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Non-arginine-aspartate (non-RD) kinases are associated with innate immune receptors that recognize conserved microbial signatures

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An important question in the field of plant–pathogen interactions is how the detection of pathogens is converted into an effective immune response. In recent years, substantial insight has been gained into the identities of both the plant receptors and the microbial molecules they recognize. Likewise, many of the downstream signaling proteins and transcriptions factors that activate defense responses have been characterized. However, the early molecular events that comprise 'recognition' and how defense signaling specificity is achieved are not as well understood. In this review we discuss the significance of non-arginine-aspartate (non-RD) kinases, a subclass of kinases that are often found in association with pattern recognition receptors (PRRs).

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Introduction

Plant and animal innate immune systems depend on a diverse assortment of cell surface and cytoplasmic receptors that detect and respond to invading pathogens. In plants, these receptors are commonly classified into a group that recognizes conserved microbial signatures (called pattern recognition receptors, PRRs) and a group that recognizes highly variable effectors (nucleotide binding site-leucine rich repeat receptors, NBS-LRRs) [1–3]. The first group contains both extracellular membrane bound receptors and intracellular receptors that frequently contain (or associate with) non-arginine-aspartate (non-RD) kinases. The second group includes intracellular NBS-LRR receptors, which are often fused to additional domains but typically lack kinase domains. These two systems of microbial perception in plants are commonly referred to as PTI (Pathogen Associated Molecular Pattern [PAMP] Triggered Immunity) and ETI (Effector Triggered Immunity), respectively [1]. Understanding how microbial signals are converted into PTI or ETI remains a fundamentally important issue.

Most plant and animal PRRs identified to date either contain kinase domains or associate with kinases via adaptor molecules [4]. From a simplistic viewpoint, kinases serve as switches that are turned on or off via conformational changes induced by ligand binding. One critical regulatory feature of kinases is the activation loop, which becomes phosphorylated and structurally reoriented to enable substrate access and/or to enhance phosphotransfer efficiency [5]. We previously found that most PRR kinases or PRR-associated kinases contain a change in a conserved arginine (R) located adjacent to the key catalytic aspartate (D) (the so-called RD motif) that facilitates phosphotransfer [6]. This positively charged R residue typically lies within a charge cluster that inhibits catalysis by the neighboring negatively charged D residue. This inhibition can be removed by phosphorylation of the kinase activation loop that lies in close proximity to the RD motif (between kinase subdomains VII and VIII). Activation loop phosphorylation produces negatively charged phospho-amino acids that neutralize the positively charged R residue resulting in kinase activation [6]. Plant and animal kinases associated with recognition of conserved microbial signatures lack the R, in its place having an uncharged residue such as Cys, Gly, Phe, or Leu. Such kinases are referred to as non-RD [7]. The functional significance of non-RD motifs and whether or not charge neutralization plays a role in the catalytic activity of non-RD kinases is currently unknown.

To date, approximately 75 plant receptor-like kinases (RLKs) have been functionally characterized. Nearly one dozen of these are non-RD kinases, all of which have known or putative functions in the recognition of conserved microbial signatures characteristic of PRRs (Figure 1). Unlike their more common RD counterparts, non-RD kinases do not generally auto-phosphorylate the activation loop; presenting a potential mechanistic difference in their activation and/or function [7]. Such changes to the RD motif are likely adaptive changes that may reflect distinctive properties of PRR-mediated signaling.

