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Stress-induced ROS compartmentalization, perception, and signaling

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Abstract

Reactive oxygen species (ROS) are essential for life and involved in the regulation of almost all biological processes. ROS production is critical for plant development, response to abiotic stresses, and immune responses. ROS can also cause direct oxidative damage and organisms must therefore balance ROS production and quenching. Here, we focus on recent discoveries in ROS biology emphasizing abiotic and biotic stress responses. Recent advancements have resulted in the identification of one of the first sensors for extracellular ROS and highlighted waves of ROS production during stress signaling in *Arabidopsis*. Enzymes that produce ROS, including NADPH oxidases, exhibit precise regulation through diverse posttranslational modifications. Discoveries highlight the importance of both N- and C-terminal regulation of NADPH oxidases through protein phosphorylation and cysteine oxidation. Here, we discuss advancements in ROS compartmentalization, systemic ROS waves, ROS sensing, and posttranslational modification of ROS producing enzymes as well as identify areas where foundational gaps remain.

Introduction

Generation of reactive oxygen species (ROS) is a necessary process in all living organisms[1]. Production of ROS including singlet oxygen ($^1O_2$), superoxide ($O_2^{\bullet -}$), hydroxyl radicals ($OH^{\bullet}$), hydrogen peroxide (H_2O_2), as well as reactive nitrogen species such as nitric oxide (NO) are required for basic biological processes and stress responses[2]. However,

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Author contributions

All authors contributed to writing the manuscript and conceiving the ideas presented. GC and MW led the structure and ideas in the manuscript and finalized the writing. SK generated Fig. 1. BC generated Fig. 2 and portions of Fig. 3. D.S. generated Fig. 3a,b, S1a and S1b. MC generated the models in Fig. 3 and Fig. S1.

Competing interests

The authors declare no competing interests. No figures contain third party material.

ROS and NO can also cause direct oxidative damage to DNA, lipids, and proteins. All living organisms must therefore balance ROS production and ROS quenching. Like other eukaryotes, plants utilize ROS as signaling molecules in multiple biological processes including abiotic and biotic stress responses[3,4,5].

Multiple environmental factors induce abiotic stress in plants, including extreme temperatures, drought, salinity, osmotic stress, excess light, ozone, and heavy metals [6, 7]. ROS contribute to stress acclimation, metabolic changes, and induction of programmed cell death [8]. Biotic signals also lead to enhanced ROS production. However, ROS are not only important during the response to external cues but basal production is critical for cell expansion and localized production is important for numerous events including pollen tube elongation, root hair growth, cell polarity and Casparian strip formation[9, 10,11,12,13]. ROS production in response to pathogen infection and abiotic stimuli, including salt stress or wounding, has been linked to local and systemic signal transduction and is integrated with calcium signaling and with the deposition of callose at plasmodesmata[14]. Importantly, both abiotic and biotic stresses lead to ROS production in several different subcellular compartments and these events must be tightly coordinated and regulated [2,15,16,17].

Although ROS production is critical for growth, signaling, and development, their reactivity are a double-edged sword. To regulate ROS toxicity, plants compartmentalize ROS production, utilize ROS scavengers, and tightly regulate ROS production temporally and spatially. Previous reviews have focused on ROS production during metabolism, biotic, and abiotic stress[6,18,19]. Here, we focus on recent discoveries in ROS biology including compartmentalization, systemic ROS propagation, and sensing of extracellular ROS in plants. We also highlight recent insight into the regulation of NADPH oxidase (NOX) activity through diverse post-translational modifications.

Compartmentalized ROS production

Specificity of oxidative signaling is partially achieved by ROS production in different subcellular compartments, including the apoplast and intracellular organelles (Fig. 1)[20]. ROS compartmentalization can also reduce the deleterious nature of highly reactive species. Intracellular ROS accumulation is in large part due to metabolic byproducts produced by chloroplasts and mitochondria[17]. Plastid produced ROS are also important signaling messengers within cells. Eukaryotic cells have evolved a complex network of signals that allow plastids to regulate gene expression in the nucleus, a process known as retrograde signaling. In many cases, ROS reactivity and permeability allows these molecules to relay signaling to the nucleus[21]. H_2O_2 originating from chloroplasts and mitochondria operates as a retrograde signal by interacting with transcription factors (TFs) affecting nuclear gene expression. The *Arabidopsis* NAC DOMAIN-CONTAINING PROTEIN17 (ANAC017) TF is localized to the ER and released upon increased levels of H_2O_2, where it alters nuclear gene expression[22]. RADIDAL-INDUCED CELL DEATH1 (RCD1) also interacts with ANAC017 and is proposed to inhibit ANAC017 activity, potentially in a ROS dependent manner[23]. The chloroplast can also act as an environmental sensor, regulating cellular communication and gene expression. Most chloroplast ROS-dependent retrograde signaling is associated with singlet oxygen, which is primarily generated by photosystem II as a

photosynthesis by-product and functions near its site of generation[24]. Singlet oxygen can trigger chloroplast-to-nucleus retrograde signaling, resulting in alteration of nuclear gene expression, stress response and programmed cell death[25].

Apoplastic ROS production is primarily produced by cell wall peroxidases and plasma membrane-localized flavin-containing NAPDH oxidases (NOXs), referred to as RESPIRATORY BURST OXIDASE HOMOLOGS (RBOHs) in plants[6,26]. Plants possess 2–10 NOX isoforms, which produce superoxide. Extracellular ROS can modify cell wall components, extracellular proteins, and plasma membrane proteins[18]. Plant NOXs play distinct roles in developmental processes such as pollen tube growth, root hair formation, leaf cell expansion and Casparian strip formation as well as defense responses[10,11,18,27,28,29]. During pathogen invasion, plants use pattern recognition receptors (PRRs) that recognize conserved molecular features, so-called microbe-associated molecular patterns (MAMPs), resulting in pattern-triggered immunity (PTI); plant immune receptors can also recognize pathogen effectors delivered inside plant cells resulting in effector-triggered immunity (ETI)[30]. PRR activation leads to apoplastic ROS production through NOX and peroxidase activity[18,26,31]. *RBOHD* is the highest expressed NOX in *Arabidopsis* and its ability to produce ROS has been linked to callose deposition, cell wall lignification, stomatal closure, and systemic acquired resistance (SAR)[17, 18, 32]. Homologs of *RBOHD* in monocots and dicots play similar roles[33,34].

