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REVIEW

ROS-Mediated ABA Signaling

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Abstract In plants, reactive oxygen species (ROS) are short-lived molecules produced through various cellular mechanisms in response to biotic and abiotic stimuli. ROS function as second messengers for hormone signaling, development, oxygen deprivation, programmed cell death, and plant–pathogen interactions. Recent research on ROS-mediated responses has produced stimulating findings such as the specific sources of ROS production, molecular elements that work in ROS-mediated signaling and homeostasis, and a ROS-regulated gene network (Neill et al., Curr Opin Plant Biol 5:388–395, 2002a; Apel and Hirt, Annu Rev Plant Biol 55:373–399, 2004; Mittler et al., Trends Plant Sci 9:490–498, 2004; Mori and Schroeder, Plant Physiol 135:702–708, 2004; Kwak et al., Plant Physiol 141:323–329, 2006; Torres et al., Plant Physiol 141:373–378, 2006; Miller et al., Physiol Plant 133:481–489, 2008). In this review, we highlight new discoveries in ROS-mediated abscisic acid (ABA) signaling.

Keywords Abscisic acid · Homeostasis · NADPH oxidase · Reactive oxygen species

Reactive oxygen species (ROS) are produced in plant cells primarily as a by-product of aerobic metabolism (Slesak et al. 2007). ROS comprise hydrogen peroxide (H_2O_2), superoxide anion (O_2^-), hydroxyl radical ($HO^{\cdot}$), and singlet oxygen ($^1O_2$), all of which can cause cellular toxicity or damage (Miller et al. 2008). Mechanisms for their generation include photosynthetic electron transport, oxalate oxidases, glycolate oxidases, xanthine oxidases, fatty acid β-oxidation, amine oxidases, cell wall-bound peroxidases, and respiration in the mitochondria, chloroplasts, and peroxisomes (Apel and Hirt 2004; Mittler et al. 2004; Miller et al. 2008). Plants have also developed mechanisms by which ROS molecules are removed from cells (Apel and Hirt 2004). ROS, which are often produced in response to pathogens, phytohormones, and environmental cues, mediate specific cellular activity that depends on the signal (Pei et al. 2000; Foreman et al. 2003; Kwak et al. 2003; Apel and Hirt 2004). The balance between ROS production and scavenging can be perturbed by various environmental factors (Apel and Hirt 2004; Laloi et al. 2006; Miller et al. 2008).

ROS, NADPH Oxidases, and ABA Signaling

A rapid rise in ROS levels is called an oxidative burst. In mammals, NADPH oxidases (NOXs) are responsible for the respiratory oxidative burst in phagocytes (Bokoch and Knaus 2003). Several studies have suggested that NOXs also function in defense and hormone signaling in plants (Keller et al. 1998; Pei et al. 2000; Jiang and Zhang 2002, 2003). Plant NOXs are localized to the plasma membrane and share structural similarities with animal NOXs (Keller et al. 1998; Torres et al. 1998; Torres and Dangl 2005). Plant NOXs have two Ca^{2+}-binding EF hands in the N-terminus, six transmembrane helices, and a cytosolic NADPH binding motif (Torres et al. 1998). Ten NOX catalytic subunit genes exist in the *Arabidopsis* genome and nine in the rice genome (Groom et al. 1996; Torres and Dangl 2005). The activity of NOXs in plants is apparently regulated by several cytosolic factors, e.g., Ca^{2+}, protein kinases, and small G proteins (Keller et al.

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1998; Sagi and Fluhr 2001; Kobayashi et al. 2007; Nuhse et al. 2007; Wong et al. 2007; Ogasawara et al. 2008). In animal cells, the small G protein Rac is a cytosolic factor activating NOXs (Sumimoto 2008). Rac homologs in plants function in ROS signal transduction. *OsRac1* in rice enhances pathogen-induced ROS formation and ROS-mediated cell death (Kawasaki et al. 1999; Ono et al. 2001). Furthermore, a direct protein–protein interaction has been detected between OsRac1 and an N-terminal region, containing the EF-hand motifs, of OsrbohB (Wong et al. 2007). Transient co-expression of OsRac1 and OsrbohB also increases ROS production in tobacco, suggesting that the small G protein Rac1 acts as a positive regulator of NOX (Wong et al. 2007).

ROS function in many cellular processes, including plant–pathogen interactions, ozone and wound signaling, development, and hormonal signaling (Neill et al. 2002a; Apel and Hirt 2004; Mittler et al. 2004; Mori and Schroeder 2004; Kwak et al. 2006; Torres et al. 2006; Miller et al. 2008). One may therefore wonder which combination of mechanisms is responsible for ROS production in specific signaling cascades among all the ROS-generating cellular mechanisms. Although many questions still remain unanswered, the *AtrbohC*, *AtrbohD*, and *AtrbohF* NOX genes have been shown to function in plant development, defense signaling, and ABA signaling (Foreman et al. 2003; Monshausen et al. 2007). Cellular ROS levels are enhanced by abscisic acid (ABA) in *Arabidopsis* guard cells (Pei et al. 2000). Furthermore, ABA increases H_2O_2 levels in maize embryos and seedlings and in *Vicia* guard cells, a process that precedes stomatal closure (Guan et al. 2000; Zhang et al. 2001; Jiang and Zhang 2002, 2003). These observations further support a role for ROS in ABA signaling.

