FORUM REVIEW ARTICLE

Redox Regulation in Plant Immune Function

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Abstract

Significance: Production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) occurs rapidly in response to attempted pathogen invasion of potential host plants. Such reduction–oxidation (redox) changes are sensed and transmitted to engage immune function, including the hypersensitive response, a programmed execution of challenged plant cells. **Recent Advances:** Pathogen elicitors trigger changes in calcium that are sensed by calmodulin, calmodulin-like proteins, and calcium-dependent protein kinases, which activate ROS and RNS production. The ROS and RNS production is compartmentalized within the cell and occurs through multiple routes. Mitogen-activated protein kinase (MAPK) cascades are engaged upstream and downstream of ROS and nitric oxide (NO) production. NO is increasingly recognized as a key signaling molecule, regulating downstream protein function through *S*-nitrosylation, the addition of an NO moiety to a reactive cysteine thiol. **Critical Issues:** How multiple sources of ROS and RNS are coordinated is unclear. The putative protein sensors that detect and translate fluxes in ROS and RNS into differential gene expression are obscure. Protein tyrosine nitration following reaction of peroxynitrite with tyrosine residues has been proposed as another signaling mechanism or as a marker leading to protein degradation, but the reversibility remains to be established. **Future Directions:** Research is needed to identify the full spectrum of NO-modified proteins with special emphasis on redox-activated transcription factors and their cognate target genes. A systems approach will be required to uncover the complexities integral to redox regulation of MAPK cascades, transcription factors, and defense genes through the combined effects of calcium, phosphorylation, *S*-nitrosylation, and protein tyrosine nitration. *Antioxid. Redox Signal.* 21, 1373–1388.

Introduction

PLANTS RESPOND TO pathogen attack by activating a plethora of defense responses that include closure of stomata, strengthening of cell walls, and synthesis of antimicrobial compounds such as phytoalexins and pathogenesis-related (PR) proteins to limit damage and prevent further spread.

A conspicuous feature of these responses is the engagement of a nitrosative burst and the parallel activation of reactive oxygen species (ROS) generation (44, 131). The plant immune system is initially triggered by the recognition of microbe- or pathogen-associated molecular patterns (MAMPs/PAMPs) by a given pattern recognition receptor (52). MAMP/PAMP-triggered immunity (or basal resistance) is the result. Successful pathogens can subvert this early immune response by utilizing a suite of effector proteins delivered into potential

host cells, resulting in effector-triggered susceptibility. In response, plants have evolved a plethora of resistance (R) proteins that can recognize a given pathogen effector resulting in highly specific, effector-triggered immunity (ETI) (52).

The hypersensitive response (HR), a form of programmed cell death, is part of ETI. In the HR, limited cell death occurs in proximity to the point of invasion, restricting pathogen spread. Importantly, salicylic acid (SA) accumulation, preceding the HR, activates a molecular signal transduction pathway marked by the induction of PR proteins and the subsequent development of systemic acquired resistance (SAR). SAR primes subsequent immune responses throughout the plant against a broad range of pathogens (26, 39).

Since plants lack mobile defense cells, the innate immunity of each cell and effective signal transduction from infected cells are critical to the activation of defense genes in proximal and distal tissues. A change in cellular

reduction–oxidation (redox) status is one of the earliest responses detected in the challenged cell (21). Production of hydrogen peroxide (H_2O_2), a reactive oxygen species (ROS) and nitric oxide (NO), a reactive nitrogen species (RNS) (110) is rapidly triggered following detection of a pathogen and may synergistically activate the HR. A massive burst of H_2O_2 in the absence of NO has been reported to induce only a weak HR in tobacco and *Arabidopsis* and NO, if not preceded by H_2O_2 production, did not invoke the HR (24, 25, 32). Interestingly, however, high endogenous *S*-nitrosothiol (SNO) levels have recently been reported to drive cell death in the absence of apoplastic ROS synthesis (131). Furthermore, H_2O_2 and NO production leads to the activation of complementary defense genes, underscoring the specificity of these reactive intermediates and further implicating redox changes as fundamental to the development of the plant immune response.

ROS and RNS production and cognate signaling are complex and this review can only provide a brief overview of redox regulation in plant immunity. We will outline how, following pathogen perception, ROS and RNS are generated and how these redox changes are sensed and the signals transduced to result in the activation of defense-related genes.

Sources of ROS

The major route of ROS production, with strong oxidizing potential, is through the incomplete reduction of apoplastic oxygen to the superoxide radical, O_2^- , by nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase). In animals, NADPH oxidase forms a transmembrane complex in which the glycoprotein 91-kDa phagocyte NADPH oxidase (gp91 phox) and p22 phox catalytic subunits generate O_2^- from molecular oxygen by electron transfer from NADPH (91). Plant NADPH oxidases, termed respiratory burst oxidase homologues (Rbohs), contain only the gp91 phox subunit, which is sufficient to drive O_2^- production (47, 105). Cofactor flavin adenine dinucleotide (FAD) and NADPH interact with binding sites within the C-terminal of gp91 phox (122).

At the N-terminus, plant Rbohs possess Ca^{2+} -binding EF-hands, which are absent from mammalian gp91 phox (53). O_2^- production by *Arabidopsis* RbohD was induced as a result of the conformational change in the EF-hand region following Ca^{2+} binding (92). In rice, the functional unit of RbohB is a dimer (91) and binding of Rac1, a GTPase, at the N-terminus was found to be a requirement for its activation (91). Constitutive expression of the RAC1 GTPase has been linked with ROS production, an HR-like reaction and increased resistance to bacterial blight and rice blast (93). The mass spectrometry data of Oda *et al.* (91) suggested that the EF-1 of one RbohB partner interacted with the EF-2 of the other and the swapping of the two EF-hands created the helix-loop-helix motif that enabled the binding of Rac1. The conformational change was Ca^{2+} dependent and was abolished following the E253A mutation within residues 251–285 of the loop (91).

Arabidopsis has 10 RBOH genes with RBOHD and F being required for the full HR (118, 131). A comparison of data from metabolic profiling of *rbohD*, *rbohF*, or *catalase 2* (*cat2*)-genetic backgrounds revealed a probable specific role for each RBOH isoform in the regulation of the metabolome

following pathogen recognition (18). *RbohD* enhanced the induction of metabolites, including ascorbic acid, threonine, and phenylalanine, during the response to *Pseudomonas syringae* pv. *tomato* (*Pst* DC3000 (*avrRpm1*), but had less effect on profiles triggered by *Pst* DC3000. In contrast, metabolites were significantly downregulated in *RbohF* following infection by *Pst* DC3000 (tyrosine, uracil, isoleucine) as well as *Pst* DC3000 (*avrRpm1*) (tyrosine, arginine, glutamic acid), with some in common with those downregulated in *rbohFcat2* (phenylalanine, SA-glucopyranoside, tyrosine) (18), suggesting the association of *rbohF*-dependent changes with compromised ability to react to *Pst* DC3000 and also that RBOHF couples oxidative stress to resistance.

