Hydrogen peroxide as a signal controlling plant programmed cell death

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Hydrogen peroxide (H_2O_2) has established itself as a key player in stress and programmed cell death responses, but little is known about the signaling pathways leading from H_2O_2 to programmed cell death in plants. Recently, identification of key regulatory mutants and near-full genome coverage microarray analysis of H_2O_2-induced cell death have begun to unravel the complexity of the H_2O_2 network. This review also describes a novel link between H_2O_2 and sphingolipids, two signals that can interplay and regulate plant cell death.

Introduction

Hydrogen peroxide (H_2O_2), generated by various environmental and developmental stimuli, can act as a signaling molecule that regulates plant development, stress adaptation, and programmed cell death (PCD) (Apel and Hirt, 2004). H_2O_2-induced PCD itself is essential for a number of developmental processes and environmental responses, including aleurone cell death, the hypersensitive response to pathogens, and allelopathic plant–plant interactions (Bethke and Jones, 2001; Bais et al., 2003; Apel and Hirt, 2004). The mechanisms of H_2O_2 generation and detoxification are well studied, but little is known as to how the H_2O_2 signal is perceived and then channeled downstream the signaling network in order to achieve the regulation of these processes (Apel and Hirt, 2004). Recently, mutants in the H_2O_2 signaling pathway were identified, providing a breakthrough in our understanding of how the signaling network functions (Nakagami et al., 2004; Rentel et al., 2004). In addition, mutants that are more tolerant to both reactive oxygen species and to perturbations in sphingolipid metabolism were obtained, thus revealing a new link between redox and sphingolipid signaling leading to plant PCD (Danon, A., and K. Apel, personal communication; unpublished data). These genetic studies were further substantiated by molecular and biochemical data bringing new insights into the interplay between H_2O_2 and sphingolipids during PCD (Gechev et al., 2004). Complementing these findings, recently published transcriptional analyses highlighted biochemical pathways and discovered new H_2O_2-responsive genes (Vandenabeele et al., 2003, 2004).

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Abbreviation used in this paper: PCD, programmed cell death.

The H_2O_2 signaling network is emerging: mutants in the H_2O_2 pathway

As H_2O_2 is both an important signaling molecule and a toxic byproduct of cell metabolism, its cellular levels are under tight control, and their maintenance has hallmarks of homeostatic regulation. The cell can sense sublethal doses of H_2O_2 and activate peroxide-detoxifying mechanisms; alternatively, upon different cell death stimuli various H_2O_2-producing mechanisms can be activated, and as a result of this deliberate H_2O_2 production a self-destructive PCD is triggered (Gechev et al., 2002; Bais et al., 2003; Apel and Hirt, 2004). H_2O_2 produced by NADPH oxidases, for example, has multiple effects ranging from growth promotion or ABA signaling to cell death (Torres et al., 2002; Foreman et al., 2003; Kwak et al., 2003). Studies with exogenously applied H_2O_2 confirm the role of H_2O_2 as a cell death trigger and show that high concentrations can cause necrosis instead of PCD (Yao et al., 2001). In agreement with these observations, overexpression of the H_2O_2-detoxifying enzyme ascorbate peroxidase can suppress the cell death induced by H_2O_2 or nitric oxide (Murgia et al., 2004). Biochemical evidence indicated that a plant MAPK cascade is responsible for relaying the H_2O_2 signal, much alike in other eukaryotes (Kovtun et al., 2000). However, plants possess an unusually high number of MAPKs, and the kinase network can be a convergence as well as a divergence point for different stress factors (Ichimura, 2002). The recent identification of the serine/threonine kinase, oxidative signal-inducible1 (OXI1), as an essential component in H_2O_2 signaling in *Arabidopsis* provided new insights into the complexity and specificity of the H_2O_2-relaying kinase network (Rentel et al., 2004). The *Arabidospis* oxi1-null mutant showed enhanced susceptibility to pathogen infection, data consistent with a role of H_2O_2 in PCD during pathogen responses, and abnormal root hair growth, a process that is also mediated by H_2O_2 (Rentel et al., 2004). The pleiotropic role of OXI1 in plant stress response(s) and development is consistent with its activation not only by H_2O_2, but also by other signals, including cellulose and various abiotic stresses. OXI1 is needed for full activation of two stress MAPKs, AtMPK3 and AtMPK6 (Rentel et al., 2004). Interestingly, the two MAPKs are involved in both abiotic and biotic stress responses and they are also activated by the H_2O_2-regulated