ROS Regulation of Ion Channels in Guard Cells

Guard cells undergo a large change in volume in response to environmental cues while the stomata are closing. This closure involves the activation and inactivation of ion channels in the plasma membrane or endomembrane for influx and efflux of K^+, Cl^-, Ca^{2+}, and malate^{2-} (Schroeder et al. 2001). ROS trigger cytosolic calcium transients (McAinsh et al. 1996; Allen et al. 2000). These fluctuations in $[Ca^{2+}]_{cyt}$ result from calcium release from intracellular Ca^{2+}stores and by calcium influx across the plasma membrane. ROS also induce stomatal closure (Lee et al. 1999; Pei et al. 2000; Zhang et al. 2001; Kwak et al. 2003). Hyperpolarization-activated plasma membrane channels have been identified in the guard cells of *Arabidopsis* and *Vicia* (Hamilton et al. 2000; Pei et al. 2000). These Ca^{2+}-permeable channels are activated by ABA (Hamilton et al. 2000; Pei et al. 2000; Murata et al. 2001) and hydrogen peroxide (Pei et al. 2000; Murata et al. 2001). Interestingly, H_2O_2 activation of the plasma membrane Ca^{2+}-permeable channels and stomatal closure in response to H_2O_2 and ABA are impaired in the ABA-insensitive *gca2* mutant, suggesting that *GCA2* functions upstream of the plasma membrane Ca^{2+}-permeable channels in ROS-mediated ABA signaling in guard cells (Pei et al. 2000). It remains unknown what protein is encoded by *GCA2*.

Two mutations, *abi1* and *abi2*, act as negative regulators working upstream or close to ABA-induced $[Ca^{2+}]_{cyt}$ increases in early ABA signaling events in guard cells (Allen et al. 1999). However, it was unclear exactly where these *PP2C* mutants act and whether they affect ABA activation of I_{Ca} channels. By analyzing stomatal movements, anion channel activation, and ABA-induced ROS production in guard cells, Murata et al. (2001) have provided the relative locations of *abi1-1* and *abi2-1* type 2C protein phosphatases in guard cell ABA signaling. In the *abi1-1* mutant, ABA activation of the plasma membrane Ca^{2+}-permeable channels is disrupted, whereas H_2O_2 activation of those Ca^{2+} channels and H_2O_2-induced stomatal closure is not disturbed, suggesting that *abi1-1* works upstream of ROS in guard cell ABA signaling (Murata et al. 2001). The *abi2-1* mutation also interferes with ABA activation of the Ca^{2+}-permeable channel and, in contrast to *abi1-1*, disrupts both H_2O_2 activation of the Ca^{2+} channels and H_2O_2-induced stomatal closure, suggesting that *abi2-1* functions downstream of ROS (Allen et al. 1999).

Many cellular mechanisms are responsible for ROS generation in plants (Apel and Hirt 2004). It had remained unknown which one is utilized to produce ROS in response to ABA in guard cells. Kwak et al. (2003) have identified mutations in two of the ten NADPH oxidase (NOX) catalytic subunit genes in *Arabidopsis*—*AtrbohD* and *AtrbohF*—that abolish ABA-induced stomatal closure, ABA promotion of ROS production, ABA-induced cytosolic Ca^{2+} increases, and ABA activation of the plasma membrane Ca^{2+}-permeable channels, thereby demonstrating that these two NOXs are sources for ABA-triggered ROS production in guard cells. *AtrbohD* and *AtrbohF* also function in plant defense signaling and methyl jasmonic acid signaling in guard cells (Torres et al. 2002, 2005; Suhita et al. 2004). Another NOX gene, *AtrbohC*, has been shown to be required for ROS production to mediate root hair growth and polarized cell expansion (Foreman et al. 2003; Monshausen et al. 2007). These studies indicate that NOX proteins play a central role in various cellular responses in plants by producing ROS in response to stimuli.

ABA also increases cytosolic calcium (Allen and Sanders 1994; Grabov and Blatt 1998; Allen et al. 2001), which results in the activation of two different types of anion channels: slow-activating sustained (S-type) and rapid transient (R-type) (Hedrich et al. 1990; Schroeder

and Hagiwara 1990). The activation of both anion channels induces anion release to the apoplasts from guard cells and depolarizes membrane potential (Roelfsema et al. 2004; Roelfsema and Hedrich 2005). Although anion channels are an essential component of guard cell ABA signaling, their molecular nature had remained unknown until *SLAC1* was identified (Negi et al. 2008; Vahisalu et al. 2008). SLAC1, containing ten predicted transmembrane domains, is localized to the plasma membrane (Vahisalu et al. 2008). It shows distant similarity to fungal and bacterial dicarboxylate/malic acid transport proteins (Vahisalu et al. 2008). Null mutations in *SLAC1* have impaired stomatal responses to CO_2, ABA, ozone, changes in humidity, Ca^{2+}, light–dark transition, H_2O_2, and NO (Negi et al. 2008; Vahisalu et al. 2008). Furthermore, ABA and Ca^{2+} activation of the S-type anion channels are abolished in the *slac1* mutant, whereas R-type anion channel activity remains functional, suggesting that *SLAC1* encodes a subunit of the S-type anion channels (Negi et al. 2008; Vahisalu et al. 2008).

