The ROS Signaling Network of Cells

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Abstract Reactive oxygen species (ROS) are toxic derivatives of atmospheric oxygen used by plant cells to control many different biological processes, including growth, development, and response to biotic and abiotic stimuli. Because of their toxicity, as well as their important signaling role, the steady-state level of ROS in cells is tightly regulated by a network of genes termed the "ROS gene network". In the flowering plant *Arabidopsis thaliana*, the ROS gene network includes more than 150 genes that manage the level of ROS in cells. The ROS network is highly dynamic and redundant, and encodes for ROS-scavenging as well as ROS-producing proteins. Recent studies have unraveled some of the key players of the network and shed light on some of the questions related to its mode of regulation, its protective roles, and its modulation of signaling networks that control growth, development, and stress response. In this chapter we will describe some of these findings.

1 Introduction

Ever since the introduction of molecular oxygen (O_2) into the earth's atmosphere by O_2-evolving photosynthetic organisms about 2.7 billion years ago, reactive oxygen species (ROS) have been the unwelcome companions of aerobic life (Halliwell 2006; Halliwell and Gutteridge 1999). In contrast to O_2, these partially reduced or activated derivatives of oxygen ($^1O_2$, O_2^-, H_2O_2, and HO•) are highly reactive and toxic, and can lead to the oxidative destruction of cells (Asada 2006; Asada and Takahashi 1987). Consequently, the successful evolution of plants, as well as all aerobic organisms, on the earth has been dependent upon the development of efficient ROS-scavenging mechanism. These allow organisms to overcome ROS toxicity and to use some of these toxic molecules as signal transduction molecules (Van Breusegem et al. 2008; Mittler et al. 2004; Bailey-Serres and Mittler 2006). ROS, therefore,

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play a dual role in plants as both toxic compounds and key regulators of many biological processes (Foyer and Noctor 2005; Apel and Hirt 2004; Mittler et al. 2004).

The use of ROS as signaling molecules by plant cells suggests that, during the course of evolution, plants were able to achieve a high degree of control over ROS toxicity and are now using ROS as signaling molecules. The delicate balance between ROS production and scavenging that allows this duality in function to exist in plants is thought to be orchestrated by a large network of genes termed the "ROS gene network", which includes more than 150 genes in the model plant *Arabidopsis thaliana*, tightly regulating ROS production and scavenging (Mittler et al. 2004).

2 Production of ROS in Plants

Organelles with a highly oxidizing metabolic activity or with an intense rate of electron flow, such as chloroplasts, mitochondria, and peroxisomes, are a major source of ROS production in plant cells. Localized ROS production in these organelles and in specific regions, e.g., apoplastic space, or apex of polarized cells, may trigger different signaling cascades. Production of ROS by the Mehler reaction and the antenna pigments in the chloroplasts is enhanced in plants by conditions limiting CO_2 fixation, such as drought, salt, and temperature stress, as well as by the combination of these conditions with high-light stress (Miller et al. 2008). During pathogenesis or wounding, ROS production is mainly apoplastic, whereas during salt stress, ROS can also be produced from internalized membranes (endosomes) by NADPH oxidase in a phosphatidyl inositol 3 kinase (PI3K)-dependent manner (Leshem et al. 2006). In C3 plants, limiting CO_2 conditions can also activate the photorespiratory pathway (del Río et al. 2006). As part of this pathway, H_2O_2 is generated in peroxisomes by the enzymatic activity of glycolate oxidase. In mitochondria, over-reduction of the electron transport chain is the main source of O_2^- production under specific stress conditions (Møller 2001). Additional sources of ROS in plant cells are the detoxifying reactions catalyzed by cytochromes in both the cytoplasm and the endoplasmic reticulum, as well as superoxide production by xanthine oxidase in peroxisomes (Corpas et al. 2008; Mittler et al. 2004; Van Breusegem et al. 2008).

