



## REACTIVE OXYGEN SPECIES AND THEIR ROLE IN PLANT DEFENSE AGAINST PATHOGEN INGRESS

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### ABSTRACT

Pathogen infections induce a rapid accumulation of Reactive Oxygen Species (ROS) in plant tissues termed as oxidative burst which is the earliest event in the plant defense response. In non-stressed plant tissues enzymatic and non-enzymatic antioxidants are able to neutralize the harmful effects of ROS. The Oxidative burst at the plant cell surface drives rapid peroxidase-mediated oxidative cross-linking of structural proteins in the cell wall, thereby reinforcing this physical barrier against pathogen ingress. ROS serve as a second messenger in a systemic signaling network in plant immunity and leading to the expression of defense genes, phytoalexin production, and triggering of a hypersensitive response (HR). Rapid production of ROS has been implicated in diverse physiological processes including resistance to biotic and abiotic stress. The requirement for ROS appears to be different for resistance to different pathogens.

**Key words:** ROS, oxidative burst, Defense, Signal.

### INTRODUCTION

Free radicals are the molecules containing one or more unpaired electrons in atomic or molecular orbital [1]. These unpaired electron(s) usually gives a considerable degree of reactivity to free radicals. Radicals derived from oxygen represent the most important class of radical species generated in living systems [2]. Molecular oxygen (dioxygen) has a unique electronic configuration and is itself is a radical. The addition of one electron to dioxygen, forms the superoxide anion radical ( $O_2^{\bullet-}$ ) [3]. Superoxide anion, arising either through metabolic processes or following oxygen “activation” by physical irradiation, is considered the “primary” Reactive oxygen species (ROS), and can further interact with other molecules to generate “secondary” ROS, either directly or prevalently through enzyme or metal-catalysed processes [4]. ROS are partially reduced or activated derivatives of molecular oxygen like singlet oxygen ( $O_2$ ), superoxide anion ( $O_2^{\bullet-}$ ), hydrogen peroxide ( $H_2O_2$ ) and hydroxyl radical ( $OH^{\bullet}$ ). ROS are not only important regulators of plant growth and development, but are also involved in limiting pathogen ingress, induction of cell death and signal transduction of several defense processes [5]. After pathogen infection the enzymes NADPH oxidase, peroxidases, superoxide

dismutase b (SOD), oxalates oxidases, lipoxygenases, quinone reductase-b and amine oxidases are involved in ROS generation [6]. If the abiotic or biotic stress is too severe, the antioxidants cannot neutralize the overproduction of ROS which leads to cell death and necrosis development [7]. Figure 1 shows that ROS are involved in various modifications of cell process leading to DNA damage and protein modifications.

### Hypersensitive response by oxidative burst

The HR restricts pathogen growth and is highly effective against biotrophic pathogens, since, with the death of host cells, the nutrient supply is removed [8]. In addition, toxic substances like ROS and phytoalexins produced in these cells apparently help to kill the pathogen [9]. ROS may originate primarily from chloroplasts, mitochondria and peroxisomes [10]. The HR is often not effective against necrotrophic pathogens because these usually kill host cells to feed on them [11]. In addition, there is a group of pathogens, often considered to be necrotrophic, which are infact inhibited to some extent by HR, e.g., *Magnaporthe grisea* [12]. The production of ROS is the first response detected within minutes of an

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attack by virulent or avirulent pathogen [13]. This weak and transient ROS generation is due to a biologically non-specific reaction. After some hours, a second, massive and prolonged ROS production, called oxidative burst, occurs in cells attacked by avirulent pathogens. This two-phase kinetics of ROS production is typical of incompatible plant–pathogen interactions that are characterized by HR [14]. Apoplastic superoxide dismutase (SOD) isoenzymes are then responsible for H<sub>2</sub>O<sub>2</sub> production by means of superoxide (O<sub>2</sub><sup>-</sup>) dismutation. Evidence for different sources of ROS has also been provided, as a lipoxygenase acting on polyunsaturated fatty acids derived from membrane lipids [15]. Extra-cellular H<sub>2</sub>O<sub>2</sub> could be directly produced by means of apoplastic enzymes such as copper amine oxidase, flavin polyamine oxidases and oxalate oxidase [16]. The consequent increase in mechanical barriers slows down pathogen penetration allowing plant cells to arrange defences that require more time to be activated molecule in biological membranes; it also acts as intracellular signal, which is able to activate defence responses [17].