ABA inhibits light-induced stomatal opening by inhibiting the plasma membrane H^+-ATPases and inward-rectifying K^+ channels that play a central role in that process (Schroeder et al. 1987; Schroeder and Hagiwara 1990; Goh et al. 1996; Pilot et al. 2001; Merlot et al. 2007). ROS produced by ABA inhibit plasma membrane H^+-ATPase via dephosphorylation of H^+-ATPase and subsequent binding of 14-3-3 proteins (Zhang et al. 2004b). Furthermore, the prevention of blue light-dependent H^+-pumping by ABA is restored by the addition of ascorbate, implying that ROS positively regulate ABA inhibition of stomatal opening (Zhang et al. 2004b).

Decreases in membrane potential through the activation of anion channels induce the inactivation of inward-rectifying K^+ channels and the activation of outward-rectifying K^+ channels, resulting in K^+ efflux from the guard cells (Schroeder et al. 1987). This continuous efflux of both anions and K^+ contributes to a loss in turgor, which leads to stomatal closing (Schroeder et al. 2001). The inward-rectifying K^+ channel protein KAT1 is localized to the plasma membrane of guard cells and induces the influx of potassium ions from apoplasts to the cytoplasm during stomatal opening (Schachtman et al. 1992; Sutter et al. 2006, 2007). ABA triggers the internalization of KAT1 from the plasma membrane to the cytoplasm in *Vicia* guard cells, indicating that ABA regulates KAT1 channel activity as well by modulating the number of proteins at the plasma membrane (Sutter et al. 2007). ROS generated by *RHD2/AtrbohC* trigger a Ca^{2+} influx into the cytoplasm, which induces endocytosis in the root hairs during their development (Takeda et al. 2008). However, it remains unknown whether ROS regulate endocytosis of the potassium channel proteins in response to ABA in guard cells.

ROS and Ca^{2+} in Other Signaling Pathways

ROS function in polarized tip growth by activating Ca^{2+}-permeable channels, which leads to tip-focused Ca^{2+} influx in *Fucus* rhizoid cells (Coelho et al. 2002). Moreover, in growing root hairs and pollen tubes, calcium influx occurs at the tips, resulting in polarized tip growth (Pierson et al. 1996; Bibikova et al. 1997; Wymer et al. 1997). This influx is mediated by the activation of Ca^{2+}-permeable channels by ROS (Demidchik et al. 2003; Foreman et al. 2003). In *rhd2/atrbohC* root hair cells, the tip-focused Ca^{2+} gradient is destroyed, indicating that ROS are required to induce tip-focused Ca^{2+} influx mediated by Ca^{2+}-permeable channels (Foreman et al. 2003). Plant NOXs contain Ca^{2+}-binding EF hands (Torres et al. 1998; Bedard et al. 2007). NOX activity is increased by calcium (Sagi and Fluhr 2001; Ogasawara et al. 2008). Furthermore, when HEK293T cells expressing *RHD2* are treated with an ionophore that causes Ca^{2+} influx, ROS production is enhanced (Takeda et al. 2008). Mutation of the EF hands in RHD2 reduces the effect of ionomycin in HEK293T cells and *Arabidopsis* (Takeda et al. 2008), suggesting a feedback regulatory loop between NOXs and cytosolic Ca^{2+}.

Protein Kinases and Phosphatases Working Upstream and Downstream of ROS in ABA Signaling

Pharmacological studies have suggested that protein de/phosphorylation plays an important role in regulating signaling cascades in response to ABA and H_2O_2 in guard cells (Schmidt et al. 1995; Mori and Muto 1997). The *Arabidopsis* genome has 76 type-2C protein phosphatase (PP2C) genes (Schweighofer et al. 2004). One of the subgroups includes ABI1, ABI2, HAB1, and PP2CA, all of which function in ABA signaling (Meyer et al. 1994; Leung et al. 1997; Saez et al. 2004; Schweighofer et al. 2004; Kuhn et al. 2006). Genetic research with *abi1-1* and *abi2-1* has shown that ABI1 and ABI2 act as negative regulators of ABA signaling (Armstrong et al. 1995; Sheen 1998; Gosti et al. 1999; Merlot et al. 2001). Localization of abi1-1 protein is required for the ABA-insensitive response of the *abi1-1* mutant, suggesting a possible target protein for abi1-1 in the nucleus (Moes et al. 2008). ABI1 interacts with AtGPX3, ATHB6, PLDα1, and ATK3 (Vranova et al. 2001; Himmelbach et al. 2002; Ohta et al. 2003; Zhang et al. 2004a; Miao et al. 2006). ABI2 physically interacts with the PKS3 protein kinase and the SOS2 protein kinase, which is disrupted by the *abi2-1* mutation (Guo et al. 2002; Ohta et al. 2003). Both ABI1 and ABI2 physically interact with the glutathione peroxidases AtGPX3, which regulate the redox state of guard cells (Miao et al. 2006). ABI1 binds to phosphatidic acid (PA), generated by phospholipase D