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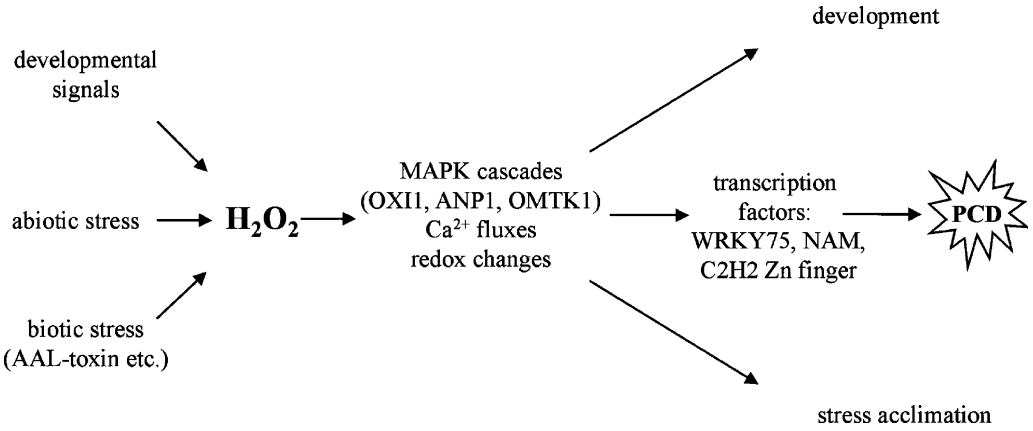

Figure 1. **Biological processes leading to and regulated by H_2O_2.** Various developmental or environmental signals (plant hormones, abiotic or biotic stress) can lead to H_2O_2 accumulation, which in turn triggers a variety of biological responses as developmental processes, stress acclimation, or PCD. The H_2O_2 signal is mediated through alterations in Ca^{2+} fluxes, redox changes, activation of MAPK cascades, and interactions with other signaling molecules like salicylic acid and nitric oxide.

MAPK kinase kinase ANP1 (Kovtun et al., 2000; Menke et al., 2004). Oxidative stress-activated MAP triple-kinase 1 (OMTK1) is a more specific MAPK kinase kinase that can be activated only by H_2O_2 and not by abiotic stresses or hormones in alfalfa (Nakagami et al., 2004). OMTK1 can specifically activate the downstream MAP kinase MMK3, which results in cell death. MMK3 can be activated also by ethylene and elicitors, thus serving as a convergence point of the cell death network (Nakagami et al., 2004).

Other components of the H_2O_2 signaling network

In addition to the MAPK cascade network, the H_2O_2 signal can also be transmitted through alterations in calcium ion fluxes and cellular redox state (Fig. 1). Both Ca^{2+} and redox alterations are very early events that follow the rises in H_2O_2 levels (Rentel and Knight, 2004). A specific calcium signature in turn can lead to various downstream effects, including cell death, through the numerous Ca^{2+}-interacting proteins, including calmodulins and the big family of calcium-dependent protein kinases (Harper et al., 2004). Plants possess a unique set of Ca^{2+}/calmodulin-regulated proteins with different biological functions. Although some of these proteins like NAD kinase aid to the production of H_2O_2 and enhance cell death (Harding et al., 1997), others like catalase have the opposite effect (Yang and Poovaiah, 2002). Catalase is of paramount importance for regulating H_2O_2 homeostasis, as it can function as a cellular sink for H_2O_2. Catalase deficiency leads to elevation of H_2O_2 levels and triggering PCD (Gechev et al., 2004; Vandenabeele et al., 2004). Calcium is therefore not only essential for PCD, but also for maintaining H_2O_2 levels that ensure cell survival (Yang and Poovaiah, 2002). In addition to Ca^{2+}/calmodulin, catalase activity may also be modulated by nucleoside diphosphate kinase (NDK). This notion is suggested by the observations that AtNDK1 interacts with the three *Arabidopsis* catalases in a yeast two-hybrid system and that transgenic plants overexpressing AtNDK1 exhibited enhanced ability to detoxify H_2O_2 (Fukamatsu et al., 2003).

