

## Review

# Intertwined roles of reactive oxygen species and salicylic acid signaling are crucial for the plant response to biotic stress

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**Abstract:** One of the earliest hallmarks of plant immune response is production of reactive oxygen species (ROS) in different subcellular compartments, which regulate plant immunity. A suitable equilibrium, which is crucial to prevent ROS over-accumulation leading to oxidative stress, is maintained by salicylic acid (SA), a chief regulator of ROS. However, ROS are not only acting downstream of SA signaling, but were also proposed to be a central component of a self-amplifying loop that regulates SA signaling as well as the interaction balance between different phytohormones. The exact role of this crosstalk, the position where SA interferes with ROS signaling and ROS interferes with SA signaling and the outcome of this regulation depend on the origin of ROS but also on the pathosystem. The precise spatiotemporal regulation of organelle specific ROS and SA levels determine the effectiveness of pathogen arrest and is therefore crucial for a successful immune response. However, the regulatory interplay behind still remain poorly understood, as up till now, the role of organelle specific ROS and SA in HR-conferred resistance has mostly been studied by altering the level of a single component. In order to address these aspects, a sophisticated combination of research methods for monitoring the spatiotemporal dynamics of key players and transcriptional activity in plants is needed, and will most probably consist of biosensors and precision transcriptomics.

**Keywords:** plant immune response, reactive oxygen species, salicylic acid, reactive oxygen species-salicylic acid crosstalk, programmed cell death, hypersensitive response-conferred resistance, RBOH NADPH oxidases, chloroplastic redox state, biosensors, precision transcriptomics

## 1. Reactive oxygen species as one of the earliest hallmarks of plant immune response

Plants have evolved sophisticated mechanisms to perceive pathogen attack and trigger an effective immune response through two distinct but inherently intertwined mechanisms [1]. More general layer, pathogen-associated molecular-pattern-triggered immunity (PTI) is based on the recognition of pathogens through cell surface-localized pattern recognition receptors (PRRs). More specific layer of immunity is mediated by intracellular nucleotide-binding domain leucine-rich repeat receptors (NLRs) [2]. NLRs, also known as R proteins, detect pathogen effector proteins within cells and activate effector triggered immunity (ETI). Successful ETI often results in HR-conferred resistance. In HR, restriction of pathogens to the infection site is associated with a form of localized programmed cell death (PCD) which is manifested as the formation of necrotic lesions on inoculated leaves [3]. HR-conferred resistance is preceded by a series of biochemical and cellular signals. Whereas the mechanism of R proteins activation is well known, the downstream signaling mechanisms leading to the restriction of the pathogen have been less studied. One of the earliest hallmarks of HR is rapid and intense production of reactive oxygen species (ROS) in different subcellular compartments [4]. The first, transient phase with low amplitude occurs within minutes after infection and is mostly apoplastic, tightly linked to posttranslational activation of plasma-membrane RBOH NADPH oxidases and cell-wall peroxidases and is attributed to PTI [5]. In arabidopsis, RBOH activity is regulated through phosphorylation and ubiquitination [6–8]. Superoxide produced by NADPH oxidases is spontaneously or by superoxide dismutase (SOD) converted to hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) [9], which crosses plasmalemma via free diffusion or aquaporin-facilitated diffusion to enter