(PLDα1), and its interaction promotes stomatal closure (Zhang et al. 2004a). PLDα1 also interacts with GPA1, the Gα subunit of a heterotrimeric GTP-binding protein, and this interaction mediates ABA inhibition of stomatal opening (Zhao and Wang 2004). These results suggest that PLDα1 functions bifurcate at *ABI1* and *GPA1* to mediate ABA signaling on both stomatal closure and opening (Mishra et al. 2006).

Two additional PP2Cs, HAB1/AtP2C-HA and PP2CA, have been identified as negative regulators of ABA signaling (Saez et al. 2004). They function in ABA regulation of seed germination, root elongation, stomatal closure, and/or gene expression (Leonhardt et al. 2004; Saez et al. 2004; Kuhn et al. 2006; Yoshida et al. 2006b). Expression of *HAB1* is up-regulated by ABA; a recessive *hab1* mutant shows ABA-hypersensitive inhibition of germination (Saez et al. 2004). Overexpression of *HAB1* impairs stomatal closure, promotes ABA-tolerance in root growth, and diminishes ABA-induced gene expression (Saez et al. 2004, 2006). SWI3B, a homolog of the yeast SWI3 subunit of the SWI/SNF chromatin-remodeling complex, is an interacting partner of HAB1 (Saez et al. 2008). The *swi3b* mutants show an insensitive ABA response in seed germination and reduced *RAB18* and *RD29B* expression (Saez et al. 2008). *ABA-hypersensitive germination3* (*ahg3*) encodes PP2CA (Yoshida et al. 2006b). Another T-DNA insertion mutant, *pp2ca-1*, has increased sensitivity to ABA in seed germination and stomatal movements, whereas overexpression of *PP2CA* impairs ABA-induced stomatal closure (Kuhn et al. 2006). Yet, it has not been tested whether these PP2Cs function downstream or upstream of ROS in ABA signaling.

Okadaic acid, an inhibitor of type-1 and type-2A protein phosphatase (PP1 and PP2A), enhances ABA-induced stomatal closure in fava bean (Schmidt et al. 1995) but reduces such closure in *Arabidopsis* (Pei et al. 1997). This suggests that PP1 and/or PP2A function as both negative and positive regulators of ABA signaling. Disruption of the PP2A regulatory A subunit *RCN1* confers ABA insensitivity in seed germination, stomatal closure, ABA-activation of anion channels, and ABA-induced cytosolic calcium increases in *Arabidopsis*, implying that *RCN1* is a positive transducer of ABA signaling (Kwak et al. 2002). Hydrogen peroxide promotes stomatal closure in the *rcn1* mutant, indicating that RCN1 may act upstream of ROS in guard cells (Kwak et al. 2002).

A guard cell-specific ABA-activated protein kinase (AAPK) from *Vicia faba* positively regulates ABA-induced stomatal closure by inhibiting plasma membrane anion channels (Li et al. 2000). The *Arabidopsis* AAPK ortholog OST1 has been identified from a genetic screen via infrared thermal imaging (Mustilli et al. 2002). ABA, but not H_2O_2, fails to trigger stomatal closure, and ABA-induced ROS production is disrupted in *ost1*, indicating that OST1 acts upstream of ROS production in guard cell ABA signaling (Mustilli et al. 2002). Moreover, OST1/SRK2E physically interacts with ABI1, and ABA activation of OST1/SRK2E is blocked in *abi1-1* but not *abi2-1*, suggesting that abi1-1 works upstream of OST1 (Yoshida et al. 2006a).

Pharmacological studies have suggested a role for MAP kinase in ABA signaling in guard cells (Burnett et al. 2000; MacRobbie and Kurup 2007; Jiang et al. 2008). The MAPK kinase inhibitor PD98059 and the MAP kinase inhibitor SB203580 partially block ABA-induced H_2O_2 generation and stomatal closure and reduce ion efflux in epidermal peels of *V. faba* (MacRobbie and Kurup 2007; Jiang et al. 2008). However, no specific MAPK kinase genes controlling stomatal movements have yet been identified. In *Arabidopsis*, *MPK3* regulates ABA-responsive genes and is activated by ABA and hydrogen peroxide (Lu et al. 2002). Gudesblat et al. (2007) have taken an antisense approach to show that diminished *MPK3* expression results in partial insensitivity to ABA in the inhibition of stomatal opening and a reduced sensitivity to exogenous hydrogen peroxide, indicating that MPK3 acts downstream of H_2O_2.

