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PLANT & CELL PHYSIOLOGY

Invited Review

Regulation of the NADPH Oxidase RBOHD During Plant Immunity

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(Received March 11, 2015; Accepted April 17, 2015)

Pathogen recognition induces the production of reactive oxygen species (ROS) by NADPH oxidases in both plants and animals. ROS have direct antimicrobial properties, but also serve as signaling molecules to activate further immune outputs. However, ROS production has to be tightly controlled to avoid detrimental effects on host cells, but yet must be produced in the right amount, at the right place and at the right time upon pathogen perception. Plant NADPH oxidases belong to the respiratory burst oxidase homolog (RBOH) family, which contains 10 members in the model plant *Arabidopsis thaliana*. The perception of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) leads to a rapid, specific and strong production of ROS, which is dependent on RBOHD. RBOHD is mainly controlled by Ca^{2+} via direct binding to EF-hand motifs and phosphorylation by Ca^{2+}-dependent protein kinases. Recent studies have, however, revealed a critical role for a Ca^{2+}-independent regulation of RBOHD. The plasma membrane-associated cytoplasmic kinase BIK1 (BOTRYTIS-INDUCED KINASE1), which is a direct substrate of the PRR complex, directly interacts with and phosphorylates RBOHD upon PAMP perception. Impairment of these phosphorylation events completely abolishes the function of RBOHD in immunity. These results suggest that RBOHD activity is tightly controlled by multilayered regulations. In this review, we summarize recent advances in our understanding of the regulatory mechanisms controlling RBOHD activation.

Keywords: Calcium • Innate immunity • Pathogen-associated molecular patterns • Pattern recognition receptors • Phosphorylation • Reactive oxygen species.

Abbreviations: BAK1, BRI1-ASSOCIATED RECEPTOR KINASE 1; BIK1, BOTRYTIS-INDUCED KINASE1; BRI1, BRASSIN OSTEROID INSENSITIVE1; BSK1, BRASSINOSTEROID-SIGNALING KINASE1; CBL, CALCINEURIN B-LIKE MOLECULE; CEBiP, chitin elicitor-binding protein; CERK1, CHITIN ELICITOR RECEPTOR KINASE 1; CIPK, CBL INTERACTING PROTEIN KINASE; CPK (or CDPK), Ca^{2+}-dependent protein kinase; DUOX, dual oxidase; EFR, EF-Tu receptor; ETI, effector-triggered immunity; FLS2, FLAGELLIN-SENSING-2; GAP, GTPase-activating protein; GEF, guanine nucleotide exchange factor; HEK293, human embryonic kidney 293; LYK5, LYSM-CONTAINING RECEPTOR-LIKE KINASE 5; LysM, lysin motif; MAPK, mitogen-activated protein kinase; NLR, nucleotide-binding domain and leucine-rich repeat-containing receptor; NOX, NADPH oxidase; PA, phosphatidic acid; PAMP, pathogen-associated molecular pattern; PBL, PBS1-LIKE KINASE; PLD, phospholipase D; PRR, pattern recognition receptor; PRX, peroxidase; PTI, PAMP-triggered immunity; RBOH, respiratory burst oxidase homolog; RK, receptor kinase; RLCK, receptor-like cytoplasmic kinase; RLP, receptor-like protein; ROS, reactive oxygen species; SRC2, SOYBEAN GENES REGULATED BY COLD 2; XA21, XANTHOMONAS RESISTANCE 21.

Introduction

Reactive oxygen species (ROS) are produced as by-products of the normal metabolism in peroxisomes, chloroplasts and mitochondria, because they have oxidizing activity and/or electron transfer chains (Tripathy and Oelmuller 2012). However, higher organisms also autonomously produce ROS and use them as signaling molecules to control a variety of biological processes. In plants, the autonomous ROS production was first documented in 1983 during infection of potato tubers with the pathogenic oomycete, *Phytophthora infestans* (Doke 1983). Since then, numerous studies have shown that ROS production is involved not only in plant immunity, but also in a variety of biological processes such as abiotic stress responses, growth and development. NADPH oxidases play a crucial role in ROS production. They transfer electrons from cytosolic NADPH or NADH to apoplastic oxygen, leading to the production of superoxide (O_2^-). The produced O_2^- can be converted to hydrogen peroxide (H_2O_2) by superoxide dismutase (Suzuki et al. 2011, Marino et al. 2012, Suzuki et al. 2012).