By comparison with mammalian systems, ROS production in the apoplast, mediated by NADPH oxidase activities encoded by the *Rboh* gene family, has been long considered as a central feature of the HR. Plants usually contain several *Rboh* genes (ten in Arabidopsis) which are transcriptionally upregulated by pathogens, and whose products display a certain degree of functional overlap [18]. Extracellular ROS production has been linked to direct lipid peroxidation, to the alkalinisation of the apoplast, thereby propagating the signal by alkali-responsive peroxidases, or to alterations in the levels and/or redox status of antioxidant pools [19]. Interestingly, down regulation or elimination of *Rboh* genes could lead to variable effects on the HR. For example, although Arabidopsis *RbohD* and *RbohF* mutants exhibited lower ROS accumulation, they displayed enhanced HR when introduced into a *lesion stimulating disease 1* mutant background, or when challenged with avirulent bacteria [18]. These results indicate that while NADPH oxidase activity is required for pathogen induced ROS production in the apoplast, these ROS might serve different signaling purposes during the HR [18].

### **Oxidative bursts in plant defense against pathogen ingress**

H<sub>2</sub>O<sub>2</sub> is an electron-accepting substrate for a wide-variety of Phenol oxidase (POX) -dependent reactions, thus POXs are generally considered to be merely ROS-detoxifying enzymes. The breakdown of H<sub>2</sub>O<sub>2</sub> by the POX reaction is highly active especially in the presence of ROS-scavenging POX substrates such as flavonoids [20]. Studies have shown that the SA- induced extracellular POX-dependent transient bursts in the generation of ROS trigger increases in Ca<sup>2+</sup> [21](fig2). H<sub>2</sub>O<sub>2</sub> is involved in the restriction of pathogen growth and induction of phytoalexins and PR proteins [22]. The expression of H<sub>2</sub>O<sub>2</sub>-induced enzymes in transgenic plants has provided an innovative approach to study the plant defence resistance. Expression of a gene encoding glucose oxidase

in transformed potato led to H<sub>2</sub>O<sub>2</sub> accumulation and increased resistance to soft rot and potato late blight. Direct injection of H<sub>2</sub>O<sub>2</sub> enhanced expression of PR genes [23], antioxidant enzymes, phytoalexins [24] and enhanced accumulation of signaling component, salicylic acids [25]. However, on certain occasions, the plant POXs actually produces ROS. ROS generation by extracellularly secreted POX in the elicitor-treated plants has been documented [26], although responsible electron-donating substrates are obscure. The elicitors are recognized by putative receptors, and ion channels are activated. The movements of H<sup>+</sup> and other ions may then contribute to a transient alkalinization of the extracellular matrix, ultimately activating the pH dependent POX (fig.3) Since this reaction solely depends on pH changes and requires no specific substrate, ROS generation involving SA, AMAs and chitoooligosaccharides (COSs) requires alternative mechanisms. It is now widely accepted that SA signaling is mediated with ROS production and an increase in Ca<sup>2+</sup> [27]

### **Microtubule depolymerization by H<sub>2</sub>O<sub>2</sub>-dependent signaling**

Plant interactions with pathogens are known to stimulate cytoskeleton reorganization [28]. The plant cytoskeleton readily remodels in response to various intracellular and external stimuli. Cortical microtubules are intimately associated with the plasma membrane and are implicated as targets of signaling networks [29].It is therefore not surprising that certain signaling pathways are interconnected and are used to regulate the dynamic microtubule cytoskeleton simultaneously. However, specific knowledge about upstream signaling pathways that regulate cortical microtubule dynamic is limited. Microtubule destabilization occurs independently of the production of ROS in tobacco cells in response to cryptogein [30]. In contrast, it has been suggested that the actin cytoskeleton is a central signaling component that couples the accumulation of ROS to programmed cell death in yeast [31]