Two calcium-dependent protein kinases, *CPK3* and *CPK6*, function positively in ABA- and Ca^{2+}-induced stomatal closure (Mori et al. 2006). Furthermore, activation of both S-type anion channels and plasma membrane Ca^{2+}-permeable channels by ABA is disrupted in guard cells of *cpk3cpk6* double mutants (Mori et al. 2006). Two recent studies have shown that plant NADPH oxidases are also phosphorylated by protein kinases. Biochemical and transient-expression research has demonstrated that the expression of a constitutive Ca^{2+}-dependent protein kinase elicits ROS production, and phosphorylation by StCDPK5 is necessary for StrbohB activation (Kobayashi et al. 2007). A phosphoproteomics investigation has revealed that phosphorylation of two serine residues in the N-terminus is required for AtrbohD activation (Nuhse et al. 2007). Together, these results imply that CDPKs are likely to contribute to guard cell ABA signaling by regulating guard cell NADPH oxidases and Ca^{2+}-permeable channels. Furthermore, the histidine kinase AHK5 functions in stomatal movements; an *ahk5* null mutation leads to reduced stomatal closure in response to H_2O_2 (Desikan et al. 2008).

Figure 1 presents various molecular elements and their regulatory mechanisms in ROS-mediated ABA signaling in guard cells.

ROS Scavengers and ROS Homeostasis

The extent of oxidative stress in a cell is determined by the amounts of superoxide, hydrogen peroxide, and hydroxyl

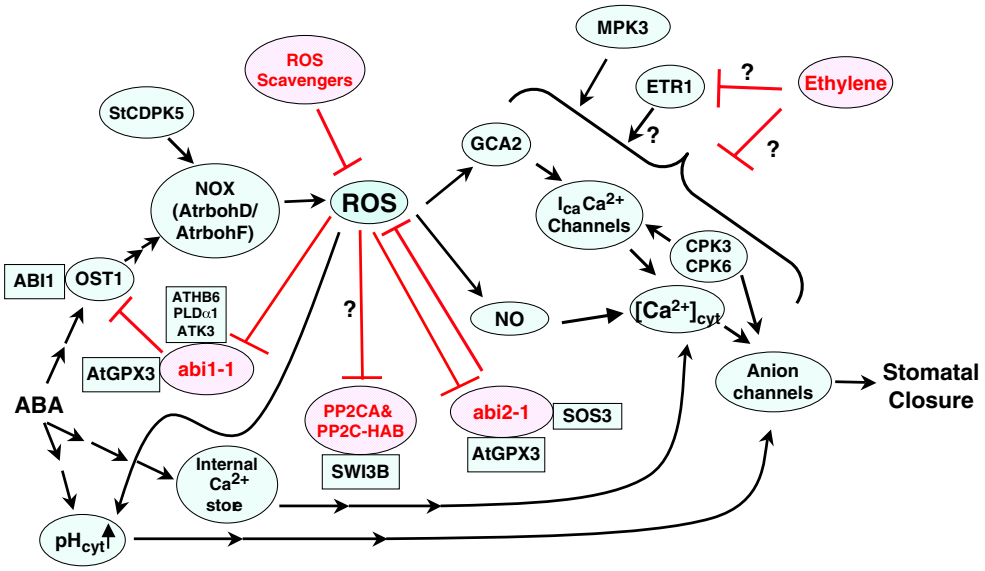

Fig. 1 Current working model for ROS-mediated ABA signaling in guard cells. Positive regulators are shown in *blue*, negative regulators in *red*. *Pointed arrows* indicate activation, *blunted arrows* inhibition. Protein–protein interactions are shown by direct contact between signaling elements. Please note that not all experimentally examined links and interactions are shown in this simplified model

radicals that are produced by different cellular mechanisms in response to endogenous and environmental cues (Apel and Hirt 2004). Plant cells have well-developed defense systems against ROS and are able to remove them through non-enzymatic and enzymatic antioxidant processes. The non-enzymatic process involves antioxidants such as ascorbate, glutathione, tocopherol, carotenoids, and flavonoids, which are important cellular redox buffers (Conklin et al. 1996; Apel and Hirt 2004). The major ROS-scavenging enzymes in plants consist of superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione peroxidase (GPX), and peroxiredoxin (PrxR) (Apel and Hirt 2004; Mittler et al. 2004).