Plasma membrane NADPH-dependent oxidases have been the subject of intense investigation (Torres and Dangl 2005; Torres et al. 2006). They are thought to play a key role in ROS signaling, and contain a multimeric flavocytochrome that forms an electron transport chain capable of reducing O_2 to superoxide. In addition to NADPH oxidases, pH-dependent cell wall peroxidases, germin-like oxalate oxidases and amine oxidases have been proposed to generate ROS at the apoplast (Mittler et al. 2004; Van Breusegem et al. 2008). Although much attention has been given to NADPH oxidases, other ROS-producing mechanisms in the mitochondria, apoplast, and peroxisomes are likely to play a role in ROS signaling in response to different stimuli or developmental signals. For example, 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 responses to pathogen attack (Bindschedler et al. 2006; Choi et al. 2007). Intriguingly, a previously unidentified nuclear source of ROS production has been observed during elicitor treatment (Ashtamker et al. 2007). These reports add additional subcellular sources of ROS production in plant cells, thereby upgrading the complexity of the ROS signaling network.

3 Scavenging of ROS in Plants

ROS-scavenging enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), glutathione peroxidase (GPX), and peroxiredoxin (PrxR) together with the antioxidants ascorbic acid and glutathione, provide cells with highly efficient machinery for detoxifying O_2^- and H_2O_2. (Foyer and Noctor 2005; Apel and Hirt 2004; Mittler et al. 2004). These mechanisms, together with the sequestering of metal ions such as free iron by ferritin and other metal-binding proteins, prevents the formation of the highly toxic HO• via the metal-dependent Haber–Weiss or the Fenton reactions (Asada 2006; Asada and Takahashi 1987). The cellular pools of the antioxidants ascorbic acid and glutathione are maintained in their reduced state by a set of enzymes capable of using NAD(P)H to regenerate oxidized glutathione or ascorbic acid (e.g., monodehydroascorbate reductase, dehydroascorbate reductase, and glutathione reductase). In addition, monodehydroascorbate radicals can be reduced back into ascorbic acid via ferredoxin using electrons diverted from the photosynthetic apparatus in the water–water cycle in chloroplasts (Asada 2006). Scavenging of H_2O_2 can also be mediated in plants by "classical" plant peroxidases (class III) using a variety of reductants. Membranes are highly susceptible to oxidative stress. In plant cells, they are protected by the activity of specific phospholipid glutathione peroxidases and by α-tocopherol (vitamin E), which is kept at its reduced state by the pool of reduced ascorbic acid. Protection of cells against singlet oxygen is generally believed to be mediated by carotenoids (Asada and Takahashi 1987).

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 PrxII E 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 crosstalk between NO and ROS (Romero-Puertas et al. 2007; Hong et al. 2008; Wilson et al. 2008). Moreover, peroxiredoxins were also 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 platform for future studies that will unravel the complexity of these networks. Insight into the crosstalk between different ROS and ROS scavenging mechanisms was also 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 crosstalk between distinct ROS such as singlet oxygen and H_2O_2 (Laloi et al. 2007). These studies not only expose the redundancy of the ROS-scavenging network, but also suggest 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.

4 ROS Signaling and its Modulation by the ROS Gene Network

Plants constantly sense and assess the level of ROS in cells and reprogram their enzymatic activities and gene expression to optimally respond and acclimate to the changing conditions in their environment. ROS signaling is thought to be controlled by two opposing processes of production and scavenging. At least three different mechanisms were proposed for sensing of ROS in plant cells: (1) receptor proteins, (2) redox-sensitive transcription factors, and (3) direct inhibition of phosphatases by ROS (Mittler 2002; Mittler et al. 2004; Apel and Hirt 2004; Miller et al. 2008). In addition, ROS can be sensed due to their effect on different metabolic pathways and the state of oxidation or redox potential of specific cellular proteins. These could be membrane-associated or soluble, and may be present in different cellular compartments. Different developmental or environmental signals feed into the ROS signaling network and perturb ROS homeostasis in a compartment-specific or even a cell-specific manner. As described above, perturbed ROS levels are perceived by different proteins, enzymes, or receptors, and modulate different developmental, metabolic, and defense pathways. ROS production is mediated by different cellular pathways, including respiration and photosynthesis, as well as by different proteins and enzymes, e.g., NADPH oxidases, amine oxidases, and xanthine oxidase (Mittler et al. 2004). In contrast, ROS scavenging is mediated by different ROS-scavenging enzymes and antioxidants that include ascorbate peroxidases, catalases, peroxiredoxins, and superoxide dismutases (Mittler 2002; Mittler et al. 2004; Apel and Hirt 2004). The intensity, duration, and localization of the different ROS signals in cells are therefore determined by interplay between these two opposing forces, i.e., ROS scavenging and ROS production, and the decoding of this signal will determine the cellular response to the original cue, modulating different developmental, metabolic, and/or defense pathways. The process described above requires a tight mode of regulation and might involve amplification and/or feedback inhibition loops. In addition to regulating the intensity and duration of the different ROS signals, the ROS-scavenging pathways are also responsible for maintaining a low steady-state "base line" of ROS on which the different signals can be registered.