Of the many produced species, H_2O_2 is considered to be the key signaling molecule, due to its relatively long half-life, its ability to oxidize proteins and move across the plasma membrane via aquaporins[35, 36], and its similar molecular properties to H_2O (Fig. 1). The *Arabidopsis* PLASMA MEMBRANE INTRINSIC PROTEIN (PIP) 1;4 and PIP2;1 aquaporins are implicated in the influx of apoplastic H_2O_2 to the cytosol during plant immune signaling and ABA-dependent stomatal closure, respectively[37,35]. Interestingly, not all aquaporins are permeable for H_2O_2, but the biochemical basis for this selectivity remains elusive[36,38]. Once inside the cell, ROS produced in the extracellular space can react with intracellular proteins or can be detoxified via intracellular scavenging systems. Notably, in animal erythrocytes, superoxide can pass the cellular membrane through anion exchange 1/solute carrier 4A1 (SLC4A1)[39]. Plant homologs of SLC4A11 mediate borate transport[40], but so far there is no evidence for superoxide transport by anion channels in plants. Rather, NOX activation leads to production of superoxide which is thought to either act in the extracellular space or to be dismutated to H_2O_2, which can enter the cytosol through aquaporins [17, 18, 35, 36].

Not only is ROS compartmentalized by subcellular localization, but there are accumulation hotspots within a compartment, akin to previous reports of hotspots of Ca^{2+} ions[41,42,43,44]. Localized ROS hotspots likely provide signaling specificity within the cytosol and chloroplast. However, the ability to visualize and measure distinct ROS with high spatial, temporal, and quantitative resolution remains a major challenge in biological research. Current methods for measuring ROS production include the use of histochemical stains, chemiluminescent and fluorescent probes, electrochemical detection, and the detection of protein oxidation via mass spectrometry [45, 46, 47, 48, 49]. Currently, no single technique combines all the desired aspects of specificity, sensitivity and quantification. Future

development of new and innovative detection platforms, including direct detection, will provide a deeper understanding of ROS production and compartmentalization.

ROS Waves: Mediators of plant systemic signaling

Over the past decade, multiple studies have demonstrated the presence of rapid long distance auto-propagating ROS signals, known as the ROS wave (Fig. 1)[50]. The ROS wave can be induced by biotic and abiotic stimuli and is followed by gene expression changes in distal tissue. The function of the ROS wave has been viewed as (1) an essential signal that alerts cells and tissues to an impending stress, (2) a link between abiotic and biotic signals, and (3) a coordinator of the whole-plant systemic stomatal response to different stresses[50].

The ROS wave is dependent on NOX activity and is integrated with systemic Ca^{2+} and pH signaling[51]. Apoplastic H_2O_2 activates the ROS sensor HYDROGEN-PEROXIDE-INDUCED CA^{2+} INCREASES 1 (HPCA1) and induces Ca^{2+} influx into cytosol[52]. Ca^{2+}-binding to CALCIUM-DEPENDENT PROTEIN KINASES (CPKs) and NOXs leads to amplification of apoplastic ROS production which may result in the propagation of a systemic signal. Due to the high cytosolic ROS scavenging capacity it is unlikely that intracellular ROS contribute directly to symplastic cell-to-cell communication via plasmodesmata. Nevertheless, NOXs can localize to plasmodesmata and apoplastic ROS production participate in the regulation of symplastic signaling by inducing callose deposition at plasmodesmata, which results in a restriction of the plasmodesmal aperture, thereby limiting cell-to-cell communication[53] (Fig. 1).

A contribution of the ROS wave in systemic acquired acclimation (SAA) has been previously suggested[54] and recent work proposes that vascular bundles play a major role in transmitting systemic ROS signals[55]. ROS production is also required for systemic acquired resistance (SAR), but the relationship between the ROS wave and SAR is not obvious. An avirulent pathogen causing local programmed cell death (PCD) can induce SAR through generation of the mobile metabolite N-hydroxypipecolic acid (NHP) and subsequent accumulation of the phytohormone salicylic acid (SA) in distal tissues[56,57]. SA regulates SAR via the redox-sensitive transcriptional co-regulator NONEXPRESSER OF PR GENES 1 (NPR1; see ROS perception section). Activation of SAR requires the aquaporin AtPIP1;4 for the cytoplasmic import of apoplastic H_2O_2[35]. Local inoculation with the avirulent bacterial pathogen *Pseudomonas syringae* pv. tomato avrRpt2 triggered ROS accumulation in distal *Arabidopsis* leaves, which was reduced in *rbohD* and *rbohF* single mutants[8, 58]. In *rbohD* and *rbohF* single mutants, the levels of the NHP precursor pipecolic acid in the distal tissue are significantly lower than in wild type plants[32]. These results suggest a function of ROS as an inducer or mediator of SAR. While the ROS wave is a very early response and starts within minutes of the perception of a local stimulus[59], the production of mobile NHP occurs hours after pathogen infection in the primary leaves, which may represent the time required for effector delivery.

