Reactive Oxygen Species Signaling in Response to Pathogens[1]

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The production of reactive oxygen species (ROS), via consumption of oxygen in a so-called oxidative burst, is one of the earliest cellular responses following successful pathogen recognition. Apoplastic generation of superoxide (O_2^-), or its dismutation product hydrogen peroxide (H_2O_2), has been documented following recognition of a variety of pathogens (Doke, 1983; Auh and Murphy, 1995; Grant et al., 2000b). Avirulent pathogens, successfully recognized via the action of disease resistance (*R*) gene products in plant immune system, elicit a biphasic ROS accumulation with a low-amplitude, transient first phase, followed by a sustained phase of much higher magnitude that correlates with disease resistance (Lamb and Dixon, 1997). However, virulent pathogens that avoid host recognition induce only the transient, low-amplitude first phase of this response, suggesting a role for ROS in the establishment of the defenses. In line with this conclusion, elicitors of defense responses, often referred to as microbe-associated molecular patterns (MAMPs), also trigger an oxidative burst. Initial characterization of the oxidative burst left unclear whether ROS acted as executioners of pathogen, host cells (in the form of the familiar hypersensitive response [HR]), or both, or, alternatively, as signaling molecules that were not directly involved in the mechanisms that actually stopped pathogen growth.

In the plant cell, ROS can directly cause strengthening of host cell walls via cross-linking of glycoproteins (Bradley et al., 1992; Lamb and Dixon, 1997), or lipid peroxidation and membrane damage (Lamb and Dixon, 1997; Montillet et al., 2005). However, it is also evident that ROS are important signals mediating defense gene activation (Levine et al., 1994). Additional regulatory functions for ROS in defense occur in conjunction with other plant signaling molecules, particularly with salicylic acid (SA) and nitric oxide (NO; see Fig. 1). However, ROS also regulate additional plant responses in relation to other signals. Here, we discuss these roles of ROS with a focus on the response to pathogen infection.

MECHANISMS OF ROS PRODUCTION IN RESPONSE TO PATHOGENS

Several enzymes have been implicated in apoplastic ROS production following successful pathogen recognition. The use of inhibitors pointed to plasma membrane NADPH oxidases (inhibited by diphenylene iodonium [DPI] but not by cyanide or azide; Grant et al., 2000a) and cell wall peroxidases (inhibited by cyanide or azide but not by DPI; Grant et al., 2000a; Bolwell et al., 2002) as the two most likely biochemical sources. The NADPH oxidase, also known as the respiratory burst oxidase (RBO), was initially described in mammalian neutrophils as a multicomponent complex mediating microbial killing (Lambeth, 2004). $gp91^{phox}$ is the enzymatic subunit of this oxidase and transfers electrons to molecular oxygen to generate superoxide. Arabidopsis (*Arabidopsis thaliana*) has 10 *Atrboh* (Arabidopsis RBO homolog) genes homologous to $gp91^{phox}$ (Torres and Dangl, 2005). Several recent reports demonstrate that members of the *Rboh* family mediate the production of apoplastic ROS during the defense responses, as well as in response to abiotic environmental and developmental cues (Torres and Dangl, 2005). However, we know very little about either the precise subunit structure of the plant NADPH oxidase or its activation. Both are likely different than in mammalian neutrophils (Torres and Dangl, 2005).

Peroxidases form a complex family of proteins that catalyze the oxidoreduction of various substrates using H_2O_2. In particular, pH-dependent peroxidases in the cell wall can also be a source of apoplastic H_2O_2 in the presence of a reductant released from responding cells (Wojtaszek, 1997; Bolwell et al., 1998). The expression of these enzymes is induced following recognition of bacterial and fungal pathogens (Chittoor et al., 1997; Sasaki et al., 2004). A French bean (*Phaseolus*

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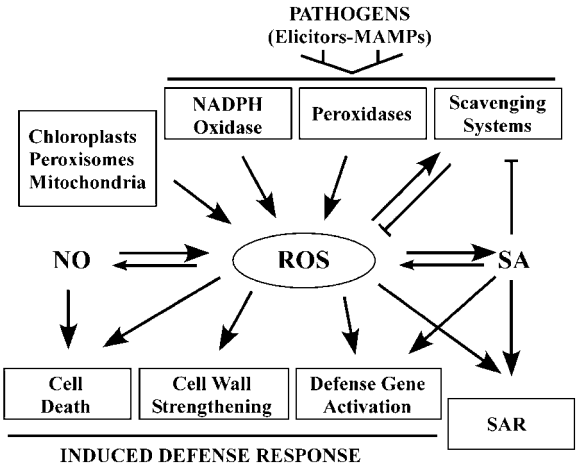

Figure 1. ROS production and functions in response to pathogens.

vulgaris) cationic peroxidase and H_2O_2 (detected by cerium chloride staining) were colocalized in the vicinity of invading bacteria together with other components of the papillae (Brown et al., 1998). These results suggested that H_2O_2 generation by this enzyme could lead to the generation of subcellular, polarized physical barriers at infection sites.