### **Gene expression by ROS**

Potential regulatory molecules modulating gene expression, which are affected by increased ROS levels, are GSH and GSSG or their derivatives. Exogenous application of GSH increased Phenyl-ammonia-lyase and chalcone synthase transcripts in bean cell suspensions [32]. A later study reported that both GSH and GSSG elicit Phe- ammonia lyase enzyme activity and phytoalexin accumulation [33]. Whereas elicitor treatment was shown to increase GSH and other thiols, experiments using an artificial precursor for glutathione suggested that an increase in intracellular GSH alone was insufficient to cause phytoalexin accumulation in bean and alfalfa cells[33].In carrot, inhibition of glutathione synthesis induced phytoalexin accumulation [34]. Lipid peroxides generated by nonenzymic reaction of AOS with lipids may serve as precursors in the synthesis of jasmonic acid, a known regulator of several defense-related genes expressed during the HR [35]. This ROS-dependent source of lipid peroxides may augment an enzymic pathway for

their production involving phospholipases and lipoxygenase [36]. Salicylic acid, which is widely believed to be the inducer of specific plant defense genes during the development of SAR, acts by inhibiting catalase activity [23]. The somewhat increased H<sub>2</sub>O<sub>2</sub> concentrations detected in salicylic acid-treated leaves, presumably resulting from catalase inhibition, was suggested to serve as a second messenger in the transcriptional activation of pathogenesis-related protein genes.

### ROS and pathogen signaling

Pathogen-induced ROS themselves are considered as signaling molecules. Elevated ROS levels are perceived by different receptors, proteins or enzymes. Although ROS receptors are largely unknown at present, plant cells sense ROS via at least three different mechanisms: (i) unidentified receptor proteins; (ii) redox-sensitive transcription factors, such as NPR1 (nonexpressor of pathogenesis-related protein 1) or heat shock factors; and (iii) direct inhibition of phosphatases by ROS [37]. The earliest reactions of plant cells include changes in plasma membrane permeability, which leads to Ca<sup>2+</sup> and proton influx and K<sup>+</sup> and Cl<sup>-</sup> efflux [37]. Ion fluxes subsequently induce extracellular production of ROS catalysed by enzymes that act as secondary messengers for the HR and defense gene expression [14]. Calcium has been shown to be important in signaling. H<sub>2</sub>O<sub>2</sub> induces calcium influx-mediated stomatal closure in *Commelina communis* and *Arabidopsis thaliana* [38]. Furthermore, it was shown that this gene could function as a Ca<sup>2+</sup>-conducting channel and that calcium ions were important for the observed cell death. Different models for the action of calcium in the regulation of ROS have been proposed. One model (Fig. 3) suggests that an elicitor interacts with a receptor coupled with a G-protein, which leads to Ca<sup>2+</sup> influx that activates a Ca<sup>2+</sup> dependent protein kinase and ultimately NADPH oxidase [39]. Another model, based on studies of innate immunity in *Arabidopsis*, suggests that pathogens or PAMPs are recognised by (unknown) receptors which trigger an ion (calcium) channel, leading to increases in cytosolic Ca<sup>2+</sup> and subsequent NO generation [40]. NO generation, together with other required factors such as an avirulent pathogen and an oxidative burst, could lead to the HR and potentially, diffusion of NO to neighboring cells could act as a signal that thereby activates further calcium channels. Activation of the oxidative burst is governed by phosphorylation/dephosphorylation [14].

Three signaling molecules, SA, JA and ET, are known to play key roles in various aspects of plant defense. These include defense against abiotic stresses, such as wounding and exposure to ozone, as well as against insect attack and microbial infection. It is widely accepted that SA induces defense generally against biotrophic pathogens, whereas JA activates defense against necrotrophic pathogens and against wounding, ozone, as well as insect pests, however, there are clear exceptions [41]. It has also been claimed recently that hormone crosstalk in plant disease and defense means more than just JA-SA antagonism and that a key pathogen virulence

strategy involves modulation of hormone signaling [42].(Fig.4). It is also suggested that plants tightly control crosstalk between SA- and JA-dependent defenses in a spatial and pathogen type-specific fashion [41]. ET and JA are often placed together in a single signaling pathway, but these models are probably too simple, since the JA and ET signaling pathways can also modulate each other. Furthermore, hemibiotrophic pathogens in different stages of infection can induce different and partly antagonistic signaling, and make the picture more complex [43]. Although the mechanisms of recognition and host responses to necrotrophs are poorly understood, it is widely accepted that there are significant differences in the case of biotrophic and necrotrophic attack [44]. In general, plants recognize pathogens via specific effectors or non-specific PAMPs/MAMPs by plant R proteins or pattern-recognition receptors (PRRs), respectively. As a consequence a cascade of defense reactions are induced including the oxidative burst [45]. Specific effectors and corresponding R proteins are rather characteristic for plant-biotrophic pathogen interactions, but there are exceptions [46]. Navarro *et al.* 2000 demonstrated that the growth-repressing DELLA proteins promote susceptibility to biotrophic pathogens but resistance to necrotrophic pathogens by modulating the SA and JA signaling pathways [47]. ROS can induce the accumulation of SA, JA and ET, and these three stress hormones, which are involved in pathogen signaling, have generally mutually antagonistic interactions [48]. After pathogen infection the following enzymes were suggested to be involved in ROS generation; NADPH oxidase, peroxidases, superoxide dismutase (SOD), oxalates oxidases, lipoxygenases, quinone reductase-b and amine oxidases [6]. In non-stressed plant tissues enzymatic and non-enzymatic antioxidants are able to neutralize the harmful effects of ROS. If the abiotic or biotic stress is too severe, the antioxidants cannot neutralize the overproduction of ROS which leads to cell death and necrosis development. Consequently, elevation of antioxidant capacity of plants should increase their tolerance to the development of necroses caused by pathogens or abiotic stresses [7].