Two peaks of ROS production have been described during PTI[60] (Fig. 1). The first is a rapid ROS burst largely mediated by NOXs[60] that participates in the ROS wave (Fig. 1). Recently it has been reported that ETI enhances the second PTI-induced ROS burst, which

begins several hours after pathogen perception and is suppressed by diphenyleneiodonium chloride (DPI), an inhibitor of flavin-containing proteins[60]. Oxidation of apoplastic proteins is also temporarily enhanced during ETI[61]. Moreover, ETI induces an accumulation of components associated with PTI such as receptor like cytoplasmic kinases (RLCKs), CPKs, Ca^{2+}-permeable channels and NOXs, which are also involved in the propagation of the ROS wave[62]. These data suggest that NOX activity is required for the second ROS burst which could propagate systemically as a "second ROS wave". Research on the ROS wave has so far mostly focused on rapid signal propagation (maximum 30 min). Hence, it would be highly interesting to investigate whether avirulent pathogens induce not only a biphasic ROS burst but also a biphasic ROS wave in the future. In addition to NOXs, a strong chloroplastic contribution to the second ROS burst has been described in response to microbial features and ozone. Chloroplastic ROS also play a role in ETI-induced cell death in response to pathogen attack[63,64,65]. For example, the chloroplast-localized redox-sensitive THIMET OLIGOPEPTIDASE 1 (TOP1) binds to SA and regulates ETI-induced PCD[66,61, 67]. Thus, redox-sensitive TOP might play dual roles in induction of cell death and SA-dependent SAR. These data demonstrate tight integration of systemic signaling during ETI over multiple time scales. Compared to the biphasic ROS burst in response to biotic stimuli, the presence of distinct ROS peaks in response to abiotic stress and wounding is less clear. However, in response to stress, apoplastic ROS accumulation also lead to changes in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{cyto}$) and amplifies stress responses. Thus, multiple mechanisms collectively contribute to the initial, systemic, and sustained ROS production in response to biotic and abiotic stimuli.

ROS perception

For a signaling molecule to function, it must be sensed. Unlike peptides or other non-reactive molecules, it is unlikely that ROS are perceived in a receptor-ligand manner. Instead, ROS are likely perceived through redox modifications, resulting in altered protein structure, localization, activity, and protein-protein interactions. While apoplastic ROS could be perceived in the extracellular space, its diffusion across the plasma membrane through aquaporin and anion channels[38] can also facilitate perception in the cytosol (Figs. 1 and 2). Intriguingly, these extra- and intracellular ROS sensing mechanisms are not mutually exclusive and could enhance sensitivity or the temporal resolution of ROS as a signaling molecule [17, 68]. A main mechanism for ROS sensing is the oxidative modification of cysteines, which has been extensively investigated and reviewed[69, 70]. The availability of different oxidation states results in a diverse range of cysteine oxidative posttranslational modifications (PTMs). In addition to cysteine, oxidation of methionine and tyrosine can affect protein structure and function[71, 72].

Within ROS-sensitive proteins, the reactivity of cysteines with ROS is strongly correlated with the local electrostatic environment that affects the pKa of cysteine residues. Cysteines are prone to oxidation at pH levels higher than their pKa. The resting pH of the cytosol and apoplast is considered to be approximately 7.5 and 5, respectively[73]. This suggests that cysteines in the apoplast could be less readily oxidized compared to those in the cytosol. However, biotic and abiotic stimuli trigger a transient alkalization of the apoplast to pH 6 −7[74], increasing the reactivity of cysteines with ROS. Inhibition of plasma membrane (PM)-

H^+-ATPase activity, by phosphorylation and by accumulation of cations in the apoplast, is responsible for apoplastic alkalization[74] (Fig. 2). Interestingly, the duration and magnitude of apoplastic alkalization differs between salinity, drought, and attack by powdery mildew[74]. Thus, stimulus-dependent apoplastic alkalization may be an important component of ROS signaling specificity. More localized modulation of pH is also possible, where distinct pH microenvironments impact oxidation of cysteine thiols[75].

The first identified ROS sensors are the prokaryotic OxyR and SoxR transcription factors that are directly oxidized by ROS[76, 77]. The functional homolog of OxyR in yeast, Yap, is not directly oxidized by ROS but is targeted by the thiol-peroxidase Oxidant receptor peroxidase (Orp1). Oxidized Orp1 oxidizes Yap1, potentially masking its nuclear export signal, resulting in nuclear localization of Yap1 and upregulation of antioxidant gene expression[78]. This thiol-peroxidase based redox relay has also been implicated in ABA-induced stomatal closure. It has been proposed that *Arabidopsis* GLUTATHIONE PEROXIDASE 3 (GPX3) is oxidized by H_2O_2 and relays oxidizing equivalents to ABA-INSENSITIVE 2 (ABI2) in the cytosol to inhibit phosphatase activity[79]. NPR1 is a SA receptor whose localization and self-association is another intriguing example of ROS-mediated protein regulation[80, 81]. NPR1 participates in global reprogramming towards defense and re-localizes to the nucleus in a redox dependent manner. At a resting state, NPR1 forms oligomers in the cytoplasm[82]. However, after the ROS burst triggered by pathogen perception, SA induces a reduction of the cytoplasm[82] leading to activation of the thioredoxin TRX-5h, which in turn catalyzes the release of NPR1 monomers via reduction of inter-molecular disulfide bonds[83]. The nuclear localization signal is unmasked in monomeric NPR1, enabling nuclear localization, transcription factor binding, and global defense gene expression[84].

Cysteine oxidation can also regulate the activity of metabolic enzymes, such as GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE (GAPDH), a conserved glycolytic enzyme catalyzing the conversion of glyceraldehyde-3-phosphate (G3P) and NAD into NADH and 1,3-bisphosphoglycerate (1,3-BPG)[85]. Oxidation of the catalytic cysteine in the active site of GAPDH blocks its catalytic activity, alters its subcellular localization and affects protein-protein interactions[85, 86]. Reducing conditions in the cytoplasm increase the affinity of cytosolic GAPDH isoforms (GAPCs) for the mitochondrial voltage-dependent anion-selective channel (VDCA) leading to localization of the GAPCs to the outer mitochondrial membrane. Oxidizing conditions, on the other hand, induce GAPC translocation to the nucleus by cysteine oxidation[87]. Under oxidizing conditions GAPCs can also activate plasma membrane-localized PHOSPHOLIPASE D δ (PLDδ), which plays a role in ABA- and H_2O_2-induced stomatal closure[88].