Figure 1

Kinase	Class	Subfamily	Plant	Pathogen	Reference(s)
LRK10	non-RD	LRK10L-2	Wheat	Fungal	[71]
PR5K	non-RD	LRK10L-2	*Arabidopsis*	?	[72]
TaRLK 1,2,3	non-RD	LRK10L-2	Wheat	Fungal	[73]
BSR1	non-RD	LRK10L-2	Rice	Fungal/bacterial	[74]
XA21	non-RD	LRR XII	Rice	Bacterial	[14]
XA26	non-RD	LRR XII	Rice	Bacterial	[75]
FLS2	non-RD	LRR XII	*Arabidopsis*	Bacterial	[15]
EFR	non-RD	LRR XII	*Arabidopsis*	Bacterial	[16]
DS1	non-RD	LRRXII	*Sorghum*	Fungal	[76]
CERK1	RD	LysM-I	*Arabidopsis*	Fungal/bacterial	[44]
RPG1	non-RD	RLCK-OS2	Barley	Fungal	[25]
Pi-d2	non-RD	SD-2b	Rice	Fungal	[77]
LecRK1	non-RD	SD-2b	tobacco	Hornworm	[78]
NgRLK1	non-RD	SD-2b	tobacco	Fungal	[79]
WKS1	non-RD	WAKL-OS	Rice	Fungal	[29]
RLP class PRRs					
CEBiP	none	LysM RLP	Rice	Fungal	[49]
LYM1/3	none	LysM RLP	*Arabidopsis*	Bacterial	[47]
Ve1	none	LRR RLP	Tomato	Fungal	[38]
LeEIX1/2	none	LRR RLP	Tomato	Yeast	[39]
DAMP Receptors					
WAK1	RD	WAK	*Arabidopsis*	Oligogalacturonides	[57]
PEPR1	RD	LRR-XI	*Arabidopsis*	Plant peptide	[54,55]
THESEUS	RD	CrRLK1L-1	*Arabidopsis*	?	[56]
ETI Receptors					
RPG5	Non-RD	NBS-LRR	Wheat	Fungal	[64]

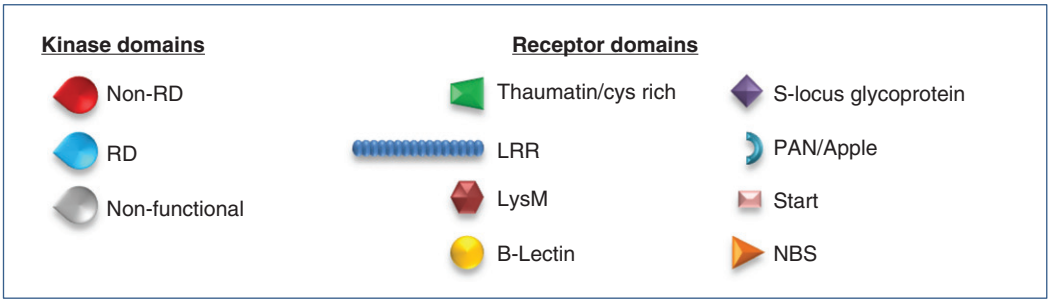

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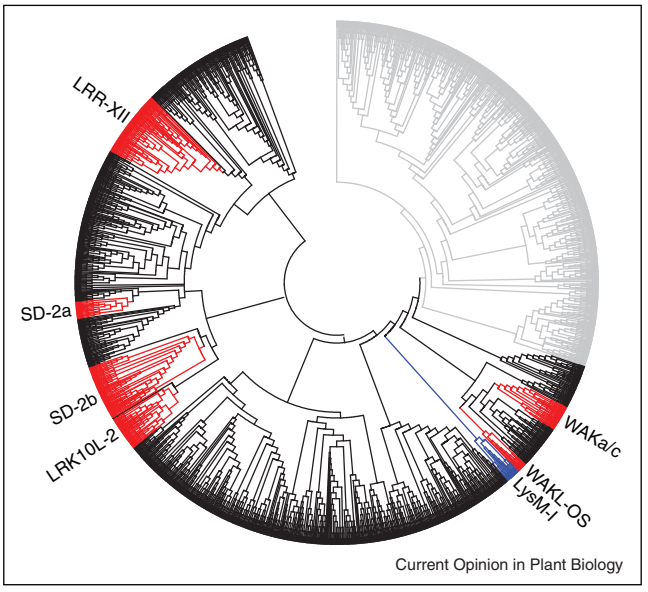
Table of known and putative PRR receptors along with their domain organizations and kinase functionality (when applicable) [71–79].

Evolution of plant PRR kinases

The kinase domains found in plant PRRs belong to the same family as those used by their animal counterparts. This kinase family has been dubbed the IRAK/Pelle family in animals (after the human and fly members) and the RLK/Pelle family in plants [8,9]. Animals have very few IRAK/Pelle kinases. Humans have four and flies have just one [10]. Nevertheless, they are still central to both innate immune signaling and development. But unlike plants, in animals, the IRAK/Pelle kinases are all cytoplasmic and lack receptor domains. For example, the cytoplasmic non-RD kinase IRAK1 physically associates with both membrane bound and cytoplasmic PRRs via adaptor proteins to transduce defense signals. A series of phosphorylation events trigger the formation of secondary IRAK1 signaling complexes that dissociate from the receptor complex and ultimately lead to activation of the transcription factor NFkB and mobilization of the innate immune response [11,12•].