In mammals, NADPH oxidases are divided into three subfamilies: NOX1–NOX4 (NADPH oxidase 1–4), NOX5 and DUOX (dual oxidase; Bedard and Krause 2007, Rada and Leto 2008). All enzymes share FAD- and NADPH-binding sites, and a functional oxidase domain responsible for O_2^- production. NOX2, the best-characterized NADPH oxidase, forms a multiprotein complex with many regulators such as $p22^{phox}$, $p47^{phox}$, $p40^{phox}$, $p67^{phox}$ and the Rac small GTPase (Canton and Grinstein 2014). These proteins regulate the activity and

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translocation of NOX2 to the plasma membrane. The mutations in NOX2 or its regulatory proteins cause chronic granulomatous disease whose patients suffer from bacterial and fungal infections (Bedard and Krause 2007), showing their crucial roles in immunity. In contrast to NOX1–NOX4, NOX5 and DUOX have additional EF-hand motifs at the N-terminus, suggesting their regulation by Ca^{2+} binding (Canton and Grinstein 2014). In addition, calcium-dependent kinases, such as protein kinase Cα and/or Ca^{2+}/calmodulin-dependent protein kinase II, are known to phosphorylate and activate NOX5 and DUOX.

In plants, NADPH oxidases belong to the respiratory burst oxidase homolog (RBOH) family, which contains 10 members in Arabidopsis (Torres and Dangl 2005). Similar to mammalian NOX proteins, all RBOHs have FAD- and NADPH-binding sites, six transmembrane domains and a functional oxidase domain. Plant RBOHs also have Ca^{2+}-binding EF-hand motifs in their N-terminal domain similar to NOX5 and DUOX (**Fig. 1**), suggesting the similar regulation by Ca^{2+}. In contrast, none of the known regulators of NOX2 has clear homologs in plants, except for Rac small GTPase.

Numerous genetic studies have shown that plants use different RBOHs to control different biological processes. For example, RBOHH and RBOHJ are involved in pollen tube growth (Boisson-Dernier et al. 2013, Kaya et al. 2014, Lassig et al. 2014), and RBOHB is involved in seed ripening (Muller et al. 2009). RBOHC regulates focal production of ROS in trichoblasts and regulates root hair formation (Foreman et al. 2003, Takeda et al. 2008), and RBOHC is also important for mechanosensing in root (Monshausen et al. 2009). The most highly expressed RBOHD plays important roles in cell death control, cell wall damage-induced lignification and systemic signaling in response to biotic and abiotic stresses (Torres et al. 2005, Miller et al. 2009, Denness et al. 2011). RBOHF may work redundantly with RBOHD in some responses because the *rbohD rbohF* double mutant shows a stronger phenotype in defense responses against pathogens, cell death control as well as ABA-induced stomatal closure (Kwak et al. 2003, Torres et al. 2005, Torres et al. 2006). RBOHF also has specific roles in some responses. It plays crucial roles in protecting shoot cells from transpiration-dependent accumulation of excess Na^{+} (Jiang et al. 2012) and in lignin deposition during casparian strip formation in endodermis, a diffusion barrier that directs water and solutes from the soil to the water-conducting tissues (Lee et al. 2013).

In this review, we focus on the best characterized RBOHD and its regulation during immunity, and discuss how plants may use these mechanisms to produce the right amount of ROS at the right place and time.

Plant Immune Signaling and RBOHD-Dependent ROS Production

Plants have two layers of plant immune recognition (Dodds and Rathjen 2010). The first layer of innate immunity is initiated by the perception of pathogen-associated molecular patterns (PAMPs) by surface-localized pattern recognition receptors (PRRs), leading to PAMP-triggered immunity (PTI). The second layer involves intracellular immune receptors, which are most often nucleotide-binding domain and leucine-rich repeat-containing receptor (NLR) proteins. These proteins directly or indirectly recognize virulence effectors secreted in host cells by pathogens and thereby inducing effector-triggered immunity (ETI).

Plant PRRs are either receptor kinases (RKs) or receptor-like proteins (RLPs) (Zipfel 2014). The Arabidopsis leucine-rich repeat RKs (LRR-RKs) FLS2 (FLAGELLIN-SENSING-2) and EFR [elongation factor-Tu (EF-Tu) receptor] recognize bacterial flagellin and EF-Tu, respectively, and are the best characterized plant PRRs (Zipfel 2014). Binding of flg22 or elf18 (the immunogenic peptides of flagellin or EF-Tu in Arabidopsis, respectively) to FLS2 or EFR, respectively, induces their instant association with the co-receptor LRR-RK BAK1, phosphorylation of both proteins and initiation of downstream responses (Chinchilla et al. 2007, Heese et al. 2007, Roux et al. 2011, Schwessinger et al. 2011, Sun et al. 2013). Perception of the fungal PAMP chitin in Arabidopsis depends on the LysM (lysin motif) RKs CERK1 (CHITIN ELICITOR RECEPTOR KINASE 1) and LYK5 (LYSM-CONTAINING RECEPTOR-LIKE KINASE 5) (Miya et al. 2007, Wan et al. 2008, Liu et al. 2012, Cao et al. 2014), while in rice (*Oryza sativa*) it depends on a ligand-induced complex between CEBiP (chitin elicitor-binding protein) and OsCERK1 (Kaku et al. 2006, Shimizu et al. 2010, Hayafune et al. 2014).