The perception of apoplastic ROS in plants has remained enigmatic until recently. CYSTEINE-RICH RECEPTOR-LIKE KINASES (CRKs) have been proposed as candidate ROS sensors based on their extracellular cysteine-rich regions as well as their transcriptional induction in response to stress[89, 90]. Structural analyses of the cysteine-rich extracellular region of plasmodesmata localized proteins, close relatives of CRKs, suggest that the cysteines stabilize the ectodomain structure and may not be accessible for efficient ROS perception[91]. Recently, another receptor-like kinase (RLK) with extracellular leucine-

rich repeat (LRR) domain, HPCA1, has recently been identified to be involved in direct ROS sensing[52] (Fig. 2). HPCA1 possesses a unique extracellular domain, the hydrogen peroxidase (HP) domain, which contains four cysteines. Extracellular H_2O_2 modifies cysteine residues in HPCA1's HP domain, resulting in increased kinase activity. Subsequently, active HPCA1 triggers activation of guard cell Ca^{2+} channels and stomatal closure. RLK-mediated MAMP perception also induces extracellular ROS production and activates a calmodulin-gated calcium channel leading to an increase in $[Ca^{2+}]_{cyto}$[92]. However, while HPCA1 is required for the extracellular H_2O_2-induced increase in $[Ca^{2+}]_{cyto}$, HPCA1 is not involved in the increase in $[Ca^{2+}]_{cyto}$ in response to perception of the MAMPs flg22 or elf26[52]. Thus, it is likely that other extracellular ROS sensors exist which mediate extracellular ROS perception in response to MAMPs and pathogen infection. Protein function can be exquisitely modulated by PTM cross-talk and the role of ROS in these processes is an emerging field of interest in both plants and animals. For example, the activity of the human serine/threonine kinase AURORA A is regulated by oxidation of cysteine 290 which inhibits autophosphorylation of threonine 288 in the conserved activation loop (T-loop)[93]. Comparative evolutionary analysis across kingdoms showed that ~11.5% of all protein kinases contain an analogous Cys residue to Cys290 in Aurora A[93]. Phosphorylation can also trigger a switch of protein conformation resulting in the exposure and consequent oxidation of cryptic redox-sensitive cysteines[94]. In plants, the direct interplay between phosphorylation and cysteine oxidation at a molecular level has not yet been described. However, multiple kinases are redox-regulated. For example, H_2O_2 activates the *Brassica napus* MAP kinase MPK4, which in turn positively regulates ROS production[95]. Oxidation of redox-sensitive cysteine residues is crucial for activation of MPK3 and MPK6 in both rice and *Arabidopsis*[96, 97]. Moreover, cysteine oxidation inactivates the calcium-dependent kinase *At*CPK21[98] . Future research will likely reveal extensive cross-talk between phosphorylation and cysteine oxidation in plants.

Recent advances in understanding redox modifications and ROS perception in plants highlight additional outstanding biological questions. What is the importance of localized ROS production and pH for perception and redox modification of proteins? Can cysteine oxidation directly regulate NOXs or modulate the activity of the kinases involved in NOX activation? What are the identities of additional ROS receptors beyond HPCA1 and is there specificity in perception in different tissue types? Finally, how is ROS perception by apoplastic sensors or sensor domains integrated with ROS influx through aquaporins?

Regulation of NOX Activity

Not only can ROS directly regulate diverse biological processes and protein activity, but ROS producing enzymes are also subject to diverse PTMs. PTMs enable precise and rapid regulation of protein activity, stability, and interaction profiles. Additional layers of regulation can be achieved through PTM interplay, or cross-talk, where different modifications work in concert to achieve signaling specificity. Here, we highlight diverse PTMs that regulate NOX activity, both positively and negatively, in plants.

NOX and superoxide production

Plant NOXs are not only central enzymes for stress response, but also play a role in development[18]. *RBOHD* is the most highly expressed NOX in *Arabidopsis* and is required for ROS production upon immune perception and abiotic cues. *RBOHF* is also involved in regulating stomatal closure, the responses to abiotic signals and Casparian strip formation[18]. Other plant NOXs are involved in various developmental processes[99].

NOXs produce superoxide, which is highly reactive and unstable making it difficult to accurately measure[19]. Superoxide can be dismutated spontaneously or more rapidly by the action of superoxide dismutase (SOD) to H_2O_2[17]. The existence of an extracellular SODs in plants has been proposed[100, 101]. However, the high dismutation rate of superoxide even in the absence of a SOD might offer sufficient protection its toxic effects[102]. H_2O_2 is frequently considered to be the key signaling molecule following NOX activation and NOX activity is often assessed by measuring H_2O_2 levels after dismutation. In plants, many studies focus on the activity of H_2O_2 (Fig. 1). Gene expression studies suggest a certain amount of specificity between different types of ROS[19] and the importance of extracellular superoxide as a signaling molecule is an underexplored area. In animals, superoxide can react with tyrosyl radicals resulting in the generation of tyrosine hydroperoxide[103]. These findings raise multiple outstanding questions. Does NOX-produced superoxide carry biological activity on its own? What is the relative importance of the balance between extracellular superoxide and H_2O_2 levels? Are apoplastic SODs involved in rapid conversion of extracellular superoxide?