It is possible that the use of ROS as versatile signaling molecules originated from their proposed use to sense biotic or abiotc stress. Most forms of stress disrupt the metabolic balance of cells, resulting in enhanced production of ROS. Simple

organisms, such as bacteria or yeast, sense the enhanced production of ROS by redox-sensitive transcription factors and other molecular sensors, activate different ROS-defense pathways, and regulate their metabolic pathways to lower the production rate of ROS (Mittler et al. 2004). This "basic cycle" of ROS metabolism maintains a low steady-state level of ROS in cells. Variations on this pathway could have originated during evolution and contributed to the use of ROS as signaling molecules to control more specialized processes, such as plant growth and defense, hormonal signaling, and development. For example, pathogen infection could alter plant metabolism causing the accumulation of ROS due to suppression or activation of different pathways (Mittler 2002). A specialized pathway used for pathogen sensing via ROS could have evolved and resulted in the pathway we now know in which the identification of a pathogen via a plant receptor will trigger an *R* gene-dependent pathway that will result in the enhanced production of ROS by plasma membrane-localized NADPH oxidases (Torres et al. 2006). This will result in a ROS signal that will activate several different defense pathways. The activity of ROS-scavenging enzymes is important in this case because these enzymes can modulate the ROS signal and determine the intensity, duration or even the type of response (Mittler et al. 1999). Similarly, an abiotic stress such as osmotic stress will result in enhanced ROS production due to altered metabolic balance (Miller et al. 2008; Mittler 2006). A signaling pathway that senses and responds to osmotic stress was recently shown to involve the activation of ROS production via NADPH oxidases and other ROS-producing signals (Mittler 2002, 2006).

5 A Model for ROS Signaling Developed from the Study of Plants Lacking Apx1

Mutants lacking cytosolic ascorbate peroxidase 1 (*apx1*) have been used in the past several years to study ROS signaling in *Arabidopsis*. Using a time-course microarray analysis comparing these plants to wild-types under conditions of moderate light stress, we have identified several key regulators involved in ROS signaling in plants, and using different mutants we studied several of these proteins, independently assessing their role in ROS signaling and responses to abiotic stress conditions (Rizhsky et al. 2004; Pnueli et al. 2003; Davletova et al. 2005a, b; Suzuki et al. 2005, 2008; Mittler et al. 2006; Ciftci-Yilmaz et al. 2007). Our analysis suggests that cytosolic APX1 functions as a buffer to control the levels of ROS that reach the nuclei and activate gene expression (Fig. 1). Thus, in the absence of APX1, the expression of different light stress response transcripts such as that of the key regulator Zat12 was facilitated (Davletova et al. 2005a).

