 levels are controlled by CPK28-mediated phosphorylation of BIK1 residues that facilitate its proteasomal degradation. In turn, the heterotrimeric G protein complex XLG2-AGB1-AGG1/AGG2 counteracts BIK1 proteasomal degradation by a yet unidentified mechanism. BAK1 phosphorylates the E3 ligases PUB12 and PUB13 in a flg22-dependent manner, which in turn ubiquitinate and target FLS2 for degradation, likely via the endocytic route; whether FLS2 degradations contributes to PTI negative regulation remains a matter of debate. (**B**) In rice, the PP2C XB15 dephosphorylates XA21 and the ATPase XB24 promotes autophosphorylation of inhibitory XA21 residues. During *Xoo* infection, XB24 dissociates from XA21. XB15 is phosphorylated by XA21, but the relevance of this modification is not clear.

Glossary

Receptor kinases: Plasma membrane-localized proteins characterized by a ligand-binding ectodomain, a single-pass transmembrane domain and an intracellular signalling kinase domain. Different types of ectodomain determine their ligand-binding specificity. Receptor kinases may act as the main receptor, or as co-receptor or regulatory protein.

Receptor-like proteins (RLPs): Surface-localized proteins similar to receptor kinases but lacking an obvious intracellular signalling domain. RLPs typically require regulatory receptor kinases to initiate signalling.

Plasmodesmata: Intercellular cytoplasmic bridges equivalent to Gap junctions that allow communication and transport of molecules between plant cells. During pathogen infection, plasmodesmata can be sealed by deposition of callose layers to isolate infected areas.

Callose: (1,3)-β-glucan polymer present in the plant cell wall. Deposition of callose occurs upon pathogen recognition, forming cell wall thickenings.

Stomata: Natural openings in the leaf epidermis formed by two guard cells that enable gaseous exchange, and are often used by pathogenic microbes to enter the leaf.

Phytoalexin: Antimicrobial compounds produced by plants during pathogen infection.

EF-hand motifs: Helix-loop-helix protein motifs involved in Ca^{2+}-binding.

Exocyst complex: An octameric complex involved in the tethering of exocytic vesicles to their site of fusion in the plasma membrane.

Camalexin (3-thiazol-2′-yl-indole): Typical *Arabidopsis* phytoalexin produced in response to pathogen infection.

VQ proteins (VQPs): Class of plant-specific proteins with a conserved FxxΦVQxΦTG amino acid motif (VQ motif; x representing any amino acid and Φ hydrophobic residues).

Salicylic acid: Phenolic plant hormone with a major role in plant defence against biotrophic pathogens. Its acetylated form (acetylsalicylic acid) is commonly known as aspirin, a widely prescribed anti-inflammatory drug.

Jasmonic acid: Best-studied member of the jasmonates family of oxylipin plant hormones. Jasmonates are typically synthesized during responses against necrotrophic pathogens and herbivores.

Auxin: Class of plant growth hormones, existing mostly as free or conjugated forms of indole-acetic acid (IAA), a tryptophan derivative. Auxin plays a pivotal role in various key developmental processes, such as cell expansion and division, root and stem elongation, and flowering.

Cytokinins: Class of plant growth hormones derived from adenine known to promote cell division and differentiation.

Brassinosteroids: Class of polyhydroxysteroid plant hormones required for several developmental and physiological processes. Brassinosteroids are perceived at the cell surface by the LRR-receptor kinase BRI1, which recruits the co-receptor BAK1 to initiate brassinosteroid-mediated signalling.

Gibberellin: Gibberellins are diterpene-type plant growth hormones involved in several developmental processes, such as seed germination, stem elongation and fruit maturation.