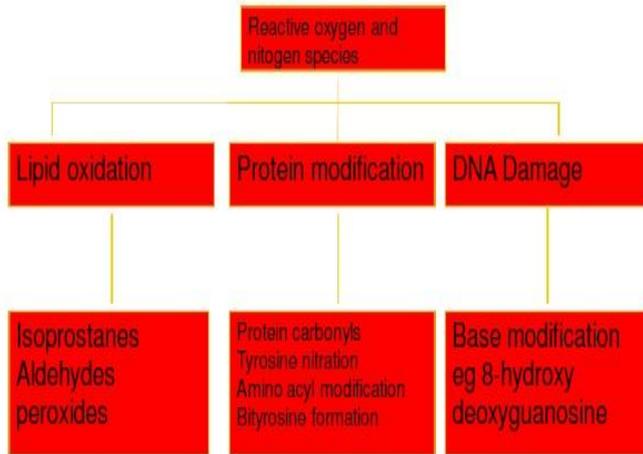
### Programmed Cell Death (PCD)

Perception of an extracellular signal activates a MAPK, which in turn can facilitate translocation of the signal to the nucleus where it can phosphorylate and activate transcription factors, thereby modulating gene expression [49]. For example, it has been reported that two tobacco MAPKs, namely salicylic acid-induced protein kinase (SIPK) and wound-induced protein kinase (WIPK), are regulated by a common upstream MAPK, which is involved in signaling for PCD [50]. MAPK, Ntf4, with a similar function to SIPK and WIPK, which, when expressed in transgenic tobacco plants, accelerated the PCD when treated with the elicitor cryptogen from *Phytophthora cryptogea* [51]. This indicates a role in signaling for PCD. The combined activation of SIPK, Ntf4 and WIPK induced an HR-like PCD [52]. Further evidence for a role of ROS in signaling has come from the fact that addition of low doses of ROS inducers stimulates the

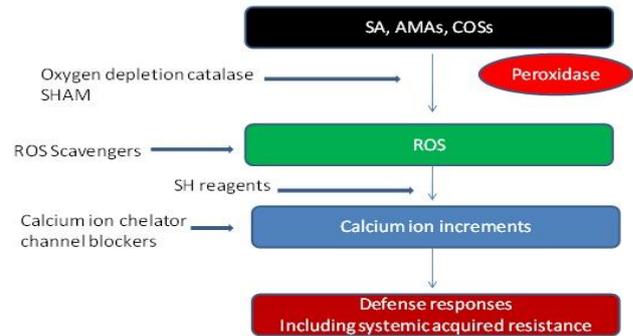
induction of detoxification mechanisms, such as SOD and glutathione-S-transferase, and activation of other defense mechanisms in neighbouring cells [53]. Pharmacological and genetic studies [53]. Dat *et al.* in 2003 support the existence of positive amplification loops involving NADPH oxidases in ROS signaling [54]. These loops might be activated by low levels of ROS and result in enhanced production and amplification of the ROS signals. It has been reported that a small GTP-binding protein, Rac, regulates ROS production in rice, most likely through an NADPH oxidase, and induces cell death in rice cells with biochemical and morphological features similar to apoptosis in mammalian cells [55]. Together, MAPK and calcium dependent protein kinases seem to play central roles in the regulation of pathogen-responsive NADPH oxidases at the transcriptional and post-transcriptional levels, respectively [56]. It has been suggested that the HR

is triggered only by balanced production of NO and ROS [57]. Little is known about signaling pathways downstream of NO/ H<sub>2</sub>O<sub>2</sub>. Nevertheless, it has been shown that NO signaling during both PCD and defense responses requires cyclic GMP and cyclic ADP ribose, two molecules that can serve as secondary messengers for NO signaling in mammals [58]. SA has been shown to be an important signaling molecule involved in defense responses to pathogen attack in many plant-pathogen interactions. SA levels increase dramatically in tobacco cells surrounding infection sites when infected with Tobacco mosaic virus [59]. ROS act synergistically in a signal amplification loop with SA to drive the HR and the establishment of systemic defences [60]. The activation of a redox-signaling pathway possessing a MAPK module has also been reported in response to infection by avirulent pathogens in Arabidopsis [61].