Novel link between redox and sphingolipid signaling during plant PCD

The fungal toxins fumonisin and AAL toxin inhibit ceramide synthase, which causes disruptions in sphingolipid metabolism and subsequent PCD (Spassieva et al., 2002). In tomato, the PCD can be prevented by a disease resistance gene called Asc. Recently, a knockout of the *Arabidopsis* homologue of Asc rendered plants sensitive to AAL toxin–induced PCD (Gechev et al., 2004). A fine balance of sphingolipids is crucial for maintaining cell survival, as not only depletion but also accumulation of ceramides can trigger PCD (Spassieva et al., 2002; Liang et al., 2003). The connection between sphingolipid metabolism and PCD was also indicated with the earlier cloning of the accelerated cell death 11 (acd11) mutant in *Arabidopsis*, a putative sphingosine transfer protein (Brodersen et al., 2002). Biochemical and molecular data demonstrated that PCD triggered by AAL toxin is associated with H_2O_2 (Gechev et al., 2004). The novel link between sphingolipid and redox signaling was further substantiated by isolating mutants that are more tolerant to both the fungal toxins that cause such perturbations in sphingolipid metabolism and to reactive oxygen species (unpublished data; Danon, A., and K. Apel, personal communication). One of these mutants, called EXECUTER1 (AT4G33630), has recently been identified as a nuclear-encoded chloroplast protein with no apparent homology to any other proteins (Wagner et al., 2004). Atr1 mutant, initially isolated in our group as AAL toxin resistant (unpublished data), was also more tolerant to H_2O_2-induced PCD (Fig. 2). Thus, sphingolipids have emerged as important signals whose interactions with ROS can regulate plant PCD.

Comprehensive analysis of gene expression during H_2O_2-induced cell death

H_2O_2-derived signals initiate global changes in gene expression through regulation of a specific subset of transcription factors and, as a result of those changes, different genetic programs including PCD are executed. The first noninvasive in planta sys-

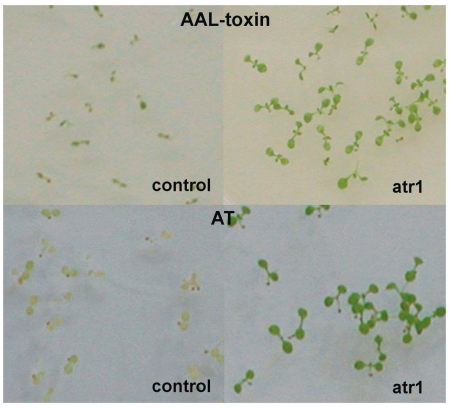

Figure 2. **AAL toxin–resistant mutant *atr1* is also more tolerant to the catalase inhibitor aminotriazole, which triggers H_2O_2-induced PCD.** On the left, control plants (*Arabidopsis thaliana* ecotype Wassilewskija, AAL toxin–sensitive background; Gechev et al., 2004) were germinated on media supplemented with 40 nM AAL toxin or 7 μM aminotriazole (AT). The plant seedlings are very small and dying on the AAL toxin–containing media or dead (yellow) on media with AT. On the right, the AAL toxin–resistant mutant *atr1*, which is in the same background, grows normally on both AAL toxin and AT-supplemented media.

