Unraveling the Tapestry of Networks Involving Reactive Oxygen Species in Plants

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The *Plant Physiology* Special Issue on Reactive Oxygen Species (ROS) published in June 2006 marked the early efforts to resolve the tapestry of mechanisms influenced by ephemeral singlet oxygen, superoxide ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\cdot}$), peroxynitrite ($ONOO^-$), and nitric oxide (NO) in plant cells. The *Updates* and research articles in the issue focused on ROS as messengers in signal transduction rather than simply unavoidable toxic by-products of metabolism or environmental perturbation. The issue was inspired by the growing perception that a delicate interplay between localized ROS production and amelioration is fundamental to responses to biotic and abiotic cues as well as development (Mittler et al., 2004). The Special Issue on ROS was the first of the *Plant Physiology* Focus Collection (Ort, 2006) to feature online papers published in the journal for the 2 years prior to and following the Special Issue (http://www.plantphysiol.org/cgi/collection/reactive_oxygen_species). Since June 2006, more than 50 publications have further explored the regulated production and scavenging of ROS in plants and the contribution of these reactive molecules to cellular processes ranging from response to pathogens to polar cell elongation. The recent investigations on the functions of ROS have been augmented by development of methods that allow direct visualization of ROS formation and artful utilization of genetic and genomic resources from multiple plant models. Here we consider some of the newly visualized fibers of the complex networks that include ROS and ROS signaling in plant cells. We apologize for any work that we were unable to include due to space limitations.

ADVANCES IN ROS IMAGING

The 2006 Special Issue on ROS included the introduction of redox-sensitive GFP as a tool to monitor the redox state of the plant cell (Jiang et al., 2006). Since then researchers have taken advantage of this tool to monitor the redox state involved in glutathione metabolism (Meyer et al., 2007) or the removal of ROS by manganese superoxide dismutase in mitochondria (Morgan et al., 2008), and additional papers are anticipated. Other valuable methods for in vivo monitoring of ROS were also introduced, including autoluminescence (Havaux et al., 2006) and biophotone imaging (Kobayashi et al., 2007) as well as luciferase imaging (Shao et al., 2007). Dichlorofluoroscein dyes have been widely used to monitor ROS levels in plants. Recently, the dyes Amplex Red and Amplex Ultra Red were added to the toolbox for detection/imaging of H_2O_2 and successfully used to demonstrate ROS production in nuclei (Ashtamker et al., 2007). An interesting application for the measurement of ROS and photosynthesis was reported by Hideg and Schreiber (2007), who used 3-(*N*-dansyl)aminomethyl-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrole, coupled with fluorescence imaging, to monitor in parallel in vivo photosynthesis and ROS formation. Monitoring intracellular versus extracellular ROS production was recently reported by Monshausen et al. (2007), who used OxyBURST and dichlorofluoroscein to monitor extracellular and intracellular ROS production in root hairs, respectively. These new tools expand the choices available to researchers attempting to monitor ROS in vivo.

RECOGNITION OF ADDITIONAL SOURCES OF ROS PRODUCTION

Localized ROS production in organelles (i.e. plastids, mitochondria, and peroxisomes) and in specific regions (apoplastic space and apex of polarized cells) may trigger different signaling cascades. With a diverse arsenal of ROS-generating enzymes, such as the plant orthologs of the respiratory burst NADPH oxidases (RBOHs), plant cells, like mammalian cells, can initiate and most probably amplify ROS production for the purpose of ROS signaling. A newly identified extracellular peroxidase and two type III peroxidases play an active role in H_2O_2 production and subsequent cell death in the local and systemic response to pathogen

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attack (Bindschedler et al., 2006; Choi et al., 2007). During pathogenesis or wounding, ROS production is mainly apoplastic, whereas during salt stress ROS also can be produced from internalized membranes (endosomes) by NADPH oxidase in a phosphatidylinositol 3-kinase-dependent manner (Leshem et al., 2006, 2007). In addition, inhibition of the fusion of these ROS-containing endosomes with central vacuoles leads to increased salt tolerance. Intriguingly, a previously unidentified nuclear source of ROS production has been observed during elicitor treatment (Ashtamker et al., 2007). These reports add two extra subcellular sources of ROS production in plant cells, thereby adding to the complexity of the ROS signaling network.

ROS SIGNAL PERCEPTION AND TRANSDUCTION

Through its relative stability and ability to diffuse through membranes, H_2O_2 already had been recognized as the most potent signaling ROS in plants. At present, specific aquaporins have been demonstrated to channel H_2O_2 actively across membranes (Bienert et al., 2007). Despite the current lack of evidence for an in planta action, these data point to aquaporins as possible regulators of intercellular signaling in plants.