Interestingly, it is becoming increasingly clear that the direct substrates of PRR complexes are receptor-like cytoplasmic

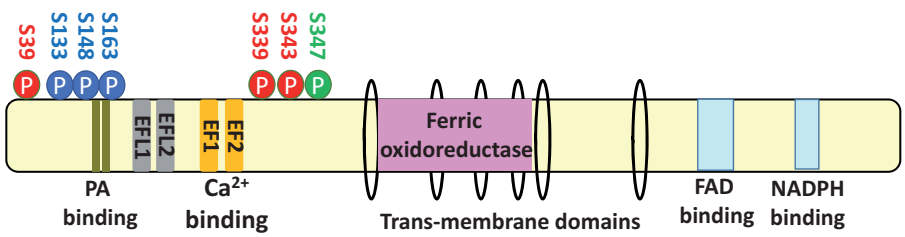

Fig. 1 Schematic representation of RBOHD domains. RBOHD has two EF-hand motifs, two EF-hand-like motifs and possible phosphatidic acid-(PA) binding sites in its N-terminal part, followed by six transmembrane domains encompassing the functional ferric oxidoreductase domain, one FAD-binding site and one NADPH-binding site. BIK1 specifically phosphorylates the residues Ser39, Ser339 and Ser343 (indicated in red), while CPKs phosphorylate the residues Ser133, Ser148 and Ser163 (indicated in blue). The residue Ser347 (indicated in green) is phosphorylated by both BIK1 and CPKs.

kinases (RLCKs). For example, BAK1 and FLS2/EFR constitutively associate with the RLCK BIK1 (BOTRYTIS-INDUCED KINASE1), which becomes rapidly phosphorylated and released from the FLS2 and EFR complexes upon PAMP perception to activate downstream immune responses (Lu et al. 2010, Zhang et al. 2010). Notably, BIK1 also associates with CERK1 and is required for chitin-induced responses (Zhang et al. 2010), indicating that BIK1 is the first convergent component for different PRR-mediated pathways. Recently, several other RLCKs such as PBL1, PBL2, PBL5 (PBS1-LIKE KINASES) and BSK1 (BRASSINOSTEROID-SIGNALING KINASE1) were also shown to be involved in PTI signaling (Zhang et al. 2010, Liu et al. 2013, Shi et al. 2013). Rice OsRLCK185 and its closest ortholog in Arabidopsis, PBL27, are involved in CERK1-mediated signaling and control the activation of mitogen-activated protein kinases (MAPKs) in response to chitin treatment (Yamaguchi et al. 2013, Shinya et al. 2014).

PAMP perception leads to a series of signaling outputs, including ion fluxes, ROS production, activation of MAPKs and Ca^{2+}-dependent protein kinases (CPKs or CDPKs), transcriptional reprogramming, callose deposition and, ultimately, immunity (Boller and Felix 2009, Segonzac and Zipfel 2011). Among these readouts, ROS production is one of the earliest responses, starting only a few minutes after PAMP treatment. ROS is also produced during ETI, but at a much slower pace. ROS have been proposed to act as antimicrobials, cross-linkers of the plant cell wall to block pathogen entry, and to act as local and systemic secondary messengers to trigger additional immune responses, such as gene expression or stomatal closure (Lamb and Dixon 1997, Suzuki et al. 2011, Nathan and Cunningham-Bussel 2013, Gilroy et al. 2014). During both PTI and ETI, ROS production is predominantly dependent on RBOHD. The Arabidopsis *rbohD* mutant does not produce ROS upon PAMP treatment or barely produces ROS in response to infection by pathogenic strains triggering ETI (Torres et al. 2002, Nühse et al. 2007, Zhang et al. 2007). Also, RBOHD orthologs in other plant species, such as tobacco (*Nicotiana tabacum;* NtRBOHD and *Nicotiana benthamiana;* NbRBOHD), rice (*Oriza sativa;* OsRBOHB) and potato (*Solanum tuberosum*; StRBOHB), play an important role in ROS production in response to PAMPs or pathogens (Simon-Plas et al. 2002, Yoshioka et al. 2003, Kobayashi et al. 2007, Wong et al. 2007).