Although the primary oxidative burst following pathogen recognition occurs in the apoplast, ROS produced in other cellular compartments may also have functions in defense. High levels of ROS can be produced inside the plant cell as by-products of metabolic processes, in particular, light-driven production of ROS as a by-product of photosynthesis (Karpinski et al., 2003; Apel and Hirt, 2004). Uncoupling, or inhibition, of the photosystem machinery in the chloroplast and photorespiration associated with chloroplast and peroxisome function can lead to the formation of high levels of ROS that can dramatically affect cellular homeostasis. It is important to recall the nearly ubiquitous requirement for light in the HR (Goodman and Novacky, 1994), as illustrated by the requirement of high-intensity light for cell death mediated by resistance gene proteins (Tang et al., 1998). Under high-light conditions, photorespiratory ROS mediate different mechanisms of lipid peroxidation leading to cell death than in the dark, underscoring the importance of light during the HR (Montillet et al., 2005).

Various ROS-scavenging systems, including ascorbate peroxidases, glutathione, superoxide dismutases, and catalases, maintain ROS homeostasis in different compartments of the plant cell (Mittler et al., 2004). These enzymes could restrict the ROS-dependent damage or finely tune ROS-dependent signal transduction. High-intensity light stress in plants with down-regulated scavenging systems leads to an ectopic oxidative burst and cell death that is phenotypically similar to HR (Chamnongpol et al., 1998; Dat et al., 2003). Differential regulation of these enzymes, in part mediated by SA, may contribute to increases in ROS and activation of defenses following infection (Dorey et al., 1998; Mittler et al., 1999; Klessig et al., 2000). In tobacco, the reduction of catalase and ascorbate peroxidase activities resulted in plants hyperresponsive to pathogens (Mittler et al., 1999), whereas the overexpression of catalase leads to more disease-sensitive plants (Polidoros et al., 2001). Collectively, these results suggest that the ROS-scavenging systems can have an important role in managing ROS generated in response to pathogens. Further, compartmentalization of both ROS production and activation of ROS-scavenging systems could contribute to fine-tuning of ROS levels and their signaling properties.

FUNCTIONS OF ROS FOLLOWING INFECTION

Pharmacological approaches also suggest that different parts of the overall ROS production in response to infection appear to be mediated by different mechanisms. Though the involvement of an NADPH oxidase has been predominant in most cases (Bolwell et al., 1998; Grant et al., 2000b; Torres and Dangl, 2005), both NADPH oxidases and cell wall peroxidases might mediate ROS production in response to the same pathogen (Grant et al., 2000a). A more detailed temporal resolution of the activity of each system may reveal that the pools of ROS produced by each mechanism do not functionally overlap. For example, differential effects of DPI on ROS accumulation during the HR- and MAMP-mediated basal defense responses were reported, with the latter being considerably less attenuated by DPI (Soylu et al., 2005). These results suggest that alternative mechanisms might be activated to produce ROS during some basal defense responses, while NADPH oxidases might have later effects following *R*-mediated pathogen recognition. However, the use of inhibitors in this work, as in other research, needs to be validated with genetic approaches.

ROS were proposed to orchestrate the establishment of plant defense response and HR following successful pathogen recognition (Apostol et al., 1989; Levine et al., 1994). Genetic proof for NADPH oxidase-*Rboh* function in the pathogen-induced oxidative burst came from the analysis of *rboh* mutants and antisense lines (Simon-Plas et al., 2002; Torres et al., 2002; Yoshioka et al., 2003). Down-regulation or elimination of *Rboh* leads to elimination of extracellular peroxide formation. Yet, this lack of ROS has variable effects on pathogen growth and HR. For example, a double mutant of the Arabidopsis *atrbohD* and *atrbohF* genes displays reduced HR in response to avirulent bacteria (Torres et al., 2002). Similarly, *Nbrboh*-silenced *Nicotiana benthamiana* plants are more susceptible to avirulent oomycete *Phytophthora infestans*, and HR is suppressed (Yoshioka et al., 2003). By contrast, the Arabidopsis *atrbohF* mutant is more resistant to a weakly virulent strain of the oomycete *Hyaloperonospora parasitica* and actually displays enhanced HR (Torres et al., 2002). There is also evidence of functional

overlap between different Rboh proteins. For example, in Arabidopsis, various phenotypes of the individual *atrbohD* and *atrbohF* mutants are accentuated in the double mutant *atrbohD atrbohF* (Torres et al., 2002; Kwak et al., 2003). Thus, while the Rboh proteins are required for ROS production following successful pathogen recognition, these ROS may serve diverse signaling functions in disease resistance and HR.

