Commentary

Convergence of cell-surface and intracellular immune receptor signalling

Plant immune responses are initiated by recognition of pathogen invasion through immune receptors. The pathogen sensing system is mainly composed of two structurally different proteins that are located on different subcellular compartments. One is the plasma membrane-localized pattern recognition receptors (PRRs) that detect pathogen-associated molecular patterns (PAMPs) (Boutrot & Zipfel, 2017). PRRs perceive extracellular PAMPs to activate PAMP-triggered immunity (PTI). In turn, adapted pathogens interfere with or modulate host signalling by virulence factors (called effectors) for successful infection. The other is intracellular nucleotide-binding domain and leucine-rich repeat proteins (NLRs) that recognize these effectors (Jones *et al.*, 2016). Activation of an NLR induces a robust immune response called effector-triggered immunity (ETI), which is often accompanied by hypersensitive response (HR) cell death. Animals encode both plasma membrane and intracellular immune receptors, which share similar structures with plant PRRs and NLRs for recognition of PAMPs; but plant and animal immune receptors evolved independently (Ronald & Beutler, 2010; Jones *et al.*, 2016). Interestingly, although PRRs and NLRs are structurally different and localize in distinct subcellular compartments, they share substantial downstream signalling, such as Ca^{2+}, mitogen-activated protein kinase (MAPK), reactive oxygen species (ROS), and phytohormone signalling as well as massive transcriptional reprogramming (Peng *et al.*, 2018). However, it is not known how PRRs and NLRs activate similar signalling outputs. In this issue of *New Phytologist*, Kadota *et al.* (2019, pp. 2160–2175) investigated protein phosphorylation dynamics upon NLR activation by phosphoproteomics. By comparing with previously published phosphoproteomics data for PTI, they discovered that phosphorylation occurred in the same residues of an NADPH oxidase, RESPIRATORY BURST OXIDASE HOMOLOGUE D (RBOHD), which is activated during both PTI and ETI. Thus, Kadota *et al.* (2019) precisely defined a signal convergent point between PRR and NLR signalling at the molecular level.

A long-standing question in plant immune signalling is how immune receptors activate downstream signalling pathways. Several recent studies have revealed that receptor-like cytoplasmic

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kinases (RLCKs) link PRR activation to downstream signalling such as MAPK cascades via kinase-mediated phospho-relay (Yamada *et al.*, 2016; Bi *et al.*, 2018). Likewise, NLRs activate kinase signalling, such as MAPK and calcium-dependent protein kinase (CPK) (Peng *et al.*, 2018). However, it remains unknown how activated NLRs initiate the kinase signalling. Therefore, the identification of several new phosphorylation sites by Kadota *et al.* (2019) will pave the way to explore NLR signalling, an uncharted research area.

'The finding of convergent phosphosites by Kadota et al. *(2019) will certainly stimulate further studies that compare kinase signalling networks activated by different types of immune receptors.'*

Quantitative phosphoproteomics reveals convergent points between PRR and NLR signalling

NLR-mediated phosphorylation signalling is less well understood than PRR signalling. Kadota *et al.* (2019) used a transgenic dexamethasone-inducible *avrRpt2* Arabidopsis system, which allowed them to dissect NLR-mediated protein phosphorylation without other signal inputs, such as PRR-mediated signals. Their phosphoproteome data revealed 84 increased phosphorylation sites (phosphosites) that are triggered by activation of an Arabidopsis NLR RESISTANT TO P. SYRINGAE-2 (RPS2), the corresponding receptor for AvrRpt2. For example, 32 and 10 phosphosites out of the 84 phosphosites were within MAPK and CPK phosphorylation motifs, respectively. Those enriched phosphosites are consistent with the previous data that MAPKs and CPKs are activated by NLR signalling. Importantly, Kadota *et al.* (2019) revealed that at least 14 phosphosites were commonly regulated by RPS2 and FLAGELLIN SENSING-2 (FLS2), which is a PRR for bacterial flagellin recognition to activate PTI. The overlapping phosphosites were found in plant immunity-related proteins, such as RBOHD, an ABC-transporter PENETRATION-3 (PEN3), a plasma membrane-localized calcium-ATPase AUTOINHIBITED Ca^{2+} ATPASE-8 (ACA8), and noncanonical Gα protein EXTRA-LARGE GTP-BINDING PROTEIN-2 (XLG2), which represent convergence points between PRR and NLR signalling.