Ca^{2+}-Dependent Regulation of RBOHD During Immunity

Although pathogen-induced ROS production was documented > 30 years ago (Doke 1983), the detailed regulatory mechanisms of RBOH activation are still unclear. Since RBOHs have Ca^{2+}-binding EF-hand motifs in their N-terminal regions, Ca^{2+} was thought to be important for their regulation. Actually, pharmacological experiments showed that apoplastic Ca^{2+} is required for PAMP-induced ROS production (Kadota et al. 2004, Ranf et al. 2011, Segonzac et al. 2011, Kadota et al. 2014). Recent structural and biochemical analysis showed that OsRBOHB has two EF-hand-like motifs in addition to two EF-hand motifs, but Ca^{2+} only binds to the first EF-hand motif (Oda et al. 2010). Mutational analysis in EF-hand motifs also showed that Ca^{2+}-binding is required for ROS production (Ogasawara et al. 2008).

CPKs were also shown to be important for the regulation of RBOHD. Potato CPKs StCDPK4 and StCDPK5 directly phosphorylate the N-terminal region of StRBOHB in a Ca^{2+}-dependent manner (Kobayashi et al. 2007). These CPKs phosphorylate Ser82 and Ser97 on StRBOHB, which correspond to Ser133 and Ser148 in AtRBOHD. In Arabidopsis, CPK4, CPK5, CPK6 and CPK11 (which are related to StCDPK4/5) are positive regulators of the PAMP-induced ROS burst (Boudsocq et al. 2010), and CPK5 was recently shown to phosphorylate RBOHD and to regulate its activity (Dubiella et al. 2013). CPK5 phosphorylates Ser148, Ser163 and Ser347 of RBOHD. Although Ca^{2+}-based regulation is clearly important, the roles of Ca^{2+} binding or CPK-mediated phosphorylation in RBOHD activation remain to be further elucidated.

Ca^{2+}-Independent Regulation of RBOHD During Immunity

Although Ca^{2+}-based regulation is required for the activation of RBOHD, other regulations probably exist. Indeed, changes in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) and activation of CPKs are induced by many different stimuli, but PAMP-induced ROS production has a unique feature as it is fast, transient and potent. Recent studies have indeed revealed the existence of an additional, Ca^{2+}-independent, direct regulation of RBOHD by the PRR complex (Kadota et al. 2014, Li et al. 2014). Biochemical analyses showed that RBOHD associates with the PRR complex in vivo, and that BIK1 directly phosphorylate RBOHD upon PAMP perception (Kadota et al. 2014, Li et al. 2014). While this association was proven constitutive in one study (Kadota et al. 2014), the other study reported that RBOHD dissociates from BIK1 after PAMP treatment (Li et al. 2014), which may reflect differences in experimental conditions. In both cases, however, it was shown that activated BIK1 phosphorylates the residues Ser39, Ser339, Ser343 and Ser347 within the N-terminal part of RBOHD. Notably, a quantitative proteomics approach using selected reaction monitoring showed that BIK1, but not CPKs, BAK1 or EFR, specifically phosphorylates Ser39, Ser339 and Ser343, while both BIK1 and CPKs phosphorylate Ser347 (**Fig. 1**). These phosphorylation events are induced by several PAMPs, such as flg22, elf18 and chitin, and start within minutes after PAMP treatment, preceding ROS production. Additionally, PAMP-induced phosphorylation at positions Ser39 and Ser343 is reduced in a *bik1 pbl1* double mutant, showing the requirement for BIK1 and PBL1 for the phosphorylation of these residues. Other RLCKs, such as PBL2 and PBL5, are, however, also genetically required for full PAMP-induced ROS production (Zhang et al. 2010, Liu et al. 2013), suggesting the redundant role of BIK1 and other PBLs.

BIK1-mediated phosphorylation also occurred in the absence of Ca^{2+} or in the *cpk5 cpk6 cpk11* mutant, showing that BIK1-mediated phosphorylations at S39 and S343 are

independent of Ca^{2+}-based regulations. Conversely, Ser163 phosphorylation is Ca^{2+} dependent and it is not phosphorylated by BIK1, BAK1 or EFR, suggesting that Ser163 phosphorylation is CPK specific. Phospho-dead mutations of BIK1-mediated phosphorylation sites suppress ROS production induced by flg22, elf18 and chitin, or the damage-associated molecular pattern, AtPep1. In contrast, phospho-mimetic mutations in BIK1-mediated phosphorylation sites lead to an increased ROS production in response to PAMP treatment and also complement the loss of BIK1 and PBL1 (Kadota et al. 2014, Li et al. 2014), showing that BIK1-mediated phosphorylation is required for ROS production. Notably, the residues phosphorylated by BIK1 are highly conserved among functional RBOHD orthologs in different plants (Simon-Plas et al. 2002, Yoshioka et al. 2003, Kobayashi et al. 2007, Kadota et al. 2014). Thus, it appears that the regulation imposed by BIK1 (and related RLCKs) is a common regulatory mechanism for RBOHD orthologs in different plant species. Importantly, RBOHD also represents the first example of a downstream substrate for any RLCK involved in plant immunity.

