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ROS around RIPK

Plants possess a multi-layered immune system (Zhang et al., 2020). Upon pathogen infection, plasma membrane-localized pattern-recognition receptors (PRRs) recognize microbe-/damage-associated molecular patterns (MAMPs/DAMPs) or immunological phytocytokines to activate pattern-triggered immunity (PTI). Intracellular nucleotide-binding leucine-rich repeat (NLR) proteins recognize pathogen-secreted effectors directly or indirectly to activate effector-triggered immunity (ETI). PTI and ETI mutually potentiate each other, and confer largely overlapping immune responses, including the production of reactive oxygen species (ROS), calcium influx, activation of mitogen-activated protein kinases (MAPKs), and transcriptional reprogramming (Ngou et al., 2021; Yuan et al., 2021). Local PTI and ETI responses can further induce systemic immunity in distal tissues, resulting in so-called systemic acquired resistance (SAR) (Zhang et al., 2020).

ROS function not only as antimicrobial agents but also, more importantly, as key signaling molecules to relay immune responses (Castro et al., 2021). ROS production in *Arabidopsis* immunity is mainly mediated by RESPIRATORY BURST OXIDASE HOMOLOG D (RBOHD), an NADPH oxidase (NOX) (Castro et al., 2021). The activity of RBOHD is tightly controlled by diverse post-translational modifications, including phosphorylation. Several protein kinases, including calcium-dependent protein kinases (CPKs), MAP4 kinase SIK1, and receptor-like cytoplasmic kinase (RLCK) VII members, have been shown to phosphorylate RBOHD at the conserved and unique sites and regulate RBOHD activity and abundance (Castro et al., 2021). It has been suggested that different kinases regulate RBOHD phosphorylation in different types of plant immunity. However, a recent study by Li et al. (2021) reported that RPM1-induced protein kinase (RIPK), a plasma membrane-associated RLCK VII member, positively regulates ROS production in multiple layers of plant immune signaling, including PTI, ETI, and SAR, through interaction with and phosphorylation of RBOHD (Figure 1).

The authors started with a genetic screen for the mutants with a defect in the lipopolysaccharide (LPS)-triggered biphasic ROS burst, and identified two alleles of *ripk* mutants, *ripk-2* and *ripk-3*. The mutants are deficient in kinase activity (Li et al., 2021), consistent with the importance of RIPK phosphorylation for its function. They went on to test the requirement of RIPK for the ROS production in PTI triggered by other MAMPs or immunological phytocytokines, including flg22, elf26, nlp20, chitin, and Pep1. Remarkably, the *ripk* mutants were compromised in ROS production triggered by all elicitors tested (Li et al., 2021). This finding is different from those in other characterized RLCK VII members that redundantly regulate ROS production in response to a specific subset of elicitors (Rao et al., 2018). Among the 46 RLCK VII members, RLCK VII-5 (PBL34/35/36), RLCK VII-7 (PBL30/31/32), and RLCK VII-8 (BIK1 and PBL1/9/11) are required for ROS production induced by flg22, elf18, or chitin, whereas RLCK VII-4 (PBL19/20/37/38/30/40) is specifically required for ROS production in response to chitin (Rao et al., 2018). Interestingly, some RLCK VII members play opposite roles in regulating ROS production in response to different MAMPs. For instance, BIK1 positively and negatively regulates ROS production in response to flg22 and nlp20, respectively, with a yet elusive mechanism (Wan et al., 2019). Of note, *ripk* mutants did not affect MAPK activation in response to elicitors (Li et al., 2021), indicating that RIPK specifically regulates ROS production. This is also different in some other RLCK VII members that regulate multiple PTI outputs (Rao et al., 2018; Wan et al., 2019).

RIPK was originally identified as a key kinase regulating NLR RPM1-mediated ETI by phosphorylating immune regulator RIN4 in response to bacterial effectors AvrB and AvrRpm1 (Liu et al., 2011). Li et al. (2021) revealed that RIPK is required for ROS production triggered by various avirulent bacteria for ETI, expanding the function of RIPK in ETI. Furthermore, *ripk* mutants are compromised in ROS production in response to plant hormone salicylic acid (SA) and a non-protein amino acid pipecolic acid (Pip), two activators of SAR (Li et al., 2021). Importantly, the *ripk* mutants are defective in SAR, suggesting that RIPK has a critical role in SAR that has not been reported for other RLCKs (Li et al., 2021).

To understand how RIPK regulates the ROS production induced by a wide range of elicitors, the authors investigated the connection of RIPK with RBOHD, a key NOX in plant immunity. Not surprisingly, RIPK interacts with RBOHD in a manner independent of immune elicitation, and directly phosphorylates RBOHD (Li et al., 2021). A total of 10 residues in RBOHD were identified to be phosphorylated by RIPK *in vitro* (Li et al., 2021). Among them, S39, S163, S339, S343, S347, T123, T161, and T179 (T177) have been previously identified as phosphorylation sites by BIK1 (Kadota et al., 2014; Li et al., 2014), CPKs (Dubiella et al., 2013; Kadota et al., 2014), or SIK1 (Zhang et al., 2018). With the phosphorylation-specific antibodies, the authors showed that S343 and S347 phosphorylation activated by flg22, Pep1, SA, and avirulent bacteria was significantly compromised in *ripk* mutants, suggesting that RIPK and other kinases, including BIK1, CPKs, and SIK1, synergistically phosphorylate RBOHD at these conserved sites during PTI, ETI, and SAR (Li et al., 2021). Consistently, S343 and S347 in RBOHD were previously identified to be phosphorylated during ETI triggered by an inducible expression of AvrRpt2 effector without activating PTI (Kadota et al., 2019). It is worth noting that the function of two

