Hydrogen peroxide—a central hub for information flow in plant cells

Veselin Dimitrov Petrov1,2,3 and Frank Van Breusegem2,3*

1 Department of Plant Physiology and Plant Molecular Biology, University of Plovdiv, 24 Tsar Assen str., Plovdiv 4000, Bulgaria
2 Department of Plant Systems Biology, VIB, Technologiepark 927, B-9052 Gent, Belgium
3 Department of Plant Biotechnology and Bioinformatics, Ghent University, Technologiepark 927, B-9052 Gent, Belgium

Received: 26 January 2012; Returned for revision: 4 March 2012; Accepted: 14 April 2012; Published: 18 April 2012

Abstract

Background Hydrogen peroxide (H2O2) was initially recognized as a toxic reactive oxygen species, able to cause damage to a variety of cellular structures. However, it became clear in the last decade that H2O2 can also act as a potent signalling molecule, involved in a plethora of physiological functions.

Scope In the present review, we offer a brief summary of H2O2 signalling events and focus on the mechanisms of its perception and signal transduction, the factors that act downstream, as well as H2O2 interference with other information transfer mechanisms.

Conclusion The significant scientific effort in the last 10 years to determine the position of H2O2 in signal transduction networks in plants demonstrated that it is essential for both the communication with external biotic and abiotic stimuli and the control of developmentally regulated processes. In addition, H2O2 complements, synergizes or antagonizes many cellular regulatory circuits by active interaction with other signals and plant hormones during growth, development and stress responses. Therefore, further understanding of H2O2 signal transduction is not only of fundamental, but also of practical importance, since this knowledge may contribute to improve agricultural practices and reduce stress-induced damage to crops.

Introduction Reactive oxygen species (ROS) are produced either after incomplete reduction of oxygen (hydrogen peroxide—H2O2; superoxide radical—O2•−; hydroxyl radical—HO•) (Gechev et al. 2006) or energy transfer to its chemically inert triplet ground state (singlet oxygen—1O2) (Kim et al. 2008). These oxygen derivatives possess a strong oxidizing potential that leads to damage to a variety of biological molecules and are therefore unwelcome byproducts of normal metabolic processes in all aerobic organisms (Halliwell 2006). During periods of biotic or abiotic stress, ROS levels can rise excessively, leading to an oxidative stress state (Apel and Hirt

* Corresponding author's e-mail address: frank.vanbreusegem@psb.ugent.be

Published by Oxford University Press. This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (http://creativecommons.org/licenses/by-nc/3.0/) which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.
Since plants are sessile organisms and cannot simply escape from adverse environmental conditions, they have developed an elaborate system to control cellular ROS concentrations (Mittler et al. 2011). In addition, plants have evolved a way to utilize lower concentrations of ROS as signalling molecules for a number of regulated processes during plant growth and development, like cell elongation (Foreman et al. 2003) and differentiation (Tsukagoshi et al. 2010), as well as in responses to a variety of environmental stimuli (Dat et al. 2000; Gapper and Dolan 2006).

Among the ROS compounds, \( \text{H}_2\text{O}_2 \) is the one that received most of the attention of the scientific community in the last decade. Hydrogen peroxide is the result of a two-step reduction of molecular oxygen (the first step leading to superoxide radical) and has a relatively long lifespan in comparison to other ROS. The long half-life (1 ms) of \( \text{H}_2\text{O}_2 \) and its small size allow it to traverse cell membranes and migrate in different compartments, which facilitates its signalling functions (Bienert et al. 2006). As a result, it is now well proved that \( \text{H}_2\text{O}_2 \) is a regulator of a multitude of physiological processes like acquiring resistance, cell wall strengthening, senescence, phytoalexin production, photosynthesis, stomatal opening and the cell cycle. The multi-functionality on the one hand, and the danger it presents in elevated concentrations on the other hand, require the very strict control of \( \text{H}_2\text{O}_2 \) concentration in plant cells.

