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Regulation of Plant NADPH Oxidases: Roles in Development, Cell Polarity, and Stress Responses

Regulace rostlinných NADPH oxidáz: funkce v buněčné polaritě, vývoji a reakcích na stres

Bachelor’s thesis

Supervisor: Ing. Martin Potocký, Ph.D.

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Declaration:
I officially declare that I have written this bachelor's thesis myself and have cited all the resources and references used. This thesis or any part of it has not been previously used for the purpose of obtaining any other academic degree.

Prohlášení:
Tímto oficiálně prohlašuji, že jsem tuto bakalářskou práci vypracovala samostatně a citovala všechny použité zdroje a literaturu. Tato práce ani žádná její část nebyla v minulosti použita k získání jiného akademického titulu.

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Abstract:

This bachelor's thesis investigates direct regulatory mechanisms of plant NADPH oxidases and summarizes current knowledge about the principles underlying their activity. It provides a detailed overview of Rboh activation by calcium binding to the EF hands of the enzymes, and Rboh phosphorylation by various kinases at specific target sites. Explanation of the roles of protein and lipid partners in mediating Rboh activity is also discussed. It summarized the known information about the interplay of these regulatory pathways and their involvement in plant immunity, stress responses, and development. The function of Rbohs during polar growth and their significance for pollen tube development are also explored. Additionally, special emphasis is laid on demonstrating how the coordination of regulatory mechanisms influences activation, localization, and termination of Rboh enzymatic activity. The feedback loops between calcium signaling and ROS production are also highlighted. Furthermore, an evolutionary perspective is included to contextualize the functional diversification of Rboh isoforms across plant lineages. Finally, the physiological significance of Rboh-mediated ROS production is summarized.

Keywords: NADPH oxidases, calcium-dependent regulation, phosphorylation, ROS production, plant immunity, pollen tube growth, regulatory interplay, lipid partners, protein partners

Abstrakt:
Tato bakalářská práce prozkoumá přímé regulační mechanismy rostlinných NADPH oxidáz a shrnuje současné poznatky o principech, které stojí za jejich aktivitou. Poskytuje podrobný přehled aktivace Rboh vazbou vápníku na EF-domeny těchto enzymů, a fosforylace Rboh různými kinázami na specifických cílových místech. Vysvětlení rolí proteinových a lipidových partnerů při zprostředkování aktivity Rboh je rovněž diskutováno. Shrnuje známé informace o vzájemném působení těchto regulačních drah a jejich zapojení do rostlinné imunity, odpovědí na stres a vývoje. Funkce Rboh během polárního růstu a jejich význam pro vývoj pylových láček jsou také prozkoumány. Kromě toho je věnována zvláštní pozornost, jak koordinace regulačních mechanismů ovlivňuje aktivaci, lokalizaci a ukončení enzymatické aktivity Rboh. Jsou rovněž zdůrazněny zpětné vazby mezi signalizace vápníkem a produkcí ROS. Práce zahrnuje také evoluční perspektivu, která poskytuje kontext pro funkční diverzifikaci izoforem Rboh mezi rostlinnými liniemi. Na závěr je shrnuta fyziologická významnost produkce ROS zprostředkované Rboh.

Klíčová slova: NADPH oxidázy, regulace závislá na vápníku, fosforylace, produkce ROS, rostlinná imunita, růst pylových láček, regulační souhra, lipidoví partneři, proteinoví partneři

List of Abbreviations

ABA – Abscisic acid
ALR1 – Aluminum resistance 1
At – *Arabidopsis thaliana*
BAK1 – BRI1-Associated Kinase 1
BIK1 – Botrytis-Induced Kinase 1
BiFC – Bimolecular Fluorescence Complementation
BY2 – Bright Yellow 2
CBD – Calmodulin-binding domain
CCaMK – Calcium and Calmodulin-dependent protein Kinase
CAX1/3 – Cation exchanger
CaMBD – Calmodulin-binding domain
CaM – Calmodulin
CBL – Calcineurin B-Like protein
CDPK/CPK – Calcium-Dependent Protein Kinase
CIPK – CBL-Interacting Protein Kinase
CRK2 – Cysteine-rich Receptor-like Kinase 2
CW – Cell Wall
CYS6 – Cystatin 6
DGK5 – Diacylglycerol kinase 5
DORN1 – Does Not Respond to Nucleotides 1
DPI – Diphenyleneiodonium
DRM – Detergent Resistant Membrane
DTI – Damage-Triggered Immunity
Duox – Dual oxidase
EGTA – Ethylene glycol tetraacetic acid
Elf18 – Elongation Factor Thermus 18
ETI – Effector-Triggered Immunity
FAD – Flavin adenine dinucleotide
FER – FERONIA
FLS2 – Flagellin Sensing 2
Flg22 –Flagellin 22
FRO – Ferric Reduction Oxidase
GTP – Guanosine triphosphate
GDP – Guanosine diphosphate
GEF – Guanine nucleotide Exchange Factor
GSNO/SNO – S-nitrosoglutathione
IP_3 – Inositol 1,4,5-trisphosphate
JA – Jasmonic acid
KAB1 – Potassium channel beta subunit
LecRLK – Lectin receptor-like kinase
LKS4 – Low-K+ sensitive 4
MAPK/MAP/MPK – Mitogen-Activated Protein Kinase

MCA – Mid1-Complementing Activity channel
Mp – *Marchantia polymorpha*
MRLK – Malectin-like receptor-like kinase
MSL – Mechanosensitive channel of Small conductance-Like
Mt – *Medicago truncatula*
NADK – Nicotinamide adenine dinucleotide Kinase
NDH – NADH dehydrogenase
NMR – Nuclear Magnetic Resonance
Nox – NADPH oxidase
Nt – *Nicotiana tabacum*
OSCA1 – Osmotic stress-activated Ca^{2+} channel 1
OST1 – Open Stomata 1
Os – *Oryza sativa*
OsDMI3 – Rice ortholog of Doesn't Make Infections 3
PA – Phosphatidic acid
PAMP – Pathogen-Associated Molecular Pattern
PB1 – Phox and Bem1 domain
PB1CP – PB1-containing protein
PBL1 – PBS1-Like 1
PBL13 – PBS1-like kinase 13
PIP_2 – Phosphatidylinositol 4,5-bisphosphate
PIRE – PBL13 interacting RING domain E3 ligase
PI3K – Phosphoinositide 3-kinase
PI3P – Phosphatidylinositol 3-phosphate
PLC2 – Phospholipase C 2
PLD – Phospholipase D
PM – Plasma membrane
PP2C – Protein Phosphatase 2C
PRR – Pattern Recognition Receptor
PS – Phosphatidylserine
PTI – PAMP-Triggered Immunity
RAE1 – REGULATION of AtAlMT1 EXPRESSION 1
RACK – Receptor for Activated C Kinase
Rboh – Respiratory Burst Oxidase Homolog
RIC – ROP-Interactive CRIB motif-containing protein
RHO GTPases – Rho-family Guanosine triphosphatases
RhoGDI – Rho GDP Dissociation Inhibitor
RLK – Receptor-Like Kinase
RLS4 – ROOT HAIR DEFECTIVE SIX-LIKE 4
Rop/Rac – Rho of plants / Rac GTPase
ROS – Reactive Oxygen Species
SA – Salicylic acid
SAR – Systemic Acquired Resistance
SIK1 – Salt-Inducible Kinase 1

SnRK – SNF1-Related Kinase
SRC2 – Stress-Related Calmodulin-binding protein 2
St – *Solanum tuberosum*
STOP1 – Sensitive to Proton Rhizotoxicity 1
Ta – *Triticum aestivum*
TPC1 – Two Pore Channel 1
WGD – Whole Genome Duplication
XCP1 – Xylem Cysteine Peptidase 1
Y2H – Yeast Two-Hybrid
Zm – *Zea mays*

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1 Introduction

Plant-specific NADPH oxidases or Rbohs (Respiratory Burst Oxidase Homologs) are transmembrane enzymes producing ROS by transferring electrons from NADPH to molecular oxygen:

$$NADPH + 2O_2 \rightarrow NADP^+ + H^+ + 2O_2^-.$$

Their domain structure includes cytosolic N- and C-terminus and six membrane-integral α-helices interconnected by five loops. The N-terminal region exhibits two Ca^{2+}-binding EF-hand motifs, while the C-terminus has FAD and NADPH binding domains (Figure 1). The NADPH domain serves as the primary donor of electrons, which are then carried by the FAD domain and transferred to two heme groups that are associated with conserved His residues in the third and fifth transmembrane helices; their main function is to ensure directional electron transport (Finegold et al., 1996; Kaur et al., 2018).

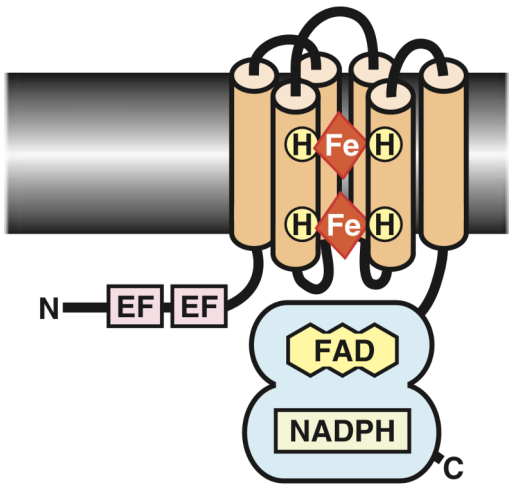

Figure 1. *A Structural model of Rboh enzyme from land plants. Taken from* Sumimoto (2008).

Rbohs and Noxs (NADPH oxidases) share a common origin, yet they differ in structure, roles within a cell, and activation mechanisms. NADPH enzymes in non-plant eukaryotic cells consist of two core proteins, gp91phox and p22phox, that are bounded to the membrane, three regulatory proteins, p47phox, p67phox, p40phox, and also Rac GTPase that is associated with them (Sumimoto, 2008).

Plant Rbohs are homologs of mammalian gp91phox (Groom et al., 1996), yet they lack the mammalian regulatory proteins and have an additional cytosolic N-terminal region with EF-hand motifs that bind Ca^{2+}, which shows that plants have gained their own regulatory mechanisms. Notably, EF-hands on the N-terminus of NADPH enzyme are not exclusive to the plant Rboh family, as non-plant Nox5, Duox1, and Duox2 also possess these motifs. However, these oxidases typically contain four EF-hand motifs and carry additional structural parts, for example, peroxidase homology region in Dual oxidase (Duox) isoforms, therefore, their regulation differs from the classical plant ones (Lambeth et al., 2007). There is still an ongoing debate if the presence of EF hands represents a novel feature in the ancestral enzyme structure or if other family members have lost these motifs over time. Another unique feature

of plant Rboh is the presence of a longer extended upstream N-terminal region, which plays a crucial role in both Ca^{2+}-dependent and independent regulation of the enzyme.