Highly toxic O_2^- is rapidly converted to H_2O_2 and O_2 by the enzyme superoxide dismutase (SOD) found in the apoplast and cytosol (16, 95, 115). Activity of SOD has been proposed to be critical for the HR response since it might be H_2O_2 , and not O_2^- , which in cooperation with NO triggers cell death development (25). Furthermore, the dismutation of O_2^- to H_2O_2 reduces the loss of NO through reaction with O_2^- (25).

Heme proteins, such as class III peroxidases, use H_2O_2 as the substrate in redox reactions, but are also able to produce H_2O_2 directly from iron–superoxide reactions at high pH in the presence of reductants (90). During H_2O_2 consumption (peroxidase cycle), transfer of O_2 to the heme group of the ferric (FeIII) enzyme forms compound I; reaction with an electron donor forms compound II, and a further electron returns the ferric enzyme to the original ground state ready for another cycle. With a strong electron donor, however, the oxidase cycle can instead produce the ferrous enzyme (FeII), which reacts with O_2 to produce compound III. Gain of one electron results in H_2O_2 and returns the enzyme to the ferric state (90).

Class III peroxidase 1 (FBP1) was first characterized in French bean (*Phaseolus vulgaris*) (14). *Arabidopsis* transformed with antisense FBP1 (13) exhibited reduced H_2O_2 accumulation and increased susceptibility to pathogens. Simultaneously, *Arabidopsis* class III peroxidases, *AtPRX33* and *AtPRX34* transcripts, were downregulated. By using *AtPRX33* and 34 knockdowns, diphenylene iodonium (DPI), an inhibitor of NADPH oxidases, and sodium azide, an inhibitor of H_2O_2 formation by heme proteins, it was established that peroxidases accounted for half the H_2O_2 in response to MAMPs in *Arabidopsis* (22). The use of DPI or azide and potassium cyanide enabled insight into the source of H_2O_2 production in several other plant species. For example, H_2O_2 is NADPH oxidase dependent in soybean and tobacco, is apoplastic peroxidase dependent in French bean, cotton, and wheat, and is dependent upon both in alfalfa and sunflower (90).

Peroxisomal catalase (CAT) is another route of H_2O_2 catabolism (76). In *Arabidopsis*, loss-of-function mutations in the Class I catalase, *CAT2*, gave a conditional photorespiratory oxidative stress phenotype, high SA levels, HR-like lesions, and enhanced disease resistance (18). In tobacco leaves, infiltration with the Tobacco mosaic virus or a glycoprotein fungal elicitor caused a reduction in transcripts of *CAT1* and *CAT2*, an increase in H_2O_2 , and loss of catalase activity in the zone of HR development (30). In contrast, cells immediately adjacent to the HR zone showed marked upregulation of *CAT2* and diminished H_2O_2 . (30). These

examples illustrate that not only ROS production, but relative production and breakdown rates of ROS are important in the establishment of the immune response.

Given the potential multiple sources of H_2O_2 from antioxidants in the apoplast, cytosol, peroxisomes, mitochondria, and chloroplasts (95), the relative contributions of ROS from the different cellular compartments leave further questions on the combination and integration of signals from numerous potential sources during both disease and resistance.

Sources of RNS/NO

Regarded as the major RNS signaling molecule, in animals, NO is synthesized by the calmodulin (CaM)-dependent enzyme nitric oxide synthase (NOS). Present in three isoforms as eNOS, nNOS, and iNOS, NOS catalyzes the NADPH-dependent oxidation of L-arginine to NO and citrulline (126). Inhibitors based on analogues of L-arginine suppress NO production in plants and have produced the argument for an NOS-like enzyme (24, 32). Indeed, an NOS possessing catalytic domains with significant similarity to animal NOS has been found in *Ostreococcus tauri*, a green alga of a class diverging early within the plant lineage (36). However, the completion of several plant genome sequencing projects has ruled out a structurally similar, higher plant NOS, with the obvious implication that such NOS was not further perpetuated during the evolution to higher plants. The focus is now moving away from the assumed activity of an NOS ortholog. A recent review exemplifies potential alternatives to NOS (Fig. 1) that might account for NO synthesis in subcellular locations throughout the plant (38).

The formation of RNS via the activity of nitrate reductase (NR) is one such alternative route of NO production (Fig. 1). NR is located in the cytosol and catalyzes the NADPH reduction of nitrate (NO_3^-) to nitrite (NO_2^-), but can also convert NO_2^- to NO at low oxygen tensions (74). Although this route to NO is generally described as of low efficiency, NO emission via NR within guard cells was detectable in *Arabidopsis* at a concentration considered ample for signal-

ing function during ABA-induced stomatal closure (27, 85). In *Arabidopsis*, NR is encoded by two genes, *NIA1* and *NIA2*. In the NR-deficient double mutant *nial1 nia2*, NO production was impaired and the HR response to avirulent *P. syringae* claimed to be blunted. However, NO production and the HR response could be restored in *nial1 nia2* by leaf infiltration with NO_2^- , the downstream product (79).

In *nial1 nia2*, NO also resulted following addition of L-arginine, also implicating an NOS-like activity for NO production. NO could have, however, resulted from the lesser known route of production from hydroxylamines and polyamines (PAs) (104, 129). PAs, synthesized from arginine and ornithine by decarboxylases are ubiquitous in plants and may act by inducing enzymic NO synthesis or may act as a substrate for NO release (34). Inhibition of arginase activity increased NO production, whereas reduction decreased production in *Arabidopsis*. The exogenous application of the PA putrescine, spermidine, or spermine, induced rapid NO production in *Arabidopsis* seedlings, possibly mediated by H_2O_2 , a reaction product of both PA- and diamine-oxidases (127). The authors also found that knockout mutants in CuAO1, a copper amine oxidase gene in *Arabidopsis*, were impaired in PA-induced and ABA-induced NO synthesis (128). Whereas expression of ABA stress responsive genes was reduced, there was no investigation of the direct effect of biotic interactions.

Under anoxic conditions, another possibility exists: heme proteins or proteins with molybdo-cofactors or iron-sulfur clusters in the electron transport chain of mitochondria, in the endoplasmic reticulum, peroxisomes, or cytosol, can also reduce NO_2^- to NO (49). In oxygenated tissues, the same proteins are highly efficient NO scavengers. Despite this, it has been proposed that NO formation would still be sufficient to fulfill a signaling role (49).

S-Nitrosylation of Proteins

It has been increasingly appreciated that not only the generation, but also the turnover, of the cellular pool of redox responsive molecules has consequences for signaling

FIG. 1. Potential sources of NO in the immune response of plants.

NO production can occur through the NADPH-dependent oxidation of L-arginine to NO and citrulline by the action of NOS-like enzymes or by activity of decarboxylases on arginine/ornithine to PAs/hydroxylamines and thence to NO by copper amine oxidase. Reductive routes from NO_3^- to NO_2^- to NO can occur as a result of heme protein or NR activity. Compartmentation of reactions in the cell is as indicated. NADPH, nicotinamide adenine dinucleotide phosphate; NO, nitric oxide; NO_2^- , nitrite; NO_3^- , nitrate; NOS, nitric oxide synthase; NR, nitrate reductase; PA, polyamine.

functions. In this respect, the *S*-nitrosylation of proteins continues to emerge as a key mediator of NO signaling, with many examples in support of its importance in transducing the NO signal (130).

