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Hierarchical regulation of NADPH oxidase by protein kinases in plant immunity☆

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ABSTRACT

Plant immune responses are attributable to hierarchical immunities, pattern-triggered immunity (PTI) and effector-triggered immunity (ETI). The plant NADPH oxidase, RBOH, is a major player in the pathogen-responsive reactive oxygen species (ROS) burst. *Nicotiana benthamiana* RBOHB is responsible for both the transient PTI ROS burst and the robust ETI ROS burst. Our understanding of the activation mechanisms controlling RBOH during PTI and ETI has increased in recent years. MAPKs are involved in the ETI ROS burst by transactivation of *NbRBOHB*, but not in the PTI ROS burst. We also introduce mechanisms of RBOH regulation at the post-transcriptional level by diverse protein kinases.

1. Introduction

Plants have developed secure defense mechanisms that are activated after sensing of pathogens, initially on the cell surface [1]. Pattern recognition receptors (PRRs) in the plant plasma membrane recognize pathogen-associated molecular patterns (PAMPs) and induce basal immunity referred to as pattern-triggered immunity (PTI). Detected PAMPs include chitin [2], flagellin [3] and lipopolysaccharides [4]. Pathogens deliver effectors into plant cells that suppress PTI, thereby promoting virulence. In some cases, dominant resistance (*R*) gene products of plants recognize these effectors or their activities and induce robust effector-triggered immunity (ETI) [1]. The typical structure of R proteins includes nucleotide-binding and leucine-rich repeat domains. ETI-based intense active defense is known as gene-for-gene resistance and is often accompanied by a hypersensitive response (HR), a form of cell death.

Rapid production of reactive oxygen species (ROS), called ROS burst, has been implicated in diverse physiological processes [5–8]. Biphasic ROS bursts during PTI and ETI are often referred to as first and second bursts, respectively [9]. The main source of ROS in plant immunity is thought to be the plasma membrane-located respiratory burst oxidase homolog (RBOH) [10–17]. The Arabidopsis (*Arabidopsis thaliana*) genome contains 10 members *RBOH* genes [7]. Several lines of evidence suggest that each member of the RBOH family has a pivotal role in resistance to pathogens, hormonal signaling, or development in plants [10,18–21]. AtRBOHD and AtRBOHF function in ROS production during pathogen responses [10], abscisic acid-induced stomatal closure in guard cells [18], and cell growth [19]. In root hair development, ROS production by AtRBOHC/RHD2 controls cell expansion through activation of Ca^{2+} channels [20]. AtRBOHH and AtRBOHJ are essential for proper pollen tube tip growth [21].

Knockdown of *NbRBOHB* in *Nicotiana benthamiana* attenuates ROS production and resistance to the potato late blight pathogen, *Phytophthora infestans* [15,22]. Previous reports showed that defense-related MAPKs are involved in transactivation of *NbRBOHB* in *Nicotiana benthamiana* [15,22]. Overexpression of the *RBOH* gene did not trigger ROS burst, suggesting that post-transcriptional regulation is required for RBOH activation [23]. Our recent studies indicated that a calcium-dependent protein kinase (CDPK/CPK) activates RBOH by direct phosphorylation of its N-terminal region

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[23]. Other studies also reported that the involvement of a receptor-like cytoplasmic kinase (RLCK) or (calcineurin B-like protein/calcineurin B-like interacting protein kinase) CBL/CIPK in the RBOH-mediated ROS bursts [24–26]. In this review, we discuss the molecular mechanisms affecting regulation of RBOH at the transcriptional and post-transcriptional levels, focusing on the actions of protein kinases in response to defense signals.

2. Regulation of RBOH by direct phosphorylation and interacting proteins

RBOH is known to contain six transmembrane domains and localize in the plasma membrane [27]. The N-terminus includes two Ca^{2+}-binding EF-hand motifs and suggests participation of Ca^{2+} signaling in the activation process [12]. Transient influx of Ca^{2+} into the cytoplasm after recognition of pathogen signals is an early event in the signaling cascades that trigger ROS burst and HR cell death [28–31]. The separated tobacco (*Nicotiana tabacum*) and tomato (*Solanum lycopersicum*) RBOHs in the polyacrylamide gel, or a heterologous expression system using mammalian HEK cells showed that Ca^{2+} could directly activate RBOH enzymatic activity [32,33]. Ectopic expression of Arabidopsis AK1 (AtCPK1, encoding a CDPK) increases NADPH oxidase activity and ROS production in tomato protoplasts [34]. CDPKs are Ser/Thr protein kinases that include Ca^{2+} - binding calmodulin-like domains and are the best characterized calcium sensors in plants. They are encoded by a large multigene family and have possible redundancy or diversity in their functions [35,36]. Biotic and abiotic stresses induce NtCDPK2-mediated ROS bursts and HR-like cell death [37]. Phosphoproteomics analyses in Arabidopsis indicated that the N-terminal region of AtRBOHD is phosphorylated *in vivo* when treated with flg22 (a 22-amino-acid peptide of flagellin) or xylanase [38], and loss of the phosphorylation sites reduces the flg22-triggered ROS burst [39]. AtRBOHD is synergistically activated by Ca^{2+} binding and phosphorylation in the heterologous expression system using the HEK cell line [33]. Some CDPKs activate potato RBOHs by direct phosphorylation of N-terminal regions [40,41].