New insights have been gained into the modes of action and regulation of previously identified molecular targets of ROS signaling. EXECUTER1 was proposed earlier as a candidate for singlet oxygen perception within the chloroplasts. Lee et al. (2007) provided genetic evidence that EXECUTER1 acts in concert with the highly similar EXECUTER2 to transfer stress-related signals from the plastid to the nucleus. The primary function of EXECUTER2 is that of a modulator that attenuates and controls the activity of EXECUTER1 dependent upon enzymatic lipid peroxidation events (Przybyla et al., 2008). Another key component of the chloroplast-to-nucleus signaling, GUN1, that mediates ROS and/or redox responses in Arabidopsis (*Arabidopsis thaliana*) also has been identified recently (Koussevitzky et al., 2007), and its role in the plant's response to heat stress has been demonstrated (Miller et al., 2007). Lesions simulating disease resistance 1 (LSD1), the first negative regulator of plant cell death identified, seems to act as a cellular hub that keeps a positive cell death regulator, the Arabidopsis basic Leu zipper (bZIP) transcription factor AtbZIP10, outside the nucleus under oxidative stress conditions. Hence, in *lsd1* mutants, AtbZIP10 transits freely into the nucleus and triggers the uncontrolled cell death phenotype (Kaminaka et al., 2006). The oxidative signal-inducible 1 (OXI1) Ser/Thr kinase is required for oxidative burst-mediated signaling and first was characterized as a downstream signaling component of the phosphoinositide-dependent protein kinase 1 (PDK1). Another Ser/Thr kinase, PTI-2, was identified as an OXI1 substrate. Specific lipid signals have been shown to activate the OXI1-PTI-2 tandem through PDK1, whereas H_2O_2 signals to OXI1-PTI-2 via a PDK1-independent pathway (Anthony et al., 2006).

The plant heterotrimeric G protein complex is involved in cell death signaling during the unfolded protein response and in ozone-induced activation of NADPH oxidases. Because of its involvement in light transmission, hormone signaling, and regulation of ion channels, this complex is a potential interface between ROS and these processes (Wang et al., 2007). Recently H_2O_2 has been found to activate G protein signaling through the promotion of dissociation of the Gα-subunit from the G protein macromolecular complex (S. Wang et al., 2008). Mitogen-activated protein kinases (MAPKs) act both upstream and downstream of the oxidative burst, and the regulation of their activity is necessary to adequately transduce stress signals to downstream targets. The Arabidopsis MAPK kinase kinase MEKK1 protein levels are both stabilized and activated by H_2O_2 in a proteasome-dependent manner and thereby positively regulate ROS-induced activation of the MAPK MPK4 (Nakagami et al., 2006). A MAPK phosphatase (AtMKP2) deactivates MAPK3 and MAPK6 and, hence, serves as a regulator of the plant response to oxidative stress (Lee and Ellis, 2007). MAPK3 and MAPK6 have been reported to be activated in response to H_2O_2 and to be a potential integrating point of environmental and developmental signals that regulate stomatal development (H. Wang et al., 2008). The activity of MAPK3 and MAPK6 can be stimulated by the nucleoside diphosphate kinase 2 (NDPK2). Verslues et al. (2007) showed that NDPK2 interacts with the salt stress-signaling salt overlay sensitive 2 (SOS2) kinase. NDPK2 interacts also with catalase, hereby further illustrating the importance of H_2O_2 signaling during the salt stress response. Besides phosphorylation, *S*-nitrosylation, the covalent attachment of an NO group to the thiol side chain of Cys residues, has been established further as a component of stress signal transduction. Together with a proteomic survey of *S*-nitrosylation targets during the hypersensitive response, new insights were gained into the regulation of individual proteins by *S*-nitrosylation (Belenghi et al., 2007; Romero-Puertas et al., 2008). Of particular interest is the *S*-nitrosylation of proteins related to the antioxidant system (germin-like protein, monodehydroascorbate reductase, and type II peroxiredoxin [PrxIIE]). *S*-nitrosylation of PrxIIE inhibits both its peroxidase and peroxynitrite reductase activities, suggesting that NO might regulate the effects of its own radicals through *S*-nitrosylation of crucial antioxidant systems components (Romero-Puertas et al., 2007).

