From Pathogen Recognition to Plant Immunity: BIK1 cROSses the Divide

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A rapid production of reactive oxygen species (ROS) represents a hallmark of plant immune responses to infection. However, how pathogen perception induces ROS production remains unclear. Li et al. (2014) fill a missing link by showing that a pattern recognition receptor complex directly associates with and activates a ROS-producing enzyme.

Plants and animals rely on innate immunity to defend themselves against potential microbial pathogens. The first line of defense is mediated by the membrane-localized pattern recognition receptors (PRRs), which perceive conserved microbial signatures, called microbe-associated molecular patterns (MAMPs). A major MAMP of bacterial pathogens is flagellin. In *Arabidopsis thaliana*, the active epitope of flagellin, flg22, is readily recognized by directly binding to the cognate PRR FLS2 and the coreceptor BAK1 (Sun et al., 2013). The activation of PRR complexes initiates transphosphorylation events between FLS2 and BAK1, as well as a cytoplasmic kinase BIK1 (Monaghan and Zipfel, 2012). Eventually, these early molecular events lead to a variety of antibacterial responses, including stomatal closure, cell wall thickening, reactive oxygen species (ROS) production, and antimicrobial compound secretion.

The oxidative burst is one of the early defense responses that occur within minutes after the perception of MAMPs. This transient ROS production is mainly dependent on the activity of a membrane-localized enzyme called respiratory burst oxidase homolog D (RbohD) (Torres et al., 2002). Previous studies suggest that RbohD is regulated by calcium and calcium-dependent protein kinases (CDPKs). MAMPs, including flg22, can induce a potent, but transient, calcium influx shortly after pathogen perception. This increased level of cellular calcium may directly regulate RbohD, which contains the EF-hand motifs that usually function as calcium-binding pockets (Kobayashi et al., 2007). Furthermore, RbohD can be phosphorylated by specific CDPKs (Kobayashi et al., 2007; Dubiella et al., 2013), which are important for flg22-triggered ROS production (Boudsocq et al., 2010). Therefore, it was proposed that MAMP-triggered calcium influx activates a specific group of CDPKs, which then activate ROS production by phosphorylating RbohD. However, a direct link between the activation of PRR complexes and the ROS production was missing.

In this issue of *Cell Host & Microbe*, Li et al. (2014) present compelling evidence suggesting a calcium-independent pathway that is important for the activation of RbohD upon flg22 perception. In particular, the authors report a thorough analysis on the activation of RbohD by BIK1, a key PRR-associating cytoplasmic kinase, through direct protein-protein interaction and phosphorylation. As such, the PRR complex directly regulates ROS production.

Initially, the authors identified RbohD as a potential BIK1-interacting protein using coimmunoprecipitation followed by mass spectrometry. In vitro and in vivo pull-down assays confirmed the interaction of RbohD not only with BIK1, but also with FLS2, suggesting that RbohD is associated with the FLS2 PRR complex. Furthermore, flg22 induces the disassociation of RbohD from FLS2. This is analogous to the flg22-induced disassociation of BIK1 with the PRR complex. Therefore, it appears that RbohD, as an inactive form, associates with the FLS2 receptor complex in the absence of flg22. Binding of flg22 with FLS2 and BAK1 induces the phosphorylation of BIK1; as the phosphorylated form of BIK1 disassociates from the PRR complex, so does RbohD.

RbohD was known to be activated by phosphorylation (Kobayashi et al., 2007; Ogasawara et al., 2008; Dubiella et al., 2013). Since BIK1 is a kinase, Li et al. (2014) next determined whether RbohD is a substrate of BIK1. They identified three residues (S39, S343, and S347) that are phosphorylated by BIK1 in vitro and further demonstrated that the phosphorylation of S39, and possibly S343, occurs in planta upon flg22 perception. Intriguingly, the phosphorylation of the RbohD S39 residue by BIK1 is not affected by cellular calcium. Furthermore, these phosphosites are important for flg22-triggered ROS production. These data suggest that BIK1 directly regulates RbohD by phosphorylating specific residue(s) in a calcium signaling-independent manner.