Other studies suggest participation of additional interactors in the process of RBOH activation. Rac1 (a small GTPase) is implicated in regulation of RBOH by direct interaction with the N-terminus of rice RBOH as reported by Wong et al. [42]. Activation of rice RBOHs was linked to interaction between the N-terminal region of RBOH and RACK1 (receptor for activated C-kinase 1) [43]. Tobacco cells transformed with an antisense construct targeting a 14-3-3 protein, which interacts with the C-terminus of NtRBOHD, showed reduced accumulation of ROS after cryptogein treatment [44]. Binding of phosphatidic acid to the N-terminus of RBOH results in RBOH-mediated ROS production in guard cells [45]. Suppression of phosphatidic acid phosphatase accelerates the NbRBOHB-mediated ROS burst in response to *Ralstonia solanacearum* in *N. benthamiana*, also suggesting involvement of phosphatidic acid in activation of NbRBOHB [46]. Heterotrimeric G protein seems to positively regulate AtRBOHD and AtRBOHF, conferring resistance to pathogen infection [47]. Biosynthetic enzymes also affect RBOH activity. RibA, a key enzyme required for the biosynthesis of flavins, such as riboflavin, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) [48], and glucose-6-phosphate dehydrogenases (G6PDHs) in the plastids and cytosol have been implicated in the supply of reduced nicotine amide cofactors for RBOH [49,50].

2.1. Direct regulation of RBOH by CDPKs

Pathogen infection or elicitor treatment causes an immediate phase I ROS burst, and then a massive phase II ROS burst. A protein synthesis inhibitor abolishes only the phase II ROS burst in potato leaves and tubers [14,51]. We previously isolated *StRBOHA–D* from potato (*Solanum tuberosum*) [52]. In potato tubers, *StRBOHA* is constitutively expressed at a low level, and *StRBOHB* is induced by treatment with cell wall elicitor from *Phytophthora infestans* [14]. In leaves, *StRBOHA, StRBOHB* and *StRBOHD* are expressed at low levels, but *StRBOHC* is markedly induced in response to *P. infestans* [52]. The NADPH oxidase inhibitor diphenylene iodonium blocks both bursts, and pretreatment of potato tubers or leaves with the protein synthesis inhibitor cycloheximide abolishes only the second burst [14,51]. On the other hand, both bursts are suppressed by protein kinase inhibitors or calcium inhibitors [28,40]. We identified Ser82 and Ser97 in the N-terminus of potato StRBOHB as potential phosphorylation sites by in-gel kinase assays using mutated N-terminal fragments of StRBOHB [40]. An anti-phosphopeptide (pSer82) antibody indicated that Ser82 is phosphorylated in response to pathogen signals in plants. We cloned StCDPK5 by cDNA expression screening using the anti-pSer82 antibody and cells expressing an N-terminal fragment of StRBOHB, and mass spectrometry analyses showed that StCDPK5 phosphorylates Ser82 and Ser97 in a calcium-dependent manner. *In vivo* interaction of StCDPK5 and StRBOHB, which were expressed in *N. benthamiana* leaves, was shown by co-immunoprecipitation assay [41]. Knockdown of *NbRBOHB* in *N. benthamiana* disrupts ROS production provoked by ectopic expression of the constitutively active mutant of StCDPK5. This loss-of-function in *N. benthamiana* is complemented by heterologous expression of wild-type potato StRBOHB, but not mutant S82A/S97A.

A comparison of predicted amino acid sequences of the four StRBOHs showed that StRBOHA, StRBOHC and StRBOHD also have presumable phosphorylation motifs corresponding to those of StRBOHB, and the recombinant N-terminal peptides of the four StRBOHs are phosphorylated by StCDPK5 *in vitro* [40]. Analyses by the bimolecular fluorescence complementation (BiFC) method indicated that StCDPK5 interacts with StRBOHB and StRBOHC on the plasma membrane, and that mutations of myristoylation and palmitoylaytion sites of StCDPK5, which are responsible for localization on the membrane, eliminate these interactions [23,41]. Domain-swapping experiments between StCDPK5 and tomato SlCDPK2, which cannot induce a ROS burst in *N. benthamiana*, showed that the N-terminal variable domains of the CDPKs confer substrate specificity *in vivo* by dictating proper subcellular localization of CDPKs [41]. These results suggest that StCDPK5 induces phosphorylation of RBOHs and thereby triggers ROS bursts.

Many studies have shown that the effects of RBOH-dependent ROS production on defense responses appear to be diverse in plant-pathogen interactions. Transgenic potato plants expressing the constitutively active StCDPK5 fused to a pathogen-inducible promoter show high resistance to *P. infestans*, but high susceptibility to the necrotrophic pathogen *Alternaria solani* [23]. The results support the idea that ROS might have a negative role in disease resistance to necrotrophic pathogens and/or a positive role in expansion of disease lesions. In addition, silencing of *NbRBOHB* confers resistance to another necrotrophic pathogen *Botrytis cinerea*, in *N. benthamiana* leaves [53]. However, suppression of *RBOH* may not always result in increased resistance to necrotrophic pathogens, possibly depending on the extent of gene silencing, because complete loss of RBOH proteins seems to be compensated by ETI-like responses [24].

AtCPK4, AtCPK5, AtCPK6 and AtCPK11, members of the CDPK I subfamily, were identified as positive regulators of flg22-induced ROS burst by functional genomic screening using protoplasts transformed with a flg22 responsive reporter [54]. Double, triple and quadruple mutant plants exhibit increasingly diminished flg22-triggered ROS bursts and pathogen resistance, indicating redundant functions of the CDPK I subfamily members in rapid PTI