Kadota *et al.* (2019) then focused on the overlapped phosphosites in RBOHD by selected reaction monitoring mass spectrometry. Previously, several studies have indicated that the

post-translational modification of RBOH including phosphorylation was required for the activity to produce ROS at apoplast (Adachi & Yoshioka, 2015; Kadota *et al.*, 2015). Kadota *et al.* (2019) found that serine 343 (S343) and S347 residues in RBOHD were highly phosphorylated during ETI triggered by inoculation with avirulent pathogens carrying AvrRpt2 or AvrRpm1 effectors, the latter of which is recognized by an Arabidopsis NLR RESISTANCE TO P. SYRINGAE PV. MACULICOLA-1 (RPM1). Moreover, the S343 and S347 were required for ROS production during ETI. Note that S343/S347 in RBOHD are phosphorylated by an Arabidopsis group VII RLCK, BOTRYTIS-INDUCED KINASE-1 (BIK1) during PTI (Adachi & Yoshioka, 2015; Kadota *et al.*, 2015). RBOHD$^{S343A/S347A}$ variant lost FLS2-mediated ROS production, pointing to the biological significance of S343/S347 during PTI. Kadota *et al.* (2019) tested the Arabidopsis double mutant of *bik1* and the functionally redundant RLCK *pbs1-like-1* (*pbl1*) for ROS production during ETI. The result showed that *BIK1* and *PBL1* were not required for the phosphorylation of S343/S347 in RBOHD during ETI. Thus, activation of PRR FLS2 and NLR RPS2 induces phosphorylation at the same residues (S343/S347) in RBOHD via different mechanisms, suggesting that RBOHD phosphorylation is a physiologically relevant signal convergent point between PRR and NLR signalling.

How is RBOHD phosphorylation regulated in NLR signalling?

How can we explain distinct dependencies on *BIK1* and *PBL1* between PTI and ETI? One explanation is that other RLCK family members might phosphorylate the S343/S347 independently or redundantly with BIK1/PBL1 during ETI. Interestingly, the RPS2-activated phosphosites included an amino acid residue S433 in RPM1-INDUCED PROTEIN KINASE (RIPK), which belongs to the RLCK VII subfamily, like BIK1 and PBL1. Thus, RIPK may play an important role in phosphorylating RBOHD during ETI. Another well-studied kinase for RBOH phosphorylation is the CPK family, which regulates RBOH via phosphorylation (Adachi & Yoshioka, 2015; Kadota *et al.*, 2015). Specifically, RBOHD S347 site is in CPK phosphorylation motif (φ-X-X-X-X-S/T-X-B; Kadota *et al.*, 2019), and CPK5 was involved in the phosphorylation (Adachi & Yoshioka, 2015) while RBOHD S343 is not in the typical CPK phosphorylation motif. A more recent study suggested that Arabidopsis MAP4 kinase SIK1 positively regulated ROS production by phosphorylating RBOHD S347 during PTI (Zhang *et al.*, 2018). Thus, these mentioned kinases may phosphorylate RBOHD during ETI, perhaps in a redundant manner.

The direct activation mechanism of downstream signalling components after NLR activation is not understood in plants. In animal systems, receptor-interacting serine/threonine protein kinase 2 (RIPK2) is known as a direct downstream component of the immune receptor NLRs, NOD1 and NOD2 (Kanneganti *et al.*, 2007). Thus, it is speculated that plant NLRs may directly activate protein kinases such as RLCKs and MAPK cascade components. Immune responses activated by NLRs are highly overlapping with those activated by PRRs, but NLR signalling is generally more prolonged and amplified compared to PRR signalling (Tsuda *et al.*, 2013). Therefore, NLR signalling likely uses and boosts PRR signalling networks. Recent time-series transcriptome analysis also supports this idea (Mine *et al.*, 2018). The difference in the signal amplitude can be explained by the fact that PRRs and NLRs activate the same kinase pathways with different phosphorylation dynamics and/ or intensities. Furthermore, NLRs might activate a greater number of kinases, which would explain the described robustness of NLR signalling. The finding of convergent phosphosites by Kadota *et al.* (2019) will certainly stimulate further studies that compare kinase signalling networks activated by different types of immune receptors.

How is signalling converged after immune receptor activation?

An interesting and emerging concept is that PRR- and NLR-mediated signalling share not only molecular pathways but also network property. Recent studies have shown that both immune receptors form similar network structures with 'co-receptor' or 'helper' (Nobori *et al.*, 2018; Wu *et al.*, 2018). Such receptor-network architecture could underpin adaptability against a wide variety of pathogen molecules and robustness of immune signalling. A hypothesis is that different receptors have evolved under similar selective pressures for recognizing pathogen molecules, and then might form similar receptor and signalling networks in the current plant immune system. In the future, more comparative studies on PRR and NLR signalling would contribute to understanding the complete picture of PRR- and NLR-mediated immunity.

Plant NLRs show distinct subcellular localization such as the cytosol and nucleus, yet they activate similar immune outputs such as HR cell death and transcriptional reprogramming (Cui *et al.*, 2015). For instance, plasma membrane-associated RPM1- and nuclear-localized RPS4-mediated transcriptional reprogramming is similar (Bartsch *et al.*, 2006). However, several studies artificially altering NLR localization showed that mis-located NLRs lost their signalling functions (Cui *et al.*, 2015). Based on these findings, we speculate that direct downstream components are more specific to each NLR at least according to subcellular localization, and then the specific signals converge at similar signalling pathways. Kadota *et al.* (2019) used only plasma membrane-associated RPS2 for their phosphoproteome analyses; further phosphoproteomics studies for NLRs that show distinct subcellular localizations would help to improve our understanding of the convergence of different immune receptor signalling pathways.

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