Oxidative Stress: Antagonistic Signaling for Acclimation or Cell Death?[1]

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Severe environmental stress imposed on plant tissues induces changes in oxygen (O_2) metabolism that cause oxidative stress. Oxidative stress occurs when reactive oxygen species (ROS) are not rapidly scavenged and the rate of repair of damaged cell components fails to keep pace with the rate of damage. If this situation persists, irreversible damage results in a loss of physiological competence and eventual cell death. However, ROS production in leaves resulting from moderate environmental stresses, within the adaptive range of the plant, also has important local and systemic signaling roles. In these circumstances, ROS production induces defense mechanisms that protect the plant but do not result in oxidative stress. We shall illustrate ROS involvement in signaling in both of these situations by considering the role of chloroplasts in initiating cellular responses to environmental perturbations, choosing examples where this organelle interacts with specific signaling pathways.

It is perhaps helpful to use a stress-strain response diagram, commonly used in mechanics, to illustrate the possible relationships between increasing imposition of environmental stress, physiological perturbations, ROS production, oxidative stress, and cell death (Fig. 1). Moderate environmental stresses on leaves can result in increased rates of ROS production and physiological changes that are reversible when the stress is removed. With increasing stress, the rate of ROS production increases and oxidative stress and irreversible damage occur, which, if sufficiently great, eventually lead to cell death. Identification of the factors that set the threshold at which a cell or tissue makes the transition from successful acclimation/resistance to oxidative stress-induced cell death is critical. However, such outcomes should not be regarded as a success (for acclimation) or a failure (for death). This may be so from a cellular perspective, but at the level of the organ or organism the processes of cell death and acclimation are inextricably linked, and both are essential for a successful response to environmental change. This may explain why many studies show that oxidative stress-induced cell death is under genetic control and not simply a consequence of ROS toxicity. Plant cells have evolved the ability to actively move up or down the curve shown in Figure 1 by regulating the balance between acclimation and cell death responses.

WHEN DOES OXIDATIVE STRESS SIGNALING OCCUR?

There are many studies of signaling networks in plants in which ROS production has been elicited or ROS have been applied. Various stresses, which elicit sufficient ROS production to cause oxidative stress and cell death, can lead to a very similar foliar pathology (e.g. chlorosis, lesion formation) and the induction of similar sets of genes. These include diverse treatments such as challenges with pathogen-derived elicitors, exposure to high chronic levels of ozone, exposure to excess light, and induction of singlet oxygen ($^1O_2$) production by photodynamic dyes or in the Arabidopsis (*Arabidopsis thaliana*) *fluorescence1* (*flu1*) mutant. In all of these cases, chloroplasts can be argued to play a prominent role in signaling. Comparisons of such treatments with those less likely to have produced any oxidative stress but that cause alteration in ROS metabolism indicate a much lower overlap of responsive genes common to all treatments (Gadjev et al., 2006). Some of this lack of commonality is likely to be due to signaling initiated by specific ROS, but this is hard to discern when key information on cellular physiological states is lacking. The signaling in cells producing ROS but not suffering oxidative stress may be quite different from that in cells suffering oxidative stress and consequent cell death.

THRESHOLDS FOR SIGNALING FOR DEATH OR ACCLIMATION

In those experimental systems studied in sufficient detail, cell death induced by oxidative stress is nearly always under genetic control. There is evidence that control of this response includes the opposing action of pro- and anti-cell death signaling. The latter is linked to the induction of a range of defense-associated genes, including those coding for the antioxidant network. The balance between these opposing systems makes for highly sensitive and dynamic systems of response, which can be influenced by ROS produced in response to additional external or internal stimuli. Two examples associated with chloroplast-sourced oxidative stress responses, involving signaling initi-