Rboh enzymes are the key players in plant signalling as they maintain different levels of ROS in response to physiological processes. This principle enables plant defense mechanisms as the oxidative burst generated by plant Rboh proteins triggers Pathogen-Associated Molecular Patterns (PAMPs), for instance, MAPK immune cascades (Raja et al., 2017a); polar growth of root hairs and pollen tubes, as elevated levels of ROS participate in cell wall loosening (Foreman et al., 2003; Kaya et al., 2014); seed germination through connection to hormone signalling (Müller et al., 2009); lateral roots differentiation (Li et al., 2015); responses to biotic and abiotic stresses (Rivas et al., 2024); and overall plant development and cell morphogenesis.

1.1 Main discoveries

The presence of NADPH oxidases in plants was first identified by complete sequencing of RbohA gene in rice (Groom et al., 1996), providing the first evidence that plants possess a similar NADPH oxidase system to a mammalian one. After that, more isoforms were identified in other model crops (Amicucci et al., 1999; Lin et al., 2009; Yoshioka et al., 2003). Shortly after, 6 and later all 10 Rboh genes with their spatial and functional features in *A. thaliana* were experimentally characterized (Torres et al., 1998). Comparative analyses demonstrated a remarkable similarity between *O. sativa* and *A. thaliana* Rboh homologs, despite them being classified into different evolutionary subclasses - monocots and dicots, respectively (Keller et al., 1998). Further studies extended the characterization to other Plantae species, including not only various Angiosperms and Gymnosperms, but also Pteridophyta and Bryophyta, providing strong evidence for the evolutionary conservation of plant Rboh enzymes. Overall, it was confirmed that these enzymes serve as the main source of ROS in plants and subsequently play a crucial role in plant signalling as ROS are considered to be second messengers.

Concurrently, studies have focused on revealing regulatory mechanisms of Rboh proteins as well as their localization, it was stated that these enzymes are directly activated by Ca^{2+} via EF-hand motifs (Sagi and Fluhr, 2001). Later, N- and C-terminus phosphorylation by various kinases, interaction with protein partners and lipids were recognized as modulators, contributing to the hypothesis of a complex interconnected regulatory network. In recent years, scientific interest was significantly driven towards the phylogeny of Rboh enzymes across the plant kingdom, uncovering lineage-specific adaptations, mechanisms of Rboh family expansion and the extent of core functional domains conservation.

1.2 Evolutionary context

No Nox isoforms can be found in prokaryotic cells, however, they do have similar enzymes (NDH), and some claim that they can use ROS in signal transduction (Pomposiello and Demple, 2001). Ferric Reduction Oxidases (FROs), which are more present in non-land plants, tend to be considered as functional analogs to classical Rbohs. FROs and Rbohs likely

share the same ancestor, with FRO I being the closest to the Rboh family (Figure 2). Notably, plants that appeared earlier in evolution possess fewer Rboh isoforms in their genome than Angiosperms, and some of them lack specific domains, such as FAD_binding_8 in Bryophyta (Zhang et al., 2023). There is still an ongoing debate about whether Rbohs in non-land plants, particularly those in algae, can be considered the same as the ones in terrestrial plants due to their structural limitations and reduced complexity. Based on known information, it can be assumed that algae isoforms are relatively distinct; in some researches they are clustered in an individual subgroup (Kaur et al., 2018), other results state that there are no typical Rboh genes in Algae species, with some exceptions (Chang et al., 2016), instead they are categorized as Ferric Reduction Oxidases (FROs). In addition to that, the absence of EF-hands in those isoforms (Hervé et al., 2006) raises more doubt about their classification, as all Rbohs from higher plants do possess EF-hand motifs.

The structural and functional differences between FROs and Rbohs, as well as between members of the Rboh family itself, are probably a result of multiple evolutionary events including whole genome or gene duplication and gene fusion events (Chang et al., 2016). They might be connected to evolutionary adaptations to perform more complex physiological processes, such as hormonal regulation, pollen fertilization, etc. Interestingly, in monocots the main duplication mechanism was Whole Genome Duplication (WGD), and representatives of this group possessed more orthologous genes, what can be used to hypothesize if duplication events occurred after dicots and monocots division (Zhang et al., 2023). In addition to that, intron loss and gain probably occurred among all Nox subfamilies after duplication events (Chang et al., 2016), which could lead to a more specialized diversification and higher stress resistance.

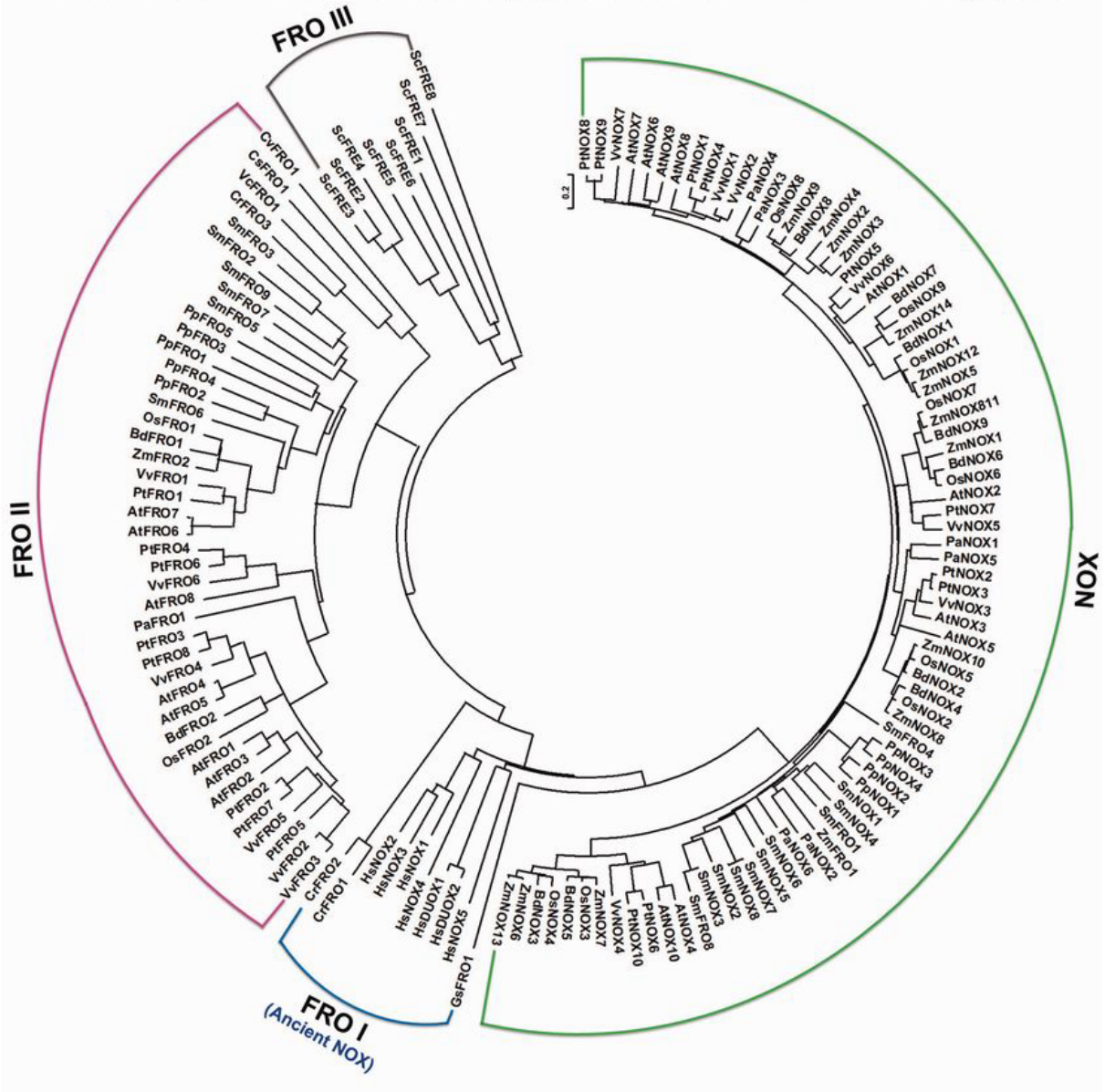

Figure 2. *Phylogenetic relationships between FROs and NOX/RBOHs. Taken from Chang et al. (2016).*

1.3 Functional diversities within the Rboh subfamily

Rboh isoforms from different species can be divided by their functional properties and main localization sites in the plant body. For simplicity, *A. thaliana* Rbohs will be used as a model for functional characterization (Table 1).

Table 1. *Functional diversification of Rbohs in Arabidopsis thaliana.*

Isoform	**Function**	**References**
RbohA	Not known	-
RbohB	Seed ripening, might be involved in thermotolerance, pathogen resistance	(Hawamda et al., 2020; Müller et al., 2009; W. Wang et al., 2021)
RbohC	Root hair tip growth, root hair formation, ROS production in trichoblasta, root hydrotropism and mechanosensing, Casparian strip maturation	(Krieger et al., 2016; Monshausen et al., 2009; Takeda et al., 2008; F.-L. Wang et al., 2021)
RbohD	Wound-induced responses, damage-induced lignification, response to biotic and abiotic stress, cell death control, ABA- and JA-mediated stomatal closure, lignin formation during senescence, lateral root development, microbial homeostasis in leaves, Casparian strip maturation	(Denness et al., 2011; Kadota et al., 2015; Kwak et al., 2003; Lee et al., 2018; Li et al., 2015; Maruta et al., 2011; Miller et al., 2009; Pfeilmeier et al., 2021; Rivas et al., 2024; Torres et al., 2005; F.-L. Wang et al., 2021)
RbohE	Tapetum cell death	(Xie et al., 2014)
RbohF	Pathogen responses, ABA- and JA-mediated stomatal closure, cell death control, Casparian strip maturation, tolerance against excess Na^+, lateral root development, cold tolerance, lignin formation during senescence**,** damage-induced lignification	(Denness et al., 2011; Jiang et al., 2012; Kawarazaki et al., 2013; Kwak et al., 2003; Lee et al., 2018; Li et al., 2015; Maruta et al., 2011; Torres et al., 2005; F.-L. Wang et al., 2021)
RbohG	Not known	-
RbohH	Specific to pollen, tip growth	(Kaya et al., 2014)
RbohI	Root abiotic responses, drought tolerance, seed germination under drought	(He et al., 2017; Lin et al., 2017)
RbohJ	Specific to pollen, tip growth	(Kaya et al., 2014)

2 Regulation of Rboh activity

Rboh regulation is a complex, multifaceted system with multiple layers of interconnected control mechanisms. This chapter focuses mainly on direct regulation like calcium ions binding to EF-hands, phosphorylation of the extended N- and C-terminus, activity of protein partners, such as small Rac GTPases, interaction with lipid compounds, and others.

2.1 Direct Ca^{2+} binding to EF-hands

Calcium ions play a key role in the regulation of the enzymatic activity of Rbohs. Their influence is complex: they bind directly to the EF-hand motifs of the N-terminal region of Rboh, take part in kinase cascades, serve as second messengers, and change conformation of Rboh, which enables upregulation of protein enzymatic activity (Figure 3).