NEW PERSPECTIVES ON ROS SCAVENGING

Specific attention was given in recent years to the participation of NAD(P)H and ATP regenerating mechanisms in the ROS scavenging network. Proteins that function to reduce NAD(P) to NAD(P)H or alter the level of ATP were found to be essential for plant tolerance to oxidative stress (Chai et al., 2006; Valderrama et al., 2006;

Marino et al., 2007; Noctor et al., 2007; Rodriguez et al., 2007; Vidal et al., 2007). These reports strengthen the link between ROS scavenging and metabolism and demonstrate that proper regulation of ROS requires reducing power that can be supplied by photosynthesis, respiration, and/or different carbon utilization pathways. Insight into the cross talk between different ROS and ROS scavenging mechanisms was gained from studies of double or triple mutants that lack key ROS scavenging enzymes in different subcellular locations (Giacomelli et al., 2007; Miller et al., 2007) and exploration of cross talk between distinct ROS such as singlet oxygen and H_2O_2 (Laloi et al., 2007). These studies not only exposed redundancy of the ROS scavenging network but also suggested that different antioxidant enzymes and different ROS in the same or different compartments mediate signature signals that control chloroplast function and plant response to various environmental stimuli.

The importance of peroxiredoxins, glutaredoxins, and thioredoxins as scavengers of ROS has gained significant support in recent years (Cheng et al., 2006; Dos Santos and Rey, 2006). A novel function was recently assigned to the peroxiredoxin PrxIIE in detoxifying $ONOO^-$, a potent oxidizing and nitrating species formed in a diffusion-limited reaction between NO and O_2^-, suggesting a key role for peroxiredoxins in mediating the cross talk between NO and ROS (Romero-Puertas et al., 2007; Hong et al., 2008; Wilson et al., 2008). Moreover, peroxiredoxins also were reported to function as redox sensors, linking the redox signaling and ROS networks of cells (Dietz, 2008). These studies suggest integration of ROS, NO, and redox signaling in cells and provide an excellent primer for future studies that will unravel the complexity of these networks.

There is new information on the fate of oxidized proteins (Møller et al., 2007) from investigations that link protein oxidation and autophagy (Bassham, 2007; Xiong et al., 2007a, 2007b) and describe the role of protein oxidation in seed dormancy (Oracz et al., 2007). It was found that protein oxidation is followed by controlled degradation and recycling and that these processes are essential for cellular viability, signaling, development, and recovery from oxidative stress.

ROS IN BIOTIC INTERACTIONS AND RESPONSES

Involvement of ROS in the interaction of plants with biotic agents has been extensively documented, with specific focus on plant responses to pathogen attack (Torres et al., 2006). Three papers have brought to the fore the importance of ROS production and scavenging by the pathogen. Egan et al. (2007) reported that the generation of ROS by the rice blast fungal NADPH oxidase is required for infection, whereas Molina and Kahmann (2007) documented that activation of ROS scavenging mechanisms by the fungal *Ustilago maydis Yap1* gene is essential to overcome the plant defense mechanisms and allow infection. In addition, Mittapalli et al. (2007) reported that the ROS scavenging mechanisms of the Hessian fly (*Mayetiola destructor*) could play an important function in the interaction with its wheat (*Triticum aestivum*) host.

ROS formation in the apoplast by the plant NADPH oxidases is well documented, and recent reports implicate other sources of ROS in biotic defense responses. These studies highlight apoplastic peroxidases (Bindschedler et al., 2006; Choi et al., 2007) as well as polyamine oxidases (Yoda et al., 2006; Angelini et al., 2008) as important for ROS production involved in wound and pathogen responses. This adds weight to previous reports (e.g. Allan and Fluhr, 1997) and supports a model in which multiple sources of ROS production are involved in the response of plants to pathogen or insect interactions. Interestingly, ROS accumulation in response to elicitation was observed in nuclei (Ashtamker et al., 2007), chloroplasts (Y. Liu et al., 2007), and mitochondria (Vidal et al., 2007). In addition, the distribution of iron within the different subcellular compartments was linked to ROS production and activation of plant defenses during the oxidative burst (G. Liu et al., 2007). These studies suggest that ROS production during pathogen responses occurs at multiple subcellular locations (and not exclusively at the apoplast) and that ROS production at these subcellular compartments has an important function in the activation of defense responses and programmed cell death (PCD).

Several proteins were added in the past 2 years to the list of possible mediators of defense responses and PCD in response to pathogens in plants (see also below). These include Bax inhibitor 1 (Watanabe and Lam, 2008), BAP1 and BAP2 (Yang et al., 2007), the receptor kinase CERK1 (Miya et al., 2007), and BAK1 (He et al., 2007; Kemmerling et al., 2007). Cyclic nucleotide-gated channel 2 (also called DND1) also was described as a key mediator of NO and hypersensitive response cell death (Ali et al., 2007). These studies provide important links between signaling events that occur upstream or downstream of ROS production during pathogen attack and expose the chronology of events that imparts successful defense against pathogens.