S-nitrosylation is a post-translational regulatory mechanism during which NO is covalently and reversibly bonded to the sulfhydryl groups of rare, low pKa cysteine residues to form an SNO, typically altering protein function (114). *S*-nitrosoglutathione (GSNO) reductase 1 (GSNOR1) was found to control cellular SNO levels in *Arabidopsis*. Mutants with increased GSNOR1 activity had lower cellular concentrations of SNO and enhanced basal resistance, whereas mutants with impaired GSNOR1 activity not only had elevated global SNO, but exhibited compromised basal, *R*-gene and nonhost resistance responses (33). The implication was that GSNOR1 regulated multiple modes of plant disease resistance. The modulation of SNO through GSNOR1 regulated SA accumulation and expression of SA-dependent genes (33, 59). The importance of GSNOR1 in modulating the pool of (S)NO in diverse physiological processes is now being appreciated in the wider plant research community.

To summarize, *de novo* production, as well as turnover, of NO has implications for plant immunity. Given the properties of NO as a small, freely diffusible molecule, collective production/turnover from several sources could accumulate sufficient NO to trigger a signal. Mirroring ROS, future studies will be needed to shed light on how multiple sources of NO are coordinated, and how NO is pooled and trafficked in the cell as a direct result of attempted pathogen colonization.

S-Nitrosylation of Proteins in the HR

The HR prevents further ingress of biotrophic pathogens from the initial site of infection by causing a localized cell death, leading to discrete lesions. Evidence suggests that *S*-nitrosylation may have a role in regulating this programmed execution of pathogen-challenged plant cells.

Salicylic acid binding protein 3 (SABP3), found in the tobacco chloroplast, exhibits a high affinity for SA and also has carbonic acid anhydrase (CA) activity (111). That CA exhibits an antioxidant activity and is a requirement for HR was shown by suppression of the *avrPto*-induced HR by virus-induced gene silencing (VIGS) (111). In *Arabidopsis*, the CA function of AtSABP3 is also required for the expression of *R*-gene resistance against attempted infection by *Pst* DC3000 expressing *avrB* (124). Growth of *Pst*DC300 (*avrB*) was enhanced in *sabp3-1* and *sabp3-2* compared with wild type. Following investigation of the effect of *S*-nitrosylation on SABP3 activity, a GSNO concentration-dependent decrease in CA activity and a parallel decrease in SA binding have been described (124). The effect was much greater in *atgsnor1-3* plants, which exhibit elevated levels of *S*-nitrosylation (33). Following biotin switch, mass spectra, and site-directed mutagenesis studies, the Cys 280 residue of SABP3 was determined as the site responsible for suppression of the CA activity, decreased binding by SA, and diminished and delayed defense reactions following *S*-nitrosylation. The authors suggest that control of CA activity by *S*-nitrosylation of SABP3 may serve as a negative feedback loop to dampen defense signaling.

Yun *et al.* (131) uncovered an alternative mechanism involving *S*-nitrosylation that could provide an explanation of how ROS-potentiated cell death may be limited (Fig. 2). Using *Arabidopsis* with mutations in GSNOR1, inoculation with *Pst* DC3000 (*avrB*) or (*avrRPS4*) revealed differences that correlated with SNO concentrations. In *gsnor1-3*, where SNO was elevated, H₂O₂ production and SA accumulation were reduced, the HR response accelerated, and cell death increased compared with the wild type, whereas in *gsnor1-1*, with SNO lower than wild type, the HR was delayed and cell death was less than in the wild type. These data imply that SNO may drive the HR independently of H₂O₂ and SA.

Membrane-bound NADPH oxidases (Rboh) catalyze the production of O₂^{•−} by reduction of molecular oxygen using NADPH as the electron donor. Yun *et al.* (131) therefore considered the effect of SNO on RbohD and F. Mutants *rbohD*, *rbohF*, and *rbohDF* exhibited a reduction in HR cell death compared with wild type showing dependency on H₂O₂ for the HR. However, the HR of *gsnor1-3rbohD*, *gsnor1-3rbohF*, and *gsnor1-3rbohDrbohF* were comparable with *gsnor1-3*, in spite of reduced H₂O₂, endorsing the idea of high SNO driving the HR. GSNO was also demonstrated to reduce RbohD activity *in vitro* and *in vivo* and this was shown to be through *S*-nitrosylation of the Cys 890 residue of Rboh (Fig. 2), abolishing O₂^{•−} synthesis, probably by disrupting the binding of cofactor FAD (131). ROS accumulation was reduced and excessive cell death by H₂O₂ blunted as a result. Similar to the proposed modulation of CA activity by SNO, this may suggest a mechanism that uses rising SNO concentration, or a threshold level, to dampen H₂O₂ production and control cell death by *S*-nitrosylation of RBOH. Intriguingly, *S*-nitrosylation of NADPH oxidase at Cys 890 also occurs in both humans and flies, implying that this mechanism may serve to regulate immune function across

FIG. 2. *S*-nitrosylation of RBOHD regulates H₂O₂ production. Binding of cofactor FAD is a requirement for O₂^{•−} synthesis by RBOHD, leading to H₂O₂. Following pathogen recognition, SNO levels rise due to activation of a nitrosative burst. Increasing SNO levels drive HR cell death in conjunction with ROS. During the later stages of HR development when SNO levels reach a threshold level, the Cys 890 residue of RBOHD is *S*-nitrosylated and binding of the essential cofactor, FAD, is blunted, leading to a reduction in ROS production. This molecular crosstalk may therefore provide a potential mechanism to curb the extent of cell death during the immune response. FAD, flavin adenine dinucleotide; H₂O₂, hydrogen peroxide; HR, hypersensitive response; O₂^{•−}, superoxide; RBOH, respiratory burst oxidase homologue; ROS, reactive oxygen species; SNO, *S*-nitrosothiol.

kingdoms (131). Furthermore, these data may explain the long standing observation that NO can function as an anti-inflammatory in a number of animal disease models.

Role of S-Nitrosylation in Reprogramming of the Transcriptome: NPR1 and TGA

A key function of redox signaling is to orchestrate the reprogramming of the transcriptome, resulting in the activation of target defense-related genes (82). A well-studied example, the immune coactivator nonexpressor of pathogenesis-related genes 1 (NPR1), which interacts with the partially, functionally redundant, TGACG motif-binding, basic domain leucine zipper (bZip) transcription factors, TGA1 and 4, in the nucleus, enabling binding by TGA at the promoter of *PR1* and presumably other SA-responsive genes (54, 66, 73, 83, 113, 114, 116) (Fig. 3). The NPR1-TGA interaction is not constitutive, but is regulated by SA-induced redox change (29).

In the absence of pathogen challenge, GSNO-mediated oxidation maintains the intermolecular disulfide bonding between conserved cysteine-156 residues of NPR1 (83, 116) causing the oligomeric state to predominate (Fig. 3). The NPR1 protein contains a functional bipartite nuclear localization signal, and nuclear localization of NPR1 is required for

activation of SA-dependent gene expression (114). As an oligomer, NPR1 is unable to translocate to the nucleus to fulfill its coactivator function. In *Arabidopsis*, *gsnor1-3* that exhibits increased GSNO and S-nitrosylation, SA-induced monomerization and nuclear transportation of NPR1 were inhibited and SA-dependent gene expression suppressed (116).