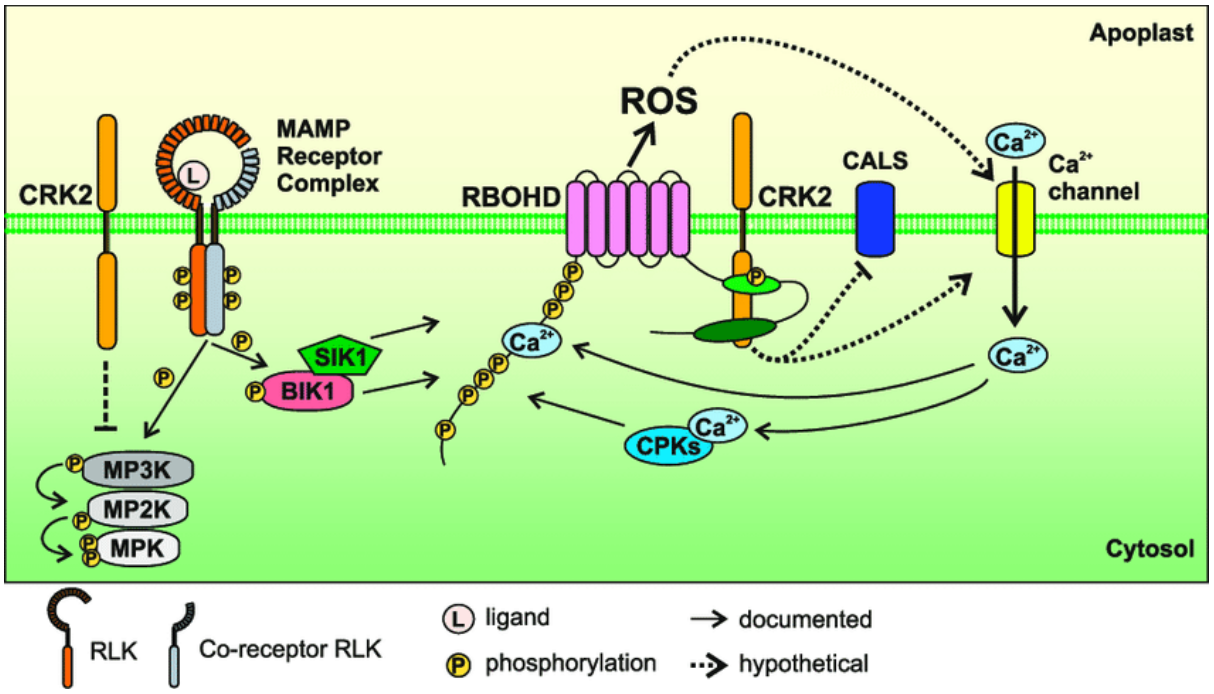

Figure 3. *Model for MAMP-triggered RbohD activation, showing the calcium-mediated and phosphorylation-mediated regulation of RbohD activity. Taken from Kimura et al. (2020).*

All plant Rboh isoforms have two EF-hand motifs on their cytosolic N-terminus that have a structure of two alpha helices connected by a calcium-binding loop (Figure 4). The X- and -Z-positioned residues in the loop are essential for calcium coordination (Grabarek, 2006). The conserved residues at these positions are often aspartic acid and glutamate, respectively (Day et al., 2002). It is well-known that EF-hands of Rboh bind calcium ions, which in turn "translate" conformational change to the enzyme and thus regulate its activity (Ogasawara et al., 2008). This regulation is conserved among all plant Rboh proteins (Kaya et al., 2014). Ca^{2+} influx upregulates ROS production by Rboh proteins, but levels of ROS produced are not the same among isoforms (Kaya et al., 2019; Potocký et al., 2012), which can be due to different Ca^{2+}-binding affinities. There is research claiming that OsRbohB has two additional

EF-hand motifs that make this enzyme similar to calcineurin B and other classical EF-hand-containing enzymes (Oda et al., 2010), yet complete structure of these additional motifs and their function are to be investigated.

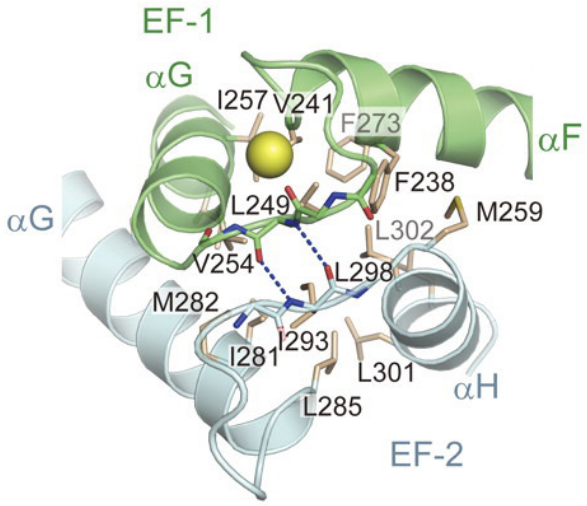

Figure 4. *EF-hand motifs of OsRbohB(138–313), Ca^{2+} is represented as a yellow sphere. Taken from Oda et al. (2010).*

The issues regarding proper understanding of direct plant Rboh calcium regulation is that only a few studies on this topic were made using X-ray crystallography, and more were made using Nuclear Magnetic Resonance (NMR) techniques, but they still do not give enough information to characterize the precise structure of N-terminus with EF-hands and its changes during Ca^{2+}-dependent regulation. The majority of research is based on bioinformatic methods that only suggest the potential conformation and structure.

Further difficulties also occur regarding different isoforms as amino acid sequences vary from one isoform to another, and it is hard to say how exactly these variations influence the activity and functions of the exact enzyme. For instance, AtRbohI does not have aspartic acid in EF-hand 1, which is conserved and essential for Ca^{2+} binding (Oda et al., 2010), yet it has two additional amino acids in EF-hand 2. These modifications may result in a higher functional specificity of this Rboh isoform. Surprisingly, the insertion of aspartic acid into the EF-hand 1 sequence of AtRbohI had an inhibitory effect on enzymatic activity (Kaya et al., 2019), which can be due to the disruption of EF-hand motifs geometry and subsequent loss of conformational interference between them. In addition, the contribution of EF-hand 2 to conformational change of the secondary structure of AtRbohD is more significant than EF-hand 1, which is more important in calcium binding (Ogasawara et al., 2008). The same results were obtained from pollen-specific AtRbohH/J, suggesting that these isoforms need a conformational change in the EF-hand 2 facilitated by calcium binding for activation. This leads to the conclusion that EF-hand motifs 1 and 2 have distinct functional roles in coordinating regulation of Rboh by calcium ions. Furthermore, the role of EF-hands for pollen tip growth was examined: it was stated that AtRbohH and -J are the main sources of ROS during pollen tube growth and Ca^{2+}-binding to EF-hand motifs is essential for polar tip growth (Jimenez-Quesada et al., 2019; Kaya et al., 2015, 2014; Potocký et al., 2007).

Based on these facts and other data, it is not completely clear whether Ca^{2+}-dependent regulation with other regulatory mechanisms acts in precisely the same way among all Rboh isoforms because even small changes in amino acid sequences can lead to different dimer conformations and N-terminus binding properties.

2.2 Phosphorylation

Another important regulatory mechanism of Rboh is post-translational phosphorylation. The protein is phosphorylated at N- and C-terminus by various kinases, which leads to the adjustment of its ROS-producing activity (Figure 3). This mechanism ensures various responses of plant cells to different stresses and pathogens and also assists adjustments to developmental needs.

2.2.1 Overview of N-terminus phosphorylation

Phosphorylation of Rboh can be depicted as the process of transmission of a phosphatic group to specific sites in the N-terminus sequence by different protein kinases that use ATP as a donor, which leads to conformational changes of the enzyme. These sites are considered to have serine (S), threonine (T), and tyrosine (Y) amino acid residues. Studies suggest that S133, S163, S343, S347, and T912 corresponding to positions in AtRbohD (Figure 5) are the most conserved between plant Rbohs, S39 is medium conserved, while S148 possesses the weakest conservation (Zhang et al., 2025). They can also be divided into either broadly targeted or highly specific to an exact kinase. Computer-based analysis revealed that most phosphosites are located in the N-terminal region upstream of EF-hands, where serine has a higher affinity for phosphorylation among all Rboh isoforms (Kaur and Pati, 2018). This localization of phosphosites can indicate a tight interconnection between phosphorylation and Ca^{2+}-dependent regulation through EF-hands, as some studies imply the effect of phosphorylation on increased affinity for calcium ions (Chu et al., 2023).

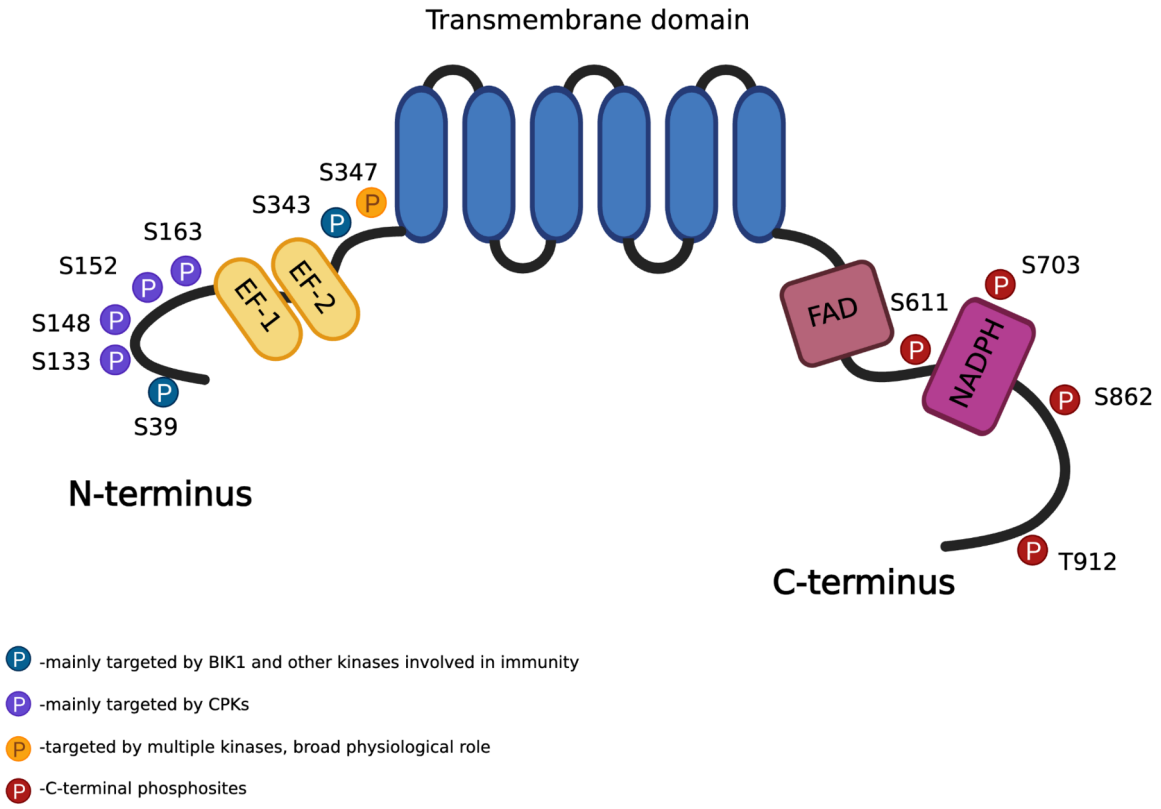

Figure 5. *Schematic model for AtRbohD structure with phosphorylated sites. Created by the author in BioRender, based on Kadota et al., (2015).*

For a better understanding of the roles of N-terminus phosphorylation on the Rboh enzymatic activity and plant cellular processes, it is important to examine protein kinase families that are associated with this process.