The RLK/Pelle family in plants underwent a dramatic expansion during early in plant evolution. Concurrent with this expansion, the IRAK domain was fused to numerous extracellular receptor and signaling domains to produce an extremely large repertoire of genes [9,13••]. The Arabidopsis genome encodes over 600 proteins that contain IRAK kinase domains (76% RLKs) while the rice genome encodes over 1000 (83% RLKs) [4,9]. Of these, IRAK kinases with non-RD motifs make up a minority portion [47 in Arabidopsis (8%) and 371 in rice (35%)] yet are abundant in a handful of subfamilies that contain many of the known PRRs. Collectively these PRR subfamilies do not form a distinct clade and are instead scattered among numerous receptor and cytoplasmic kinase clades involved in a wide range of biological functions (Figure 2). Thus, the phylogenetic history of these genes suggests that PRR function probably evolved multiple times. Kinases that function as PRRs or in association with PRRs are nearly indistinguishable from those that control numerous other biological processes, both with respect to their structure and their evolutionary history. To date, the only distinguishing feature that has been identified is the non-RD motif [4]. This implies that there is the potential for kinases that normally function in other processes to adopt PRR functions. It stands to reason that upon adopting a PRR function, additional fine-tuning would be required either through the swapping of kinase domains via recombination or the accumulation of mutations in residues associated with kinase function and/or protein–protein interactions. The finding that many PRRs have adopted changes in the otherwise highly conserved RD kinase motif is probably a consequence of such adaptations. This strict association between the presence of the non-RD motif in kinases associated with PRRs suggests that PRR signaling may have unique requirements that differ from other biological surveillance programs.

Figure 2



PRR RLK subfamilies carrying non-RD kinase domains do not form a unified clade and are scattered among numerous RLK clades involved in various biological functions. Depicted is a reconstructed topological phylogenetic tree from [4] using all known Arabidopsis and rice kinase sequences. RLK/Pelle kinase nodes are in black while all non-RLK/Pelle kinase families are shown in gray. Non-RD PRR subfamilies are colored red and labeled. The LysM-I family containing the RD kinase CERK1 is shown in blue.

Known PRRs contain non-RD kinases

Based on both the presence of non-RD kinase motif and whether or not the subfamilies had undergone lineage specific expansion, we previously predicted that a handful of the >50 RLK subfamilies in rice (ca. 37% of RLKs) and *Arabidopsis* (ca. 7.5% of RLKs) function as PRRs [4]. These include the subfamilies LRR-XII, SD-2a, SD-2b, RLK10L-2 (also known as PR5K), WAK (non-RD subgroups a, c), and WAKL-OS. Since that time, additional confirmed or putative PRRs have been identified for the LRR-XII, SD-2b, RLK10L-2, and WAKL-OS RLK subfamilies (Figure 1). Surprisingly, the largest of these subfamilies including LRR-XII, SD-2b, and RLK10L-2 have remained small in Arabidopsis (10, 7, and 13 genes, respectively) relative to rice and poplar that contain >40–100 genes for each of these three subfamilies [4,13••]. Both the LRR-XII and SD-2b subfamilies are more ancient and have also remained relatively small in the more primitive bryophyte *Physcomitrella patens* that has 4 LRR-XII and 13 SD-2b kinases [13••].

Differences in the expansion rates of RLK subfamilies associated with immune and/or stress responses have been attributed to diversifying selection pressures such as those imposed by pathogens, though substantial direct functional evidence is lacking [4,8,9,13••]. The most well

characterized PRR subfamily is LRR XII that includes several known rice and Arabidopsis PRRs including *Xanthomonas* resistance 21 (XA21), Flagellin-Sensing 2 (FLS2), and Elongation Factor-Tu Receptor (EFR) [14–17]. Functional orthologs of Arabidopsis FLS2 have been found in rice, tomato, and tobacco [18–20]. While these orthologs all detect flagellin, their precise specificities vary with regard to the epitope and bacterial strain. The closely related PRR, XA21 recognizes the conserved sulfated protein, Ax21, which is produced by the bacterial pathogens in the genera *Xanthomonas*. Ax21 plays a critical role in bacterial communication (aka 'quorum sensing') [21]. Arabidopsis FLS2 recognizes exogenously applied Ax21 peptide derivatives [22]. However, the converse is not true. Rice varieties lacking Xa21 but containing FLS2 do not recognize Ax21. The observation that flagellin and Ax21 perception require distinct receptors in rice suggest that PRRs have diversified in rice as compared with Arabidopsis [3,14,22]. However, it is currently unclear how many different ligands each of these receptors are capable of recognizing and just how specialized they really are. Arabidopsis FLS2 was recently shown to recognize the Clavata 3-peptide (CLV3-p), which is also perceived by the CLV1 and CLV2 RLKs to maintain homeostasis of the shoot apical meristem (SAM). [23••]. CLV3-p recognition by FLS2 in the SAM renders the stem cells immune to pathogen infection. This finding suggests that each LRR-XII receptor may be able to recognize more than one ligand. The animal PRR counterparts, called Toll-like receptors (TLRs), also carry extracellular LRR domains and recognize a diverse array of pathogen and host ligands. Structural studies have shown that each of the variable repeats among the extracellular LRRs of TLRs can possess unique ligand binding specificities [24••]. Further characterization of plant LRR-XII PRRs should shed light on why PRR clades have expanded in some plants and not others.