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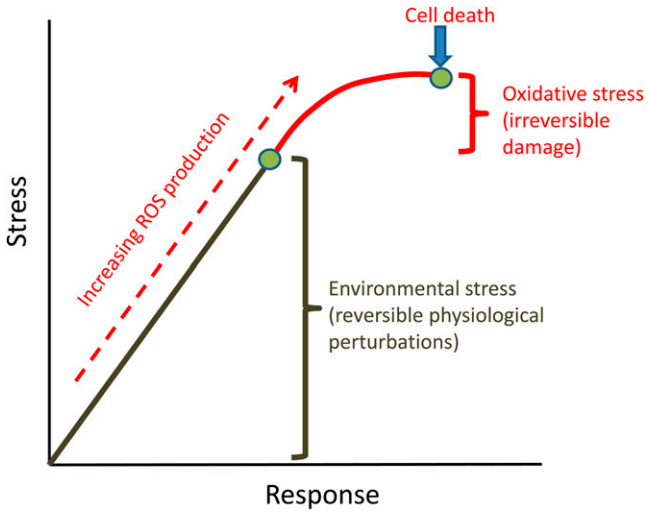

Figure 1. Model for the response of a plant system to the application of increasing stress in the context of ROS production, oxidative stress, and cell death.

ated by $^1O_2$ and superoxide anion ($O_2^{\cdot -}$), are described below.

$^1O_2$ Signaling

$^1O_2$ is highly reactive and promotes rapid photooxidative stress. $^1O_2$ generation is associated with production of lipid (hydro)peroxide radicals, which can initiate signaling and propagation of cellular damage (Triantaphylidés et al., 2008). However, cell death primarily associated with $^1O_2$-induced oxidative stress has been shown to be under genetic control and to initiate specific signaling pathways (Wagner et al., 2004). Damaging levels of $^1O_2$ are produced in response to excess excitation of PSII when photosynthetic metabolism is drastically diminished by stress or inhibitors. In such situations, excitation energy is not quenched sufficiently rapidly in PSII by reaction center photochemistry or carotenoids, and this can result in an increase in the activity of pro- and anti-cell death pathways.

Most of the information on the genetic control of $^1O_2$ signaling and cell death has so far come from studying *flu1*. $^1O_2$ production can be manipulated in *flu1* by altering the degree of light exposure and the preceding dark period. This means that in *flu1*, cell death can be due either to direct overwhelming ROS-induced necrosis or, with a lesser rate of production, activation of a cell death signaling pathway. This mutant generates $^1O_2$ in the vicinity of the thylakoid membrane (Przybyla et al., 2008), but it must be noted that, unlike in wild-type plants, $^1O_2$ production is not associated with excess excitation of PSII. The activation of cell death signaling in *flu1* is controlled by two chloroplast-located proteins, EXECUTER1 (EXE1) and EXE2 (Wagner et al., 2004; Lee et al., 2007; Przybyla et al., 2008). In wild-type plants treated with 3-(3,4-dichlorophenyl)-1,1-dimethylurea, an inhibitor of oxidation of the primary quinone electron acceptor of PSII and, consequently, photosynthetic electron transport, the production of $^1O_2$ in PSII reaction centers is increased, and in these circumstances, the EXE1/EXE2 pathway promotes cell death (Wagner et al., 2004). The presence of an antagonistic anti-cell death system, involving signaling by the ROS hydrogen peroxide (H_2O_2), has been demonstrated that counteracts the EXE1/EXE2 pro-cell death pathway (Laloi et al., 2007). The H_2O_2 anti-cell death pathway may control the capacity of the cell to quench $^1O_2$ signaling by regulating lipid-soluble antioxidant levels and control of the repair of photodamaged D1 protein, a component of the PSII reaction center.

$^1O_2$ generation also activates jasmonic acid- and salicylic acid (SA)-directed signaling pathways that control the expression of many defense-associated genes but are not part of the EXE1/EXE2 pathway. These observations may reflect a convergence of ROS signaling pathways from the chloroplasts that feed into another dynamic antagonistically regulated system that is now considered.