the cell [10,11]. The second, high amplitude, and sustained phase is initiated a few hours after infection in different compartments, including apoplast, chloroplasts, mitochondria, and peroxisomes, requires transcriptional activation of RBOH genes and is associated with the establishment of defense responses and the HR [9,12]. However, it has recently become clear that both layers have been intertwined and share common regulatory mechanisms [13]. The role of RBOHD in PTI is well known in diverse pathosystems [14], while its involvement in HR has been less studied. However, several studies have pointed to the essential role of RBOH-generated ROS also in HR cell death and/or HR-conferred resistance. This includes viral pathosystems [15], such as potato-potato virus Y interaction [16] and one of the most studied viral pathosystems *Nicotiana tabacum* - tobacco mosaic virus (TMV) [17,18] bacterial pathosystems [19–23], oomycete pathosystems [19,24,25] and fungal pathosystems [23,26–29]. The results of above mentioned studies show that the role of RBOH-generated ROS in immunity is pathosystem-, but also RBOH isoform-dependent [14]. Moreover, it has been shown that different RBOHs isoforms perform different roles within the same host or in different hosts. Some are involved in cell death induction and/or resistance, while others are not, some are involved in early, while others in late ROS production, they regulate different signaling pathways in the plant immune response or they function together in the resistance to some pathogens [19,22,28,30,31].

## **2. Crosstalk between RBOHD-derived reactive oxygen species and salicylic acid in programmed cell death and resistance**

Although ROS regulates plant immunity, biotic stress can cause ROS over-accumulation leading to oxidative stress [32]. Therefore, a suitable equilibrium is crucial for redox homeostasis in the plant. Salicylic acid (SA) is known as a chief regulator of ROS, however, underlying mechanisms are still largely unexplored [33]. In the early 1990s, it was discovered that SA affects ROS production in response to stress [34]. To date, it became clear that SA is required for the restriction of bacterial, oomycete, fungal, and viral pathogens during HR in various pathosystems [3,35,36], including tobacco mosaic virus (TMV) [37,38] and PVY [16,39]. However, the exact role of SA and the position where SA interferes with ROS signaling depends on the origin of ROS but also on the pathosystem [40].

One of the main targets of SA to mediate ROS signaling induction is NADPH oxidase RBOH [40]. However, this regulation seems pathosystem dependent. In *Arabidopsis*, treatment with SA induced ROS production, which resulted from the regulation of the PRRs and is most probably AtRBOHD-dependent [41]. This is also supported by the presence of SA-responsive cis-regulatory elements in the promoters of different RBOHD isoforms in *Arabidopsis* and rice [42,43]. On the other hand, in potato, SA-induced ROS production is rather related to ETI, as in potato HR-conferred resistance against potato virus Y (PVY), RBOHD was under transcriptional regulation of SA [16]. Similarly, in another study on potato, SA treatment induced RBOHB expression, which was also responsible for the second, ETI-related ROS burst phase [44]. In SA-treated pear fruits, the expression of RBOH was isoform dependent [45], which is in agreement with the study performed in potato [16].

Under stressful conditions, ROS are not only acting downstream of SA signaling, but were also proposed to be a central component of a self-amplifying loop that regulates SA signaling as well as the interaction balance between different phytohormones [33,46]. RBOHD knockout *Arabidopsis* mutant plants accumulated higher levels of SA following interaction with pathogens [47]. On the other hand, Chaouch et al. did not detect any difference in SA accumulation in the RBOHD mutant compared with wild-type *Arabidopsis* [20]. The inconsistency in the results might be connected to tight spatial regulation of SA accumulation as it was shown in potato HR-conferred resistance against potato virus Y (PVY), where accumulation of SA was spatially regulated by RBOHD [16]. Interestingly, however, SA biosynthesis was not controlled by RBOHD-generated ROS in this interaction [16].

There have also been a few indications of intertwined role of RBOHD and SA in spatial regulation of cell death induction or suppression. Pogany et al. showed that in *Arabidopsis*, RBOHD triggers death in cells damaged by fungal infection, but simultaneously inhibits death in neighboring cells through the suppression of free SA and ET (ethylene) levels [47]. The developed lesions mimicked HR during incompatible interactions. Consistent with the results of this study, Torres et al. showed that in *Arabidopsis*, AtrBOHD-dependent ROS production at infection sites with elevated levels of SA suppressed SA-dependent HR cell death in neighboring cells using cell death and RBOHD double mutants [26].