Cytoplasmic non-RD PRRs

While most identified PRRs are membrane bound RLKs, some cytoplasmic non-RD kinases also appear to meet the criteria for PRR functions. For example, barley Rpg1 confers broad-spectrum resistance to stem rust caused by the fungal pathogen *Puccinia graminis* [25]. Rpg1 lacks a canonical receptor domain and predominantly resides in the cytoplasm [26]. It consists of two tandem kinase domains; a catalytically active non-RD kinase domain that is preceded by a catalytically inactive kinase domain. Mutations in either kinase domain abrogate resistance function [27]. Nirmala *et al.*, 2011 identified two conserved *Puccinia* proteins that, when applied in combination, were capable of triggering Rpg1 mediated resistance. Rpg1 was found to interact with both proteins *in vitro* suggesting that despite the lack of an identifiable receptor domain, Rpg1 still functions as a PRR [28]. In another example, wheat Kinase-Start 1 (WKS1) was identified as conferring broad-spectrum resistance in a temperature dependent fashion to the fungal pathogen *Puccinia striiformis* that causes wheat stripe rust. WKS1 is also a cytoplasmic protein that carries a non-RD kinase domain and a START lipid/sterol binding domain similar to the previously identified Enhanced Disease Resistance 2 (EDR2) protein from Arabidopsis [29]. While the mechanism with which WKS1 confers resistance is still unknown, these examples suggest that PRR function is not limited to detection of extracellular conserved microbial signatures.

Cooperation between RD and non-RD kinases

In humans, the RD kinase IRAK4 is recruited to PRR receptor complexes upon PAMP perception and is required for activation of the non-RD kinase IRAK1. While not yet confirmed *in vivo*, it appears that IRAK4 directly phosphorylates the IRAK1 activation loop [30]. In Drosophila, the IRAK1 counterpart, called Pelle, is likewise critical for TOLL mediated innate immune signaling [31,32]. However, the Drosophila counterpart of IRAK4 lacks the kinase domain that was apparently lost at some point during its evolutionary history [33•]. Instead, evidence suggests Pelle is not trans-phosphorylated but rather auto-phosphorylated in a concentration dependent manner upon ligand recognition by the associated TOLL receptor [34]. In plants, growing evidence suggests that, like humans, PRR signaling often requires the cooperation of regulatory RD kinases.

A subfamily of RLKs that contain RD kinase domains, called Somatic-Embryogenesis Receptor-Like Kinases (SERKs), have been shown to play critical roles in PRR function. BAK1 (also known as SERK3) is known to physically associate with several PRRs including FLS2, EFR, and XA21 [Chen and Ronald, submitted]. Tomato BAK1 orthologs also associate with tomato receptor-like proteins (RLPs) that function in the immune response including the LeEIX1/2 (tomato ethylene-inducing xylanase) and Ve1 (Verticillium resistance) resistance proteins [35–38,39•,40]. BAK1 association with these RLPs appears to be triggered upon pathogen perception. LeEIX1/2 bind a conserved fungal molecule. The association of BAK1/SERK3 with LeEIX1 attenuates defense signaling mediated by LeEIX2 [39•,40]. In the case of Ve1, both BAK1/SERK3 and SERK1 are required for defense signaling. Together with the EIX observations, these results indicate that SERKs can play both positive and negative roles in PRR signaling.