$O_2^{\cdot -}$ Signaling

Under excess light conditions that would produce a strong burst of $^1O_2$ and other ROS in the chloroplast, there is now good evidence of the activation of SA- and ethylene-induced defenses that confer resistance to biotrophic pathogens and are associated with ROS-induced local lesions, similar to those produced in the hypersensitive response (HR) to avirulent biotrophic pathogens (Mühlenbock et al., 2008; Straus et al., 2010). One of the key genes emerging from such studies is *ENHANCED DISEASE SUSCEPTIBILITY1* (*EDS1*), which plays a role in development of the HR and mediates EXE1/EXE2 $^1O_2$-regulated cell death (Ochsenbein et al., 2006). It is becoming clear that EDS1 plays a pivotal role in a mutually antagonistic system, integrating ROS signals from chloroplasts in cells suffering photooxidative stress (Straus et al., 2010). In plants treated with methyl viologen (paraquat), oxidative stress is associated with increased production of $O_2^{\cdot -}$ and predominant induction of a suite of $O_2^{\cdot -}$-responsive genes. EDS1 antagonizes the protective effect of the *NUDIX HYDROLASE7* (*NUDT7*)-encoded pyrophosphohydrolase (Straus et al., 2010). NUDT7 acts by promoting poly-ADP Rib-controlled regulation of antioxidant and general cellular defenses and raises the threshold at which EDS1 can trigger cell death. To tip the cell toward death may require additional production of ROS (principally $O_2^{\cdot -}$), which can come from plasma membrane-located NADPH oxidases or other environmental sources, such as from ozone. The balance between the accumulation of specific ROS and their scavenging by enhanced antioxidant defenses is controlled by EDS1 and NUDT7 and decides the fate of the cell by pushing it toward death

or toward resistance. Very few details of linking steps in these pathways have been identified, but there is strong evidence for the involvement of mitogen-activated protein kinases in transducing signals derived from chloroplast-sourced ROS (Liu et al., 2007).

In the chloroplast, a signaling source of $O_2^{\cdot -}$ under natural conditions has been argued to be the Mehler reaction (Fryer et al., 2003). In algae, there is good evidence that the Mehler reaction serves to dissipate significant levels of excess excitation energy (Waring et al., 2010). However, in higher plants, its role as a major sink for the dissipation of excess excitation energy has been questioned (Badger et al., 2000), but this does not exclude that the Mehler reaction activity that is present serves to initiate ROS signaling.

ROS SIGNALING IN CELLS NEIGHBORING CELL DEATH LESIONS

EDS1, in response to chloroplast-sourced ROS, also activates SA and other hormone-controlled signaling, which produces intercellular signal(s) that stimulate a further burst of ROS, leading to cell death and the development of lesions (Mühlenbock et al., 2008; Straus et al., 2010). However, at some point, the spread of lesions is contained and the signaling changes to produce a response that pushes the cell into defense mode. This raises the threshold beyond which cell death can occur and instead triggers resistance or acclimation. This signaling is associated with the most mobile and least reactive of the ROS, H_2O_2.

In animal cells, rapid and highly localized accumulation of H_2O_2, in excess of 100 μM, at the cytosol side of the plasma membrane is important for the response to external stimuli and motility (Ushio-Fukai, 2006). This H_2O_2 arises as a dismutation product of $O_2^{\cdot -}$, which is generated in a reaction catalyzed by plasma membrane-bound NADPH oxidases. H_2O_2 accumulation is facilitated by transient inactivation of H_2O_2-scavenging peroxiredoxins by phosphorylation. This occurs within subcellular microdomains, and the accumulated H_2O_2, for a short period, inhibits signaling-associated protein Tyr phosphatases (Woo et al., 2010). In plants, the best described source of plasma membrane-associated NADPH oxidase is that encoded by the respiratory burst oxidases (Rbohs) homologous to the $gp91^{phox}$ subunit of mammalian neutrophil NADPH oxidases (Sagi and Fluhr, 2006). In Arabidopsis, AtrbohD has been implicated in the generation of H_2O_2 for intracellular signaling, leading to successful lesion containment and the establishment of enhanced cellular defense against oxidative stress (Torres et al., 2005). SA is involved in this response and may be linked to increased production of glutathione, thus enhancing cellular antioxidant capacity (Mou et al., 2003; Mateo et al., 2006). However, a second regulatory protein, LESION-SIMULATING DISEASE1 (LSD1), also prevents lesion spread and enhances ROS-scavenging capacity by increasing superoxide dismutase and catalase activities (Kliebenstein et al., 1999; Mateo et al., 2004). In cells neighboring the lesion, an alternative EDS1-based antagonistic system between pro-death and anti-death pathways is established. ROS production and cell death are promoted via EDS1 and AtrbohD, while antioxidant defenses that prevent oxidative stress are mediated by LSD1 and SA. The default response favors resistance, in contrast to the EDS1-NUDT7 interaction in the lesion zone, which appears to favor cell death. How signal transduction is mediated from the burst of AtrbohD-catalyzed ROS to signaling pathways is not known, but it could involve glutathione peroxidases, as described for abscisic acid (ABA)-mediated signaling (see below). AtrobhD is also central to localized cell-to-cell signal propagation, presumably by providing apoplastic $O_2^{\cdot -}$, which dismutates in the apoplast to H_2O_2 (Miller et al., 2009). The effect of such localized H_2O_2 signal propagation is to contain the spread of the HR/photooxidative stress-induced lesion by raising the threshold of cell death initiation by boosting antioxidant defenses in adjacent cells. LSD1 is critical for this lesion containment and appears to control the configuration of antioxidant defenses. Current understanding does not explain how a cell finds itself switching between NUDT7-EDS1 and LSD1-EDS1 control to form lesions when the whole leaf has been treated with paraquat or other uniformly applied external stimuli. Presumably, subtle and highly localized cell-to-cell differences in photosynthetic physiology or some form of "quorum" sensing operates that requires some, but not all, cells to die as part of an integrated whole leaf response.