Upon pathogen attack, an increase in SA mediates redox changes that promote NPR1 oligomer to monomer formation through the reduction of the disulfide bonds within NPR1. Cytosolic thioredoxins, TRX-h3 and TRX-h5, were revealed by Tada *et al.* (116) to counter the effect of GSNO-facilitated oligomerization. Both TRX-h3 (constitutively expressed in *Arabidopsis*) and TRX-h5 (upregulated in response to *P. syringae* pv. *maculicola* [Psm]) were necessary for full induction of *PR* genes. In pull-down experiments, NPR1 binding with TRX was increased by SA and TRX enzymic activity was inversely correlated with binding (116). Correspondingly, in a cell lysate system, the NPR1 monomer increased and NPR1 oligomer decreased within 15 min of addition of TRX-h5 (116). Tada *et al.* (116) gave further evidence for the role of TRX by showing that in the TRX reductase knockout mutant, *ntra*, in which regeneration of cytosolic TRX is blocked, NPR1-dependent SAR against *Psm* was partially impaired. In aggregate, to maintain

FIG. 3. Role of S-nitrosylation in reprogramming of the transcriptome: NPR1 and TGA. In the absence of pathogen challenge, GSNO-mediated oxidation maintains disulfide bonding within immune coactivator NPR1 and the oligomeric state predominates. Following pathogen challenge, SA-mediated redox changes enable TRX reduction of disulfide bonds, releasing the NPR1 monomers that are capable of translocation to the nucleus. Within the nucleus, reduction of the disulfide bonds (-SS-) in the TGACG motif-binding transcription factor, TGA1, by GSNO-mediated redox change enables interaction of TGA with NPR1 and, subsequently, binding of DNA by TGA. Transcription proceeds. The promoter is cleared of exhausted NPR1 at the end of transcription by ubiquitin-mediated degradation by the proteasome to enable recruitment of further fresh NPR1 coactivator. As targets of phosphorylation, the Ser 11 and Ser 15, within a conserved phosphodegron motif of NPR1, are an inherent part of the promoter clearance process, enabling recruitment of an adapter to the site and interaction with the CUL3 ubiquitin ligase. NPR1, nonexpressor of pathogenesis-related genes 1; redox, reduction-oxidation; SA, salicylic acid; TRX, thioredoxin.

oligomer/monomer cycles of NPR1, transient oxidative/reductive fluctuations involving GSNOR1 and TRX may be required (66, 73, 116).

In the nucleus, SA-mediated redox changes promoted denitrosylation and reduction of the disulfide bonds in TGA1 and TGA4 (66, 73). Cys 260 and Cys 266 of TGA were shown as the redox-sensitive residues (66, 83). Monomeric NPR1 interacted with TGA1/TGA4 to form a transcriptionally active complex that was able to bind the cognate motifs within the *PR1* promoter (66, 83).

To enable transcription by TGA to continue, NPR1 turnover might constitute a requirement. As targets of phosphorylation, the Ser 11 and Ser 15 residues within a conserved phosphodegron motif of NPR1 were shown as an inherent part of the promoter clearance process, enabling recruitment of an adapter to the site and interaction with CUL3 ubiquitin ligase, promoting NPR1 turnover (113). In this way, the promoter can be cleared of exhausted NPR1 by ubiquitin-mediated degradation *via* the proteasome, enabling recruitment of fresh NPR1 to drive a further round of transcription.

Protein Tyrosine Nitration

Protein tyrosine nitration, in which the nitro group, NO_2^- , combines with tyrosine residues to form 3-nitrotyrosine creating an irreversible alteration to protein configuration, is another redox-based post-translational regulatory mechanism. Considered a relevant biomarker of NO-dependent oxidative stress, 3-nitrotyrosine has been associated with disease in animals (100). Tyrosine nitration is mediated by RNS such as the peroxynitrite (ONOO^-) anion and nitrogen dioxide (NO_2^-), formed rapidly as secondary products of NO

metabolism in the presence of oxidants, including O_2^- , H_2O_2 , and transition metal centers (2, 100). In animals, ONOO^- is highly toxic, a dose-dependent cell death occurs from 1 μM of ONOO^- , whereas concentrations of up to 1 mM did not cause the death of soybean cells (25).

Data have been presented suggesting that NO, not ONOO^- , in synergy with H_2O_2 induces the HR (25). Thus, the equilibrium between the relative rates of O_2^- dismutation to H_2O_2 and reaction of O_2^- with NO to form ONOO^- might be critical for regulating HR development (Fig. 4) (25). However, why plants should be so tolerant of ONOO^- , while animal cells are so sensitive to this ordinarily highly toxic RNS, remains to be established. Perhaps, plants have evolved unique enzyme systems to more efficiently degrade ONOO^- . In *Arabidopsis* and yeast, two plastid-located peroxiredoxins (PRXs), PRXIIIE and 2-Cys-Prx, respectively, detoxify the ONOO^- ion through hydroperoxide-reducing peroxidase activity (2, 84, 103, 107). S-nitrosylation of *Arabidopsis*, PRX at Cys 121 (Fig. 4) abolished the activity of this protein. Thereby, ONOO^- detoxification was inhibited, allowing ONOO^- formation to increase thus removing NO and O_2^- , driving protein nitration (5). Hence, PRXIIIE function might regulate HR formation.

The high diffusion rate and low stability of ONOO^- , have made it difficult to study. However, evidence of a role for nitration in plant defense is beginning to accumulate. Tobacco BY-2 cells treated with the fungal elicitor, INF1, from *Phytophthora infestans* produced ONOO^- after 1 h with production peaking at 6–12 h. INF1-induced production of ONOO^- could be abolished using the ONOO^- scavenger, urate. Following treatment with an ONOO^- donor, sydnonimine hydrochloride, nitrated proteins of 20 and 50 kDa were recognized in a BY-2 cell extract using anti-nitrotyrosine

FIG. 4. Peroxynitrite in defense and signaling. O_2^- can react with NO sources resulting in peroxynitrite, ONOO^- . However, NO, not ONOO^- , in combination with H_2O_2 may lead to HR development. Thus, the balance between SOD-catalyzed dismutation of O_2^- to H_2O_2 , and reaction of O_2^- with NO to ONOO^- is critical. PRXIIIE detoxifies peroxynitrite, but is deactivated by S-nitrosylation of Cys 121. Thereby, ONOO^- accumulates and NO and O_2^- are removed. The ONOO^- anion reacts with tyrosine residues of proteins, a process termed protein tyrosine nitration, altering protein activity. It is speculated that nitrated proteins may be subject to proteolysis or denitrase activity to render nitration reversible and suggesting a putative signaling function. ONOO^- , peroxynitrite; SOD, superoxide dismutase.

monoclonal antibodies (106). Since proteins of the same molecular weights were similarly identified in INF1-treated cells, the authors concluded that tyrosine nitration had occurred in response to INF1. In *Arabidopsis* leaf extracts, ONOO⁻ was identified with the use of the peroxynitrate-sensitive fluorescent dye, HKGreen-2, following infection with *Pst* DC3000 expressing *avrB*. ONOO⁻ accumulation was evident at 3–4 h postinoculation and peaked at 7–8 h postinoculation and correlated with the increase in protein tyrosine nitration and the onset of HR (17, 41). Identified in 2D western blots, proteins that were nitrated following inoculation with *Pst* DC3000 (*avrB*) included FBA1 and FBA2 (glycolysis and Calvin Cycle enzymes), the RuBisCO large subunit and RuBisCO activase, chloroplastic ATP synthase, and isoforms of the oxygen-evolving protein of photosystem II (17).