Schwessinger *et al.*, 2011 recently showed that BAK1 kinase activity is required for innate immune signaling by FLS2 and EFR but not for complex formation with either FLS2 or EFR upon exposure to their respective ligands [35,41••,42]. As is the case with human IRAK1/IRAK4, it is possible that BAK1 and/or other SERK proteins phosphorylate the activation loops of PRR non-RD kinase domains. In this scenario, phosphorylation of the activation loop upon receptor hetero-dimerization may

serve as a switch for the formation of signaling complexes. A similar model has been proposed for IRAK1 function [43]. Once phosphorylated, the activation loop is reoriented to expose the ATP binding pocket, which also appears to be required for interaction with downstream signaling proteins. Phosphorylation would thus affect the ability of the non-RD kinase to form complexes required for biological function.

PRRs with RD kinase domains

While the vast majority of intracellular and extracellular PRRs contain non-RD kinase domains, there is at least one exception that carries an RD kinase. The Arabidopsis Chitin Elicitor Receptor Kinase (CERK1) carries an extracellular plant lysine domain (LysM) and an RD kinase domain [44,45]. AtCERK1 binds fungal chitin, a conserved microbial signature. AtCERK1 is also required for perception of a chemically related compound from bacteria called peptidoglycan (PGN), which is also highly conserved. Unlike the case with chitin, PGN is not directly bound by AtCERK1 [46,47•]. Instead PGN binds two LysM extracellular proteins, LYM1 and LYM3, which lack kinase domains. Chitin perception in rice appears to be more like Arabidopsis PGN perception in that chitin binds the membrane bound receptor Chitin Elicitor Binding Protein (CEBiP) that carries two extracellular LysM domains but lacks a kinase domain [48]. To transduce the immune response upon chitin perception, CEBiP still requires OsCERK1 for signaling [49•].

Thus far it is unclear whether chitin and PGN perception associate with a not-yet-identified non-RD kinase like other characterized PRRs. Closely related RLKs that contain LysM motifs also mediate recognition of Nod factors that are derivatives of chitin oligosaccharides produced by symbiotic rhizobacteria [50,51]. Domain swapping studies between the Nod Factor Receptor NFR1 and CERK1 showed that innate immunity vs. nodulation signaling specificity is conferred by two regions within their kinase domains [52••]. The first is a short stretch of amino acids within the kinase activation loop. The second is an adjacent YAQ motif within the αEF/αF domain known to regulate the conformation of the kinase activation loop. This finding suggests that the switch between innate immune signaling specificity by CERK1 and nodulation by NFR1 is mediated, at least partly, by changes to the activation loop [52••]. Thus, in addition to the RD motif, other residues associated with activation loop function may also be under adaptive selection pressures to specify PRR signaling. This presents the possibility that CERK1 has adopted changes directly to the activation loop in lieu of alterations to the RD motif.

Relationship between DAMP and PAMP signaling

Like animal innate immune systems, plants also contain receptors that respond to Damage Associated Molecular Patterns (DAMPs), which are produced as a consequence of pathogen infection and perception [53]. DAMPs function to amplify or reinforce innate immune signaling. Characterized plant DAMP receptors all contain RD kinase domains. These DAMP receptors include the Arabidopsis proteins Wall Associated Kinase (WAK1), Pep Receptor (PEPR1), and Theseus [54–56,57•] as well as the predicted rice DAMP receptor, WAK25 [58]. The close relationship of DAMP-mediated and PAMP-mediated responses is reflected in the fact that DAMP perception also often requires BAK1/SERK3 and also in the observations that PAMP and DAMP signals trigger overlapping transcriptional responses. In the case of WAK25, down regulation compromises XA21-mediated Immunity, indicating that WAK25 is a positive regulator of this process [58]. Despite these similarities, important differences have been described. For example, DAMP detection by WAK1 triggers a response that is similar to FLS2 but is weaker with regard to the amplitude of defense gene expression, is not as comprehensive, and does not induce key Salicylic Acid (SA) dependent genes such as PR1, which are a hallmark of PRR-mediated signaling [59,60]. Domain swapping experiments between the RD kinase domain of WAK1 and the non-RD kinase domain of EFR demonstrated that activation of MAMP and PAMP pathways are specific to their respective kinase domains [61•]. Rice contains a large subfamily of WAKs containing non-RD kinase domains (WAKa,c) that have not yet been characterized [4]. These non-RD WAKs may present an opportunity to better understand how changes in the RD motif influence MAMP vs. PAMP signaling.