The Relationship Between Ca^{2+}-Dependent and Ca^{2+}-Independent Regulations of RBOHD

The fact that phospho-mimetic mutations of BIK1-mediated phosphorylation sites of RBOHD enhance PAMP-induced ROS production but do not trigger the ROS production in the absence of PAMPs suggests that BIK1-mediated regulation is required but not sufficient to induce ROS production. As mentioned in the previous section, treatment with a Ca^{2+} chelator and mutations in EF-hand motifs inhibit PAMP-induced ROS production, showing that Ca^{2+}-based regulation is also required (Kadota et al. 2004, Ogasawara et al. 2008, Segonzac et al. 2011, Kimura et al. 2012). Thus, we propose a two-step regulation of RBOHD during which the rapid BIK1-mediated phosphorylation primes RBOHD activation by increasing the sensitivity to the Ca^{2+}-based regulation (**Fig. 2**). For example, BIK1-mediated phosphorylation may trigger conformational changes leading to an increased Ca^{2+} binding affinity for the EF-hand motifs in the N-terminal region of RBOHD, and/or an increased ability of CPKs to phosphorylate RBOHD. It is also theoretically possible that BIK1-mediated phosphorylation affects the homodimerization and/or intramolecular interaction between the N- and C-terminal regions of RBOHD. Since many endogenous and environmental stimuli induce $[Ca^{2+}]_{cyt}$ changes, this two-step regulation may be important to ensure that the immune-related strong ROS production is only triggered when BIK1 becomes activated upon PAMP perception. The prerequisite role of BIK1-mediated phosphorylation prior to (or concomitantly with) Ca^{2+}-based regulation is also suggested by pharmacological experiments. Pre-treatment of human embryonic kidney 293 (HEK293) cells transfected with RBOHs with a protein phosphatase inhibitor dramatically enhances ROS production induced by Ca^{2+} ionophore treatment (Ogasawara et al. 2008, Kimura et al. 2012). Conversely, pre-treatment with a Ca^{2+} ionophore does not affect small ROS production induced by a protein phosphatase inhibitor.

BIK1 and PBL1 may also regulate Ca^{2+} signaling directly or indirectly. PAMP induces biphasic $[Ca^{2+}]_{cyt}$ changes, but, interestingly, *bik1* and *pbl1* single or double mutations partially inhibit $[Ca^{2+}]_{cyt}$ changes (Li et al. 2014, Ranf et al. 2014, Monaghan et al. 2015). Although the identity of the Ca^{2+} channel(s) involved in PTI is still unclear, these results suggest that BIK1 and PBL1 may directly phosphorylate and activate Ca^{2+} channels or important positive regulator(s) thereof, or that ROS themselves play an important role in the activation of this channel(s). Interestingly, electrophysiological studies have shown that H_2O_2 can activate Ca^{2+} influx in Arabidopsis through the plasma membrane, and that this Ca^{2+} influx plays an important role in ABA-induced stomatal closure (Pei et al. 2000). The same Ca^{2+} influx is also activated by treatment with yeast (*Saccharomyces cerevisiae*) elicitor and chitosan, showing the potential involvement of an H_2O_2-activated Ca^{2+} channel in PTI (Klusener et al. 2002). Notably, *rbohD* mutation specifically suppresses the second peak of PAMP-induced biphasic $[Ca^{2+}]_{cyt}$ changes (Ranf et al. 2011), suggesting a positive feedback activation of Ca^{2+} signaling by ROS. This ROS-induced Ca^{2+} influx may in turn enhance Ca^{2+} interaction with RBOHD, CPK activation and CPK-mediated phosphorylation, leading to boosted ROS production. This is consistent with the fact that H_2O_2 treatment activates CPK5 and CPK-mediated phosphorylation of RBOHD (Dubiella et al. 2013). Notably, a similar positive feedback regulation was also recently proposed for the activation of RBOHH and RBOHJ during pollen tube growth (Wudick and Feijo 2014). Of note, it was recently shown in Arabidopsis that CPK28 indirectly regulates the ROS and Ca^{2+} bursts by controlling BIK1 protein turnover, illustrating another loop of inter-regulation between ROS and Ca^{2+} (Monaghan et al. 2014, Monaghan et al. 2015).