In order to confirm the biological significance of BIK1-mediated RbohD phosphorylation in downstream defense responses, Li et al. (2014) examined flg22- and bacterial pathogen-induced stomatal defense using a series of *Arabidopsis* mutants. Stomatal closure is an important defense mechanism of plants to restrict bacterial entry into the apoplastic space early in the infection, and it requires RbohD (Mersmann et al., 2010). Consistent with a regulatory role of BIK1 on RbohD, the *bik1* mutant *Arabidopsis* was unable to induce stomatal closure in response to flg22. Furthermore, phosphomimetic mutants of RbohD on the BIK1 phosphorylation sites, but not wild-type RbohD, can partially complement the deficiency of the *bik1* mutant on defense

against bacterial infection. These data suggest that RbohD phosphorylation is downstream of BIK1 to regulate stomatal defense.

Taken together, the results from Li et al. (2014) significantly advance our understanding of the activation of plant immunity upon MAMP perception. The finding that the ROS production enzyme RbohD associates with the FLS2 receptor complex and can be activated by BIK1 through direct phosphorylation connects a missing link between the onset of defense signaling upon flg22 perception and an early immune response, i.e., ROS production. BIK1 also associates with the PRR EFR that recognizes the bacterial elongation factor Tu (EF-Tu), as well as with PEPR1 and PEPR2, which are receptors of damage-associated molecular patterns (DAMPs) (Liu et al., 2013); therefore, it appears that BIK1 acts as a central convergent regulator that is activated by a plethora of external and internal ligands to initiate downstream stress responses. Indeed, another recent paper by Kadota et al. (2014) reporting similar conclusions showed that RbohD can associate with EFR and be phosphorylated by BIK1 in the presence of the MAMPs EF-Tu and chitin, as well as the DAMP Pep1.

These important findings warrant further investigations to fully understand the early regulatory events of plant immune responses. Most notably, the relationship between BIK1-mediated phosphorylation and calcium-dependent regulation of RbohD is still unclear. Li et al. (2014) showed that calcium ions were not required for RbohD phosphorylation (at least in the case of S39) but were indispensable to ROS production. This could be in part due to the EF-hand motifs at the N-terminal region of RbohD, which may bind to calcium and directly regulate RbohD (Ogasawara et al., 2008). In addition to the direct regulation by calcium, results from Li et al. (2014) and Kadota et al. (2014) suggest a more exciting model highlighting a hierarchical regulation with BIK1 initiating RbohD activation, whereas calcium signaling is essential for the amplification of ROS production. This is consistent with the previous result showing that the CDPK CPK5 could be activated by ROS, which forms a positive feedback loop (Dubiella et al., 2013). Another interesting observation made by Li et al. (2014) that supports this model was that BIK1 and its related cytoplasmic kinase PBL1 were required for the full activation of calcium influx triggered by flg22. This result indicates that RbohD activation by BIK1 may occur earlier than the calcium-dependent activation. While understanding the molecular mechanisms by which BIK1/PBL1 regulate calcium influx will provide key information on this regulation hierarchy, another way to dissect these processes is to determine whether BIK1-mediated phosphorylation is required for CDPK-mediated phosphorylation. Previous research suggested that CPK5 may phosphorylate RbohD at four serine residues, including S39 and S347 (Dubiella et al., 2013). Therefore, it appears that BIK1 and CPK5 phosphorylate RbohD at both common and distinctive residues. Future experiments are needed to analyze the full complements of RbohD phosphosites by BIK1, CPK5, and possibly other PBLs and CDPKs, and evaluate the timing of the phosphorylation and the biological significance of these residues. For example, an examination on how the phosphorylation of individual sites may affect the phosphorylation of other sites will help discriminate the distinct phosphorylation events of RbohD by BIK1/PBLs and CPK5/CDPKs. Answers to these questions will provide important insight into the complicated regulatory network of plant innate immunity.

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