Conclusions

Accumulated evidence supports our earlier finding that PRRs most often contain or associate with non-RD kinases. However, the reason for this association is still unresolved. A key question is whether the selection pressures that drive the adoption of non-RD motifs in PRR kinases are imposed by pathogens, by defense signaling constraints within the host, or by both. It is known that PRR kinases and those involved in downstream signaling events are common targets of pathogen effector proteins that aim to inhibit PRR-mediated signaling by blocking kinase function. For example, the kinase domains of PRRs such as FLS2 are targeted by bacterial effectors that disrupt immune signaling [62]. Such pressures could potentially lead to a diminished role for kinase function. In fact, the kinase auto-phosphorylation activities of Xa21, FLS2, and EFR are relatively weak compared to their RD counterparts like BAK1 [39,63]. While a potentially attractive hypothesis, it seems unlikely or at least incomplete given that numerous other conserved residues that are absolutely required for kinase catalytic activity remain unchanged in PRRs.

Instead of pathogen imposed pressures, innate immune signaling itself may have unique mechanistic requirements that are not satisfied by the canonical RD kinase motif. Innate immune signaling probably requires exquisite control to prevent inadvertent activation. ETI (and less commonly PTI) are often accompanied by programmed cell death (PCD). This is supported by the finding that most non-RD kinases in humans regulate innate immunity, apoptosis, and/or cell cycle control [4]. But if non-RD kinases are important for innate immune regulation in general, then it would be anticipated they would also function in mediating ETI receptor signaling. Two recent studies suggest they may. The barley fungal resistance protein Rpg5 encodes an NBS-LRR protein that also contains a C-terminal non-RD kinase domain [64••]. This finding implies that cytoplasmic non-RD kinases could potentially be involved in mediating ETI receptor signaling (at least in some cases). Likewise, the Arabidopsis ETI receptors RPM1, RPS2, and RPS5 were found to all associate with FLS2, forming a larger signaling complex [65]. Thus non-RD containing PTI receptors and ETI receptors may work in concert to trigger innate immunity.

How do non-RD kinases function differently from their RD counterparts? As stated earlier, one possibility is the lack of auto-phosphorylation of the activation loop, which does not commonly occur in non-RD kinases and signifies a potential mechanistic difference in their mode of activation. Currently, the evidence for a lack of activation loop auto-phosphorylation is meager as there are few studies in which *in vivo* phosphorylation sites for non-RD PRRs have been identified. In the case of Xa21 at least, *in vitro* phosphorylation studies failed to identify phospho-amino acids within the activation loop [63,66]. As appears to be the situation in humans, plant PRR kinases may require a regulatory RD kinase such as BAK1 to promote signaling. These regulatory RD kinases may act analogous to IRAK4 and trans-phosphorylate the activation loops of the PRR non-RD kinases. But the consequences of such a mechanism on signaling are still unclear since charge neutralization of the R residue is presumably unnecessary. A scenario proposed for human IRAK1 is that it predominantly plays a scaffolding role for signaling complex formation as data suggest PRR signaling does not depend on IRAK1 kinase activity [43]. Support for such a model in plant PRRs is mixed. FLS2, EFR, and XA21 do have relatively low kinase auto-phosphorylation activities. Likewise, mutations in key catalytic residues of the XA21 kinase domain appear to have only partial effects on resistance function [67]. However, equivalent experiments with FLS2 and EFR suggest that catalytic function is indeed required for innate immune signaling [41••,68]. These mixed findings suggest that there may be variation in PRR signaling mechanisms.

An alternative operational mechanism is that the non-RD kinases of PRRs may be constitutively active. In animals, some non-RD kinases maintain a constitutively active conformation owing to the lack of need for charge neutralization of the inhibiting R residue [69]. Constitutive activation could potentially promote a more rapid response to ligand recognition and/or a sustained signaling response; both of which are inherent properties of innate immune signaling. Alternatively, non-RD kinases may be regulated in an opposite manner with auto-phosphorylation causing a constitutively inactive state. Support for this hypothesis comes from observations in rice, where auto-phosphorylation of the XA21 kinase is promoted by the ATPase XB24. This auto-phosphorylation maintains XA21 in the inactive state. Only upon ligand binding does XB24 dissociate, which appears to be the first step in XA21 signaling [70]. Determining the precise role of kinases in PRR signaling and their mechanism(s) of activation will require further biochemical and structural studies to identify how non-RD kinase catalytic activity is regulated, what role (if any) non-RD catalytic activity plays in signaling, and the relationship between non-RD kinases and their RD regulatory partners.

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