The results of above mentioned studies suggest pathosystem-dependent correlation between SA and RBOH-derived ROS in HR cell death and HR-conferred resistance. Different mechanisms in regulating RBOH-dependent ROS production are important for maintaining signaling specificity, while the cross talk with SA signaling provides another layer of regulation.

### **3. Chloroplastic reactive oxygen species play a role in the signaling for programmed cell death and induce SA-dependent transcription of immune genes**

A growing body of evidence supports a central role of chloroplasts as integrators of environmental signals and key defence organelles, as they host biosynthesis of several key defence-related molecules, including SA and ROS and are therefore primary sites for the biosynthesis and transmission of pro-defence signals during plant immune responses [12,48,49]. Although increases in chloroplastic ROS concentration have been observed in different incompatible plant-pathogen interactions [50], the role of chloroplastic ROS during HR still remains largely unknown. Eventhough there have been a few studies proposing the role of RBOH-generated ROS in HR cell death as discussed in the previous chapter, there have been more studies suggesting chloroplastic ROS involvement in the signaling for and/or execution of HR cell death in incompatible interactions [16,51–56]. Zurbriggen et al. (2009) suggested that ROS generated in chloroplasts during non-host interaction are essential for the progression of PCD, but do not contribute to the induction of pathogenesis-related genes or other signaling components of the response, including SA signaling [52]. Similarly, Yao and Greenberg did not detect an increase in the expression of genes from SA-mediated signaling in the *acd2* mutants that show spontaneous light-dependent PCD and chloroplastic H<sub>2</sub>O<sub>2</sub> increase [57]. In contrast, Straus et al. suggested that chloroplastic ROS acts as a flexible spatiotemporal integration point leading to opposite SA signaling reactions in infected and surrounding tissue to control the propagation of PCD [53]. Interestingly, Ochsenbein et al. (2006) also observed that chloroplastic singlet oxygen activates SA-mediated signaling, although SA was not required for a singlet oxygen-mediated cell death [58]. In potato HR, ROS generated in the chloroplasts around the cell death zone are involved in SA-independent execution of cell death and SA-dependent immune signaling, which are spatially regulated [16,59].

Moreover, recent evidence suggests that chloroplastic ROS might, in addition to signaling in HR cell death, also be involved in controlling plant immune responses by reprogramming transcription of genes involved in response to pathogen attack as one of the retrograde signals [58,60–65]. Transmission of pro-defence signals is facilitated by direct connections between chloroplasts and other organelles [66,67]. It has been suggested that extensions in the form of fluid-filled tubules, known as stromules that contain soluble components of the compartments could facilitate this transmission by enabling more targeted and stronger signal transmission [68]. Stromules are induced by a variety of biotic and abiotic stresses and are involved in retrograde signaling after pathogen invasion, light stress or movement of chloroplasts within the cell [69–71]. However, their direct role in immunity is still largely unresolved [68]. In potato HR-conferred resistance against PVY, stromule formation is induced in close proximity to the cells with oxidized chloroplasts [59]. Since Stonebloom et al. (2012) showed that cell-to-cell transport is negatively regulated by an oxidative shift in chloroplasts, while reductive shift in chloroplasts causes increased cell-to-cell transport, these results could indicate on the potential role of stromules

in the signaling for HR-conferred resistance [72]. This is further supported by the fact that stromule formation is induced on the front of virus multiplication zone and is tightly spatiotemporally regulated by SA signaling [59]. Another phenomena, which indicates on the role of stromules in signaling, are connections of stromules with the plasma membrane and mitochondria as well as the nucleus, suggesting that the direct transfer of proteins and metabolites between these organelles and the apoplast could occur [73].