N-terminal NOX regulation

Plant NOXs are composed of six trans-membrane domains, which contain an active ferric oxidoreductase domain, as well as N- and C-terminal extensions which are localized in the cytoplasm and are highly similar to mammalian NOXs. The N-terminus of NOXs contain Ca^{2+} binding EF-hands and PA might contribute to the regulation of some plant NOXs[104]. In contrast, the C-terminus contains FAD and NADPH binding domains. Tissue-specific expression patterns (e.g. pollen specific *AtRBOHH* and *J*) and stress stimulus-inducible expression profiles of *NOX* genes suggest spatio-temporal transcriptional regulation[105, 106]. In addition, NOX enzyme activity is also regulated at the post-translational level. Phosphorylation of the N-terminal region at conserved residues through distinct kinases has been demonstrated to affect NOX enzymatic activity. In *Arabidopsis* RBOHD, specific N-terminal residues are phosphorylated by distinct kinases (Fig. S1a). Examples include the RLK DOESN'T RESPOND TO NUCLEOTIDES 1 (DORN1) which phosphorylates S22 and T24, BOTRYTIS INDUCED KINASE 1 (BIK1) which phosphorylates S39 and S343, and CALCIUM DEPENDENT PROTEIN KINASES (CPKs) which phosphorylate S133, S148, and S163[107, 108, 109, 110, 111].

The N-terminus of RBOHs functions as a hub for a multitude of kinases which induce ROS production. S339 is phosphorylated by BIK1 and the MAP4K SERINE/THREONINE KINASE 1 (SIK1), while S347 is phosphorylated by BIK1, CPKs and SIK1[108, 109, 110, 111, 112]. Phosphorylation of those residues (with the exception of S148) is linked to NOX activation, in particular phosphorylation of S343 and S347 during immune

responses[113]. Intriguingly, phosphorylation of S163 contributed to negative regulation of the ROS producing activity and a S163A variant of RBOHD displayed enhanced ROS production following chitin treatment[14, 113] (Fig. S1c). The interaction between multiple kinases with specific and convergent sites allows for flexibility in activation of RBOHD in response to independent stimuli and developmental stages. SCHENGEN1 (SGN1) phosphorylates unidentified residues in the N-terminus of both RBOHD and RBOHF *in vitro*[27]. OPEN STOMATA 1 (OST1), CALCINEURIN B-LIKE INTERACTING PROTEIN KINASE 26 (CIPK26) and SCHENGEN 1 (SGN1) were also found to modulate ROS-production of RBOHD and RBOHF in HEK293T cells. In particular, CIPK26-induced phosphorylation of RBOHF S13/S130/S132 and S174 residues was found to enhance and reduce ROS production, respectively (Fig. S1d). However, the phosphorylation status of RBOHF residues has not been elucidated *in planta*[15,27].

Different kinases might target key residues of RBOHs in a tissue- or stimulus dependent manner. For example, BIK1, CPKs, SIK1, DORN1 and OST1 stimulate NOX-dependent ROS production in response to MAMPs, ATP and ABA, while SGN1 regulates the Casparian strip formation in the roots[27, 37, 107, 108, 109, 110, 111, 112]. The RLCK MARIS has been implicated in activation of RBOHH/J in pollen tube growth[114]. However, the preference of distinct kinases for specific sites and the importance of kinase specificity in different tissues awaits future investigation.

C-terminal NOX regulation

Since excessive ROS accumulation is detrimental, a coordinated balance between positive and negative regulation of ROS production is required. Some of the most pressing questions in ROS biology focus on how responses are appropriately regulated to ensure robust and efficient outputs pre- and post-activation. Recent studies highlight the importance of diverse C-terminal PTMs for fine-tuning NOX activity.

S-nitrosation is the addition of a nitrosonium ion (NO^+) to the reactive thiol of a cysteine residue thereby forming S-nitrosothiol (SNO)[115]. S-nitrosothiols can be also produced by oxidation of thiol to thiyl radical followed by addition of NO (S-nitrosylation)[116]. While NO production in plants is still poorly understood, the best characterized way includes the enzymatic and non/enzymatic reduction of nitrite to NO[117]. In general, the predominant mechanism for S-nitrosothiol formation is S-nitrosation[118]. In plants, the production of nitric oxide increases upon pathogen perception and induces protein S-nitrosation with important implications in the regulation of immune responses[119]. RBOHD is S-nitrosated on C890 *in vitro* by exposure to S-nitrosoglutathione (GSNO) and Cys-NO and interestingly also *in vivo* upon infection with a bacterial pathogen[120]. Transgenic expression of a C890A variant of RBOHD ($RBOHD^{C890A}$) in the *Arabidopsis rbohD* mutant background leads to increased ROS production compared to *rbohD* plants complemented with wild type RBOHD upon pathogen infection[120]. S-nitrosation of C890 destabilizes FAD binding, thereby reducing NOX activity[120]. Together, this suggests that pathogen-induced NO production provides a negative feedback loop limiting ROS production by RBOHD. Intriguingly, C890 is evolutionarily conserved across plants and humans, corresponding to C537[121] and C694[122] in human NOX2 and NOX5, respectively, and S-nitrosation also

leads to decreased enzymatic activity (Fig. 3c,d; Fig. S1b). This conservation highlights the importance of cysteine S-nitrosation for downregulating NOX activity. Another PTM that affects NOX activity is persulfidation, the covalent addition of a thiol group to cysteine[123]. Cysteine persulfidation modulates protein structure, localization, intermolecular interactions and enzyme activities[123]. In plants, a major driver of persulfidation is hydrogen sulfide (H_2S), a gaseus molecule that is produced in the cytosol by cysteine-degrading enzymes[123]. ABA triggers persulfidation of RBOHD. Persulfidation of RBOHD is abolished in plants expressing RBOHD$^{C825A/C890A}$ and those plants also exhibit reduced ROS production following ABA treatment[123]. Cysteine persulfidation of RBOHD likely results in an increased negative charge, which enhances FAD binding and subsequently enzymatic activity. Cysteine residues might be important regulation sites for NOXs as Cysteine residues in both N and C terminal regions are conserved (Fig S1b).