ROS IN CELL DEATH

As mentioned above, the role of ROS in cell death has received attention in recent years. The evaluation of the Arabidopsis *catalase2* mutants at different day lengths has revealed that photoperiod is a critical determinant of the oxidative stress response, with lesion development being enhanced in plants grown under long-day conditions (Queval et al., 2007). Singlet oxygen-induced cell death depends on the blue light/UVA-specific photoreceptor cryptochrome and differs from PCD triggered by H_2O_2/O_2^- (Danon et al., 2006). These results reinforce the emerging concept that ROS-dependent cell death in plant cells

not only is mediated through damage caused by indiscriminative oxidation but also occurs through interactions with other signaling pathways, such as those determined by photoperiod, and illustrate that other cues impinge on the transcriptional responses provoked by increased ROS levels.

The cell death inhibitors Bax inhibitor 1 (Watanabe and Lam, 2008) and BAP2 (Yang et al., 2006, 2007) inhibit oxidative stress-dependent cell death events, while a newly identified executioner of oxidative stress-induced cell death is metacaspase 8 (He et al., 2008). BAK1 and BAK1-LIKE1 have dual roles: positive and negative regulation of brassinosteroid-dependent and brassinosteroid-independent growth and cell death pathways, respectively, through an alternating interaction with BRI1 and the LRR receptor-like kinase FLS2 (Chinchilla et al., 2007; He et al., 2007; Heese et al., 2007; Kemmerling et al., 2007). ROS also were shown to induce autophagy to degrade oxidized proteins in Arabidopsis. Down-regulation of autophagy gene 18 leads to the accumulation of oxidized proteins and subsequent increased sensitivity to oxidative stress (Xiong et al., 2007a, 2007b). Genetic evidence has been provided for an alternative route to remove oxidized proteins through 20S proteasome-dependent proteolysis (Kurepa et al., 2008).

Developmentally regulated PCD has been reported during tracheary element formation, seed development, germination, and senescence (Van Breusegem and Dat, 2006). H_2O_2 together with ethylene has been demonstrated to be involved in the hypoxia-induced lysigenous aerenchyma formation in Arabidopsis. Interestingly, this cell death event involves early root-to-shoot signaling and shoot regulatory feedback on a root-specific redox regulation (Mühlenbock et al., 2007), emphasizing the interplay between ROS and redox.

ROS IN ABIOTIC STRESS RESPONSES

Under abiotic stress conditions, increased ROS levels are associated with both signaling and oxidative damage. In water-stressed maize (*Zea mays*), sustained cell elongation in the apical root region is correlated with increased apoplastic ROS levels (Zhu et al., 2007). Accordingly, salt stress-induced inhibition of leaf expansion in maize is correlated with reduced apoplastic ROS production (Rodriguez et al., 2007). These data suggest that increased apoplastic ROS production has a positive effect on growth under water stress conditions and consolidates previous reports on ROS-mediated cell wall loosening sustaining the capability of cell walls to expand during abiotic stress conditions.

The importance of an integrative role of mitochondrial oxidative respiration in the abiotic stress response of plants has been established further with the identification of the pentatricopeptide repeat protein (PPR40) that is important for the correct ubiquinol-cytochrome *c* oxidoreductase activity of complex III and the adaptation to adverse environmental conditions (Zsigmond et al., 2008). Somewhat surprising, because previous overexpression studies in Arabidopsis made plants more resistant to oxidative stress, is the observation that overproduction of alternative oxidase in transgenic tobacco (*Nicotiana tabacum*) led to increased ozone sensitivity. This suggests that within a narrow window specific levels of mitochondrial ROS might be necessary to trigger mitochondrial retrograde signaling events required to launch a proficient defense response (Pasqualini et al., 2007).

An alternative mode by which H_2O_2 and probably other ROS mediate abiotic stress responses is stabilization of specific transcripts that encode stress-related proteins. Chung et al. (2008) demonstrated that increased ROS levels enhance the stability of the salt-induced *SOS1* mRNA. This plasma membrane Na^+/H^+ antiporter interacts via its cytoplasmic tail domain with runaway cell death 1, revealing a new function for SOS1 within the oxidative stress response (Katiyar-Agarwal et al., 2006). The plasma membrane slow anion channel-associated 1, a distant homolog of fungal and bacterial dicarboxylate/malic acid transport proteins, has been found to be essential for stomatal closure during abiotic stress, including ozone stress, and to act as an essential subunit for S-type anion channel function or regulation (Vahisalu et al., 2008; Negi et al., 2008). These findings further elucidate the intricate networks of cellular processes that are modulated by ROS during abiotic stress.