Our model suggests that, in the absence of APX1, ROS that accumulates in the cytosol is sensed by different redox-response transcription factors, such as heat shock transcription factors (HsfA4a), and triggers a cascade of different transcription factors that include members of the zinc finger protein Zat family (Zat12, 10, and 7) and members of the WRKY transcription factor family. Additional players

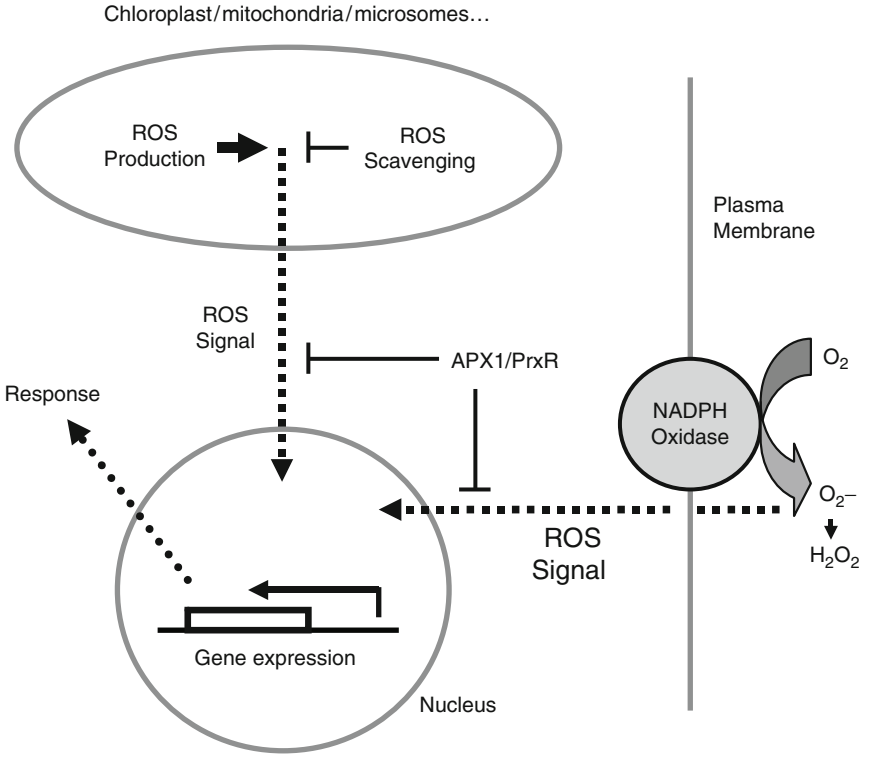

Fig. 1 A hypothetical model showing the role of cytosolic ROS-scavenging systems in the regulation of ROS signaling in plant cells. Different ROS signals originating from different organelles, or at the plasma membrane, e.g., by NADPH oxidases, reach the nucleus and regulate gene expression. Cytosolic ROS-scavenging systems such as cytosolic ascorbate peroxidase 1 (APX1) or cytosolic peroxiredoxins (PrxR) act as buffers to attenuate these signals and control the amount of ROS that reach the nucleus

in this pathway could include MBF1c that is a transcriptional coactivator and NADPH oxidase (RbohD) that is likely to be involved in amplifying the ROS signal (Rizhsky et al. 2004; Pnueli et al. 2003; Davletova et al. 2005a, b; Suzuki et al. 2005, 2008; Mittler et al. 2006; Ciftci-Yilmaz et al. 2007).

The model presented in Fig. 1 could be extended to include additional sources of ROS such as mitochondria or apoplast that generate ROS in response to specific stimuli, and include additional cytosolic ROS-scavenging mechanisms such as preoxiredoxins, glutathione peroxidase, and thioredoxins. The ROS signal generated in the different cellular compartments is hypothesized to reach the nuclei and activate gene expression in a process that will induce a response to the perceived stimuli. In the presence of APX1, the levels of ROS that reach the nuclei will be attenuated to generate the correct response, preventing, for example, misactivation of cell death or any additional unwanted responses. Additional studies are underway in our laboratory to study this model in more detail.