Broadly, across 112 NOXs presently known in the plant kingdom, the C-terminus is more conserved in amino acid sequence than full length proteins when compared *to Arabidopsis* RBOHD (Fig. 3a). Additionally, residues known to be post-translationally modified are conserved, especially in functionality, along the C-terminus of NOX homologs (Fig. 3b). While the N-terminus functions as a hub for activation, PTMs on C-terminal residues can have opposing effects on ROS production. As described above, S-nitrosation inhibits while persulfidation enhances RBOHD activity. Interestingly, phosphorylation of the C-terminal region is also critical for regulation of human NOXs. Ataxia telangiectasia mutated (ATM)-kinase-dependent phosphorylation of NOX2 S486 leads to reduced NOX2 activity[121]. Human NOX5 can be activated by phosphorylation of S475, S490, T494, and S498 by PROTEIN KINASE C (PKC) and CALCIUM/CALMODULIN-DEPENDENT KINASE II (CAMKII; Fig. 3b, c). However, CAMKII also participates in negative regulation of NOX5 activity by phosphorylation of S659[100] (Fig. 3c). Recent work has identified kinases that interact with residues in the C-terminus of RBOHD. CRK2 phosphorylates RBOHD S703 and enhances production of ROS during recognition of flg22[68] (Fig. 3b,c). The kinase AvrPphB SUSCEPTIBLE 1-LIKE 13 (PBL13) also interacts with the C-terminal region of RBOHD, however phosphorylation of RBOHD T912 by PBL13 dampens ROS production[124] (Fig. 3b,c). These results suggest that at the NOX C-terminus multiple kinases converge to achieve robust, precise, and selective control of NOX activity.

C-terminal PTMs play a key role in calibrating the enzymatic activity of RBOHD and also regulate ROS by fine-tuning the abundance of RBOHD. Ubiquitination, which is characterized by the addition of one or more ubiquitin molecules to lysine (K) residues, is involved in nearly all aspects of eukaryotic biology[125, 126] and plays a crucial role in regulating immune signalling[127]. For example, ubiquitination negatively regulates crucial molecular players of immune responses in *Arabidopsis*, including FLAGELLIN-SENSING 2 (FLS2)[128], BIK1[129], and the chitin receptor LYSIN-MOTIF RECEPTOR LIKE KINASE 5 (LYK5)[130]. Recent work demonstrates interplay between phosphorylation and ubiquitination to negatively regulate RBOHD[124] (Fig. 3c). PBL13 can phosphorylate RBOHD T912 *in vitro,* and the abundance of RBOHD$_{T912D}$ is reduced compared to wild-type RBOHD *in planta*[124]. PBL13 interacts with the PBL13 interacting RING domain E3 (PIRE) ligase, a RING domain E3 ubiquitin ligase[124]. Strikingly, RBOHD ubiquitination was enhanced in complementation lines expressing the phosphomimic RBOHD^{T912D}, while

the phosphonull RBOHD^{T912A} decreased ubiquitination levels. Importantly, deletions of *pbl13* and *pire* exhibited enhanced RBOHD protein accumulation and enhanced ROS production triggered by flg22. Furthermore, ubiquitination results in endocytosis and vacuolar-mediated degradation[124]. Both T912 and C-terminal lysine residues are conserved in other plant RBOHs, indicating that phosphorylation and ubiquitination may be a conserved mechanism for RBOH regulation (Fig. 3). These results suggest posttranslational control of NOX protein abundance through cross talk between phosphorylation and ubiquitination.

The C-terminus of RBOHs act as a conserved hub to modulate production of ROS and NOX abundance. Phosphomimetic mutants of S862 and T912 lead to decreased ROS production and abundance of RBOHD, respectively, and these residues are highly conserved across plant NOXs (Fig. 3b). A recent *Arabidopsis* screen led to the identification of a mutant defective in LPS-triggered ROS burst[131]. The mutation responsible for the decreased RBOHD activity was mapped to residue E919, which is conserved in all *Arabidopsis* RBOHs[6] (Fig. 3). Intriguingly, mutation of this residue to a lysine (E919L) had no effect on protein accumulation, localization, or association with BIK1. These finding suggest a role for E919 as an “off switch” for RBOHD. An important focus of future research will be to determine how multiple kinases dynamically regulate ROS production by interacting with conserved residues in N- and C-terminal regions. Given the conservation of C-terminal residues across diverse NOXs, it is possible that different organisms enlist specific modifying enzymes to fine-tune NOX abundance at similar residues to regulate the amplitude and duration of ROS production.

Conclusions

ROS are essential for life, but must be appropriately regulated in response to development as well as diverse stimuli. Recent scientific advances highlight the complexity of ROS effect on proteins, enzymatic activity, and cellular processes. Future advances in the field of ROS will shed light on a fundamental component of all cellular life, with significant implications for crop improvement and protection. We hope the ideas presented in this article serve as an invitation for the scientific community to push the field forward.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data availability statement

Data used to generate Fig. 3 a, b and Fig. S1 a, b as well as detailed methodology and scripts are available in the GitHub repository (https://github.com/DanielleMStevens/

ROS_production_review). The NOX C-terminal alignments are available in wasabi (http://was.bi?id=gedS1F).