In spite of the observations on nitrated proteins, in stark contrast with *S*-nitrosylated proteins, little is known about the effects of nitration on protein function *in vivo*, consequently, the impact of this redox-based, post-translational modification in immunity is still uncertain. One tantalizing hypothesis is that nitration may disturb the balance between nonphosphorylated and phosphorylated tyrosine residues on proteins, thereby potentially impeding the phosphorylation cascades of MAP kinases (80, 81). Indeed ONOO⁻, which accumulated in plants during the HR, led to protein nitration of some MAP kinases (67). However, the robust demonstration of reversibility is a critical issue for protein nitration to emerge as an accepted mechanism for cellular signaling (2, 120). In this context, denitrification activity has also been proposed but, as yet, no such activity has been demonstrated. There are other issues, not least that only a few candidate nitrated proteins have been identified to date. Therefore, at present, protein nitration leaves many pertinent questions unanswered.

Glutathione as a Redox Sensor

The extent to which ROS and NO accumulate is determined, in part, by the antioxidant system, which strives to maintain homeostasis to support protein activity. Redox-sensing components with different antioxidant buffering capacities form interconnected compartments that detect changes in ROS and RNS metabolism and transduce these redox changes to alter gene expression and cellular function (37, 87, 112). In this context, either a threshold concentration or change in ratio of reduced to oxidized small molecule redox couples would enable signaling. Glutathione (GSH), a low molecular weight thiol, and ascorbate are key moderators of cellular redox potential in many physiological processes (37). Ascorbate and GSH are ideally positioned between ROS and cellular reductants, such as NADP/NADPH, to form a gradient of redox potential and enable a signaling function (37, 73, 88, 112) (Fig. 5).

GSH is an essential metabolite in plants. Being relatively easily quantifiable in comparison to H₂O₂, for example, GSH is considered a reliable marker of altered redox status (76). Found in high (mM) concentrations in plant tissues, it participates in thiol–disulfide reactions where the reduced form (GSH) is oxidized to the disulfide form (GSSG) and reduced again to GSH by glutathione reductase (GR) in a continuous fashion (Fig. 5) (88). The reduced form of GSH predomina-

FIG. 5. GSH in redox homeostasis. The position of GSH at approximately midway on the scale of redox potential, between oxidants such as H₂O₂ and reductants, enables a signaling function. The large cellular pool of GSH, maintained in the highly reduced state as GSH, buffers oxidative changes resulting from ROS. In the process, GSH is oxidized to GSSG and recycled to GSH by NADPH-dependent GR. GSH also participates in thiol–disulfide interactions: GSNO, a reservoir of SNO bioactivity, is turned over by GSNOR to GSH. The ratio of GSH to GSSG is paramount: the catalase-deficient mutant, *cat2*, with elevated H₂O₂ and the *nudt7* mutant, both with a reduced ratio of GSH/GSSG, exhibit enhanced resistance to *Pseudomonas syringae*. GSH, glutathione; GSNO, *S*-nitrosoglutathione; GSNOR, GSNO-reductase; GSSG, glutathione disulfide; *nudt7*, Nudix domain-containing hydrolase 7. ASC, ascorbate; DHASC, dehydroascorbate.

tes, with high GSH:GSSG ratios of 20:1 commonly occurring (75). In *Arabidopsis*, the Nudix domain-containing hydrolase 7 (*nudt7*) mutation was found to cause perturbation to cellular redox homeostasis (42, 43). This was expressed in the consistently lower ratio of GSH to GSSG in *nudt7*, suggesting that more GSH molecules were being used to regenerate oxidized molecules. The redox imbalance leads to a hyper-response to *Pst* DC3000, leading to accelerated activation of defense genes in two pathways: *PR1*, dependent on both *NPR1* and SA; *PR2* and *AIG1*, independent of *NPR1*. Collectively, engagement of these two signal pathways by *nudt7* resulted in enhanced basal disease resistance. NUDT7 is, thus, a negative regulator of the basal defense response.

Arabidopsis cat2, a knockout mutant for the major leaf catalase, has elevated H₂O₂ and mimics lesion production in the absence of pathogens, a phenotype shown to be SA dependent. CAT2 function is photorespiratory linked, therefore responses are masked when grown in CO₂ and revealed when transferred to air (46). Concentrations of reduced GSH fell from ~95% to 50% in *cat2* in favor of GSSG when plants

were transferred to air, leading to SA accumulation, NPR1-dependent gene expression, and pathogen resistance (99).

The Cinnamyl alcohol dehydrogenase (CAD) and CAT2 double mutants (*cad2 cat2*) were employed as tools to dissect SA- and jasmonic acid (JA)-dependent responses in GSH-deficient *Arabidopsis* (46). In the *cad2* mutant, the blockage at γ -glutamylcysteine synthetase (γ -ECS), prevents condensation of cysteine and glutamate to γ -EC, the precursor to GSH. The upregulation of JA-responsive genes in *cat2 npr1* was expected since the antagonism between SA and JA pathways, partly mediated by NPR1 function, is abolished. However, under the oxidative stress imposed by *cat2*, but with decreased GSH, *cad2 cat2* exhibited a reduction in transcripts of JA-related and SA-related genes. Application of exogenous GSH restored expression of JA-, but not SA-related genes, suggesting GSH has an important role in determining JA gene expression that may operate without NPR1 involvement.

Crosstalk Between Calcium, H_2O_2 , and NO

The question arises of how the recognition of a putative pathogen is translated into ROS and NO production. In plants, the calcium ion, Ca^{2+} , is a ubiquitous intracellular second messenger, involved in signal propagation in numerous pathways and its role in defense has been extensively

summarized (8, 40, 62, 70, 71). Various stimuli, including exogenous H_2O_2 and pathogen elicitors, trigger changes in cytosolic Ca^{2+} concentration that are detected by Ca^{2+} sensors, such as CaM or calmodulin-like protein (CML), calcium-dependent protein kinase (CDPK), calcineurin B-like protein (CBL), and Ca^{2+} /CaM-dependent protein kinase (CCaMK) and transduced into a signal (23, 72, 94).