RBOHD Regulation by Rac Small GTPase and Oligomerization

A homolog of mammalian Rac small GTPase in rice, OsRac1, is a positive regulator of OsRBOHB and is involved in immunity (Wong et al. 2007, Oda et al. 2010). OsRac1 interacts with an N-terminal region of OsRBOHB, and overexpression of a constitutively active version of OsRac1 leads to ROS accumulation in tobacco. In Arabidopsis, AtROP1, an OsRac1 homolog, plays an important role in RBOHH/RBOHJ-regulated pollen tube growth (Qin and Yang 2011, Boisson-Dernier et al. 2013, Kaya et al. 2014, Lassig et al. 2014). Moreover, expression of the constitutively active version of tobacco NtRac5 in pollen tubes enhances ROS production, while the expression of a dominant negative version suppresses it (Potocky et al. 2012). These results suggest a close relationship between Rac small GTPase and RBOHs in other pathways.

Structural analyses of OsRBOHB, coupled with in vitro binding and NMR (nuclear magnetic resonance) titration assays, showed that OsRac1 binds the coiled-coil region generated upon homodimerization of the N-terminal region of

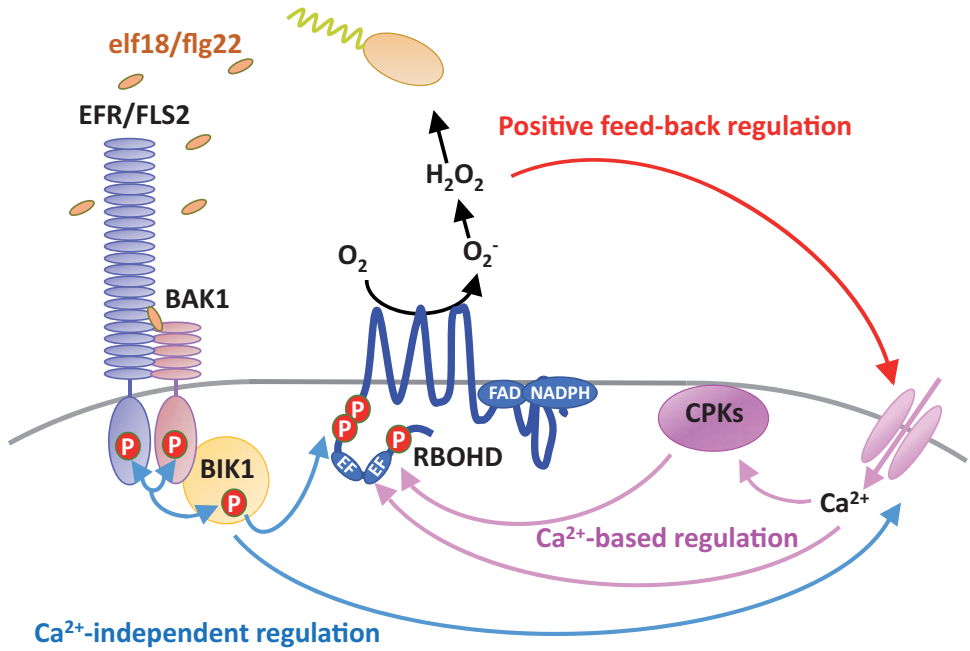

Fig. 2 A two-step activation model for RBOHD during immunity. Upon PAMP perception, PRRs, such as EFR and FLS2, and their co-receptor BAK1 directly phosphorylate and activate BIK1. Phosphorylated BIK1 has a higher binding affinity for RBOHD and phosphorylates it on some specific sites. BIK1-mediated phosphorylation may 'prime' the Ca^{2+}-based regulation of RBOHD by inducing conformational changes that could lead to increased Ca^{2+} binding affinity for EF-hand motifs and/or increased accessibility for CPK-mediated phosphorylation. At the same time, PRRs together with BIK1 also activate Ca^{2+} channel(s) and induce Ca^{2+} influx directly or indirectly. This leads to Ca^{2+} binding to an EF-hand motif in RBOHD and also activation of CPKs, which in turn phosphorylates RBOHD. The produced H_2O_2 itself may trigger further activation of Ca^{2+} channel(s), leading to the full activation of Ca^{2+} signaling and Ca^{2+}-based regulation of RBOHD.

OsRBOHB, suggesting that the homodimerization of OsRBOHB is required for Rac GTPase interaction (Oda et al. 2010, Kosami et al. 2014). Variable-angle total internal reflection fluorescence microscopy analysis showed that AtRBOHD is present in dynamic discrete spots at the plasma membrane and that a pool of RBOHD forms homodimers (Hao et al. 2014). Interestingly, flg22 or ABA treatment increases this homodimerization. Moreover, treatment with a Ca^{2+} ionophore or a protein phosphatase inhibitor also induced RBOHD homodimerization, suggesting that dimerization and subsequent Rac GTPase interaction may be activated by Ca^{2+}-based regulation and/or protein phosphorylation. Interestingly, in vitro binding assays showed that the N-terminal region of RBOHD homodimerizes even in the absence of Ca^{2+} (Oda et al. 2010). The N-terminal region of RBOHD also forms intramolecular interactions in the C-terminal region, suggesting that this region inhibits homodimerization by forming intramolecular interactions with the N-terminal region.