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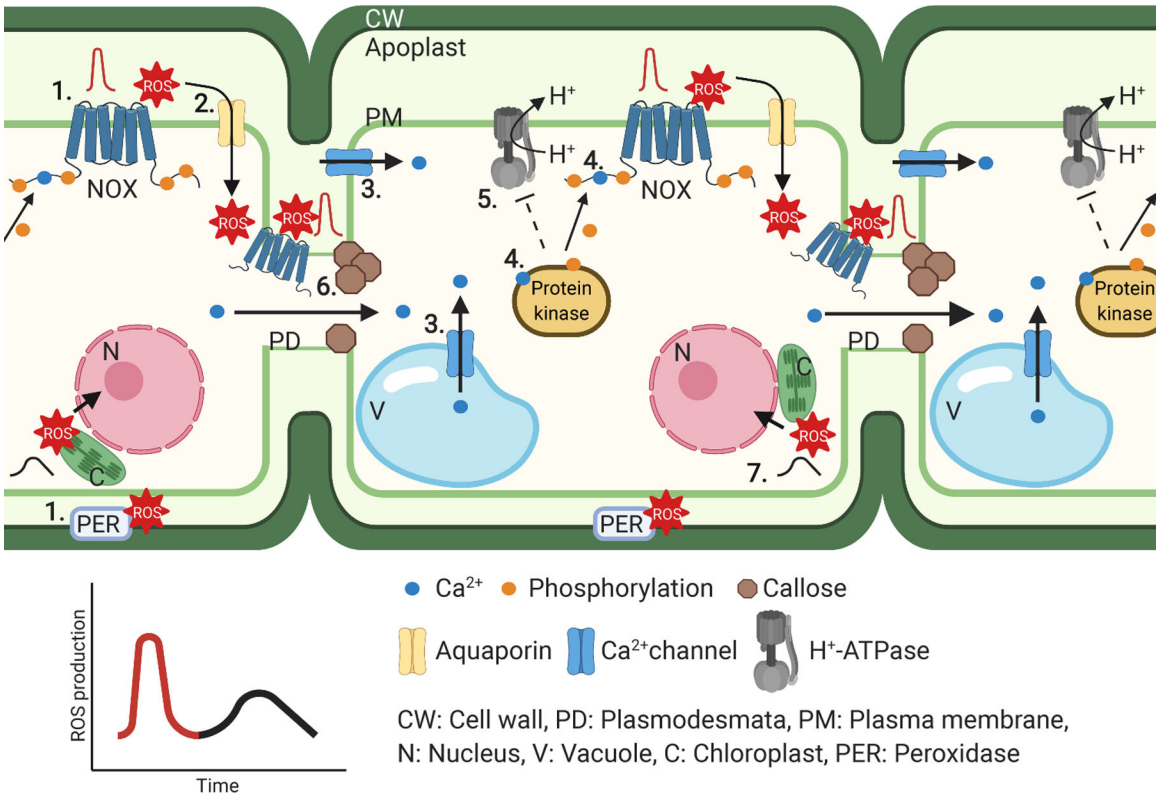

Figure 1. Biphasic ROS production and cell-to-cell communication.

1. Stimuli induce rapid initial apoplastic ROS production by NADPH oxidases (NOXs) and peroxidases (PER). 2. Apoplastic ROS can enter into the cytosol via aquaporins. 3. ROS then activates Ca^{2+} influx into the cytosol from the apoplast and vacuole. 4. Ca^{2+} binds to NOXs or protein kinases, which amplifies NOX activation. 5. Apoplastic ROS accumulation frequently coincides with extracellular alkalization by inhibition of H^{+}-ATPases. 6. NOX-dependent ROS can restrict symplastic signaling by modulating callose deposition at plasmodesma. 7. After initial rapid systemic signaling, a second ROS burst with contributions from the chloroplast may affect nuclear gene expression.

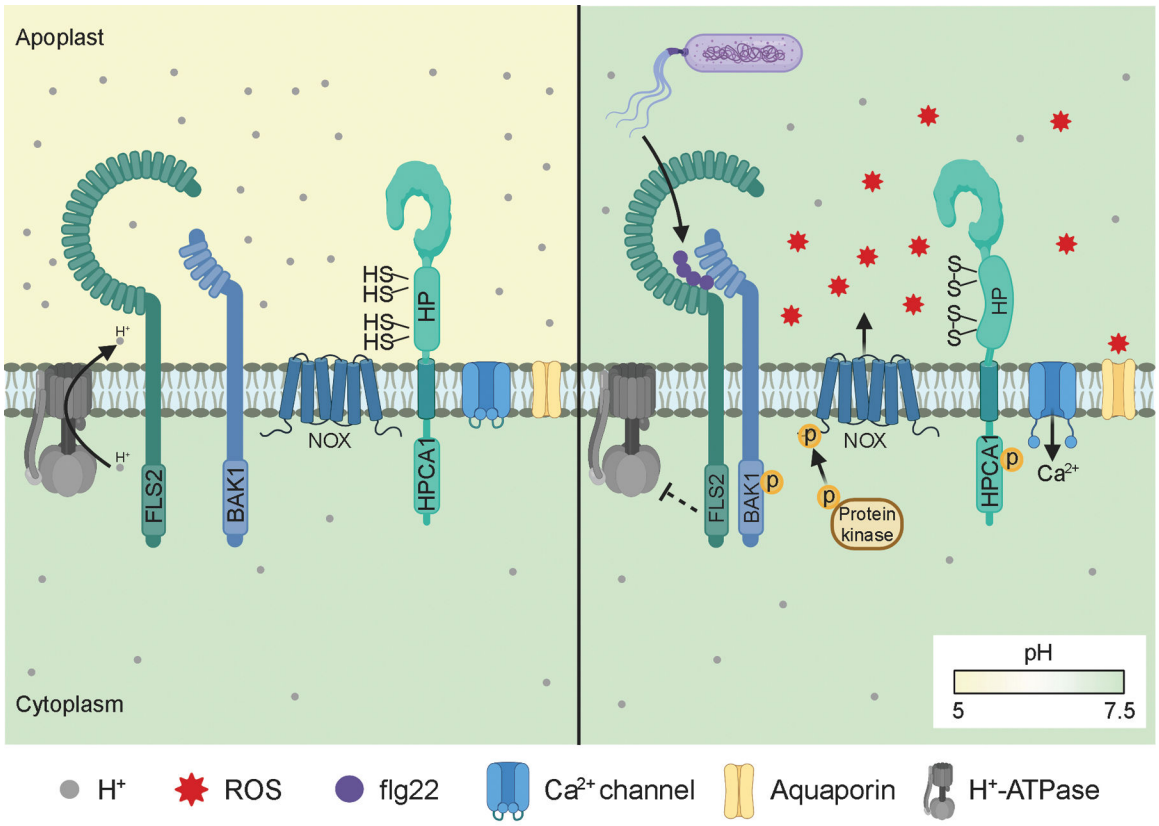

Figure 2. ROS perception by the HPCA1 receptor.