The interactions between the elicitor cryptogin from the Oomycete *Phytophthora cryptogea* and tobacco, between endopolygalacturonase 1 from *Botrytis cinerea* and grapevine, between *Pst*, carrying *avrB* or *avrRpm1* avirulence genes, and *Arabidopsis* (8, 40, 62) illustrate the general scheme of signaling. In each case, changes in permeability of the Ca^{2+} channels of the plasma membrane gave rise to a calcium signature within minutes of the given interaction. In *Arabidopsis*, functional *cNGC2* or *cNGC4* genes, encoding cyclic nucleotide-gated channels in the plasma membrane, were a requirement for the HR response to avirulent pathogens (9, 20, 71). Recognition of *Pst* isolates gave a rapid rise in cAMP and triggered a cNGC2-mediated Ca^{2+} influx (70, 71, 98) (Fig. 6). Ali *et al.* (1) found that HR was suppressed in the *Arabidopsis* defense no death 1 (*dnd1*) mutant, which is impaired in the cyclic nucleotide-gated channel (cNGC2), but that HR could be partially restored by NO donor, SNP. Other plasma membrane Ca^{2+} -permeable channels, such as glutamate-like receptors (GLRs) were necessary for

FIG. 6. Early Ca^{2+} fluxes and Ca^{2+} -dependent processes in the plant cell following pathogen/elicitor interaction. Binding of elicitor to its plasma membrane receptor stimulates cAMP release, activating plasma membrane cNGC, allowing rapid influx of Ca^{2+} into the cell. RBOH (NAPDH oxidase) is directly activated by Ca^{2+} binding at the N-terminal EF-hands and indirectly following phosphorylation by Ca^{2+} -activated CDPK leading to H_2O_2 production. CaM/CML activation following the rise in cytosolic Ca^{2+} leads to NO production. The rise in NO triggers release of Ca^{2+} from intracellular Ca^{2+} pools through the activation of cGMP and cADPR-gated channels. Positive feedback by NO upregulates CML. CaM and CDPK have been shown to be S-nitrosylated (50), however, the impact of this modification on their activities remains to be established. ACAs in the plasma membrane and tonoplast regulate Ca^{2+} efflux. ACA, autoinhibited Ca^{2+} -ATPase; cADPR, cyclic ADP ribose; CaM, calmodulin; CDPK, calcium-dependent protein kinase; cGMP, cyclic guanosine monophosphate; CML, calmodulin-like protein; cNGC, cyclic nucleotide-gated channel.

Ca²⁺ signaling and NO, but not H₂O₂, production in tobacco (121).

The calcium signal was rapidly transduced; in all interactions cited, Ca²⁺ influx led to rapid production of H₂O₂ and NO (32, 35, 72) and activation of mitogen-activated protein kinases (MAPKs). Production of H₂O₂ was through direct Ca²⁺ activation of NADPH oxidase (RBOH) (40, 65, 86, 97, 117). Complementary to Ca²⁺ activation, it was shown that RBOHA and B in potato and RBOHD in *Arabidopsis* were activated through phosphorylation of Ser 82 (potato) and Ser 39 and Ser 148 (*Arabidopsis*) by CDPK5 (31, 56). However, as the name suggests, activity of CDPK5 itself was Ca²⁺ dependent (56). Furthermore, exogenous H₂O₂ rapidly induced CDPK5 phosphorylation indicating a positive feedback loop between CDPK5 activation and H₂O₂ production, although the route of H₂O₂ perception to reiteration of the Ca²⁺ signal was not described (31).

Ma *et al.* (72) suggested that cytosolic CaM/CML activation following Ca²⁺ influx (Fig. 6) was a requirement for NO induction, since NO generation and HR were both inhibited in an *Arabidopsis* mutant with impaired Ca²⁺ sensor protein, CML24. Similarly, CML9 was activated by Ca²⁺ following interaction with *Pst* DC3000 and immunity was impaired in *cml9* mutants (61). Following entry of Ca²⁺ through cNGC2, it was found that the CaM antagonist, W7, abolished NO production (1), reiterating the involvement of CaM or CML sensors.

The observation that the NO scavenger cPTIO, and NOS inhibitors, reduced the cytoplasmic Ca²⁺ increases induced by cryptogin, led Lamotte *et al.* (60) and Wendehenne *et al.* (125) to suggest that NO production further perpetuated Ca²⁺ influx by the mobilization of intracellular pools of Ca²⁺ via cyclic guanosine monophosphate (cGMP)- or cyclic ADP ribose (cADPR)-gated Ca²⁺ channels. This extended their earlier studies (32, 55) linking expression of defense genes *PR1* and *PAL* to NO and downstream cGMP and cADPR.

Oligogalacturonide (OG) application, a process shown to trigger rapid NO synthesis (101), was used as a technique to highlight NO target genes during genome-wide expression profiling in *Arabidopsis* (50). Measured against the control, pretreatment with the NO inhibitor, cPTIO, 25 differentially expressed genes responding to NO were annotated as Ca²⁺ signaling-related genes, including CML, CDPK, CBL, CBL-interacting protein kinases (CIPKs), and autoinhibited Ca²⁺ ATPases (ACAs) (50). The genes encoding CMLs were up-regulated by NO; those encoding CIPKs were repressed (50). Previously, a CaM and a putative CDPK were revealed as S-nitrosylated following cryptogin challenge of tobacco cells, indicating the possibility of post-translational modulation of Ca²⁺ sensors by NO (7).

In *Zea mays*, the ABA-H₂O₂-NO-MAPK cascade is emerging as central to antioxidant-related stomatal closure (132) and may have implications for defense against pathogens that use stomata as the route of entry. Elaborating further, Hu *et al.* (48) showed that treatment with ABA or H₂O₂ gave a rise in cellular Ca²⁺ and CaM and led to enhanced expression of SOD4, APX, and GR (48). Whereas pretreatment with CaM antagonists eradicated ABA-related H₂O₂ production, pretreatment with ROS inhibitors did not inhibit the ABA-related CaM increase. Ma *et al.* (69) demonstrated the later occurrence of the Ca²⁺ sensor, CCaMK, in the cascade. Exogenous H₂O₂ treatment increased CCaMK gene

expression and CCaMK activity. Blocking endogenous production of ROS using DPI, an inhibitor of NADPH oxidase, prevented activation of CCaMK showing a requirement for H₂O₂. Since H₂O₂ was known to increase NO accumulation, use of the NO scavenger, cPTIO, and NOS inhibitor, L-NAME, resulting in reduced CCaMK transcripts and failure of stomata to close, demonstrated that H₂O₂-dependent NO production was critical for ABA-induced stomatal closure, and mediated the Ca²⁺ activation of CCaMK. Thus, crosstalk between Ca²⁺, H₂O₂, and NO was shown as influential in another defense context.

The balance between Ca²⁺ influx and efflux appears critical. In *Arabidopsis*, ACAs, located in the plasma membrane, ER, and tonoplast, control efflux (19). Knockouts of vacuolar ACA 4 and ACA11 enhanced the HR (15). *Arabidopsis* has 10 ACAs and the challenge will be to determine how the Ca²⁺ influxes, through cNGCs, and effluxes, through ACAs, are coordinated to regulate the Ca²⁺ signal.

In summary, following pathogen detection, the Ca²⁺ influx into the cytosol through cNGCs, acts as a signal that is rapidly transduced by H₂O₂ and NO production. Subsequently, H₂O₂ and NO transmit and reinforce the signal through positive feedback loops involving Ca²⁺ sensors, leading to further Ca²⁺ inflow from internal Ca²⁺ pools through Ca²⁺-gated channels and ROS and RNS production. Post-translational modification by S-nitrosylation may regulate CaM and CDPK activities (Fig. 6).