tem to study H_2O_2-induced cell death was based on gene silencing of catalase, which resulted in elevation of endogenous H_2O_2 levels and triggering of PCD under photorespiratory conditions (Vandenabeele et al., 2003). Initially applied to tobacco, the system was then used to trigger PCD in *Arabidopsis* and the expression during H_2O_2-induced cell death was profiled with a DNA chip representing 6,000 genes (Vandenabeele et al., 2004). This approach was developed further by using a catalase inhibitor, which allowed not only transcriptional profiling but also screening for mutants compromised in H_2O_2-induced cell death, and for rapid functional testing of knockout mutants (unpublished data). Microarray profiling using a DNA chip with near-full genome coverage performed in our group resulted in identification of many new H_2O_2-responsive genes and outlining of pathways that are likely to participate in the cell death process. The dataset with the H_2O_2-responsive genes is available at our web site (http://www.rug.nl/biologie/onderzoek/onderzoekGroepen/MolecularBiologyofPlants/onderzoek/copyofSupplementalTable1_H2O2.xls). Comparison of this dataset with the transcriptional analysis during AAL toxin–induced cell death revealed a group of genes regulated in a common fashion and a role for the proteasome and the ethylene pathways in the regulation of cell death, notions supported by functional studies with proteasome and ethylene biosynthesis inhibitors (Gechev et al., 2004; unpublished data). Among the identified genes were a number of transcription factors as well as genes encoding for putative or unknown proteins. Three transcription factors were noted in particular: WRKY75 (AT5G13080.1), which is very strongly induced also during senescence (Guo et al., 2004); the C2H2 zinc finger Zat11 (AT2G37430.1), which is induced also by singlet oxygen and the superoxide radical-generating herbicide paraquat (op den Camp et al., 2003); and a NAM protein (AT2G43000.1). Some other genes highly regulated by the two cell death triggers and presumably acting downstream from the transcription factors were a FAD-linked oxidoreductase (AT1G26380.1), an oxoglutarate-dependent dioxygenase (AT3G13610.1), and a frnE gene (AT5G38900.1). The large number of regulated genes with unknown function in these studies provides us with novel leads to search for plant-specific PCD key regulatory molecules.

Concluding remarks

Despite the recent progress in our understanding of the signaling role of H_2O_2 in plants, there are still many unanswered questions. The H_2O_2 sensor(s) in plants are still elusive. A recent report implicates the eukaryotic antioxidant enzymes, 2-Cys peroxiredoxins, as primary sensors for H_2O_2 (Wood et al., 2003). Plant, animal, and yeast 2-Cys peroxiredoxins, in contrast with bacterial ones, are much more sensitive to H_2O_2 inhibition (Wood et al., 2003). Their ubiquity ensures that in a resting cell, H_2O_2 is kept at constant low levels to prevent any signaling. However, upon an H_2O_2 burst the sensitive 2-Cys peroxiredoxins are rapidly inactivated and then the unscavenged H_2O_2 can react as a messenger with other components of the signaling network like the yeast Orp1-Yap1 sensor (Toledano et al., 2004). Crucial for the outcome of the initial H_2O_2 signal could be the ability of other antioxidant enzymes, including catalase and ascorbate peroxidases, to detoxify the excess H_2O_2. Failure to do so may lead to switching PCD instead of stress acclimation. Recent evidence demonstrates that the inactivation of the peroxiredoxins, initially thought to be irreversible, is actually reversible, thus providing a way to regulate the action of 2-Cys peroxiredoxins as floodgates for H_2O_2 (Woo et al., 2003). Other molecules like two-component histidine kinases may also serve as H_2O_2 sensors, but experimental evidence for their role in plants is still lacking (Apel and Hirt, 2004).

Regardless of the way of sensing, it is still unclear how this small molecule is able to trigger such different responses as stress acclimation or PCD and to initiate distinct developmental programs. The answer to H_2O_2 multifunctionality and the complexity of the responses can be in the H_2O_2 interaction with calcium, nitric oxide, lipid, and plant hormone signaling pathways (Thoma et al., 2003; Zhang et al., 2003; Rentel and Knight, 2004). For example, interactions between H_2O_2 and nitric oxide are essential for PCD during the hypersensitive response and the defense against pathogens, where a fine balance between the two signals modulates cell death (Delledone et al., 1998, 2001). Moreover, often several types of reactive oxygen species are produced, some of which can interact with each other. For example, superoxide radicals can be rapidly dismutated into H_2O_2; the two reactive oxygen species can form highly destructive hydrogen radicals (Dat et al., 2000). Also, the particular location of H_2O_2 and other reactive oxygen species produced in the cell may determine different physiological or developmental responses. Equally as important for H_2O_2 signaling can be the cellular redox state (Mou et al., 2003). The H_2O_2 network itself, although emerging, is far from being understood. There are still many unknown players that modulate the cellular responses to H_2O_2-derived signals. The outcome of H_2O_2 signaling depends on those interactions, which in turn are

determined by the particular cell type, cell compartments, and interacting proteins present at that particular time.

Microarrays with full genome coverage may be a useful tool for identification of genes and other components of the cell death machinery. However, many of the signaling molecules, including the potential sensors, are low-abundant proteins. The best way to identify such proteins can be genetic screening for mutants compromised in H_2O_2-induced PCD. Further functional studies will then be needed to establish the precise role of each played in the cell death network.

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