Mitogen-Activated Protein Kinases (MAPKs):

MAP cascades are activated by external stimuli like pathogens and abiotic stresses. There are only a few studies providing evidence of direct Rboh phosphorylation by MAPKs, instead, they are thought to trigger different integral signaling pathways that result in enzyme phosphorylation by other proteins. MAP4 kinases phosphorylate S347 in AtRbohD, and S797, S906 in MxRbohD, this results in activation of the protein and ROS production that facilitates immune and stress responses (Zhai et al., 2022; M. Zhang et al., 2018). It is important to note that ROS and MAPK cascades have a feedback regulatory loop, where ROS can regulate the activity of MAP kinases, however, the exact source of ROS in this loop is not defined (Son et al., 2011).

Calcium-Dependent Protein Kinases (CDPKs):

CDPKs are S/T kinases that possess EF-hands and, as a result, their activity is tightly connected with cellular concentrations of calcium ions. Their substrate specificity is dictated by a variable domain that ensures their localization (Asai et al., 2013). Further research on

this topic can provide valuable data for identifying CDPK family members that might be involved in Rboh regulation. They directly activate many Rboh isoforms among different plant species by phosphorylation of serine residues in N-terminal region: AtCPK16 phosphorylates RbohD in *A. thaliana* at S133, S148, S163, S347 and *Os*CDPK5/13 phosphorylate RbohH at S92, S107 in *O. sativa* during hypoxia, *St*CDPK4/5/14 phosphorylates RbohB at S82, S97 in *S. tuberosum* in wound healing, StCDPK23 might also phosphorylate StRbohA-E in tubers wound healing, MtCDPK5 phosphorylates RbohB/D/C in *M. truncatula* in immune responses, and TaCDPK2/4 phosphorylate TaNox7 in *T. aestivum* during wheat panicle development (Hu et al., 2023; Kobayashi et al., 2007; Li et al., 2025; Ma et al., 2021; Yu et al., 2018, 2024).

CBL-Interacting Protein Kinase (CIPKs):

CIPKs interact with Calcineurin B-Like proteins (CBLs) that bind cytosolic calcium ions via EF-hand motifs. During plant immunity responses, calcium signatures are initiated and "decoded" by CBL-CIPK recognition, resulting in signal transferring and Rboh phosphorylation (Ma et al., 2020). They also take part in maintaining homeostasis of Ca^{2+} levels inside the cell by phosphorylating Cation exchanger1/3 (CAX1/3) auto-inhibitory domain (Wang et al., 2024). Understanding this process can be important for further assumptions about the ROS-producing activity of Rboh as it is strongly dependent on calcium ions as well as phosphorylation, and the interplay between them is not thoroughly studied. CIPK26 is thought to phosphorylate AtRbohF/C, while this phosphorylation enhances ROS production by AtRbohC, the effect on AtRbohF is debatable (Drerup et al., 2013; Kimura et al., 2013; X. Zhang et al., 2018). Some studies show that CIPK11 might act as an alternative to the CIPK26 pathway, as it also phosphorylates AtRbohF, and in CIPK26/11 co-expression experiments there was no significant ROS level increase (Han et al., 2019).

Receptor-Like Kinases (RLKs):

RLKs are associated with hormone signaling and take part in plant immune responses initiated by different stimuli: PAMP-Triggered Immunity (PTI), Systemic Acquired Resistance (SAR), Effector-Triggered Immunity (ETI), and Damage-Triggered Immunity (DTI) (He and Wu, 2016). They maintain the signal transduction to downstream cascades through Rboh phosphorylation and following production of ROS as second messengers. RbohD is considered to be the main target of kinases in defense and stress response activation. Using LecRLK Botrytis-Induced Kinase 1 (BIK1) as an example, all steps of such mechanisms can be clearly visualized (Figure 7). First, Flagellin Sensing 2 (FLS2) recognizes pathogenic peptide flg22 and binds to it, which subsequently leads to conformational changes and interaction with BRI1-Associated Kinase 1 (BAK1) (Li et al., 2014). This interaction initiates the Pattern Recognition Receptor (PRR) complex that phosphorylates BIK1 directly or through downstream signaling cascades. BIK1, in turn, phosphorylates the N-terminus of RbohD at S39, S343, S347, what leads to the activation of the enzyme (Kadota et al., 2014). There are other RLKs that activate Rboh enzymes: OsMRLK63 phosphorylates OsRbohA at S26 in response to drought, Does Not Respond to Nucleotides 1 (DORN1) phosphorylates AtRbohD at S22, T24 to regulate stomatal aperture, RIPK phosphorylates AtRbohD at S343, S347, Low-K+ sensitive 4 (LKS4) phosphorylates AtRbohC/D/F at S39 that might facilitate

Casparian strip formation, Aluminum resistance 1 (ALR1) phosphorylates AtRbohD at S39 in activating aluminium detoxification, and ZmBLK1 phosphorylated ZmRboh4 in initiating immune responses (Chen et al., 2017; Ding et al., 2024; Jing et al., 2024; Li et al., 2021; F.-L. Wang et al., 2021; Zhong et al., 2024). In Bryophytes MpPBLa phosphorylates Rboh1 at S30, S55, S169, S406 in *M. polymorpha* during plant defense, and these residues are conserved among Rboh isoforms of terrestrial plants (Chu et al., 2023). Together with previous studies on the role of PBS1-Like 1 (PBL1) in Rboh-mediated ROS production during plant immune responses (Kadota et al., 2014), the evolutionary conservation of PBL-Rboh interaction in response to PAMPs can be further discussed.

SNF1-Related Kinase (SnRKs):
SnRK is another important kinase family that takes part in regulating plant energy metabolism in plant stress responses. There are only a few studies providing data about direct Rboh phosphorylation by this family of kinases: SnRK1α1 phosphorylates Rboh1 at S188, S189, S308 in tomato, resulting in improved tolerance to low nitrogen, and Open Stomata 1 (OST1) phosphorylates AtRbohF at S13, S174 during ABA-mediated stomatal closure. It was also stated that the coexpression of OST1 with CIPK26 resulted in enhanced ROS production as they both phosphorylate the enzyme, yet S174 phosphosite is thought to be more specific to OST1, showing the complexity of plant signaling phosphorylation (Sirichandra et al., 2009; Zheng et al., 2024).

Calcium and Calmodulin-dependent protein Kinase (CCaMKs):
CCaMKs are positive regulators of ABA signaling and are crucial for stress responses. SAPK8/9/10 directly phosphorylate OsRbohB at S140, and this phosphorylation is required for initiating subsequent phosphorylation of S191 by rice ortholog of Doesn't Make Infections 3 (OsDMI3), resulting in water and oxidative stress tolerance. Interestingly, S140 of the OsRbohB N-terminal region is analogous to S174 of AtRbohF which is a target phosphosite for OST1 kinase that also plays a role in ABA-mediated signaling (Wang et al., 2023). Therefore, it can be hypothesized that the importance of these residues in ABA responses is conserved among plant species, and these kinases can be functionally interchangeable, yet experimental evidence is required.

Summarizing the overview of Rboh phosphorylation by different kinases, phosphorylation of the upstream N-terminal region is the conserved mechanism of Rboh activation. The exact phosphorylation sites ensure the specificity of the response to different stimuli. In addition, the same sites can be phosphorylated by members of distinct kinase families, for example, CDPKs and RLKs at S347 (Kadota et al., 2014; Yu et al., 2024), raising a question regarding plant preferences of signaling pathways.

2.2.2 C-terminus phosphorylation and ubiquitination

While phosphorylation of the N-terminal region of Rbohs is a well-studied regulatory mechanism, phosphorylation of the C-terminus remains largely unknown. It is thought to be involved in PAMP-induced ROS production in plant immunity. Previous research has

indicated AtRbohD C-terminal phosphorylation only by PBS1-like kinase 13 (PBL13) and Cysteine-rich Receptor-like Kinase 2 (CRK2), which are members of the RLK family. PBL13 phosphorylates S862, T912 of RbohD, while CRK2 phosphorylates S611, S703 and S862. The phosphorylation of S862 in both cases is thought to negatively regulate ROS production by RbohD. On the other hand, S703 phosphorylation by CRK2 upregulates the enzyme. Interestingly, residues corresponding to AtRbohD S611, 862 were shown to be conserved among all plant Rbohs and also human Nox2/5, whereas S703 was not preserved in pollen AtRbohH/J (Kimura et al., 2020; Lee et al., 2020). These findings show that C-terminal Rboh phosphorylation might be conserved and could have evolved early in evolution and that pollen isoforms possess high specificity and are evolutionarily distinct from other Rbohs.

Moreover, PBL13 AtRbohD phosphorylation at T912 is critical for subsequent AtRbohD ubiquitination by PBL13 interacting RING domain E3 ligase (PIRE). PIRE can directly interact with the C-terminus of RbohD and ubiquitinate specific sites, which leads to enzyme stability and further endocytosis accompanied by vacuolar degradation, which might play an important role in the termination of immune responses. A recent study has shown that the activity of SlRbohB in *S. lycopersicum* is also regulated by PIRE-mediated ubiquitination, and that C-terminal T856, corresponding to T912 of AtRbohD, is essential for this process. In addition, analysis of Rboh homologs from numerous plant species revealed the conservation of residues corresponding to T912 of AtRbohD. Notably, the full PIRE protein structure first appears in Gymnosperms, whereas PBL13 is present only within *Brassicacea*. These findings suggest that cross-communication between phosphorylation and ubiquitination of Rboh homologs is an evolutionary conserved mechanism for regulating Rboh activity, although plants outside of *Brassicacea* may rely on different kinases in this crosstalk (Castro et al., 2024).

In addition, one study demonstrated intramolecular interaction between C- and N-terminus of OsRbohB, which also occurs in Nox5. This interaction might be important for enzyme stabilization and proper conformational changes during Ca^{2+}-induced activation (Oda et al., 2010). Although there is no evidence supporting its existence in other isoforms, its abundance in OsRbohb and Nox5 suggests the possibility of such interaction. Research on this topic can reveal useful information for characterizing the interconnection of Rboh regulatory mechanisms, as this interaction might be important for the accessibility of phosphosites for different kinases and, subsequently, can explain the preference for different signaling pathways.

2.3 Protein partners

Enzymatic activity and spatio-temporal properties of Rbohs are modulated by protein partners that can directly interact with the enzyme. They serve as signal intermediates between environmental stimuli and plant responses. *In silico* analyses suggest a large number of potential partners for each Rboh isoform (Kaur and Pati, 2018), yet there is only little experimental data available confirming direct interaction and regulatory effect on Rbohs.