SOD catalyzes the dismutation of superoxide to hydrogen peroxide and molecular oxygen. These SODs are categorized into three main groups, based on their metal cofactor. Cu/Zn SODs, with copper and zinc, are mainly localized to the cytosol and chloroplasts. Mn SOD possesses manganese as its cofactor and is localized to the mitochondria and peroxisomes (Alscher et al. 2002). Fe SODs are found predominantly in the chloroplasts. *Arabidopsis thaliana* has three Cu/Zn SOD isoforms, one Mn SOD and three Fe SOD isoforms (Alscher et al. 2002). Hydrogen peroxide is removed by a number of different peroxidase enzymes or cycles: catalase, the ascorbate-glutathione cycle, and/or the glutathione peroxidase cycle. Catalases (CATs) are heme-containing tetrameric enzymes that catalyze hydrogen peroxide to water and oxygen (Apel and Hirt 2004). Plant catalases are involved in photorespiratory functions and the scavenging of hydrogen peroxide during β-oxidation of fatty acids in germinating seeds (McClung 1997). In *Arabidopsis*, three CAT isozymes are found, mainly in peroxisomes (McClung 1997). Ascorbate peroxidase performs the same general function as catalase. However, unlike CAT, APX utilizes ascorbate as its specific donor to reduce hydrogen peroxide to water, with the concomitant generation of monodehydroascorbate (Shigeoka et al. 2002). GPXs catalyze the reduction of H_2O_2, organic hydroperoxides, and lipid peroxides using GSH and/or other reduction equivalents (Mittler et al. 2004).

Specific roles for antioxidant enzymes have been explored via genetic approaches. Expression of a pea *Cu/ZnSOD* in tobacco leads to a significant increase in resistance to methyl viologen (MV)-induced damage, and *MnSOD* overexpression in tobacco reduces such damage (Bowler et al. 1991; Gupta et al. 1993). However, the overexpression of tobacco *Cu/ZnSOD* in tobacco and tomato is not sufficient to provide tolerance toward oxidative stress (Allen 1995). These results suggest that SODs act as the first line of defense against superoxide by converting it to H_2O_2 (McKersie et al. 1993; Allen 1995). Transgenic *Arabidopsis* plants expressing antisense transcripts of c*APX* and *CAT1* are hypersensitive to oxidative stress induced by pathogen infection (Mittler et al. 1999). A T-DNA insertion mutation in *APX1* results in reduced photosynthetic activity and enhanced stomatal opening due to greater levels of hydrogen peroxide (Pnueli et al. 2003). Furthermore, levels of transcripts encoding different calcium-binding proteins and calmodulin-like proteins are increased in *Apx1*-deficient *Arabidopsis* plants, suggesting crosstalk between ROS and calcium signaling (Pnueli et al. 2003). Transcription levels of antioxidant genes *CAT1*, *cAPX*, and *GR1* and the total activity of these enzymes are elevated by ABA, whereas pre-treatment with the MAPK kinase inhibitors PD98059 and U0126 significantly blocks expression and the total enzymatic activities induced by ABA. This suggests that a MAPK cascade functions in gene regulation (Zhang et al. 2006). *CAT1* expression is up-regulated in response to ABA and H_2O_2 treatments via AtMKK1–AtMPK6 to down-regulate ROS levels in *Arabi-*

dopsis. This may therefore support the notion of negative feedback regulation in ROS production triggered by ABA (Xing et al. 2008). A null mutation in *AtGPX3* impairs ABA- and H_2O_2-regulated stomatal closure (Miao et al. 2006). Moreover, the knockout and overexpression of *AtGPX3* confers reduced and enhanced drought tolerance, respectively, indicating a role for ROS in that stress response (Miao et al. 2006).

Regulation of Gene Expression by ROS

Vast research has been conducted on ROS-controlled gene expression that modulates plant development, growth, hormonal signaling, and defense (Apel and Hirt 2004; Miller et al. 2008). Expression profiling is used to obtain insights into how ROS signal transduction leads to the regulation of expression networks. Desikan et al. (2001) have hybridized an *Arabidopsis* cDNA microarray representing 11,000 genes with RNA extracted from cultured cells that were treated with 20 mM H_2O_2. There, 113 genes are up-regulated, while 62 are down-regulated by the hydrogen peroxide treatment. Interestingly, some of those regulated genes have similar expression patterns when plants are treated with ROS-inducing stresses, such as wilting, UV, or harpin. This suggests that these environmental cues may utilize ROS as a second messenger to control gene expression and, thus, cellular processes.

Wang et al. (2006) have conducted hybridization of Affymetrix ATH1 chips with RNA extracted from *Arabidopsis* seedlings that were treated with 100 μM ABA or 10 mM H_2O_2. They have shown that 459 genes are up-regulated, while 221 are down-regulated by the hydrogen peroxide application. Furthermore, transcription levels of 391 genes are elevated, and 322 are repressed by ABA. Of these ABA- and H_2O_2-regulated genes, 143 and 75, respectively, are up- or down-regulated by both treatments.

More detailed information on ROS-controlled gene expression has come from analyses by Gadjev et al. (2006) in nine independent transcriptome experiments that also included publicly available data. These transcriptome analyses entailed datasets from various ROS-scavenging knockdown or knockout plants treated with exogenously applied ROS-generating chemicals. Their overall analyses of integrated data have shown that 8,056, 5,312, and 3,925 genes are up-regulated by three-, four-, or five-fold, respectively. Among them are defensin-like proteins, unknown proteins, and Toll-interleukin-1 class disease resistance proteins that were highly induced by ROS in most experiments, with at least a five-fold increase in transcript levels. Thus, these highly up-regulated genes may serve as marker transcripts for ROS-induced gene expression (Gadjev et al. 2006).