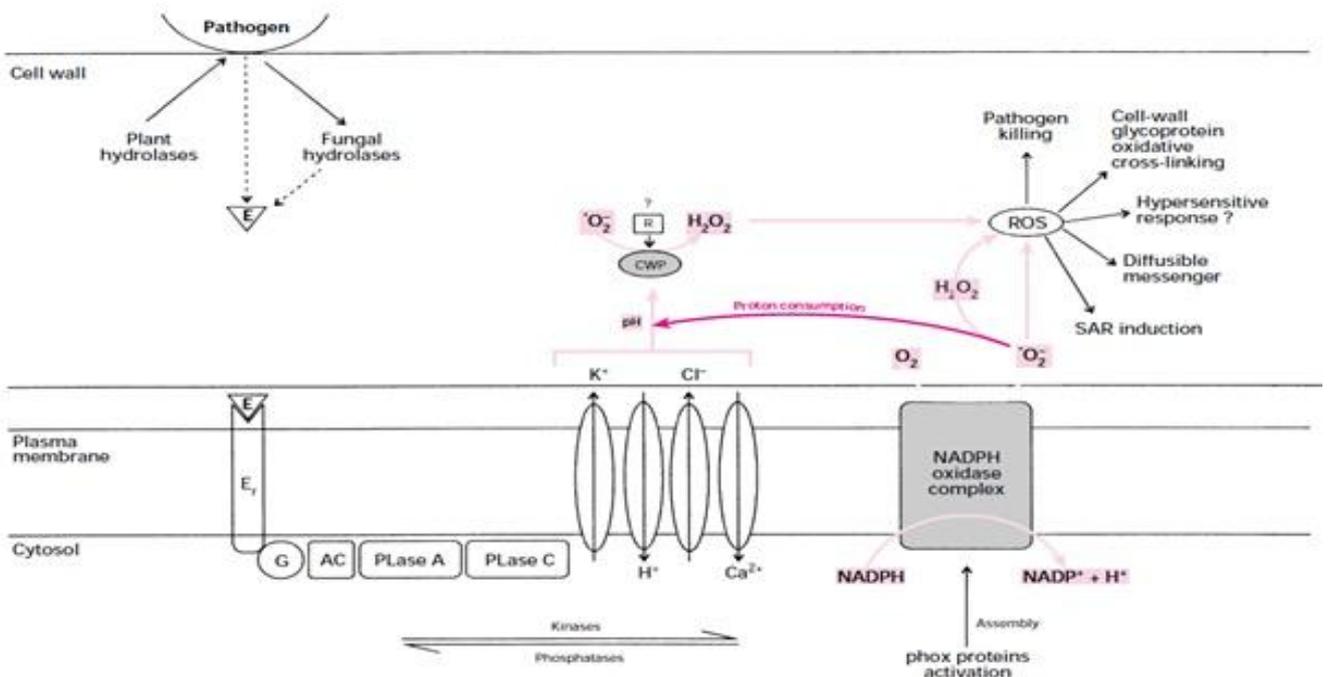
**Fig. 1. Various biomarkers of oxidative damage**



**Fig. 2. Mechanisms for the actions of SA, AMAs (aromatic monoamines) and COSs (chito oligo saccharides). The signal transduction pathways common for SA, AMAs and COSs in plants**



**Fig. 3. Possible origin of ROS building the oxidative burst**



## CONCLUSION

Pathogen infections induce rapid accumulation of ROS which are involved in limiting pathogenic ingress, induction of cell death and in signal transduction of many defense responses. ROS mediated death of plant tissues can increase susceptibility to necrotrophic, but resistance

to biotrophic pathogens. The rapid production of ROS establishes different defensive barriers against pathogens. The influence of other factors such as environment, plant hormones and activation of different signaling pathways plays an important role for the accumulation of ROS.

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