Left: The flagellin receptor complex and ROS receptor HPCA1 in the absence of pathogen perception. **Right:** Perception of the immunogenic flagellin epitope flg22 results in FLS2-BAK1 complex formation, inhibition of the H^+-ATPase resulting in alkalization of the apoplast, and activation of NOX-induced ROS production. The increase in extracellular pH enables cysteine modification of HPCA1's hydrogen peroxidase (HP) domain, increasing HPCA1's kinase activity and downstream ROS signalling.

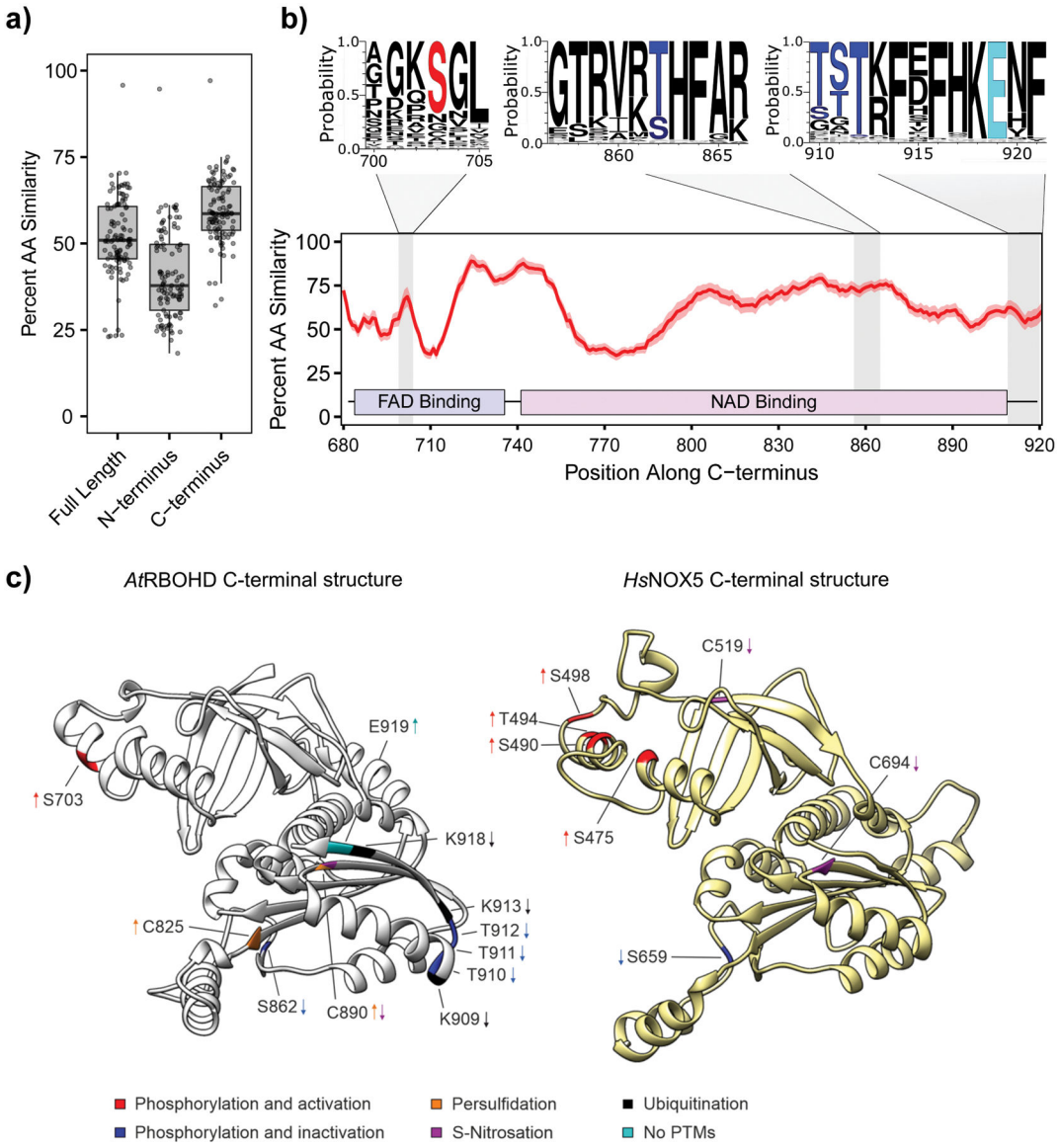

Figure 3. Conservation of the NOX C-terminus across plants and conservation of critical residues in RBOHD and human NOX5.

(a) Conservation of plant NOX homologs when comparing full length, N-terminal (residues 1–376) or C-terminal regions (residues 680–920). Percent amino acid (AA) similarity was compared using *At*RBOHD as a reference across 112 plant NOXs. **(b)** Sliding window of average amino acid similarity displayed in red with a 95% confidence interval in light red along the C-termini for plant NOX homologs. *At*RBOHD was used as a reference with a 7 amino acid sliding window. Regions known to be regulated via posttranslational modifications (PTMs) are highlighted in grey and displayed by weblogos with modified residues differentially colored. **(c)** Structural models of (left) *At*RBOHD C-terminus (residues 613–920) and (right) HsNOX5 β-isoform C-terminus (residues 401–719). Residues are labelled with different colours, based on the type of post-translational modifications (PTMs) underlying their regulatory role. Positive and negative regulatory outputs of the PTMs are specified by up- and down-arrows, respectively. Red label

indicates positive regulation by phosphorylation, blue indicates negative regulation by phosphorylation, black specify ubiquitination, purple specify S-nitrosylation and orange indicates persulfidation. Residues highlighted with light blue were not found to be post-translationally modified, but were experimentally shown to modulate ROS producing activity.