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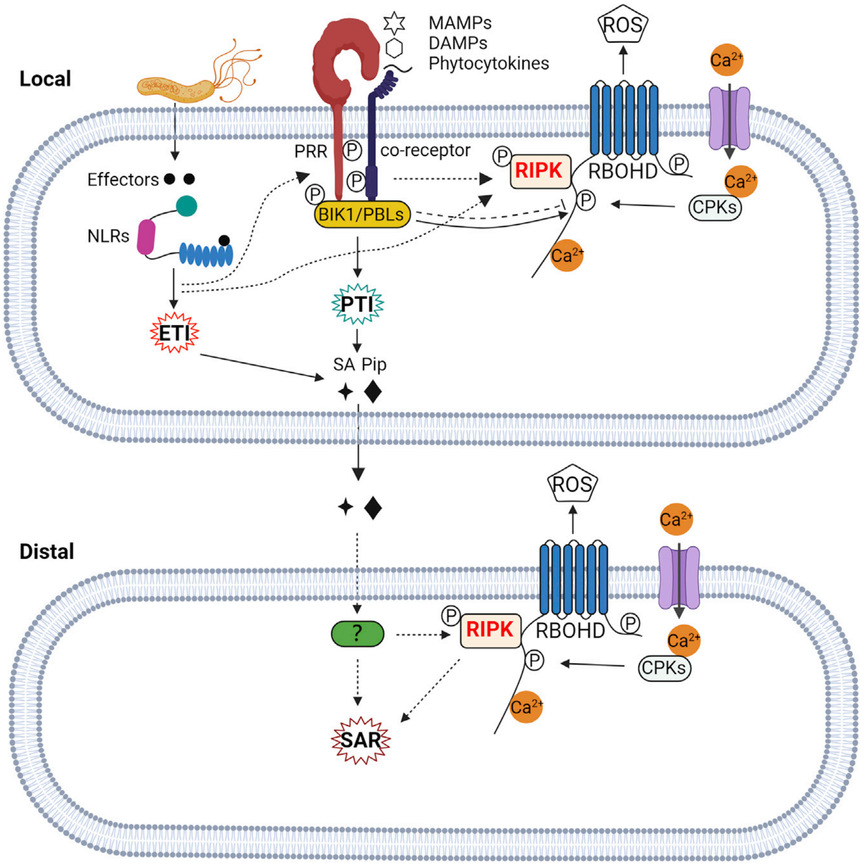

Figure 1. RIPK and other RLCKs regulate ROS production in multi-layered plant immunity.

Upon recognition of MAMPs/DAMPs/phytocytokines, PRRs recruit co-receptors to form PRR complexes, along with auto- and trans-phosphorylation. Activated PRR complexes may directly phosphorylate RIPK, which further phosphorylates and activates RBOHD to stimulate ROS production. Activated PRR complexes also phosphorylate BIK1 and PBLs, which may stimulate or inhibit ROS production in response to different elicitors. The pathogen-secreted effectors, when perceived by NLRs, trigger ETI. ETI induces ROS production via RIPK-mediated RBOHD phosphorylation. ETI-induced RIPK phosphorylation may depend on PRR complexes or unknown components. Ca^{2+} influx occurs along with ROS production. Ca^{2+} binding activates CPKs, which further phosphorylate and activate RBOHD. Local PTI and ETI can induce SAR in distal tissues. RIPK is also phosphorylated upon SAR activation with a yet elusive mechanism and phosphorylates RBOHD for ROS production in SAR. This figure was created with the software BioRender (BioRender.com).

unique phosphorylation sites in RBOHD by RIPK, S196 and S215, has not been explored.

An interesting question is how RIPK connects different layers of plant immunity. Li et al. (2021) revealed that RIPK is phosphorylated upon activation of PTI, ETI, and SAR. Upon MAMP perception, PRR complexes phosphorylate RLCK BIK1, which subsequently phosphorylates RBOHD for activation (Zhang et al., 2020; Castro et al., 2021). Presumably, a similar mechanism exists for RIPK phosphorylation in PTI. However, how RIPK is phosphorylated in ETI and SAR remains an interesting question to be addressed. RBOHD-mediated ROS production has been revealed as a critical signaling event in the mutual activation circuit of PTI and ETI to ensure robust immunity (Ngou et al., 2021; Yuan et al., 2021). Given that PRR components are required for the full activation of RBOHD during ETI (Ngou et al., 2021; Yuan et al., 2021), it is possible that RIPK might be phosphorylated by components of PRR complexes during ETI. The function of RIPK in ETI-induced ROS production is likely independent of its phosphorylation of RIN4 (Liu et al., 2011). Identifying RIPK-associated proteins, especially the kinases phosphorylating RIPK upon initiation of PTI, ETI, and SAR, may provide hints as to how RIPK is activated and regulated during different layers of plant immune responses.

NOXs are highly conserved in animals and plants and regulate a wide range of biological processes from development to stress adaptation (Castro et al., 2021). Is RIPK-mediated RBOHD phosphorylation important for ROS production in other plant physiological responses, such as polarized growth during root hair and pollen tube development? Unlike mammals, plants do not have complex regulatory subunits of NOXs to regulate their activity (Castro et al., 2021). Is it possible that RIPK is a subunit of the RBOHD enzyme complex? Another interesting question to be addressed is whether and how RIPK-mediated RBOHD phosphorylation regulates the differential waves of ROS production in different layers of plant immunity.

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