SYSTEMIC SIGNALING

Long-range (systemic) signaling occurs in which leaves or unchallenged parts of plants acquire increased resistance to subsequent environmental challenges from parts of the plant that have been exposed to a stress that provokes lesion formation. For both systemic acquired acclimation to excess light and systemic acquired resistance to biotrophic pathogens, the underlying responses seem to predominantly involve reiteration of the events in challenged leaves, but with much less intensity (Alvarez et al., 1998; Karpinski et al., 1999; Rossel et al., 2007; Mühlenbock et al., 2008). The reduced intensity of the response may represent a raised threshold that pushes cells more into acclimation or resistance and away from cell death at an earlier stage.

ROS SIGNALING WITHOUT OXIDATIVE STRESS

Responses to environmental perturbations that promote ROS production but do not provoke oxidative stress or cell death are common and important in understanding how plants adapt to their environment. However, there is very little information concerning

the underlying signaling responses involving ROS from chloroplasts in such situations. Here, we describe one emerging example.

In bundle sheath cells (BSCs) of leaves exposed to a moderate increase in light intensity and low humidity, ABA signaling is suggested to interact with a chloroplast-sourced H_2O_2 signal to drive the induction of high-light, ABA-responsive genes (Galvez-Valdivieso et al., 2009). Within 30 min of exposure to high light at ambient or lower humidity, ABA biosynthesis is activated in vascular parenchyma cells triggered by a transient lowering of leaf water potential, which is caused by a rapid increase in transpiration. The ABA secreted from vascular parenchyma interacts with BSCs and induces the antioxidant gene *ASCORBATE PEROXIDASE2* (*APX2*), which is expressed only in this tissue. An extant ABA biosynthetic capacity is required for successful physiological adjustment to repeated episodes of increased light (Galvez-Valdivieso et al., 2009). The ABA-mediated control of *APX2* expression occurs via two antagonistic pathways. Positive control is achieved by signaling involving OPEN STOMATA1 (OST1) protein kinase and the ABA-INSENSITIVE1 (ABI1) and ABI2 protein phosphatase 2Cs (Fryer et al., 2003; Galvez-Valdivieso et al., 2009; Galvez-Valdivieso and Mullineaux, 2010). Negative regulation of *APX2* expression occurs via the Gα subunit, GPA1, of the heterotrimeric G protein complex (Galvez-Valdivieso et al., 2009; Galvez-Valdivieso and Mullineaux, 2010).