Plant *Rac2* homologs (called Rop for Rho-like proteins) also regulate the production of ROS by the NADPH oxidase, as they do in animals (Kawasaki et al., 1999; Moeder et al., 2005). Interestingly, different plant Rac proteins appear to act as either positive or negative regulators of ROS production. For example, *Osrac1* is a positive regulator of ROS production and cell death (Ono et al., 2001), whereas *Ntrac5* acts as a negative regulator of ROS production via *NtrbohD* (Morel et al., 2004). These analyses suggest that combinations of Rac isoforms with specific Rboh isoforms may mediate differential regulatory outcomes and could explain the differential functions of NADPH oxidases in regulation of defense and cell death.

ROS production has been associated with the formation of defensive barriers against powdery mildew in barley (*Hordeum vulgare*; Huckelhoven and Kogel, 2003). ROS produced in the barley/powdery mildew interaction were observed in vesicles inside the cell, suggesting that the polarized delivery of ROS, among other factors, might contribute to inhibition of pathogen growth (Collins et al., 2003). Interestingly, specific granules in mammalian neutrophils are a site for assembly and activation of the oxidase enzyme system (Segal, 2005). Further verification will be needed to assess if a plant NADPH oxidase is responsible for this ROS in vesicles and its specific function in the interaction with powdery mildew.

ROS, in association with SA, were proposed to mediate the establishment of systemic defenses (systemic acquired resistance [SAR]; Durrant and Dong, 2004). The rapidity of ROS production and the potential for H_2O_2 to freely diffuse across membranes suggested that ROS could function as an intercellular or intracellular second messenger (Levine et al., 1994; Lamb and Dixon, 1997). ROS metabolism could also affect the function of NPR1, a crucial mediator of these systemic responses, by controlling NPR1 redox state (Mou et al., 2003). However, although H_2O_2 may mediate the accumulation of defense markers beyond the initial infection site, inhibitor studies indicate that it is unlikely that it is itself the translocated signal that mediates SAR (Bi et al., 1995; Dorey et al., 1999; Costet et al., 2002), and genetic proof will be needed to clearly establish the role, if any, of ROS in SAR. Interestingly, there is also evidence that NADPH oxidase mediates the systemic production of ROS in response to successful viral infection in Arabidopsis, although the functional relevance of this remains unclear (Love et al., 2005).

Although ROS usually correlates with successful disease resistance responses, some pathogens may induce production of ROS to their own advantage. For example, necrotrophs appear to stimulate ROS production in the infected tissue to induce cell death that facilitates subsequent infection (Govrin and Levine, 2000). The fungal necrotroph Botrytis triggers significant changes in the peroxisomal antioxidant system, leading to a collapse of the protective mechanism at advanced stages of infection. This process is partly related to senescence (Kuzniak and Sklodowska, 2005). Interference with the chlorophyll degradation pathway also results in overaccumulation of ROS and an increase in susceptibility to some necrotrophic pathogens (Kariola et al., 2005). In addition, there are also reports of ROS being produced, together with increased levels of ROS detoxification enzymes, during compatible interactions involving virus (Allan et al., 2001; Clarke et al., 2002). Some proteins of the Rac family also appear to function in pathogen susceptibility (Schultheiss et al., 2003). Thus, ROS is produced as part of a complex network of signals that respond to pathogen attack and mediate multiple responses, sometimes with opposite effects, in different contexts or in response to different pathogens.

INTERACTION OF ROS WITH OTHER SIGNALS

Interaction with other plant defense regulators may account for these divergent outcomes in ROS signaling. SA is a plant signaling molecule involved in defense responses, local and systemic, to pathogen attack (Durrant and Dong, 2004). SA levels increase dramatically in cells surrounding infection sites (Enyedi et al., 1992). ROS was proposed to act synergistically in a signal amplification loop with SA to drive the HR and the establishment of systemic defenses (Draper, 1997). This model was based, in large part, on experiments using submaximal doses of both exogenous H_2O_2 and pathogen to drive SA accumulation; subsequent increases in SA enhanced ROS production (Leon et al., 1995; Shirasu et al., 1997). SA accumulation can also down-regulate those ROS-scavenging systems that, in turn, can contribute to increased overall ROS levels following pathogen recognition (Klessig et al., 2000). However, ROS and SA antagonize each other's action in the regulation of cell death expansion at the margins of pathogen-triggered HR lesions in the lesion mimic mutant *lsd1* (Torres et al., 2005). *lsd1* fails to contain the initial HR following pathogen recognition (Dietrich et al., 1997). Unexpectedly, ROS produced by AtrbohD and AtrbohF are negative regulators of the unrestricted cell death expanding from the margins of an initial HR site in *lsd1*, whereas SA produced through isochorismate synthase is a positive regulator of this cell death (Torres et al., 2005). These surprising results underscore how ROS can mediate different functions in different cellular and spatial contexts, and in relation to other regulatory signals. Similarly, SA and the hormone jasmonic acid seem also to either synergize

or antagonize in their signaling functions at different concentrations. Synergy, in this case, drives ROS production and cell death (Mur et al., 2006).