Recently, it was shown in rice that the guanine nucleotide exchange factor OsRacGEF1 and the Rho GTPase-activating protein RhoGAP control the activity of OsRac1 (Akamatsu et al. 2013, Liu et al. 2015). OsRacGEF1 stimulates the exchange of GDP for GTP to generate the activated form, while RhoGAP is thought to stimulate the GTPase activity of OsRac1 and terminate signaling events. Silencing of *OsRacGEF1* compromises resistance against bacterial pathogen, while silencing of *RhoGAP* induces spontaneous cell death and enhances resistance against pathogens. Furthermore, OsCERK1 interacts with and activates OsRacGEF1 upon chitin perception, suggesting that CERK1 activates OsRac1 through OsRacGEF1 phosphorylation. However, functional orthologs of OsRac1 that may function in PAMP-induced ROS production in Arabidopsis are not known yet. It is also not clear whether OsRacGEF1-mediated regulation is conserved in other PRR signaling pathways across species. Indeed, OsRacGEF1 does not bind OsXA21 (XANTHOMONAS RESISTANCE 21), a rice PRR related to EFR and FLS2 (Akamatsu et al. 2013), despite the fact that the OsXA21 kinase domain has the same ability to induce ROS production as EFR when expressed in Arabidopsis (Holton et al. 2015).

Other Possible Regulation Mechanisms of RBOHD During Immunity

Other possible regulations of RBOHs are also suggested. Yeast two-hybrid screening using the C-terminal region of tobacco NtRBOHD identified a 14-3-3 protein as an interactor (Elmayan et al. 2007). Silencing of this 14-3-3 gene compromises PAMP-induced ROS production, suggesting a positive regulation of NtRBOHD by 14-3-3. The exact binding site and the role of the 14-3-3 interaction on the activation mechanism of RBOHD are however still unclear. A similar approach using the N-terminal region of RBOHF identified the ortholog of SOYBEAN GENE REGULATED BY COLD 2 (AtSRC2) as an interactor (Kawarazaki et al. 2013). Heterologous expression in the

HEK293 cell line showed that SRC2 positively regulates Ca^{2+}-induced ROS production by RBOHF. The same approach also identified CIPK26 [calcineurin B-like molecule (CBL)-interacting protein kinase] as a specific interactor of the RBOHF N-terminal region in yeast two-hybrid studies (Kimura et al. 2013). Co-expression of either CBL1 or CBL9 with CIPK26 strongly enhances ROS production by RBOHF in HEK293 cells, suggesting that the CBL1/9–CIPK26 complex has a positive role in RBOHF-mediated ROS production (Drerup et al. 2013). However, the sites of phosphorylation of RBOHF by CIPK26 are not identified yet, and the roles of SRC2 and CBL/CIPK in the regulation of other RBOHs are still unknown.

Phospholipase Dα1 (PLDα1)-derived phosphatidic acid (PA) has been shown to interact directly with RBOHD and enhances ROS production (Zhang et al. 2009). Paradoxically, PLDβ1-deficient plants were shown to produce less PA but enhanced ROS production in response to a virulent pathogen (Zhao et al. 2013). Moreover, the arginine residues required for PA binding in the N-terminal part are located in a variable region of RBOHs among different species, suggesting that PLDα1- and PA-mediated regulation may not be relevant for PAMP-induced ROS production in all species.

Roles of RBOHD During Plant Immunity

ROS production is thought to be directly toxic to pathogens, as shown during animal immunity (Lambeth 2004), and may also restrict pathogen entry by triggering stomatal closure, strengthening of plant cell walls via cross-linking of glycoproteins and regulating callose deposition (Bradley et al. 1992, Mersmann et al. 2010, Luna et al. 2011, Macho et al. 2012). ROS themselves also trigger ROS production in neighboring cells and help in generating a ROS wave, which results in the activation of the defense response in distal leaves (Miller et al. 2009, Dubiella et al. 2013, Choi et al. 2014, Gilroy et al. 2014). However, until recently, evidence for a clear contribution of RBOHD-mediated ROS production to immunity against pathogens was still sparse. Indeed, null *rbohD* mutation has no significant impact on disease susceptibility (Macho et al. 2012, Marino et al. 2012, Kadota et al. 2014). However, this could be due to the observation that *rbohD* plants overaccumulate salicylic acid, ethylene and antimicrobial compounds upon pathogen challenge, and exhibit increased expression of the immune marker gene *PR-1* upon bacterial perception ((Pogany et al. 2009, Chaouch et al. 2012, Kadota et al. 2014). These pleiotropic effects may mask the true function of RBOHD in immunity. Notably, constitutive or inducible overactivation of immunity in a null mutant of an otherwise positive regulator of PTI signaling has been observed before (Petersen et al. 2000, Nishimura et al. 2003, He et al. 2007, Kemmerling et al. 2007, Zhang et al. 2010). This suggests that key immune components are 'guarded' by NLR proteins (Segonzac and Zipfel 2011). Actually, this has been nicely demonstrated for the MEKK1–MKK1/MKK2–MPK4 cascade that is guarded by the NLR protein SUMM2 (Zhang et al. 2012).