Active production \( \text{H}_2\text{O}_2 \) occurs mostly at the apoplastic space and is required for triggering the ‘oxidative burst’ that is a part of the hypersensitive response to pathogens, but is also a prerequisite for normal growth, development and cell death (Miller et al. 2010). The main source of this \( \text{H}_2\text{O}_2 \) is a class of cell membrane NADPH-dependent oxidases like respiratory burst oxidase homologues (Rboh), which are regulated by a unique class of Rho-like proteins called ROPs (Rho-related GTPases from plants) (Agrawal et al. 2003), as well as cell wall-associated peroxidases (Bolwell et al. 2002). Of course, multiple other sources of \( \text{H}_2\text{O}_2 \) exist in different plant cell compartments, but these are the result of increased metabolism (like photosynthesis and fatty acid oxidation in peroxisomes and glyoxisomes, as well as overenergization of the electron transport chains in chloroplasts and mitochondria, etc.) (Fig. 1). In most cases, \( \text{H}_2\text{O}_2 \) is formed after reduction of superoxide radicals catalysed by superoxide dismutase (SOD). Simultaneously, a vast network of antioxidants is constantly on the alert for rising \( \text{H}_2\text{O}_2 \) concentrations and provides effective scavenging for it (Apel and Hirt 2004; Gechev et al. 2006; Miller et al. 2010). This antioxidant system consists of several enzymes, such as catalase (CAT), ascorbate (APX) and secretory peroxidases (POX), glutathione reductases (GR) and peroxiredoxines (Prx), and non-enzymatic compounds like tocopherols, ascorbic acid and flavonoids (Willekens 1995; Noctor and Foyer 1998; Asada 1999; Miller et al. 2010).

The biological effect of \( \text{H}_2\text{O}_2 \) is mostly dependent on its concentration, but also on the site of production, the developmental stage of the plant and previous exposures to different kinds of stress. Generally, at low concentrations it acts as a signalling molecule, while at higher concentrations it provokes the onset of cell death (Gechev and Hille 2005). Hydrogen peroxide-induced cell death is essential for some developmental processes and environmental responses, including asexual cell death, leaf senescence, hypersensitive response to pathogens, allelopathic plant–plant interactions, etc. (Bethke and Jones 2001; Bais et al. 2003; Gechev et al. 2005).

It is obvious that \( \text{H}_2\text{O}_2 \) is a molecule with enormous impact on normal plant cell functioning. Although much progress has been achieved in revealing its role in plants, there is still a lot to be unveiled. Yet, in this review we focus on the present state of the art in knowledge concerning \( \text{H}_2\text{O}_2 \) and its signalling network.

**\( \text{H}_2\text{O}_2 \) signalling network**

**Perception of \( \text{H}_2\text{O}_2 \) signals**

A wide range of environmental stimuli lead to a transient rise in cellular \( \text{H}_2\text{O}_2 \) levels. In these cases, \( \text{H}_2\text{O}_2 \) can be viewed as a signal that relays the initial stimuli to downstream effectors. The existence of \( \text{H}_2\text{O}_2 \) sensing proteins still remains enigmatic. However, it is presumed that increased \( \text{H}_2\text{O}_2 \) levels could be perceived directly by redox-sensitive transcription factors (TFs) that orchestrate downstream cascades (Miller et al. 2008). Good candidates for such TFs are class A heat shock factors (Hsfs), which are shown to be responsive to oxidative stress both in animals and plants (Miller and Mittler 2006; Kotak et al. 2007). Moreover, it was demonstrated that Hsfs of both *Drosophila* and mammalian origin are able to interact directly with \( \text{H}_2\text{O}_2 \), and as a result form DNA-binding-competent homotrimers in a reversible manner (Zhong et al. 1998; Ahn and Thiele 2003). Plants may utilize a similar mechanism to detect \( \text{H}_2\text{O}_2 \) signals.

In addition, a recent study suggested another intriguing alternative for \( \text{H}_2\text{O}_2 \) perception *in planta*: the oxidation of methionine (Met) to methionine sulfoxide (MetSO) may couple oxidative signals to changes in the protein phosphorylation state (Hardin et al. 2009). The authors provide *in vitro* and *in vivo* evidence that Met oxidation by \( \text{H}_2\text{O}_2 \) may compromise peptide phosphorylation when these residues are situated within
phosphorylation motifs. As an example, the in vitro oxidation of Met\textsuperscript{538} in leaf nitrate reductase (NR) strongly inhibits the phosphorylation of the adjacent Ser\textsuperscript{534}. Moreover, exogenously applied H\textsubscript{2}O\textsubscript{2} causes a reduction of Ser\textsuperscript{534} phosphorylation in vivo as well, while the over-expression of a cytosolic MetSO repair enzyme has the opposite effect and leads to an increase in phosphorylation rates. Such a redox methionine-dependent switch would be a fast and efficient way to relay the oxidative signal further downstream. Future research on the topic should identify and catalogue proteins with suitable target Met residues in order to unravel how phospho-
widespread this mechanism could be. A solution to achieve this is offered by a recently developed proteomic technology called COFRATIC (combined fractional diagonal chromatography) which, among its other useful applications, is able to identify oxidized methionine residues and quantify their degree of oxidation. In the first study using this new method, COFRATIC analyses on human Jurkat cells revealed that H$_2$O$_2$-sensitive methionines are preferentially located in zones exposed to the protein surface, enriched for charged and poor in hydrophobic residues (Ghesquière et al. 2011).