ROS signaling has also been linked to NO, another reactive oxygen derivative produced following pathogen recognition (Delledonne et al., 1998; Durner et al., 1998). NO seems to work in conjunction with ROS in the potentiation of the pathogen-induced cell death (Delledonne et al., 2001). Cytological studies show that ROS and NO are associated with cell death adjacent to infected cells and that both signals modulate each other's accumulation (Tada et al., 2004; Zeier et al., 2004). Interestingly, both ROS and NO collaborate to mediate abscisic acid (ABA)-induced stomata closure (Desikan et al., 2004). NO synthesis and stomata closure in response to ABA are severely reduced in the NADPH oxidase double mutant *atrbohD atrbohF*, suggesting that endogenous H_2O_2 production elicited by ABA is required for NO synthesis (Bright et al., 2006). Collectively, these data suggest that the interplay between these molecules mediates a variety of physiological responses.

Calcium metabolism is intimately related to ROS signaling. Increases in cytosolic Ca^{2+} is also one of the fastest responses upon pathogen infection, and the use of specific inhibitors show that Ca^{2+} influx is required for ROS production after elicitation (Blume et al., 2000; Grant et al., 2000b). Ca^{2+} can activate an Rboh protein in vitro (Sagi and Fluhr, 2001), and all plant Rboh proteins contain two EF-hands in their N-terminal region that may account for this Ca^{2+} regulation (Torres and Dangl, 2005). On the other hand, ROS appears to be required to prime Ca^{2+} influx after elicitation (Levine et al., 1996). Therefore, Ca^{2+} fluxes appear to function both upstream and downstream of ROS production, indicating a complex spatiotemporal Ca^{2+} regulation of these signaling networks. Phosphorylation events have also been proposed to occur both upstream and downstream of ROS production in response to pathogens (Nurnberger and Scheel, 2001; Apel and Hirt, 2004).

ROS generated via the NADPH oxidase and subsequent Ca^{2+} channel activation may represent a common signaling link in many plant responses. For example, ROS functions as an intermediate in ABA signaling during stomata closure through the activation of Ca^{2+} channels in guard cells (Pei et al., 2000). Thus, activation of Ca^{2+} channels represents a common signaling cassette in response to at least ABA and pathogen response. ROS may be the crucial signal in each system, since fungal elicitors induce both elevation of free cytosolic Ca^{2+} and stomata closure in guard cells (Klusener et al., 2002). The same *Atrboh* genes have been implicated in each system (Torres et al., 2002; Kwak et al., 2003), suggesting that the same NADPH oxidases regulate different ROS-dependent functions in different cellular contexts.

Responses associated with ROS may also interact with ethylene signaling. Ethylene can induce programmed cell death and senescence (de Jong et al., 2002). Both ROS and ethylene have been implicated in signaling in response to viral infection (Love et al., 2005). Interestingly, the ethylene receptor ETR1 can function as an ROS sensor, mediating stomatal closure in response to H_2O_2 (Desikan et al., 2005). Thus, this protein may constitute a node mediating cross talk between ethylene and H_2O_2. Thus, ROS signaling interacts with many other regulatory events in a complex network of signals that govern the response to pathogens and other factors of the environment as well as developmental cues. This cross talk may account for the multiplicity of responses mediated by ROS and explain why ROS produced by the same mechanism exert variable effects in different contexts.

CONCLUDING REMARKS

The rapid production of ROS in the apoplast in response to pathogens has been proposed to orchestrate the establishment of different defensive barriers against the pathogens. Based on genetic analysis, the NADPH oxidase appears to be the predominant enzymatic mechanism responsible for this oxidative burst. However, other mechanisms of ROS production in other compartments, as well as various ROS-scavenging systems, may modify and regulate these responses. ROS produced by the NADPH oxidase alone can mediate diverse and sometimes opposite functions in different cellular contexts, underscoring the complexity of ROS signaling. More efforts should be put toward understanding the interplay between the different pools of ROS, and the flux of information between different compartments to further understand the regulatory capabilities of ROS. We are only beginning to understand the spatiotemporal relationships of ROS generation and removal and the interaction of ROS with other signaling molecules. This promises to be an important, and technically challenging, avenue for future work.

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