2.3.1 Isoforms of Rac/Rop GTPase in regulating Rboh activity

Rops are a plant-specific subfamily of small RHO GTPases. They exist in two states: inactive in GDP-bound form and active with bound GTP. Frequently, they are associated with the Guanine nucleotide Exchange Factor (GEF), and the Rop-GEF complex initiates Rop activation by facilitating the release of GDP and binding of cytosolic GTP (Berken et al., 2005). Some studies highlight the importance of Rop-GEF interaction in spatio-temporal regulation of Rop and its subsequent role in cellular polar growth (Denninger et al., 2019). Each Rop isoform has its unique functions, for example, AtRop1/3/5 are pollen-specific and are crucial for pollen tube growth, AtRop2 is thought to be crucial for root growth and ROS formation during hypoxia, OsRac1 plays a role in ROS-mediated pathogen defense and cell death, NtRac5 is also pollen-specific and regulates ROS production during tip growth (Craddock et al., 2012; Kawasaki et al., 1999; Li et al., 1998; Ono et al., 2001; Potocký et al., 2012). Numerous studies have suggested the potential interconnection between Rop and Rboh, as they take part in the same processes, and Rboh has been proven to be the main source of ROS formation in plants. It was experimentally demonstrated that constantly active ZmRacs from maize upregulate ROS production in a mammalian system (Hassanain et al., 2000). Moreover, similar experiments were done using human Rac1 isoform in soybean (Park et al., 2000). This shows the conservation of Rop-induced upregulation of ROS production and can potentially imply the conservation of Rop-Rboh interaction.

Although a lot of research shows the interconnection between Rops and ROS formation, only a few studies confirm direct Rboh regulation by them. It was demonstrated using Yeast Two-Hybrid (Y2H) that OsRacs can directly interact with several OsRbohs, and this interaction occurs in the N-terminus upstream of EF-hands. It was also shown that Rops in active GTP form are preferential for binding (Wong et al., 2008). These results are consistent with recent research indicating that the Rboh N-terminal region upstream of EF-hands has the highest affinity for phosphorylation and protein partner interaction (Kaur and Pati, 2018). A further comprehensive study of OsRac1 regulation of OsRbohB indicated that T39, V43, F44 and D45 residues in the Switch I region of OsRac1 are essential for interaction with the N-terminus of OsRbohB, and these residues create a negatively charged region. Interestingly, this region undergoes a conformational change upon activation that might lead to its stabilization, which results in a stronger association with the positively charged binding region of OsRbohB. In addition, A273 and Y277 were characterized as critical sites in the N-terminus of OsRbohB for OsRac1 binding (Kosami et al., 2014).

In recent years, it has been frequently hypothesized that, apart from upregulating Rbohs' enzymatic activity, Rops are also involved in the spatial targeting of the enzymes. For instance, mislocalization of ROS production by pollen NtRbohs was observed during Rop inhibition in pollen tubes of *N. tabacum*, which might indicate the importance of Rop activity in the proper localization of Rboh proteins (Potocký et al., 2012). Similar results were observed during root hair growth in *A. thaliana* as Rho GDP Dissociation Inhibitor (RhoGDI), a negative Rops regulator, controls the localization of AtRbohC ROS production (Carol et al., 2005). In another study, the same results were observed for AtRbohC, yet it was

further identified that the specific localization of AtRbohC in the tip of a growing root hair is dependent on microfilaments (Takeda et al., 2008). Rops are known to be involved in modulating the dynamics of actin filaments, and they were proven to contribute to the polar growth of pollen tubes through their interaction with ROP-Interactive CRIB motif-containing proteins (RICs) (Zhou et al., 2015). Therefore, the spatial control of Rboh enzymes can be mediated by Rop-RIC-F-actin dynamics.

2.3.2 AtSRC2 in regulating RbohF activity

Stress-Related Calmodulin-binding protein 2 (SRC2) is a protein whose expression is promoted under low temperature. Y2H experiments confirmed a direct interaction between AtSRC2 and the N-terminal region of AtRbohF. This interaction leads to the upregulation of Ca^{2+}-dependent ROS production, yet does not have any effect on the activation of the enzyme by phosphorylation. Increased ROS levels trigger signaling pathways involved in cold stress response. It was also stated that AtSRC2 and AtRbohF might be localized near each other in plant cells (Kawarazaki et al., 2013). This data provides the first evidence of AtRbohF function in tolerance to low temperatures, yet further research on this topic is needed as the precise mechanism of interaction remains unclear, and no homologs in other plant species have been identified so far.

2.3.3 Regulation by 14-3-3 protein

14-3-3 proteins are well-known coordinators of signal transduction pathways during plant development and stress responses (Roberts et al., 2002). Two-hybrid screening of *N. tabacum* cDNA demonstrated an interaction between NtRbohD C-terminus and one of the isoforms of 14-3-3 proteins. In the same study, Bright Yellow 2 (BY2) cells transformed with antisense constructs for Nt14-3-3 protein did not accumulate ROS during treatment by cryptogein, confirming the role of this protein in positively regulating NtRbohD during plant defense (Elmayan et al., 2007). Although the regulatory character of this interaction has been confirmed, the mechanism of this regulation is still unclear. It is most likely that the 14-3-3 protein regulates the enzyme both directly and indirectly, taking part in other signaling pathways.

2.3.4 Role of PB1CP in inhibition of RbohD activity

As stated in previous chapters, RbohD is activated by different mechanisms during pathogen attacks. One of them includes a BIK1 RLK kinase that directly phosphorylates the N-terminal region of the enzyme, upregulating ROS production. PB1-containing protein (PB1CP) is believed to be tightly connected with BIK1-mediated immunity. In a recent study, it was demonstrated that PB1CP has a negative effect on ROS production during pathogen immunity. Furthermore, immunoprecipitation of RbohD together with PB1CP under flg22 and elongation factor thermus 18 (elf18) treatment showed their association, and subsequent pull-down assays confirmed direct binding of PB1CP to the N-terminal region of AtRbohD. It was also stated that PB1CP competed with BIK1 for binding, and they have similar binding

region. Additional analysis on the roles of PB1CP during PAMP-induced defense response has revealed the potential involvement of this protein in endocytosis of RbohD to small endomembrane compartments and its later degradation (Goto et al., 2024). This endocytosis may serve as a feedback mechanism for regulation of PAMP signaling through time-dependent modulation of RbohD levels at the plasma membrane (PM). PB1 domain is highly conserved among plants (Hsin et al., 2021), however, the BIK1-PB1CP-RbohD cascade was only characterized in *A. thaliana*, and further comparative analysis in other species is needed to define its conservation.

2.3.5 CYS6 and XCP1 in regulating RbohD by promoting its degradation

Another mechanism of regulating pathogen-induced responses through RbohD vacuolar degradation involves Xylem Cysteine Peptidase 1 (XCP1) and Cystatin 6 (CYS6). As discussed above, RbohD phosphorylation by PBL13 and its ubiquitination by PIRE leads to the internalization of the enzyme into the vacuole. XCP1 directly interacts with RbohD and promotes its degradation, while CYS6 inhibits XCP1 activity during PTI, which results in RbohD accumulation and an effective immune response (Figure 6). However, the exact RbohD region that interacts with XCP1 is not fully defined, as pull-down assays showed the interaction with both N- and C-terminus, while Bimolecular Fluorescence Complementation (BiFC) demonstrated that only the N-terminal region is sufficient for interaction. It was also confirmed that CYS6, XCP1 and RbohD signaling cascade might be conserved among plant species (Liu et al., 2024). According to these data it can be stated that, the balance between CYS6 and XCP1 regulates ROS levels produced by RbohD during pathogen defense responses. This mechanism, together with PB1CP pathway, are important regulatory mechanisms that accompany other regulatory systems and make possible the rapid switch between the resting and activated state of the enzyme, which as a result, scales back damage caused by excessive ROS accumulation.

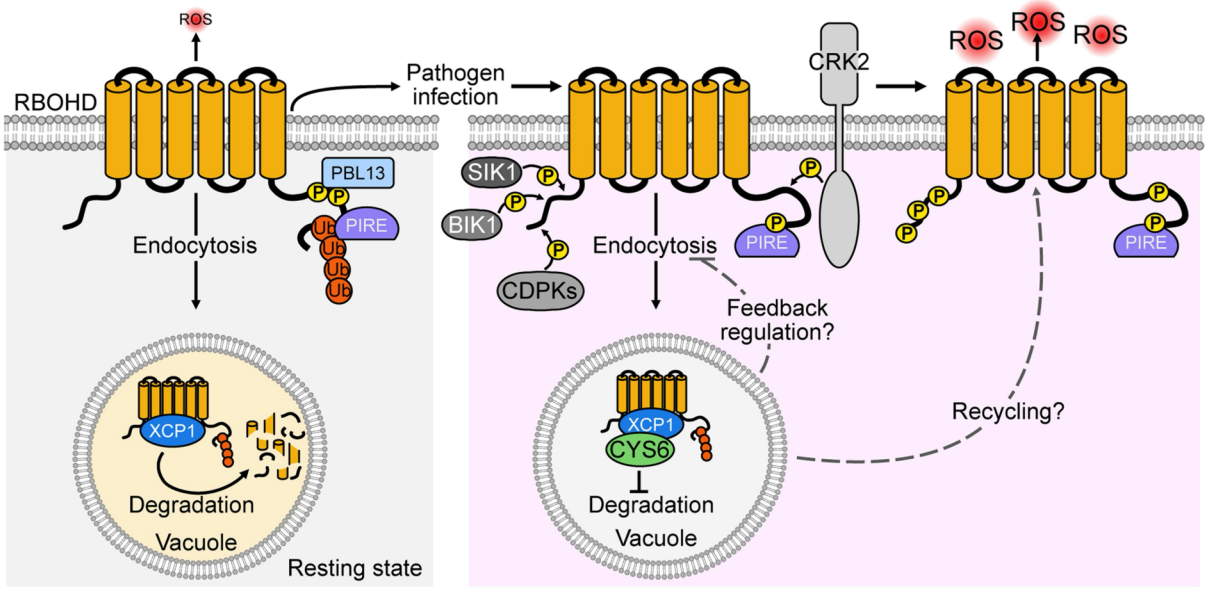

Figure 6. *Interplay between RbohD phosphorylation, ubiquitination and vacuolar degradation during PTI. Taken from Liu et al., (2024).*

2.3.6 CaM activation of Rboh by directly binding to EF-hand motifs

It can be hypothesized that CaM proteins might directly regulate Rboh activity by interacting with its N-terminal EF-hands, as a lot of proteins that possess EF-hands are targeted by calmodulin in a calcium-dependent manner. Human homolog Nox5 can be directly regulated by CaM, but it binds to the Calmodulin-binding domain (CaMBD) in the C-terminus and results in higher sensitivity to calcium ions (Tirone and Cox, 2007). In addition, CaMs regulate other enzymes that possess redox activity, such as NADK (Tai et al., 2019). Nevertheless, calmodulin is an important molecule for the indirect regulation of Rboh enzymes because it takes part in CCaMKs, CDPKs, MAPs cascades as described earlier, lipid signaling, and other mechanisms that directly regulate Rboh activity (Qi et al., 2024; Takahashi et al., 2011). These facts promote calmodulin as a potential protein partner of Rboh, yet experimental evidence on this topic is missing, and further immunoprecipitation experiments are needed to confirm this interaction.