Crosstalk Between MAPK Cascades and ROS and NO

MAPK cascades, consisting of the three modules, MAPKKK-MAPKK-MAPK, are integral to many plant responses, including stress (51). Starting with MAPKKK, sequential phosphorylation leads to activation of the terminal MAPK, with the MAPK in turn phosphorylating a target protein to regulate its activity. In plants, MAPKKs commonly act as divergence points: one MAPKK can activate more than one MAPK enabling a flexible response to multiple stressors (51).

In *Arabidopsis*, MAPK3, MAPK4, and MAPK6 have been implicated in responses to pathogens. Xylanase from *Trichoderma viride* was found to induce MAPK6 (89). The bacterial flagellin peptide, flg22, induced MEKK1 (MAPKKK1), leading to activation of MEKK4 and MEKK5 and MAPK3 and MAPK6 (28, 89). Similarly, SA-induced protein kinase (SIPK), wound-induced protein kinase (WIPK), and Nicotiana Fus-3-like kinases 4 and 6 (NTF4 and NTF6) were activated in tobacco responses (28, 96, 102). Evidence for positioning of MAPK cascades upstream of, and linking them with, H₂O₂ and NO production comes from studies of the H₂O₂ and NO bursts produced by INF1 of *P. infestans* on *Nicotiana benthamiana* (Fig. 7). Constitutively active MAPK kinase (MEK2) (3, 4) caused phosphorylation of the MAPKs, SIPK/NTF4, as well as induction of NO. NO production was compromised by VIGS of NO Associated1 (NOA1) (4). Expression of NOA1 was not induced by INF1 suggesting that post-transcriptional control of NOA1 influenced NO production by MEK2-SIPK/NTF4. MEK2-SIPK followed by NTF4 also mediated the ROS burst and ROS production was prevented by VIGS of RbohB (4). ROS generation, however, was also compromised by VIGS of SIPK or NTF6 of the cascade MEK1/SIPK/NTF6, indicating

FIG. 7. Positioning of MAPK signaling cascades upstream of NO and ROS production. The *P. infestans* elicitor, INF1, induces the tobacco MAPK cascade, MEK2-SIPK/NTF4, leading to production of NO. NO synthesis is abolished by VIGS of NOA1 even in the presence of INF1. However, INF1 does not induce expression of NOA1 suggesting post-transcriptional control of NOA1-influenced NO production by MEK2-SIPK/NTF4. Similarly, MEK2 activates SIPK, with phosphorylation of NTF4 resulting in ROS production. However, ROS production can also occur via the MEK1-SIPK-NTF6 pathway. Both routes lead to activation of RbohB, with VIGS of RbohB, SIPK, or NTF6 resulting in the absence of the Rboh-mediated oxidative burst. MAPK, mitogen-activated protein kinase; MEK2, MAPK kinase; NTF4, Nicotiana Fus-3-like kinase 4; SIPK, SA-induced protein kinase; VIGS, virus-induced gene silencing.

convergence of two MAPK cascades on Rboh. One proposed control mechanism was through activation of RibA, a flavin biosynthesis enzyme, by MEK2-SIPK/NTF4, in turn supplying flavin for NR, NOS-like enzymes, and RBOH (3).

The activation of MAPK cascades downstream of ROS production in the plant immune responses is inferred from the abundant evidence of H_2O_2 induction of MAPKs in oxidative stress responses in general (57, 108–110, 123). Ozone, used as a potent H_2O_2 generator, resulted in a rapid, transient activation of MAPK6 as well as interaction with MAPK3 in *Arabidopsis* and activation of SIPK and interaction with WIPK in tobacco (108, 109). ANP1, a member of the ANP class of *Arabidopsis* MAPKKKs, was induced by H_2O_2 (57), leading to induction of MPK3 and MAPK6. Constitutively active ANP1 mimicked the effect of H_2O_2 .

That regulatory systems are complex is exemplified by the elicitor harpin, derived from *P. syringae* pv. *syringae*. Known to trigger H_2O_2 production, harpin induced *Arabidopsis* MAPK4 and MAPK6 (6, 28, 89) and exogenous H_2O_2 could substitute for harpin to activate AtMAPK6, but not MAPK4 (28). However, the use of catalase and DPI to block H_2O_2 and O_2^- did not eradicate harpin-induced activation of either MAPK4 or MAPK6 suggesting that the harpin-triggered oxidative burst and harpin activation of MAPKs were through divergent pathways.

Tobacco SIPK, but not WIPK, was activated by NO and SA (58), the only NO-dependent MAPK in defense so far identified (8). Furthermore, tyrosine nitrated MAPKs were identified among proteins following $ONOO^-$ accumulation

during the HR in *Arabidopsis*, although they were not explicitly named (67).

The intensity and duration of activation of MAPKs are tightly controlled through the balance between phosphorylation and dephosphorylation (11, 63). In *Arabidopsis*, MAPK phosphatase 2 (MKP2) is a dual specificity phosphotyrosine phosphatase (PTP) that targets oxidant-activated MAPK3 and MAPK6. RNA-dependent gene silencing of MKP2 prolonged MAPK3 and MAPK6 activation and an ozone-hypersensitive phenotype with rapid leaf necrosis resulted (63). The phosphatase, MKP1, interacted with MAPK3, 4, and 6 in yeast-2-hybrid studies. However, in the *mkp1* mutant, only MAPK6 activity was elevated, nevertheless, plants had high SA levels and were resistant to *P. syringae* infection (10, 11). *Arabidopsis* PTP1 dephosphorylates and deactivates MAPK3 and MAPK6 (10, 11). In the *mkp1 ptp1* double mutant, defense responses such as *PR* gene expression were enhanced indicating that PTP1 and MKP1 act as negative regulators of defense responses. Furthermore, PTP1 was directly redox responsive, being deactivated *in vivo* by H_2O_2 oxidation (45). H_2O_2 did not change PTP1 concentration or stability, but induced a rapid, reversible Cys-dependent conformational change (45). Mutational analyses suggested that Cys 235 was the essential residue for redox sensing in PTP1: replacement by Ser abolished phosphatase activity (119). Illustrating the complexity of ROS/RNS cellular networks and feedback loops, both MKP1 and PTP1 were shown to bind CaM and activity was increased by CaM in a Ca^{2+} -dependent manner (64).

ROS in Systemic Signal Propagation

In addition to local signaling functions, evidence has long suggested a potential role for ROS in systemic signaling in plants. Furthermore, properties of H_2O_2 , such as small size, relatively long lifespan of ~ 1 ms, and ability to traverse membranes *via* aquaporin channels rather than by limited diffusion alone, promote the potential signaling functions of this redox-active small molecule (12). In this context, during the replication and systemic spread of Cauliflower mosaic virus (CaMV) in a compatible host-virus interaction in *Arabidopsis*, a long distance ROS signaling pathway was engaged (68). Thus, within 2 h of inoculation, during a time frame that preceded virus movement, a ROS burst occurred both locally and also systemically, as part of a phenomenon termed the rapid systemic response. Systemic, but not local, H_2O_2 accumulation was abolished in *rbohD* double mutants and in *etr1-1* and *ein2-1* mutants, implicating NADPH oxidase and ethylene signaling in the generation and transduction of the response. These data therefore highlight long distance ROS signaling as an integral feature of the immune response of *Arabidopsis* plants to CaMV infection.