#### **4. Reactive oxygen species of different source and type induce diverse transcriptional responses**

Distinct subcellular compartments produce different ROS types [74], which could all regulate gene expression [75]. Although each organelle could, in theory, locally manage its own ROS homeostasis, ROS and related signaling intermediates are also involved in interorganellar communication [9,73,76]. For example, chloroplasts-derived redox signals could be first integrated in the cytosol or directly transferred to the nucleus, either through physical nucleus-chloroplast interaction or via stromules, to control retrograde signaling [77]. Only a small number of proteins targeted to the chloroplast have been also identified in the nucleus to function as retrograde signal transducers in response to biotic and abiotic stresses, some of them being WHIRLY1, the PHD-type transcription factor PTM and NUCLEAR RECEPTOR INTERACTING PROTEIN 1 (NRIP1) [78]. For example, WHIRLY1 has been proposed to convey the redox status in chloroplasts to the nucleus in a SA-dependent manner [79]. While localization of WHIRLY1 and PTM to both chloroplasts and nucleus have been shown, how their translocation from chloroplasts to the nucleus occurs is still not known [80,81]. On the other hand, Caplan et al. suggested that after TMV inoculation, NRIP1 protein is translocated from the chloroplast to the nucleus via stromules [70].

In the nucleus, control of gene expression depends mainly on the activity of transcription factors (TFs) that interact with oxidative stress-responsive cis-regulatory elements within the gene promoters. It has been reported that the transcripts generated by increased intracellular H<sub>2</sub>O<sub>2</sub> levels encode proteins of diverse functional categories including TFs, protein kinases, heat shock proteins, glutathione S-transferases (GSTs), UDP-glucuronosyltransferases (UGTs), and cytochrome P450 monooxygenases (CYPs). Upregulated TFs belong to different stress-related TFs' families, including WRKY, AP2/ERF, MYB, NAC, heat shock factor (HSF), and ZAT [74]. Interestingly, studies have shown that the transcriptional response to apoplastic ROS produced during oxidative burst has little similarity to the effect of chloroplastic ROS [9]. The role of individual ROS species from different organelles in transcriptional responses have been studied [82], but since the signaling pathways are connected multiple effects of different organelle specific ROS on transcriptional response have to be addressed.

#### **5. Tools for studying redox state with high spatiotemporal resolution with focus on cytoplasmic and chloroplastic redox state in hypersensitive response**

Despite the new insights that have been brought into the role of redox mechanisms in plant defense response, still one of the major challenges is to monitor local, subcellular and global ROS dynamics with high selectivity, sensitivity and spatiotemporal resolution that allows for quantification [83]. Small organic molecule-based probes have been originally used to measure ROS in plants, however they have significant limitation as they do not allow for spatiotemporal resolution, since the changes in the fluorescence of the probe as the result of ROS presence are irreversible [84,85]. Nondestructive real-time measuring of redox state in plants at high spatial and temporal resolution is feasible since Jiang et al. reported the use of redox sensitive green fluorescent protein (roGFP) [86]. Measurement of roGFP fluorescence intensity following excitation at two different excitation maximum permits an evaluation of the relative proportion of roGFP in a reduced or oxidized state. The biosensors have been modified by the addition of signal sequences to target them to different subcellular organelles, while two variants roGFP1 and roGFP2 with different excitation and emission spectra were developed to allow for optimal selection according to