2.4 Lipid interactions and regulation

Lipids themselves serve as messengers in signaling networks. They modulate both the activity and localization of numerous enzymes and might play a role in promoting interaction with other proteins. For instance, PA binding to MPK6 stimulates the activity of the enzyme during salt stress, while binding to the Potassium channel beta subunit (KAB1) promotes its membrane targeting (Kolesnikov et al., 2022; Yao and Xue, 2018). Regulation of Rbohs by lipid interactions is the less studied topic, yet it has the potential to significantly enrich our knowledge about the interplay between regulatory pathways. Further exploration of this aspect can uncover key components in the coordination of signaling cascades and might lead to new insights into the fundamental principles of plant responses to biotic and abiotic stresses.

2.4.1 Role of PA and phosphoinositides

Phosphatic acid in plant cells is synthesized by various pathways. Direct regulation of Rboh isoforms was experimentally confirmed only for PA synthesized by Phospholipase D alpha1 (PLDα1) and Diacylglycerol kinase 5 beta (DGK5β). However, they take part in distinct ROS-mediated responses: PA synthesized by PLDα1 is involved in regulation of RbohD and RbohF during ABA and salicylic acid signaling, while Phospholipase C2 (PLC2)/DGK5-derived PA is responsible for mediating PAMP-induced ROS-production in *A. thaliana* (Kalachova et al., 2022, 2013; Qi et al., 2024; Zhang et al., 2009). Filter binding assays showed that PA directly binds to RbohD/F at N-terminus, and further experiments indicated R149, R150, R156, and R157 as PA binding residues (Zhang et al., 2009). Interestingly, in the same experiment, PA association with RbohD was stronger than with RbohF, which indicates that these isoforms might have slightly different functional roles, where RbohD plays a primary role in ABA-mediated stomatal closure, whereas RbohF has a supportive role.

DGK5β-derived PA binds to the N-terminus of RbohD at the same sites as during ABA-signaling. This interaction leads to protein stabilization and an increase of PM Rboh abundance, as PA might decrease vacuolar degradation of RbohD by PIRE. This leads to higher ROS accumulation in PM. In addition, it was also stated that the Calmodulin-binding domain (CBD) of DGK5β is important for PA synthesis and interaction with RbohD during PAMP-induced ROS production (Qi et al., 2024). According to this, it can be stated that CBD of DGK5β serves as a "translator" of Ca^{2+} signal to lipid signaling that results in controlling levels of RbohD at the plasma membrane and localized ROS synthesis during pathogen defense.

PA, Phosphatidylinositol 4,5-bisphosphate (PIP_2), and Inositol 1,4,5-trisphosphate (IP_3) are known to be responsible for pollen tube growth rates and direction via membrane secretion and modulation of cellular Ca^{2+} levels (Monteiro et al., 2005). A study on regulatory mechanisms of pollen Rboh in *N. tabacum* revealed that PA and PIP_2 are important for NtRbohH/J activation, their ROS production, and subsequent polar pollen tube growth. Furthermore, PIP_2 was demonstrated to be the most effective in Rboh activation (Potocký et al., 2012). Direct interaction of PIP_2 and IP_3 with Rboh isoforms has not been demonstrated yet, however, PIP_2 was shown to be important in modulating cytosolic-bound/active NtRac5 that directly upregulates Rboh-mediated ROS production in pollen tubes, and IP_3 might indirectly influence Rboh activity via regulating Ca^{2+} signatures (Franklin-Tong et al., 1996; Ischebeck et al., 2011).

Another evidence of the regulatory role of phosphoinositides is the fact that inhibition of Phosphoinositide 3-kinase (PI3K) abolished ROS production during salt stress, and application of exogenous Phosphatidylinositol 3-phosphate (PI3P) to seedlings recovered ROS synthesis (Leshem et al., 2007). This demonstrated that PI3P might also be involved in the regulation of RbohD/F under salt stress and suggested that Rboh-mediated ROS production can also occur in intracellular endomembranes, possibly following Rboh endocytosis.

It was indicated that lipids can act both upstream and downstream of Rboh-mediated ROS production, forming a feedback loop. In *A. thaliana* and pollen tubes of *N. tabacum* ROS activated PLDδ produced PA that subsequently took part in plant ROS tolerance and inhibited programmed cell death (Potocký et al., 2012; Zhang et al., 2003). It is still not completely clear how exactly PA contributes to ROS tolerance, and further investigation on this topic is needed.

2.4.2 Rboh regulation within membrane microdomains

It is known that membrane lipid composition can influence the kinetic properties and enzymatic activity of membrane proteins (Laude and Prior, 2004). Considering this, the lipid environment around Rbohs might be a major factor influencing Rboh's accessibility for interaction with other proteins, such as Rops (Ischebeck et al., 2011). The study of RbohD

dynamics revealed that this enzyme is colocalized with membrane microdomains and can be present in monomeric and dimeric states. The oligomerization of the enzyme is influenced by Ca^{2+}-binding to EF-hands and Rboh phosphorylation. These regulatory mechanisms increase the percentage of RbohD dimers within PM and upregulate its diffusion, which indicates that these regulatory mechanisms not only promote protein activation, but also affect its mobility. The same effect was also observed upon flg22 and ABA treatment (Hao et al., 2014). These results can indicate that the RbohD active form is a dimer that forms clusters within PM microdomains. A similar hypothesis was made about the conformation of OsRbohB, it was predicted to form a dimer by swapping of EF-hands (Oda et al., 2010). Based on this information and additional data, it can be hypothesized that localization within membrane microdomains can indirectly facilitate Rboh dimerization and activation as Rboh regulatory proteins, for example, several RLKs, also accumulate in membrane microdomains during plant immunity, and their lipid composition enhances protein interactions (Keinath et al., 2010).

In addition, sterol-enriched membrane microdomains were shown to affect RbohD localization and mobility, as sterols play an essential role in controlling Rboh localization (Posé et al., 2009). For instance, sterol-enriched membrane microdomains in *P. meyeri* pollen are critical for apical ROS production in pollen tube tip and subsequent pollen tube polar growth (Liu et al., 2009). Sterol-mediated localization of both RbohD and pollen Rbohs shows the conservation of this mechanism. However, the exact strategy of such regulation remains unclear. Moreover, the importance of clathrin-dependent and membrane microdomain-associated pathways was established in RbohD internalization, which might influence localized ROS bursts and ensure feedback mechanism terminating excessive ROS accumulation (Hao et al., 2014).

3 Interplay between mechanisms regulating Rboh activity

Mechanisms of Rboh regulation are multifaceted, they often strongly depend on each other or operate at different regulatory levels. Without such regulatory interplays and feedback mechanisms, accurate plant responses to the environment would not be possible, as all of them are parts of different signaling pathways, which are essential for proper spatial and temporal ROS production.

3.1 Dual nature of Ca^{2+}-Rboh regulation

Calcium ions are fundamental signal molecules. In a resting state, their cytoplasmic concentrations are kept very low, and thus even the slightest change in calcium signatures is enough to propagate fine-tuning of cellular processes. In Rboh-mediated ROS production, Ca^{2+} ions serve as both negative and positive modulators depending on the phase of signal propagation and the influence of other regulatory pathways.

3.1.1 Interconnection with protein partners (Rop-Rboh activity)

The detailed relationship between calcium ions and Rac/Rops in the regulation of Rboh activity remains largely unknown as Rac/Rop isoforms act in a different manner during interaction with Rboh isoforms from distinct plant species. A comprehensive analysis was performed in rice for OsRac1 and OsRbohB interaction, which revealed that increased cytosolic Ca^{2+} levels inhibit OsRac1 association with the N-terminal region of OsRbohB. It was also stated that EF-hand motifs are not required for their association, yet EF-hand 2 might be essential for OsRac1 binding suppression under high Ca^{2+} levels. Therefore, consistent with other studies indicating the positive role of elevated cytosolic Ca^{2+} concentrations on Rboh enzymatic activity, the dual role of calcium ions can be proposed. In other words, at the beginning of the oxidative burst, calcium ions promote Rboh activation and stabilization by directly binding to EF-hands or indirectly through kinase cascades, which results in conformational change of the enzyme. This conformational change might be essential for Rboh-Rop interaction. Rop binding upregulates the activity of Rboh, and its ROS production is further increased leading to the second wave of calcium influx. At higher calcium concentrations, Rboh-Rop association is inhibited, resulting in initial ROS production termination. Afterwards, other mechanisms for ROS synthesis downregulation may take part, such as ubiquitination (Lee et al., 2020; Wong et al., 2008). Concurrently, the excessive calcium ions are sequestered into the vacuole by the CAX pathway, which is triggered by calcium ions themselves at higher concentrations (Wang et al., 2024). Thus, ROS and Ca^{2+} create a negative feedback loop that enables proper control of oxidative bursts and calcium signatures.

3.1.2 Positive feedback loop regarding calcium-permeable channels

ROS activation of calcium-permeable channels is an essential step for signal propagation and mediation of Rboh activity. H_2O_2 is considered to be a more stable ROS, and it can freely circulate within membranes, thus it is proposed to activate calcium-permeable channels

(Henzler and Steudle, 2000). During ABA treatment, ROS were proven to activate cation channels in guard cells (Pei et al., 2000). Although the source of ROS production was not identified in the study, the key role of Rboh-derived ROS in stomatal movement and ABA signaling leads to the assumption that Rbohs are the most suitable candidates. It was also shown that AtRbohD-mediated ROS production is essential for the propagation of calcium wave through Two Pore Channel 1 (TPC1), as Diphenyleneiodonium (DPI) treatment abolished Ca^{2+}-wave transmission during salt stress (Evans et al., 2016). According to these data, it appears that Rboh-mediated ROS production and Ca^{2+} influx have a positive feedback loop, where initial calcium influx triggers ROS production probably through direct binding to EF-hands. Then ROS elevation can trigger calcium channels which promote further Ca^{2+} influx that eventually upregulates Rbohs and promote stronger ROS signal (Takeda et al., 2008). The possible channel candidates for this process were proposed, such as Osmotic stress-activated Ca^{2+} channel 1 (OSCA1), channels of Mid1-Complementing Activity channel (MCA), and Mechanosensitive channel of Small conductance-Like (MSL) family (Kurusu et al., 2015). However, the experimental evidence for validating interconnection of Rboh-mediated ROS production and activation of these channels is missing, thus further extensive research on this topic may shed light on the temporal interplay between Ca^{2+} and regulatory mechanisms of Rbohs.

3.1.3 Synergistic activation with phosphorylation

Phosphorylation of the N-terminus of Rbohs is proposed to synergistically activate the enzyme together with direct Ca^{2+}-binding to EF-hands (Ogasawara et al., 2008). Another study provides additional information claiming that Ca^{2+}-binding to EF-hands can not promote stable and long-lasting conformational change of the enzyme, thus phosphorylation is needed to ensure that the protein remains active. This process is consistent with the positive feedback loop between Ca^{2+} and ROS. Research also states that phosphorylation of RbohF is a prerequisite for Ca^{2+}-dependent regulation (Kimura et al., 2012).
Similar results were observed for RbohD as BIK1 can phosphorylate it in a calcium-independent manner during PAMP responses, and BIK1 phosphorylation can be prior to regulatory pathways that are dependent on calcium ions (Kadota et al., 2014). However, assumptions regarding the prerequisites for Rboh regulation can be a subject of debate, as only little information is available providing in-depth experimental research on the interplay of different regulatory pathways. Additional difficulties occur in characterizing interconnection between regulatory pathways involving calcium ions, as it is a highly complex and multifactoral system, where, for example, calcium ions themselves can act upstream of kinases phosphorylating Rboh. Moreover, the requirements for activation can differ among Rboh isoforms and during distinct plant responses. Nevertheless, it can be concluded that Ca^{2+}-binding to EF-hands and N-terminus phosphorylation influence each other, as, for example, it was confirmed that phosphorylation of the enzyme enhances Ca^{2+}-binding affinity of EF-hands, and this mechanism might be conserved among plants (Hashimoto et al., 2023).