Subsequently, a potential role for ROS in long distance wound signaling was uncovered (77). A bidirectional signal travelled at up to $8.4 \text{ cm} \cdot \text{min}^{-1}$ from the site of wounding in wild-type *Arabidopsis*, whereas the signal travelled upward only at $0.5 \text{ cm} \cdot \text{min}^{-1}$ in the *rbohD* mutant. The rate of ROS accumulation in the apoplastic space around the wound site in wild-type plants corresponded with the rate of progress of the systemic signal. In *rbohD*, however, ROS accumulation and long distance signaling were suppressed, implicating RbohD-derived ROS in mediating the observed long distance signaling. Interestingly,

FIG. 8. ROS as a long distance signal in plant immune function. RbohD-derived ROS is a requirement for local and long distance signal transmission. Following PAMP recognition or wounding, Ca^{2+} influx activates CDPK5 that phosphorylates RbohD with concomitant production of apoplastic O_2^- . H_2O_2 , generated by dismutation of O_2^- , facilitates CDPK5 activation upon entering the originating and neighboring cells, thus initiating a positive feedback loop involving CDPK5 and H_2O_2 . From such cell-to-cell transmission, ROS conveys the defense signal as a wave to distal tissues. The additional requirement for ethylene with H_2O_2 in the systemic response to CaMV suggests further layers of complexity. PAMP, pathogen-associated molecular pattern; CaMV, Cauliflower mosaic virus.

experiments suggested that generation of the systemic signal required both Ca^{2+} and RbohD-derived ROS, whereas propagation depended only upon RbohD-dependent ROS synthesis. An ROS wave model was proposed, in which an initial cellular burst of H_2O_2 triggered a wave of cell-to-cell communication, propagating the signal to distal sites (78).

Recently, studies on CDPK5 (see Crosstalk Between Calcium, H_2O_2 , and NO) and its target, RBOHD, extended the initial observations of Love *et al.* (68) implicating ROS as a long distance signal in plant immune function (Fig. 8). Constitutive RbohD phosphorylation, resulting from overexpression of CDPK5, gave prolonged and sustained ROS production following bacterial flg22 treatment, in both local and systemic tissues (31). Conversely, in *rbohD* or *cdpk5* mutants, flg22-induced ROS was absent and transcripts of NHL10, a rapidly induced, defense-related gene, were dramatically reduced in distal tissues. In protoplasts, exogenous application of ROS (H_2O_2) induced a rapid phosphorylation of CDPK5, with a cognate increase in activity of this kinase, suggesting a potential ROS-mediated positive feedback loop. As a result, Dubiella *et al.* (31) included CDPK5 as an additional component in a cell-to-cell signaling model, whereby PAMP recognition triggered CDPK5 activity, leading to phosphorylation and subsequent activation of RbohD. The resulting synthesized ROS might then function as a signal that could enter adjacent cells and trigger CDPK5 activation. Further iteration of this ROS-driven, positive feedback loop, could subsequently relay the signal to cells distal to the point of infection, priming plant immune responses (Fig. 8). The emerging data suggest that *S*-nitrosylation of RbohD (131) (see “*S*-Nitrosylation of Proteins in the HR” section) offers a potential mechanism for deactivation of this long distance signaling system. Thus, SNO levels rising concurrently with H_2O_2 , could eventually facilitate *S*-nitrosylation of RbohD, blunting binding of the essential cofactor, FAD, and thereby curtailing ROS synthesis.

Conclusions

The emerging data imply that there may be multiple, potential sources of ROS and RNS. Further, the cellular compartmentation often associated with the production of these radicals, gives rise to new questions relating to their specificity of induction, spatial and temporal functions, and coordination to orchestrate an effective immune response. Development of systems to microquantify H_2O_2 and NO production in subcellular compartments will increase our understanding of how redox changes are perceived, propagated, and coordinated throughout the cytosol and organelles. The profound consequences for redox regulation of the transcriptome in plant immunity will only be fully realized as more redox-responsive transcription factors and their cognate target genes are identified. This rapidly growing area is opening up more questions on control of transcriptional complexes, with the common regulatory theme of *S*-nitrosylation being reinforced. The complexity of redox regulation in plant immunity will require a systems approach to consolidate data on the regulation of MAPK cascades, MKP, transcription factors, and defense genes through the combined effects of Ca^{2+} , phosphorylation, *S*-nitrosylation, and protein tyrosine nitration.

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Abbreviations Used

ACA = autoinhibited Ca²⁺-ATPase
AIG1 = *avrRpt2*-induced gene
APX = ascorbate peroxidase
avr = avirulent
CA = carbonic acid anhydrase
CAD = Cinnamyl alcohol dehydrogenase
cADPR = cyclic ADP ribose
CaM = calmodulin
CaMV = Cauliflower mosaic virus
CAT = peroxisomal catalase
cat2 = catalase 2
CBL = calcineurin B-like protein
CCaMK = Ca²⁺/CaM-dependent protein kinase
CDPK = calcium-dependent protein kinase
cGMP = cyclic guanosine monophosphate
CIPK = CBL-interacting protein kinase
CML = calmodulin-like protein
cNGC = cyclic nucleotide-gated channel

dnd1 = defense no death 1
DPI = diphenylene iodonium
 γ -ECS = γ -glutamylcysteine synthetase
ETI = effector-triggered immunity
FAD = flavin adenine dinucleotide
FBP1 = French bean, class III peroxidase 1
GLR = glutamate-like receptors
gp91 phox = glycoprotein 91-kDa phagocyte NADPH oxidase
GSH = glutathione
GSNO = S-nitrosoglutathione
GSNOR = GSNO reductase
GSSG = glutathione disulfide
H₂O₂ = hydrogen peroxide
HR = hypersensitive response
JA = jasmonic acid
MAMP/PAMP = microbe/pathogen-associated molecular pattern
MAPK = mitogen-activated protein kinase
MEK2 = MAPK kinase
MKP = MAPK phosphatase
NADPH oxidase = nicotinamide adenine dinucleotide phosphate oxidase
NIA1, NIA2 = NR genes
NO = nitric oxide
NO₂⁻ = nitrite
NO₃⁻ = nitrate
NOS = nitric oxide synthase
NPR1 = nonexpressor of pathogenesis-related genes 1
NR = nitrate reductase
NTF4 = Nicotiana Fus-3-like kinase
nudt7 = Nudix domain-containing hydrolase 7
O₂⁻ = superoxide
OG = oligogalacturonide
ONOO⁻ = peroxynitrite
PA = polyamine
PR = pathogenesis related
PRX = peroxiredoxin
Pst = *Pseudomonas syringae* pv. *tomato*
Psm = *P. syringae* pv. *maculicola*
PTP = phosphotyrosine phosphatase
R = resistance
RBOH = respiratory burst oxidase homologue
redox = reduction–oxidation
RNS = reactive nitrogen species
ROS = reactive oxygen species
SA = salicylic acid
SABP3 = salicylic acid binding protein 3
SAR = systemic acquired resistance
SOD = superoxide dismutase
SIPK = SA-induced protein kinase
SNO = S-nitrosothiol
TGA = TGACG motif-binding basic domain/leucine zipper (bZip) transcription factor
TRX = thioredoxin
VIGS = virus-induced gene silencing
WIPK = wound-induced protein kinase