the redox state in the particular organelle. per-roGFP1 and px-roGFP2 were targeted to peroxisomes by fusing the peroxisomal targeting peptide sequence SKL [87,88], mt-roGFP1 and mt-roGFP2 are available for measuring redox state in mitochondria due to the fusion with mitochondrial localization signal peptide from the tobacco b-ATPase [86,88], er-roGFP2 was constructed by fusing roGFP2 with the ER retention signal peptide HDEL for following redox state in ER [89], while cp-GFP2, pt-roGFP2 and chl-roGFP2 were constructed by adding plastid targeting signal peptide TKTP, coding sequence for RuBisCo small subunit transit peptide or the first 74 amino acids from PRXa to roGFP2 coding sequence, respectively, for measuring change of redox state in chloroplasts [72,90,91]. Since pt-roGFP is targeted to chloroplast outer plastid envelope membrane, it also allows imaging stromules formation [69]. The above mentioned sensors were studied in tobacco, arabidopsis and potato in different developmental and environmental stresses [69,72,94,95,86–93], some of them also in HR [59,96,97]. roGFP2 expressed in the cytosol senses the redox potential of the cellular glutathione buffer via glutaredoxin (GRX) as a mediator of reversible electron flow between glutathione and roGFP2 [89]. To facilitate specific real-time equilibration between roGFP2 and the glutathione redox couple, fusion constructs with human glutaredoxin 1 (GRX1) Grx1-roGFP2 and roGFP2-Grx2 were generated [98–100]. To further extend the range of suitable probes, roGFP derivatives roGFP2-iL and roGFP1-iX with different midpoint potential and excitation properties were generated [101]. Another group of genetically-encoded sensors instead of measuring glutathione redox potential detects H<sub>2</sub>O<sub>2</sub> levels. roGFP2-Orp1, based on a redox relay between the GPX-like enzyme oxidant receptor peroxidase-1 (Orp1) from *Saccharomyces cerevisiae* and roGFP2 was generated for sensing transient changes in H<sub>2</sub>O<sub>2</sub> [92]. Another sensor, which reports on local alterations in H<sub>2</sub>O<sub>2</sub> concentrations exploits the H<sub>2</sub>O<sub>2</sub>-sensitive bacterial transcription factor OxyR for its response and was named HyPer [102,103]. Above mentioned types of sensors were also used to follow redox potential and H<sub>2</sub>O<sub>2</sub> in HR in the cytoplasm [104–109] or were further upgraded to follow redox potential in different cellular compartments, including chloroplasts [110]. Using HyPer2, Exposito-Rodriguez et al. showed that in photosynthetic *Nicotiana benthamiana* epidermal cells, exposure to high light increases H<sub>2</sub>O<sub>2</sub> production in chloroplast stroma, cytosol and nuclei, suggesting direct H<sub>2</sub>O<sub>2</sub> transfer from chloroplasts to nuclei [111]. Other genetically-encoded redox and H<sub>2</sub>O<sub>2</sub> sensors are reviewed in [83,112]. The most recent ones are biosensor CROST for measurements of the thioredoxin redox state in chloroplasts [113], FRET-based biosensors and ROS regulated promoter-FP fusions [83,114].

Another strategy for studying the role of organelle-specific redox state is by altering ROS production in the organelle of interest, here we focus on chloroplasts. Chloroplasts produce various forms of ROS during photosynthesis. One of the most reactive ones is singlet oxygen, which is produced by energy transfer from excited triplet state chlorophyll to the oxygen, mainly in the photosystem II reaction center [115]. The other major source of chloroplastic ROS is Mehler reaction in photosystem I, which reduces oxygen to superoxide anion that is further converted to H<sub>2</sub>O<sub>2</sub> by thylakoid-bound and stromal superoxide dismutases [116]. H<sub>2</sub>O<sub>2</sub> is reduced in reactions catalysed by 2-Cys peroxiredoxin (Prx) and ascorbate peroxidase (APX) [117]. Therefore, the role of chloroplastic ROS could be studied in transgenic plants with overexpressed thylakoidal ascorbate peroxidase (tAPX), which results in decreased chloroplastic ROS production [57,118] or in transgenic plants with estrogen-inducible RNAi silenced tAPX expression, which results in increased chloroplastic ROS production [62]. Inducible silencing of tAPX increased H<sub>2</sub>O<sub>2</sub> production in chloroplasts, which activated SA biosynthesis and SA inducible gene expression [62]. Interestingly, however, over-expression of stromal ascorbate peroxidase (sAPX) or treatment with photosynthesis inhibitor DCMU attenuates nuclear H<sub>2</sub>O<sub>2</sub> accumulation and high light responsive gene expression, while cytosolic ascorbate peroxidase over-expression has little effect [111]. This was explained by the direct H<sub>2</sub>O<sub>2</sub> transfer from chloroplasts to nuclei, avoiding the cytosol, which enables photosynthetic control over gene expression [111]. As Prx similarly as APX reduce H<sub>2</sub>O<sub>2</sub>, silencing of Prx results in enhanced chloroplastic H<sub>2</sub>O<sub>2</sub> accumulation. By VIGS-induced Prx silencing, Ishiga et al. showed