The zinc finger protein ZAT10 was demonstrated to be a regulator of the abiotic stress response. Transgenics with enhanced or suppressed ZAT10 levels were more tolerant to multiple stresses (Mittler et al., 2006; Rossel et al., 2007). A remarkable tolerance to drought stress has been reported recently in transgenic tobacco plants that express an isopentenyltransferase gene under the control of a drought stress-responsive promoter (Rivero et al., 2007). Tolerance to drought stress in these plants was accompanied by the enhanced ability to scavenge ROS supported by a battery of ROS scavenging mechanisms, implying a link between cytokinin accumulation and enhanced ROS scavenging capability under stress.

A ROS, ROP, RBOH, CALCIUM, AND PH NETWORK

Evidence of interplay between the monomeric GTPase Rho-like GTPase of plants (ROP), RBOH NADPH oxidases, cytosolic calcium transients, and ROS production was touched upon in several articles in the June 2006 *Plant Physiology* Special Issue (Gapper and Dolan, 2006; Kwak et al., 2006; Sagi and Fluhr, 2006; Torres et al., 2006). Recently several pieces of this remarkable tapestry have been assembled. First, it was shown that activation of the plasma membrane-localized RBOHs involves phosphorylation of two N-terminal Ser by a calcium-dependent protein kinase as well as interaction with ROP. RBOH phosphorylation as well as binding to calcium synergizes its activation, raising the possibility that it may function

as a calcium sensor (Ogasawara et al., 2008; Takeda et al., 2008). The RBOH/ROP-GTP interaction is regulated by the binding of calcium to two EF-hand motifs at the N terminus of the oxidase (Wong et al., 2007). The consequence of RBOH activation is localized production of O_2^-, which is rapidly converted to H_2O_2, presumably in the apoplastic space. These findings have raised a new question: Are ROP- and calcium-dependent protein kinase-mediated activation of RBOHs functionally independent, or does one event precede another?

Tip-localized and ROP GTPase-dependent ROS production by RBOHs is likely to be a general mechanism in the control of polarized growth of cells such as pollen tubes, root hairs, and *Fucus* zygotes (Gapper and Dolan, 2006; Jones et al., 2007; Potocky et al., 2007; Coelho et al., 2008; Takeda et al., 2008). But the recent work of Monshausen et al. (2007) adds complexity to this network through the demonstration that ROS production via Arabidopsis RBOHC negatively regulates episodes of cell expansion that occur just behind the tip of the root hair. They document temporal acidification of cytosol at the root hair tip that is tightly correlated with growth. Pulses of ROS accumulation just behind the tip apex followed a localized increase of apoplastic pH but were out of phase with cell expansion, suggesting that elongation is dampened by coordinated apoplastic alkalinization and ROS. Additional research is necessary to resolve questions regarding the role of RBOHC in establishing a tip-focused calcium gradient, pH oscillations, and generation of ROS (Knight, 2007). One enigma is that root hairs of *rbohC* mutants still produce extracellular ROS. Lee et al. (2008) provide evidence of a phosphatidylinositol-3-P-dependent mechanism that does not require RBOHC but also drives cell elongation behind the root hair tip. Another new twist in the story of RBOHs and other possible ROS generators is the finding that encapsulated endosomes produce ROS (Leshem et al., 2007; Lee et al., 2008). A chicken-and-egg dilemma is whether ROS production is the cause or an effect of localized calcium influx in elongating root hairs. Tip-localized activation of RBOHC promotes calcium channel activation and calcium influx, thereby stimulating RBOH activity and amplification of the initial signal (Takeda et al., 2008). The recent work by Demidchik et al. (2007) indicates that H_2O_2 and $OH^·$ might serve as distinct signals in the regulation of calcium influx in roots, due to the existence of calcium channels that are distinctly sensitive to specific intracellularly or extracellularly generated ROS.

The calcium and ROS connection in the regulation of stomatal aperture was studied further. It was shown previously that abscisic acid promotes ROS production that results in increases in cytosolic calcium that lead to stomatal closure (Kwak et al., 2006). Methyl jasmonate also promotes stomatal closure, and although this involves ROS formation the mechanism is distinct from that invoked by abscisic acid (Munemasa et al., 2007). Together, these ongoing studies raise further questions about the multifaceted roles of different ROS in the regulation and/or response to changes in cytosolic calcium and pH that underlie cellular mechanisms critical to development programs and environmental response in plant cells.

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