3.1.4 Upstream calcium-independent regulation of RbohD by BIK1

Studies on direct regulation of RbohD by the PRR complex revealed that RbohD can be directly phosphorylated, and this phosphorylation and activation of the enzyme does not require Ca^{2+}, as treatment with Ethylene glycol tetraacetic acid (EGTA) did not abolish BIK1 activation and subsequent phosphorylation of S39, 343 RbohD N-terminal residues. These phosphosites are strongly conserved among orthologs in all plant species, leading to the conclusion that this calcium-independent regulation of RbohD during PAMP-induced immunity is conserved among plants (Kadota et al., 2014). Notably, BIK1 is a downstream target for calcium-dependent kinases and its activity and abundance are precisely controlled by CPK28 phosphorylation. CPK28 mediates BIK1 turnover under PAMP-triggered immunity, resulting in shielding against excessive ROS accumulation (Monaghan et al., 2014). This creates a loop between BIK1 and PAMP-triggered Ca^{2+} influx. In addition, BIK1 phosphorylation of RbohD results in ROS formation that can subsequently trigger upregulation of calcium-permeable channels and lead to higher cytosolic Ca^{2+} concentrations, which might activate CDPKs that further phosphorylate the enzyme and promote its activation (Dubiella et al., 2013). Thus, despite the fact that BIK1 does not require calcium for its enzymatic activity, RbohD can not be fully activated without Ca^{2+}.

3.2 Summary of the interplay between Rboh post-translational regulatory pathways

Understanding the interconnection between different regulatory pathways of Rboh enzymes is important, as it reveals spatial and temporal aspects of ROS production in response to various environmental stimuli. It is the precise coordination between these regulatory pathways that ensures proper ROS-induced signal propagation and termination, which leads to high specificity of plant responses. Recently, the model for temporal regulation of Rbohs was proposed, characterizing the interplay of discussed regulatory mechanisms. This system consists of two consecutive steps: “Modification-Activation” and “Brake”. During the initial modification-activation phase, calcium signatures promote activation of Rboh enzymes. Later, exogenous stimuli initiates Rbohs phosphorylation by RLKs and their ROS production. Elevated ROS levels further increase calcium influx probably through the activation of Ca^{2+}-permeable channels, where the role of kinases was also proposed. An eventual rise in Ca^{2+} concentrations triggers CDPKs that further upregulate the ROS-producing activity of Rboh. In the later brake phase, excessive cytosolic Ca^{2+} promote negative feedback regulatory mechanisms. Internalization of Rbohs also occurs via different endocytic pathways as described earlier. The importance of H_2S in ROS degradation during this step is also highlighted (Zhang et al., 2025).

The interplay between regulatory mechanisms of Rboh can be summarized and illustrated using RbohD as a representative example (Figure 7).

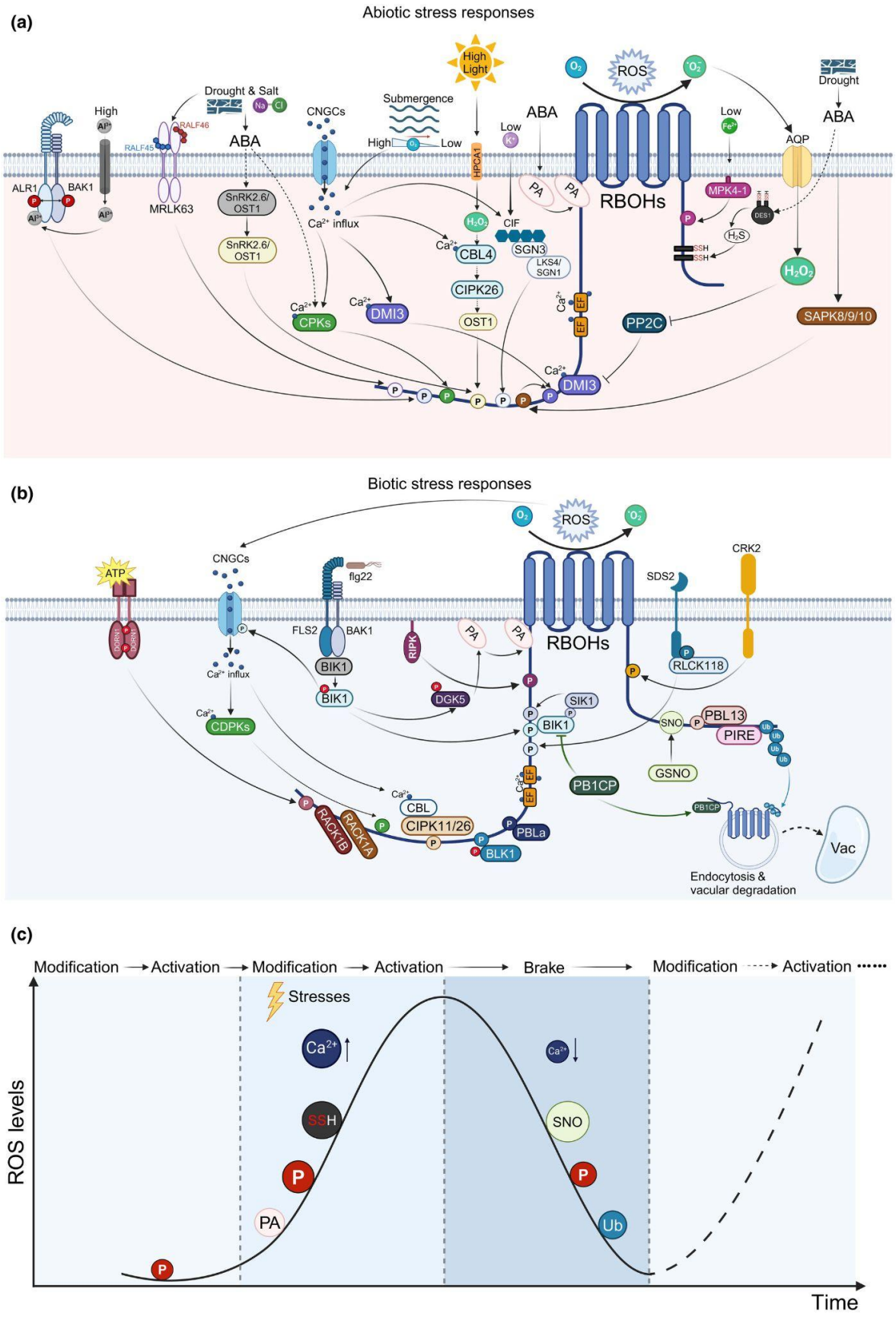

Figure 7. *Overview of interplay between regulatory pathways of RbohD during biotic and abiotic stress. Taken from Zhang et al., (2025).*

3.3 Protein partners and lipids in Rboh spatial localization and polar growth

The spatial organization of Rbohs is an important factor for their enzymatic activity and subsequent cellular signaling. As discussed earlier, Rboh enzymes are localized in membrane microdomains that are heterogeneous and consist of dominantly sterols and sphingolipids. These microdomains promote Rboh clustering and might target them to micro-environments sufficient for interaction with regulatory proteins. A study on microdomain polarization in pollen tubes identified that Rbohs are found in Detergent Resistant Membranes (DRMs), and their activity is sterol-dependent (Liu et al., 2009). Similar results were observed during the investigation of OsRac1 and OsRbohB interaction. It was highlighted that this ROP isoform interacts with OsRbohB, specifically when exposed to sphingolipids containing 2-hydroxy fatty acids. This interaction leads to Rboh activation and ROS production during immunity (Nagano et al., 2016). In addition, another research demonstrated that the rice Receptor for Activated C Kinase (OsRACK1), which interacts with OsRac1 and influences its activity, is also predominantly associated with DRMs (Nakashima et al., 2008), which leads to the conclusion that the lipid composition of microdomains influence coupling of Rboh and Rac/Rops. Furthermore, the extensive research on Rop6's role during osmotic signaling revealed that Rop6 is responsible for upstream RbohD/F activation and ROS formation. It was also stated that Rop6 nanodomains are colocalized with clustered RbohD, and these clusters are more abundant when the permanently active form of Rop6 is present (Smokvarska et al., 2020). Moreover, Rboh microdomain localization promotes ROS generation at specific sites, for example, polarized microdomain localization is essential for polar growth in pollen tubes, as the microdomain environment promotes Rop-Rboh interaction and, as a result, localized ROS production (Liu et al., 2009).

Anionic phospholipid phosphatidylserine (PS) can directly interact with Rop6 and modulate its spatial organization and nanoclustering, which might subsequently influence Rboh activity. PS localization at plasma membrane is controlled by FERONIA kinase (FER), which is a receptor of cell wall (CW) integrity in tip-growing cells (Smokvarska et al., 2023). The importance of FER in Rboh regulation and tip growth was also reported in root hairs, where FER positively regulates ROP2-RbohC interaction (Duan et al., 2010). Similarly, FER acts upstream of Rop and RbohH/J in pollen tubes, and overexpression of FER leads to excessive CW deposition and termination of tip growth (Boisson-Dernier et al., 2013). Thus, it can be hypothesized whether this mechanism of Rboh spatial regulation during polar growth is conserved, and further research on this topic can uncover sufficient details for understanding the complex spatial regulation of Rboh and ROS production.

4 Physiological impact of Rboh regulation

Rbohs produce ROS, which are important signaling molecules involved in biotic and abiotic stress responses, plant development, growth, and hormone signaling.