**H$_2$O$_2$ transport**

One important feature that facilitates H$_2$O$_2$ in its intermediary functions is its ability to diffuse across membranes. It was shown, for example, that H$_2$O$_2$ produced by the chloroplast electron transport chain can leak out of chloroplasts in a light-intensity-dependent manner (Mubarakshina et al. 2010). However, H$_2$O$_2$ is a relatively neutral solute and native membranes present a significant barrier to its free diffusion (Bienert et al. 2006). Hydrogen peroxide can be transported through specific membrane aquaporin homologues of the TIP (tonoplast intrinsic protein) and PIP (plasma membrane intrinsic protein) families. For instance, it was shown that expression of Arabidopsis thaliana AtTIP1;1 and AtTIP1;2 genes in yeast cells decreased their survival rate in the presence of H$_2$O$_2$, while blocking this aquaporin-mediated diffusion alleviated the effect of H$_2$O$_2$ (Bienert et al. 2007). Moreover, a more recent analysis suggests that the Ar/R (aromatic/arginine) regions in PIP2 proteins are critical for their selectivity towards H$_2$O$_2$ (Dynowski et al. 2008) and as all eight PIP2 proteins in Arabidopsis are conserved in these positions, presumably all of them are involved in the specific transport of H$_2$O$_2$.

In turn, H$_2$O$_2$ is able to influence the transport of water and other solutes between cells. In Phaseolus vulgaris it was found that H$_2$O$_2$ exerts concentration-dependent effects on root hydraulic conductivity, which is one of the determinants of root water uptake. Levels of H$_2$O$_2$ $<$ 1 mM increase conductivity, while those $>$ 1 mM decrease it (Benabdellah et al. 2009). In Arabidopsis root meristems, H$_2$O$_2$ treatments induce modifications of plasmodesmal flux based on H$_2$O$_2$ amounts in a similar manner—low concentrations boost plasmodesmal permeability while high concentrations inhibit it (Rutschow et al. 2011). An attractive, but still speculative way to explain these phenomena is that low levels of H$_2$O$_2$ create a signal for mild stress, which can be alleviated by increased flow of solutes and nutrients. Conversely, accumulation of higher amounts of H$_2$O$_2$ produces a message for severe stress, like during pathogen invasion, which requires that the cell is isolated (Rutschow et al. 2011).

**H$_2$O$_2$ as a second messenger**

Hydrogen peroxide possesses some features typical for second messenger molecules. For example, its production is easily upregulated by many stimuli, mainly through NADPH-oxidases and peroxidases, as already discussed. In addition, H$_2$O$_2$ is a small and relatively mobile molecule that has the potential to carry information between different cellular compartments. Moreover, H$_2$O$_2$ is able to modulate the activities of many other signalling components (thus relay signals) and intercalate in a number of signalling cascades with different biological outcomes, including the one that leads to its own synthesis (Fig. 2). In the latter case, either a positive or a negative feedback is provided by inducing or inhibiting H$_2$O$_2$ modulating systems (Mittler 2002; de Pinto et al. 2006; Van Breusegem and Dat 2006). This is mainly dependent on the H$_2$O$_2$ concentration and the timing of its synthesis. The possibility of a positive feedback provides a way to amplify the initial signal (another important characteristic of second messengers), while the negative feedback option ensures that the system can be effectively switched off in order to prevent excessive damage.

The most typical targets of H$_2$O$_2$ include effectors of calcium homeostasis, ion channels, protein kinases or phosphatases and TFs.