The functional redundancy in plant ROS-scavenging genes has been investigated through a combination of transcriptome analysis and antisense transgenic plants or knockout mutants (Mittler et al. 2004). Enzymes controlling ROS homeostasis are negatively or positively regulated by ROS levels and also contribute to ROS-regulated gene expression. The expression pattern of maize *CAT1* genes has been characterized by analyzing *cis*- and *trans*-elements binding to the *CAT1* promoter; ABA induces up-regulation of *CAT1* gene expression and ROS production (Guan et al. 2000). A transcriptome analysis study of *Arabidopsis* antisense transgenic plants with variously decreased CAT2 activity levels has shown that CAT2 contributes to the regulation of gene expression when transient H_2O_2 production is achieved through photorespiration under high-light conditions (Vandenabeele et al. 2004). The *CAT2* antisense transgenic plants are more sensitive than the wild type to increased H_2O_2 accumulation, suggesting that this gene plays a central role in scavenging hydrogen peroxide. This transcriptome analysis has revealed that ROS regulate various antioxidant, defense-related, and cell death-related genes (Vandenabeele et al. 2004).

Transcriptome and promoter analyses in ozone-treated plants have elucidated the diverse cellular functions of ROS (Mahalingam et al. 2006). Promoter elements found in ROS-regulated genes include phytohormone-, defense-, and stress-responsive elements (Mahalingam et al. 2006). Another promoter study has examined ten different sets of microarray data and identified novel *cis*-acting elements for ROS and sucrose (Geisler et al. 2006). Altogether, these expression analyses suggest that ROS regulate gene expression in a variety of cellular signaling cascades and have a broad range of roles as signal molecules in plants. They also show that multiple *cis*-acting elements are present in the promoters of genes responsive to ROS, further supporting roles in many cellular processes. These properties of ROS-regulated gene expression may contribute to the capacity of plants to cope under variable environmental conditions.

Redox-sensitive transcription factors such, as OxyR in bacteria and Yap1 in yeast, undergo conformational changes when exposed to ROS, resulting in the induction of defense genes (Georgiou 2002). A similar mechanism exists in plants. NPR1 is an important transcription cofactor for systemic acquired resistance (SAR; Mou et al. 2003; Pieterse and van Loon 2004). In its resting state, NPR1 exists as oligomers through intermolecular disulfide bonds. A more reduced cellular environment promotes the induction of SAR and causes the oligomeric form of NPR1 to change into the monomeric form, which is then transported to the nucleus to activate defense-gene expression (Mou et al. 2003). It would be interesting to examine whether such redox-sensitive transcriptional factors also exist in ROS-mediated ABA signaling.

ROS in Other Hormonal Signaling

ROS also act as second messengers in other plant hormonal signaling. Joo et al. (2001) have shown that gravitropic treatment leads to asymmetric ROS production in the roots, leading to their curvature, whereas an antioxidant application results in impaired gravitropism. Auxin-induced ROS production and the auxin-transporter inhibitor N-1-naphthylphthlamic acid do not block ROS-induced root curvature, indicating that ROS function downstream of auxin transport in such signaling (Joo et al. 2001). ROS levels are enhanced in transgenic rice over-expressing RACK1 (Receptor for Activated C-Kinase 1), which interacts with the small GTP-binding protein OsRac1 (Nakashima et al. 2008). OsRac1 also functions in ROS production that leads to a hypersensitive response and, thus, disease resistance. Furthermore, auxin induces up-regulation of *RACK1*, *CAT1*, *CAT2*, and *CAT3* transcript levels (Guan and Scandalios 2002; Nakashima et al. 2008), which also suggests ROS involvement in auxin signaling.

Ethylene signaling also appears to use ROS. H_2O_2-induced stomatal closure is abolished in *etr1-7*, a loss-of-function mutation of the ethylene receptor, suggesting a role for the ethylene receptor *ETR1* in H_2O_2-induced stomatal closure (Desikan et al. 2005). In contrast, application of either ethylene or its precursor 1-aminocyclopropane-1-carboxylic acid inhibits ABA-induced stomatal closure (Tanaka et al. 2005). Furthermore, ABA-promoted stomatal closure is reduced in the ethylene over-producing mutant *eto-1* and in two ethylene-insensitive mutants, *etr1-1* and *ein3-1* (Tanaka et al. 2005). Auxin and cytokinin partially block ABA-induced stomatal closure by promoting ethylene production (Tanaka et al. 2006). Further, genetic and cellular biological studies are required to provide more detailed information on the roles of ethylene signaling and receptors in H_2O_2- and ABA-induced stomatal closure.