Stress responses:

During salt stress, ROS wave transduces a signal to nearby cells through apoplast in roots and, together with TPC1 channels, promotes plant stress tolerance. Interestingly, the same study has identified that *A. thaliana* mutant that lacks functional RbohD possesses a decreased speed of signal transduction both in salt stress and wounding, indicating that they might share the exact mechanism of transmission (Evans et al., 2016). Salt stress also promotes Rboh internalization which leads to intracellular ROS production and improved tolerance of excessive Na^+ (Leshem et al., 2007). There is research highlighting the importance of Ca^{2+}-dependent regulation, lipid interactions, phosphorylation by various kinases, and balance between PM-localized and internalized Rboh proteins in salt-induced oxidative burst. Importantly, the disruption of one of those regulatory mechanisms does not entirely abolish ROS production, showing the complexity and robustness of these signaling pathways (Han et al., 2019). Rbohs can also promote detoxification of heavy metals through ALR1-BAK1-RbohD system: phosphorylation of RbohD and further ROS production modify REGULATION of AtAlMT1 EXPRESSION 1 (RAE1) F-box, which leads to Sensitive to Proton Rhizotoxicity 1 (STOP1) nucleus accumulation and organic acid anions secretion that chelate Al^{3+} ions (Ding et al., 2024). ROS production by Rboh enzymes can also initiate responses to ion deficiencies. For instance, ROS accumulation by phosphorylated MxRbohD2 initiates a Fe-shortage response (Zhai et al., 2022). Similarly, K deprivation triggers AtRbohC/D/F phosphorylation and emerging oxidative burst induces K^+ transporters (F.-L. Wang et al., 2021). Furthermore, ROS production by RbohF initiates cold stress response cascades, and SRC2 can directly interact with the enzyme (Kawarazaki et al., 2013). Various kinases from different families phosphorylate Rbohs in response to drought, where ROS signal propagation initiates stomatal closure through downstream cascades and hormone signaling (He et al., 2017; Jing et al., 2024). ROS waves can also enable hypoxia and high light tolerance, for instance, AtRbohI expression is induced under oxygen starvation and is regulated by auxin signaling, AtRbohF and -D are also important sources of hypoxia-mediated ROS (Lin et al., 2017; Sirichandra et al., 2009).

RbohD induction under biotic stress has already been briefly discussed in previous chapters. During PTI as well as ETI, ROS production together with Ca^{2+} act as the main signal transduction mechanism initiating PAMP-immunity (Kadota et al., 2015). In addition, it was demonstrated that ROS production is sufficient for actin remodeling during plant immunity as it acts upstream of CP, which is important for actin dynamics, this process is tightly connected with Rboh-PA interaction (Cao et al., 2022). Furthermore, some evidence shows that RbohB might be involved in immunity responses to nematodes (Hawamda et al., 2020). Moreover, termination of plant pathogen immunity is also tightly connected with RbohD ubiquitination and degradation, which shows that Rboh not only initiates such responses, but also enables proper signal restriction (Lee et al., 2020).

Hormone signaling:

Hormone signaling is tightly interconnected with ROS produced by Rboh. ROS can alter hormone gene expressions as well as modulate existing hormone levels.

Auxin:
During abiotic stress, ROS decreases auxin levels, which leads to the inhibition of plant development and growth. ROS levels can also alter the expression and localization of PINs, subsequently leading to auxin level and dynamics adjustments (Raja et al., 2017b). On the other hand, auxin itself can modulate ROS levels by upregulating the expression of ROOT HAIR DEFECTIVE SIX-LIKE 4 (RLS4), which enhances Rboh abundance and activity during polar growth (Mangano et al., 2017). Another mechanism for auxin-mediated ROS regulation involves Rops that are activated by auxin signaling (Raja et al., 2017b). They directly interact with the N-terminus of Rboh enzymes and promote their active state, thus resulting in an increase of ROS production. Recently, apoplastic ROS production activated by auxin involving rapid calcium changes and mediated by RbohC/F was also reported (Kulich et al., 2025). Together, these mechanisms demonstrate the feedback loop between auxin and ROS signaling.

Salicylic acid:
SA is involved in primary defense immunity and stomatal closure. It promotes ROS production by RbohD and results in the regulation of stomatal movement (Kalachova et al., 2013). ROS levels, on the contrary, can influence SA accumulation, leading to enhancement of defense genes. This shows that they form a feedback signaling mechanism. In addition, ROS origin, together with pathosystem, ensures the specificity of the interplay between ROS and SA signaling and enable proper immune responses (Lukan and Coll, 2022)

Jasmonic acid:
JA plays a crucial role in plant development and stress responses. Research shows that JA influences Rboh activation and subsequent ROS production, and RbohD/F take part in JA-mediated stomatal closure via regulating MeJA-inducible genes. However, ROS levels do not regulate MYC2 gene expression, leading to the conclusion that JA signaling pathways are modulated by ROS only to some extent (Maruta et al., 2011).

Abscisic acid:
ABA hormone signaling is tightly interconnected with Rboh regulation through multiple pathways including phosphorylation, lipid interaction, and others. ABA-mediated stomatal movement relies on ROS produced by Rboh enzymes, as ABA signaling promotes activation of Rboh by phosphorylation via OST1 (Sirichandra et al., 2009). It was also stated that ABA might influence RbohD homodimerization (Hao et al., 2014). Moreover, ROS and ABA function in a positive feedback loop, as ROS inhibit Protein Phosphatase 2C (PP2C) that suppresses ABA signaling through OST1 dephosphorylation (Mittler and Blumwald, 2015).

Polar growth and cell shape:

Several studies indicated the importance of localized ROS synthesis by Rboh proteins in polar growth. Experiments with *A. thaliana* double mutant lacking functional RbohH/J showed that pollen tube tip growth is dependent on Rboh-mediated ROS production, and calcium ions influence its activation (Kaya et al., 2014). In addition to that, ROS formation in pollen grain near a single aperture region was also demonstrated (Potocký et al., 2012). That might indicate the role of Rboh proteins in overall pollen grain germination. Moreover, Rboh functions in later reproductive development were identified, as ROS produced by Rbohs initiate pollen tube rupture that enables the transfer of sperm cells to the ovule (Duan et al., 2014). It was shown that RbohC is also responsible for localized ROS synthesis in the apical region of root hairs which enables their polar growth. And a positive feedback loop between ROS and Ca^{2+} levels was proposed as the main mechanism for controlling the axis of plant cell growth. In addition, it was demonstrated that proper Rboh localization at the cell tip is essential for tip growth, and microfilaments together with Rop were responsible for Rboh apical localization (Takeda et al., 2008).

Role in germination:

The involvement of RbohB in seed after-ripening was proved as well, promoting the connection between ABA signaling and RbohB ROS synthesis (Müller et al., 2009). Another evidence of Rboh-derived ROS significance during reproduction is the function of RbohE in programmed cell death in the anther tapetum (Xie et al., 2014).

Growth, development and morphogenesis:

In roots, H_2O_2 accumulation in the elongation zone can lead to bending. Experiments with *A. thaliana* mutants lacking functional RbohC revealed that ROS produced by this isoform is important for the bending and coordinated growth of roots towards water (Krieger et al., 2016). The mechanism of ROS-promoted root gravitropism was also detected, it was proved to be activated by auxin (Joo et al., 2001). Although the origin of ROS was not identified in the study, it can be hypothesized that Rboh enzymes are responsible for ROS production, as these enzymes are the main source of apoplastic oxygen radicals, and they are interconnected with auxin pathways. Furthermore, Rboh isoforms were identified as important factors for Casparian strip formation through ROS-mediated activation of K^+ transporters (F.-L. Wang et al., 2021). Conversely, RbohD/F were reported to inhibit lateral root development by increasing ROS levels in primary roots (Li et al., 2015). In addition, RbohF takes part in secondary cell wall formation, which results in stone cell deposition in pear fruit (X. Wang et al., 2021). Moreover, Rboh-derived ROS also promote early senescence (Lee and Back, 2021).

5 Future perspectives

Direct regulation of Rboh enzymes is a major field that can possess a lot of sufficient information for a further comprehensive understanding of plant signaling, responses to stress and plant developmental biology.

Future research can shed light on the evolutionary origin of the Rboh structure, especially EF-hand motifs and thus direct calcium-mediated regulation. Comparative analyses could help to clarify whether EF-hands are ancestral or lineage-specific. This finding can serve as a marker for characterizing the phylogenetic relations between species and help to trace their diversification. Furthermore, investigation of variations of residues in the N-terminus of different Rboh isoforms can extend our understanding of how these differences influence calcium-binding, phosphorylation, dimerization and other regulatory mechanisms. This subsequently can reveal new insights on the interplay of regulatory pathways, as the N-terminus is considered to be crucial for mediating signaling cascades, and even small changes in its sequence lead to alternative enzymatic activity. This investigation could possibly lead to additional information about Rboh isoform functional diversifications.

A proper understanding of alterations between phosphosites can also provide valuable details about individual phosphorylation events and how exactly different kinases compete or cooperate among themselves and with other regulatory partners to ensure the precise control of plant responses. Mutagenesis of isoform-specific residues and phosphoproteomics can help to elucidate these relationships. Further research on the variable domain of CDPKs can help to identify new kinases that can interact with Rboh proteins. This data can lead to crop improvement, as manipulating with Rboh phosphosites can fine-tune ROS production and lead to enhanced resistance to pathogens and stress.

Another future research direction is connected to calcium channels. Upregulated Rbohs produce higher levels of ROS, which can activate cation channels, leading to the second wave of Rboh activation. Considering crop improvements, this topic can be significant for evaluating ROS concentrations that enhance immunity and do not lead to cell death. As for example, manipulation with phosphosites can increase ROS production, and it is crucial to understand how it will influence downstream cascades and feedback loop between ROS and Ca^{2+} levels. Research on the possible channel candidates can also extend our knowledge about the interplay of regulatory mechanisms and the temporal aspect of Rboh modulation.

Investigation of lipid-Rac-Rboh interaction can clarify how it influences the localization of the enzyme and can uncover new aspects of polar root and pollen tube growth. Pollen tube growth is a critical part of plant fertilization, and it needs a precise signaling system for CW expansion and redeposition. Thus, regulation of localized Rboh-derived ROS is the key element to ensure the accurate growth direction. These studies may contribute to the development of techniques enhancing pollination and seed production, which are important for producing sufficient agricultural productivity with minimal input of resources.

Conclusion

This work provides a comprehensive overview of the regulatory mechanisms modulating Rboh activity in different responses to environmental stimuli and overall plant signaling and development. Ca^{2+}-dependent regulation, including direct binding to EF-hand motifs, phosphorylation of N- and C-terminus of Rboh, lipid-mediated regulation, and interaction with protein partners are thoroughly studied. The distinct roles and calcium-binding affinity of EF-hand 1 and 2 are examined, providing a better understanding of Rboh structure and activity. Additionally, the dual nature of Ca^{2+}-dependent regulation, where in the initial phase of response, calcium ions activate the enzyme and in the later phase possess inhibitory activity, is highlighted. The feedback loop regarding calcium-permeable channels and ROS production is also discussed, offering a deeper understanding of calcium homeostasis within the cell.

Members of various protein kinase families that directly phosphorylate Rboh were identified, and sites of their action were listed and compared, indicating the tight interconnection between them and proposing the hypothesis of their interchangeability. The roles of phosphorylation and ubiquitination in adjusting ROS production through Rboh internalization were discussed, illustrating a comprehensive system of initiating and terminating localized ROS generation that promotes proper plant responses. Special emphasis was laid on the interplay between regulatory pathways and their roles in mediating spatial and temporal control of the enzyme. The influence of membrane microdomains on Rboh activity was explored, showing that their lipid composition promotes protein-protein interactions.

The role of interconnected regulatory cascades in determining Rboh localization was explained, showing how complex interactions coordinate enzyme dynamics, ensuring specific cellular needs like polar growth. The mechanism of pollen tube development and how it is interconnected with Rboh-mediated ROS production was also discussed. Finally, the overall physiological roles of Rboh in different plant responses, hormonal signaling, plant development, and morphogenesis were outlined. Based on the discussed findings, several directions for future research were proposed, highlighting the potential of further investigation into this topic to improve agricultural techniques.

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