that Prx functions as a negative regulator of pathogen-induced cell death in the healthy tissue that surrounds the lesions, while chloroplastic ROS play a role in the cell death initiation [54]. The role of chloroplastic ROS could also be studied by the use of transgenic plants with chloroplast-targeted flavodoxin (Fld) [119]. Fld improves delivery of reducing equivalents to productive pathways of the chloroplast, which in turn restricts chloroplastic ROS production. The introduction of a Fld in chloroplasts of various plant species resulted in increased tolerance to different biotic and abiotic stresses [52,119–124]. Plants overexpressing glycolate oxidase (GO) are another system for studying the effects of chloroplastic H<sub>2</sub>O<sub>2</sub> [125]. By exploiting this system, Schmidt et al. showed that H<sub>2</sub>O<sub>2</sub> dosage in Arabidopsis chloroplasts regulates HR-conferred resistance to hemibiotrophic fungus by the induction of WRKY33 [126]. While above mentioned systems modulate chloroplast H<sub>2</sub>O<sub>2</sub> accumulation, arabidopsis flu mutant manifest increased singlet oxygen production in chloroplasts upon dark/light shift [127], which leads to induced SA synthesis and suppressed spread of necrotic lesions [58]. ROS production in the chloroplasts can also be enhanced by using inhibitors and redox catalysts. The herbicide methyl viologen (paraquat) acts by re-directing electrons from photosystem I (PSI) to oxygen and thereby enhancing the production of superoxide in the chloroplasts [128]. As it also inhibits APX, this leads to accumulation of H<sub>2</sub>O<sub>2</sub> in treated plants [129]. To study the role of chloroplast-derived photo-oxidative stress caused changes in H<sub>2</sub>O<sub>2</sub> and glutathione redox potential in different cellular components, Ugalde et al. treated Arabidopsis seedlings with methyl viologen and recorded dynamic changes in glutathione redox potential and H<sub>2</sub>O<sub>2</sub> levels with the genetically encoded biosensors Grx1-roGFP2 and roGFP2-Orp1 targeted to chloroplasts, the cytosol, or mitochondria [110]. Similarly, the role of chloroplastic ROS was studied by uracil, acting as chloroplast electron transport chain inhibitor, which significantly reduced ROS generation and delayed necrosis appearance in biotic stress [130].

## 6. Conclusions

The results of above mentioned studies suggest that the precise spatiotemporal regulation of key players, including organelle specific ROS and SA levels determine the effectiveness of pathogen arrest and is therefore crucial for a successful immune response. The change of SA and ROS levels and other key players alter the rate of cell-to-cell and systemic pathogen spread, rate of cell death induction and spatial transcriptional response leading to susceptibility or resistance. We suggest that only a coordinated and intertwined action of all key players enable effective immune response. However, the specific interactions between them and the regulatory interplay behind still remain poorly understood, as up till now, the role of organelle specific ROS and SA in HR-conferred resistance has only been studied by altering the level of a single component. In order to address these aspects, a sophisticated combination of research methods for monitoring the spatiotemporal dynamics of key players and transcriptional activity in plants is needed. The precise sampling of tissue sections surrounding the HR-PCD with spatial resolution, suitable for transcriptomics analyses [16] in combination with the use of biosensors [131] could enable identification of novel key players and unraveling the interconnectivity of components of immune signaling and will therefore present a step forward in studying the resistance response.

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