Accordingly, expression of RBOHD harboring mutations in BIK1-specific phosphorylation sites in the *rbohD* mutant suppresses the excessive defense responses but does not restore PAMP-induced ROS production (Kadota et al. 2014). Moreover, these transgenic plants are more susceptible to hypovirulent bacteria, revealing that RBOHD and BIK1-mediated phosphorylation contribute to immunity. The further characterization of this transgenic line will be useful to investigate the role of RBOHD in immunity against different pathogens. However, to understand fully the role of RBOHD in immunity, it will be interesting to test whether RBOHD is actually guarded by an NLR protein.

Perspectives

There are still many gaps in our knowledge of RBOH-mediated ROS networks. Most importantly, one has to validate experimentally the proposed two-step regulation model of RBOHD. Also, how ROS affect downstream PTI signaling events, such as stomatal closure or callose deposition, is also largely unclear. PAMP-induced ROS may change the redox status to affect enzymatic activities or gene expression, but these target proteins often remain to be identified. The putative H_2O_2-responsive Ca^{2+} channel(s) is also still unknown.

It is also important to compare the regulation mechanisms of RBOHD during different immune signaling pathways. For example, during ETI, effector perception induces a strong and sustained ROS production that lasts several hours, while PAMP-induced ROS production is activated very transiently and peaked at around 10 min after PAMP treatment (Torres et al. 2002, Nühse et al. 2007). CPK1, CPK2, CPK4 and CPK11 are involved in RBOHD activation during ETI (Gao et al. 2013), but it is unclear how this regulation compares with RBOHD regulation by CPKs and BIK1 during PTI.

It will also be interesting to investigate the role of other RLCKs in the regulation of other RBOHs. The BIK1-mediated phosphorylation sites Ser39, Ser343 and Ser347 are relatively well conserved among all RBOHs in Arabidopsis, suggesting the important roles of these phosphorylations. Actually, it was shown that phospho-dead mutations of RBOHC at positions Ser318 and Ser322, which correspond to Ser343 and Ser347 in RBOHD, compromise its function during root hair formation (Takeda et al. 2008), suggesting the important roles of RLCK-mediated phosphorylation in the regulation of RBOHs in general.

The negative regulation of RBOHD is also largely unknown. Plants might change RBOHD subcellular localization through endocytosis to reduce the amount of activated RBOHD at the plasma membrane (Hao et al. 2014). *S*-nitrosylation on the residue Cys890 during ETI was shown to dampen RBOHD activity probably through inhibition of FAD binding (Yun et al. 2011). It is still unknown whether a similar mechanism also occurs during PTI.

In addition to RBOHD, class III peroxidases (PRXs) are also involved in apoplastic H_2O_2 accumulation during PTI. An Arabidopsis *prx33*/*prx34* knockdown line showed reduced H_2O_2 accumulation 7 h after PAMP treatment, similar to what was observed in *rbohD* mutant plants (Daudi et al. 2012). In contrast, the rapid ROS burst that occurs almost

immediately (within 1–2 min) after PAMP treatment is entirely dependent on RBOHD (Nühse et al. 2007, Zhang et al. 2007). This may suggest that RBOHD-mediated rapid ROS production triggers a secondary, late PRX33/34-dependent ROS production. Recently, PRX33 and PRX34 were also shown to be important for salicylic acid-mediated gene expression and subsequent defense responses, showing the important roles of apoplastic ROS in immunity (Bindschedler et al. 2006, Mammarella et al. 2015). It will be important in future work to clarify the relationship of RBOHD and PRX33/PRX34 in ROS signaling during PTI.

Funding

This work was funded by the Gatsby Charitable Foundation [to C.Z.], the European Research Council [to C.Z.], KAKENHI 24228008 [to K.S.]; RIKEN Special Postdoctoral Research Fellowship [fellowships to Y.K.]; Japan Society for the Promotion of Science [Excellent Young Researcher Overseas Visit Program fellowhip to Y.K.]; the Uehara Memorial Foundation [fellowship to Y.K.].

Acknowledgments

We apologize to colleagues whose work could not be covered because of space limitations. All members of the Zipfel and Shirasu laboratories are acknowledged for stimulating discussions.

Disclosures

The authors have no conflicts of interest to declare.

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