**Mitogen-activated protein kinases and H$_2$O$_2$**

Mitogen-activated protein kinases (MAPKs) in plants form a large network implicated in a vast array of functions, including the relay of H$_2$O$_2$ signals (Zhang et al. 2006; Xing et al. 2008). Mitogen-activated protein kinases can either be activated by H$_2$O$_2$ accumulation, or trigger an H$_2$O$_2$-induced oxidative burst themselves (Nakagami et al. 2005; Pitzschke et al. 2009). Thus, while H$_2$O$_2$ activates the ANP1 (an MAPK kinase kinase) protein, which in turn initiates a phosphorylation cascade with AtMPK3 and AtMPK6 (Kovtun et al. 2000), the MEK2 pathway is part of an amplification mechanism upstream of Rboh genes, which are known to produce H$_2$O$_2$ (Yoshioita et al. 2003). This positioning of MAPK cascades up- or downstream of H$_2$O$_2$ indicates the complexity and multidirectionality of stress signaling networks.

A prominent place among the module effectors modulated by ROS metabolism in Arabidopsis holds the MEKK1–MKK1/2–MPK4 pathway. The MAPK kinase kinase MEKK1 is regulated by different stresses and H$_2$O$_2$ in a proteasome-dependent manner (Pitzschke et al. 2009). MEKK1 can activate the downstream
kinases MPK3, MPK4 and MPK6. However, only mpk4 mutants possess a similar phenotype to mekk1 and have similar changes in gene expression. Therefore, it is MPK4 that is usually considered as an H2O2-induced MEKK1 target. The signal between MEKK1 and MPK4 is transmitted by the MAPK kinases MKK1 and MKK2 (Qiu et al. 2008). These are in part functionally redundant, as single mkk1 or mkk2 mutants have normal activities of MPK4, unlike the double mkk1/mkk2 mutant (Gao et al. 2008). Analyses of gene expression reveal that of 32 TFs that are highly responsive to multiple ROS-generating conditions, 20 are regulated via the MEKK1–MKK1/2–MPK4 pathway (Pitzschke et al. 2009). This confirms the central role of these factors in oxidative stress signalling. Surprisingly, a unique shortcut in MAPK pathways has been found for MEKK1—it can interact directly with the TF WRKY53 and phosphorylate it (Miao et al. 2007). This modification increases WRKY53 DNA-binding activity and allows the immediate induction of stress and defence-related target genes by bypassing of downstream kinases. In addition, MEKK1 was demonstrated to possess the ability to bind WRKY53 promoter in a region which participates in the switch from a leaf-age-dependent to a systemic plant-age-dependent WRKY53 expression (Miao et al. 2007).

Fig. 2 A simplified schematic representation of the major signalling components in the H2O2-transduction network, their interactions and different outcomes in the plant cell. Stress conditions and a variety of environmental cues lead to accumulation of H2O2, which serves as a second messenger and relays the signal to downstream effectors, including TFs, MAP-kinases, miRNAs, etc. Some of these effectors can in turn interact with each other. The information flow in this network is rarely straightforward, as it diverges in some points and converges in others. The final output of the cascade depends on the nature of the signal, H2O2 concentration, locus of H2O2 synthesis, interaction with other active signalling pathways, previous exposure to stress, etc.
In plants, transmission of pathogen and disease signals is also governed by MAPKs in a H$_2$O$_2$-dependent manner. This function is exerted in tobacco by SIPK and WIPK kinases (Asai and Yoshioka 2008), whereas in Arabidopsis it is exerted by their homologues MPK3 and MPK6 (Nakagami et al. 2005). MPK3/6 are not only activated by ANP1, as mentioned above, but also by the serine–threonine kinase OXI1, which similarly can be induced directly by H$_2$O$_2$ (Rentel et al. 2004). Hydrogen peroxide may also be involved in MPK3/6 responses to heavy metals, since these two MAPKs are activated after cadmium (Cd) treatment by a mechanism that presumably requires accumulation of H$_2$O$_2$ (Liu et al. 2010).

Recently, it has been proved that the MAPK phosphatase MPK2 can functionally interact with MPK3 and MPK6 (Lumbreras et al. 2010). By dephosphorylating regulatory residues in these targets, MPK2 provides a negative signal with profound effects on pathogen reactions, as shown by mpk2 mutant phenotypes. Moreover, MPK2 seems to have differential functions in biotrophic versus necrotrophic responses, as mpk2 plants exhibit opposite symptoms when infected with the biotrophic Ralstonia solanacearum or the necrotrophic Botrytis cinerea. mpk2 mutants are more resistant to R. solanacearum and more susceptible to B. cinerea, suggesting that MPK2 is a negative regulator of the HR-induced (hypersensitive response) cell death which protects against biotrophic pathogens but facilitates necrotrophic infections.