By analogy with ABA-mediated signaling in guard cells (Cutler et al., 2010), the BSC ABI/OST1 pathway is most likely activated by one or more members of the PYRABACTIN RESISTANCE1 (PYR1)/PYR1-LIKE (PYL)/REGULATORY COMPONENTS OF ABA RECEPTOR1 ABA receptor family (Cutler et al., 2010). The activation of this signaling pathway by ABA binding to PYR/PYL1, which then binds to ABI1/2, consequently inhibits their protein phosphatase activity, leading directly to increased activity of OST1. This brings about phosphorylation of a number of protein substrates, including the ABA-responsive element binding factor transcription factors (Cutler et al., 2010). ABA-responsive element binding factors interact with ABA response cis-elements on the promoters of target genes, including *APX2*.

Whereas ABA signaling in BSCs is necessary for the induction of *APX2*, it is not sufficient per se to account for increased *APX2* expression within 30 min of high-light exposure (Fryer et al., 2003; Galvez-Valdivieso et al., 2009). Therefore, additional signals are required. We propose that H_2O_2, sourced from chloroplasts and plasma membrane-located NADPH oxidases, accelerates ABA signaling in BSCs. Null mutants in chloroplastic APX genes have been used to support the hypothesis that a specific H_2O_2 signal from chloroplasts influences the rate of induction of *APX2* and other high-light-responsive genes (Maruta et al., 2010). Within 10 min of exposure to high light, BSC chloroplasts produce H_2O_2, and this is not associated with irreversible photoinhibition or oxidative stress but is associated with rapid induction of *APX2* expression (Fryer et al., 2003; Galvez-Valdivieso et al., 2009; Maruta et al., 2010). However, H_2O_2 accumulation is contained within BSC chloroplasts (Fryer et al., 2003; Galvez-Valdivieso et al., 2009), suggesting that any H_2O_2 signal has to be transduced to a non-ROS signal to traverse the reducing environment of the cytosol. We suggest that this non-ROS signal is destined to activate AtrbohD and AtrbohF NADPH oxidases at the plasma membrane. The involvement of AtrbohD/F and a strong rapid accumulation of extracellular H_2O_2 in high-light signaling has been observed (Davletova et al., 2005; Bechtold et al., 2008; Galvez-Valdivieso et al., 2009; Miller et al., 2009). As with the involvement of AtrbohD and AtrbohF in ABA signaling in guard cells (Kwak et al., 2003; Miao et al., 2006), we suggest that H_2O_2 produced at the plasma membrane could oxidize glutathione peroxidase isoforms, which in turn would bind to and inhibit ABI1 and ABI2 in BSCs. This would accelerate ABA signaling via increased activity of OST1 kinase activity. This model allows for other environmental stimuli to further activate AtrbohD/F, for example, by wounding. Interestingly, AtrbohF can be phosphorylated by OST1 (Sirichandra et al., 2009), and AtrbohD has to be phosphorylated to participate in ROS production in response to the elicitor flagellin (Nühse et al., 2007). It is possible that the integration of signaling from many chloroplasts could occur via the control of AtrbohD/F activation.

Information on the antagonistic negative regulation of *APX2* induction by GPA1 is sparse. However, it is known to also involve GPA1-mediated negative control of H_2O_2 production, which is most likely from a plasma membrane-derived source (Galvez-Valdivieso et al., 2009; Galvez-Valdivieso and Mullineaux, 2010). This agrees with a link between NADPH oxidase and GPA1 postulated in the ABA network model for guard cell signaling (Li et al., 2006).

CONCLUDING REMARKS

From the examples provided, a general model emerges of systems that tend to pull cells in opposite responses irrespective of whether cells suffer oxidative stress or not. In all of our examples, chloroplast-sourced ROS can be argued to initiate this antagonistic signaling. We speculate that in all the examples provided, EDS1 plays a key integrating role in onward transduction of the signal from chloroplasts. Furthermore, we suggest that NADPH oxidase-catalyzed production of plasma membrane- and extracellular-sourced ROS acts as a major node from which redox-regulated proteins distribute the message to individual hormone-driven signaling pathways. The intrinsic instability of antagonistic signaling systems may confer a high degree of responsiveness on cells and set the thresholds for cell death or resistance necessary for sensitive responses to fluctuating environments. It is

clear from many papers we have not cited that the concept of "opposing or antagonistic forces" in plant ROS signaling is likely to be widespread and can provide a conceptual framework with which to interrogate the complexity of stress-response signaling networks using computational and modeling methodologies emerging in systems biology.

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