6 Coordination of the ROS Network

The different scavenging and producing enzymes encoded by the ROS gene network can be found in many different subcellular compartments. In addition, more than one enzymatic activity per a specific ROS can usually be found in each of the different compartments (Mittler et al. 2004). Because ROS such as H_2O_2 can diffuse between different cellular compartments (Bienert et al. 2007), ROS metabolism in a particular compartment can effect or alter the ROS homeostasis/signaling of a neighboring compartment or reach the nuclei and activate gene expression (Fig. 1). Transporters for the antioxidants ascorbic acid and glutathione are likely to be central in determining the specific concentrations of these compounds and the redox potential in the different cellular compartments. An anonymous player in the ROS signaling network is the vacuole. Its ROS-scavenging and ROS-producing potentials are unknown at present. It is possible that this organelle, because of its relatively large cellular volume, plays an essential role in the control of ROS metabolism in plants. Recent studies in *Arabidopsis* have suggested that the mode of coordination between different components of the ROS removal network of plants is complex. For example, the application of light stress to *Arabidopsis* resulted in the induction of cytosolic and not chloroplastic ROS-defense enzymes (Mittler et al. 2004), even though most ROS produced during light stress are thought to be generated in chloroplasts and/or peroxisomes. The cytosolic ROS-scavenging pathways were further shown to be required for the protection of chloroplasts during light stress (Davletova et al. 2005a). In a different study, a double mutant deficient in cytosolic ascorbate peroxidase 1 and peroxisomal catalase 1 was found to be more tolerant to light stress compared to wild-type or single mutants deficient in ascorbate peroxidase 1 or catalase 1 (Rizhsky et al. 2002). This finding was very surprising because it suggested that different cellular pathways are activated in cells in response to enhanced ROS production in the cytosol or peroxisomes. Activation of both the cytosolic and peroxisomal pathways further results in the generation of a new signal that is different from that activated by the two individual cytosolic or peroxisomal signals. How ROS metabolism and signaling are coordinated between different organelles in cells is largely unknown at present.

7 NADPH Oxidases, a Possible Link Between Calcium and ROS Signaling

NADPH oxidases play a key role in ROS signaling and plant defense responses to pathogen infection, abiotic stress and injury. They generate $O_2^{\bullet -}$ by oxidizing NADPH and transferring the electron to O_2. They represent the plant homologs to the mammalian phagocyte NADPH oxidase subunit $gp91^{phox}$ (Torres and Dangl 2005). The *Arabidopsis* genome contains ten classical NADPH oxidase genes, all of which contain a presumably cytosolic 300 amino-acid amino-terminal extension with two EF-hands that binds Ca^{2+} and at least one key phosphorylation site.

It was recently shown that activation of the plasma membrane localized NADPH oxidases involves phosphorylation of two N-terminal serines by a calcium-dependent protein kinase (CDPK) as well as interaction with Rho-like GTPase (ROP). NADPH oxidase 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 NADPH oxidase/ROP interaction is regulated by the binding of calcium to two EF-hand motifs at the amino terminus of the oxidase (Wong et al. 2007). The consequence of NADPH oxidase activation is localized production of $O_2^{\bullet -}$, which is rapidly converted to H_2O_2, presumably in the apoplastic space. The ROS signal produced could reach the nuclei (Fig. 1), activating gene expression in response to a given stimuli and generating a plant response to this stimuli. In root tips, a complex interaction between calcium, pH, and ROS oscillation was recently reported to control elongation (Monshausen et al. 2007; Van Breusegem et al. 2008).

8 Concluding Remarks

Although ROS were initially considered to be toxic byproducts of aerobic metabolism, in recent years it became obvious that plants can cope with ROS toxicity to the degree of using ROS as signal transduction molecules. ROS signaling was shown to be involved in the regulation of basic biological processes and responses to biotic and abiotic stimuli. ROS signaling and ROS toxicity are kept in check by the ROS gene network of plants. This network includes ROS-scavenging and ROS-producing enzymes that modulate the level of ROS in cells. Thus, the overall level of ROS is always kept under control and ROS are allowed to accumulate and/or oscillate for the purpose of signaling in a highly controlled manner (Mittler et al. 2004). This process is achieved by a tightly controlled balance between ROS production and ROS scavenging in the different cellular compartments. The interplay between ROS scavenging and production in the different cellular compartments, therefore, determines the intensity, duration, and localization of ROS signals, and the decoding of these signals determine the plant's response, or developmental and/or growth adaptations. A complex relationship between divergent ROS signals from different cell components can also result in cell reprogramming depending on the nature of the signals and their origin.

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