ROS are also used in methyl jasmonate (MJ)-induced stomatal closure. MJ promotes H_2O_2 production in guard cells, causing stomata to close in WT and *ost1-2* plants (Suhita et al. 2004). This indicates that, unlike in ABA signaling, *OST1* does not play a role in MJ signaling in guard cells. Furthermore, MJ-induced stomatal closure is impaired in the NOX *atrbohD/F* double mutant, suggesting that AtRbohD and AtRbohF NOXs are responsible for MJ-triggered ROS production in guard cells (Suhita et al. 2004).

Crosstalk Between ROS and NO in ABA Signaling

Nitric oxide (NO) is a reactive nitrogen molecule that functions in various cellular responses, including host–pathogen interactions, stomatal movements, flower development, and hormonal signaling (Delledonne et al. 1998; Neill et al. 2002a; He et al. 2004; Bright et al. 2006; Yan et al. 2007; Wilson et al. 2008). NO-generating mechanisms are of prime interest in this research field. Animal NO synthase-like proteins in plants have been suggested as NO-producing enzymes based on pharmacological and enzymatic assay results (Crawford 2006). However, because the specificity of NO synthase activity assay is questionable and plant NOS genes do not share high similarity with their animal counterparts, it appears that further studies are required to provide direct genetic evidence that plant NO synthases are responsible for NO production (Zemojtel et al. 2006; Tischner et al. 2007).

Nitrate reductase (NR) might be another enzymatic source of NO in plants (Wilson et al. 2008). Two NR genes in *Arabidopsis*, *NIA1* and *NIA2*, share high sequence homology. ABA fails to induce nitric oxide production and stomatal closure in *nia1/nia2* double mutants, implying that those two genes function in ABA-induced NO generation and signaling (Desikan et al. 2002; Bright et al. 2006). *NIA1* and *NIA2* are expressed in the guard cells and appear to be required for producing NO, as triggered by ABA (Bright et al. 2006). Nitric oxide promotes stomatal closure and ABA promotes the production of NO in wheat (Garcia-Mata and Lamattina 2002). In *Arabidopsis*, the NO donor SNP induces stomatal closure in a dose- and time-dependent manner (Neill et al. 2002b). Because SNP is associated with this closure in many other plants as well, including pea, tomato, and fava bean, NO might be considered a universal signaling molecule (Desikan et al. 2002, 2004; Garcia-Mata and Lamattina 2002).

Several research groups have implied that crosstalk exists between ROS and NO in ABA signaling in guard cells (Neill et al. 2002b; Desikan et al. 2004; Dong et al. 2005; Bright et al. 2006). For example, Bright et al. (2006) have shown that the application of ROS scavengers CAT or ascorbate greatly reduces ABA-induced NO generation and stomatal closure, suggesting that the accumulation of hydrogen peroxide positively regulates NO production and NO-induced stomatal closure. Measurements of the nitric oxide generated upon H_2O_2 treatment in *atnos1* and *nia1*/*nia2* have demonstrated that this process is significantly impaired in *nai1*/*nia2* but not in *atnos1* (Bright et al. 2006). NO also functions in ABA inhibition of stomatal opening in *V. faba* (Yan et al. 2007). The crosstalk between ROS and NO occurs in ABA signaling and programmed cell death (PCD) signaling. In maize mesophyll cells, ABA-induced NO production relies on ABA-induced H_2O_2 and mediates ABA activation of the MAP kinase cascade (Zhang et al. 2007). That cascade seems to be a converging point in the ABA, H_2O_2, and NO signaling networks of plants (Desikan et al. 2004; Zhang et al. 2007; Xing et al. 2008; Zong et al.

2009). NO also induces PCD with the aid of ROS in soybean cell culture and tobacco BY-2 cells (Delledonne et al. 2001; de Pinto et al. 2002). Further investigations of *NOS*-like and *NR* genes should shed more light on NO signaling and crosstalk with ROS in ABA signaling and other signaling cascades.

Concluding Remarks

These exciting, recent findings clearly demonstrate that ROS are a central part of the signaling cascades in various cellular processes in plants. One question still remaining is whether intracellular localization of ROS production has a role in the specificity and efficiency of ROS-mediated signaling. NOXs are the major ROS source in many signaling cascades. Although plant NOX proteins have been reported to be localized to the plasma membrane, some are also found in the ER and the nucleus in animal cells (Li and Shah 2002; Ambasta et al. 2004; Van Buul et al. 2005; Ushio-Fukai 2006). Therefore, it would be interesting to examine whether plant NOXs are also present in these sub-organelles and if this subcellular localization of NOX-produced ROS contributes to specificity for cellular responses mediated by ROS. A few cytosolic factors that positively regulate NOX activity and, thus, ROS production have been identified, leaving the negative regulators of NOX yet to be described. Although ABI1 and ABI2 protein phosphatases are negatively regulated in vitro by ROS, the in vivo ROS target proteins await discovery. Combined genetic, biochemical, and cellular approaches with real-time ROS imaging in living cells (Dooley et al. 2004; Hanson et al. 2004; Monshausen et al. 2007) should provide further insights into ROS-mediated cellular signaling.

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