Two recent studies show that brassinosteroids (BRs) are able to provoke apoplastic H$_2$O$_2$ accumulation, which further switches on MPKs to increase stress tolerance in different species. In cucumber, Rboh, MPK1 and MAPK3 are among the target genes that are upregulated in this BR-induced process (Xia et al. 2009); in maize, BR-stimulated ZmMPK5 induction drives an NADPH oxidase-mediated amplification loop required for the enhancement of the activities of antioxidant enzymes (Zhang et al. 2010).

Transcription factors acting downstream of H$_2$O$_2$

The last link in the chain of H$_2$O$_2$ signal mediators is a variety of TFs. They induce a massive reprogramming of the transcriptome and lead to a second wave of effectors, which in turn switch on various responses like defensive mechanisms and the assembly of the cell death machinery. The TFs that are associated with H$_2$O$_2$ signalling are members of different families, including NAC, ZAT, WRKY, DREB, bZIP and MYB.

In Arabidopsis, many NAC TFs control leaf senescence. Notably, 15 senescence-associated NAC TFs are upregulated by H$_2$O$_2$ (Balazadeh et al. 2010a), of which ANAC032 and ANAC042 are the most significantly induced (around 25 times). ANAC042 has already been reported previously as a possible target of H$_2$O$_2$ pathways (Gechev et al. 2005) and it was proposed that its activation occurs through the OXI1/MPK3/6 cascade. Another key regulator of age- and salt-promoted senescence is ANAC092. As 40 % of its salt-dependent senescence regulon is actually affected by H$_2$O$_2$, an oxidative burst obviously plays a role in ANAC092-mediated responses (Balazadeh et al. 2010a, b). More recently, a similar regulatory network, which includes cross-talk between salt and H$_2$O$_2$, was also ascribed to ORS1, the parologue of ANAC092 (Balazadeh et al. 2011). Moreover, the authors even localized a H$_2$O$_2$-responsive regulatory element in the proximal 230 bp of the ORS1 promoter region (Balazadeh et al. 2011).

The zinc-finger TFs from the Zat family are also often related to oxidative stress. For example, Zat11 and Zat12 are highly induced by ROS (Gadjev et al. 2006). In addition, overexpression of Zat12 leads to increased levels of oxidative stress-responsive transcripts, while its inhibition results in higher sensitivity to H$_2$O$_2$ (Rizhsky et al. 2003; Davletova et al. 2005). In experiments with apr1 mutants, Miller et al. (2008) nominated three Zat TFs as being involved in ROS signalling and abiotic stress–Zat7, Zat10 and the already mentioned Zat12. Remarkably, the induction of Zat7 by H$_2$O$_2$ is impaired in knockout Zat12 plants, which suggests a direct dependence of Zat7 expression on Zat12. However, this relationship is probably more complex, as Zat12-overexpressing plants fail to modulate Zat7 expression.

WRKY TFs are often described as key players in plant responses to biotic, abiotic and oxidative stresses (Eulgem and Somssich 2007). Of these TFs, WRKY52 and WRKY70 are the most widely discussed H$_2$O$_2$-inducible representatives of the family. WRKY52 is a senescence-related factor which can affect the expression of a variety of stress, defence and senescence-associated genes. Its overexpression leads to accelerated senescence, while its knockout provokes a delay in this process (Miao et al. 2004). On the other hand, WRKY70 is specific for salt stress and shows a similar expression pattern to Zat7 in apr1 mutant plants (Miller et al. 2010). It was previously demonstrated that WRKY70, together with the HASTY TF, and Zat7 actually interact in a yeast two-hybrid assay through the EAR-domain of Zat7 (Ciftci-Yilmaz et al. 2007).

Other H$_2$O$_2$-induced TFs from different families include DREB2A that are upregulated not only by oxidative stress, but also by drought, cold and heat stress (Rizhsky et al. 2003), many bZIP, ERF and MYB factors.
that configure the response of japonica rice to chilling stress (Yun et al. 2010), and the redox-sensitive Hsfs which were described above as possible direct sensors of H₂O₂.

**miRNAs and H₂O₂**

Plant miRNAs are known to be ubiquitous regulators of gene expression at the post-transcriptional level and modulate a great diversity of biological responses by directing mRNA targets for cleavage or inducing translational silencing. Recently, in the first genome-wide study of H₂O₂-regulated miRNAs in plants, seven miRNA families were found to be differentially expressed after H₂O₂ treatment in rice seedlings (Li et al. 2011). These include miR169, miR397, miR528, miR827, miR1425, miR319a.2 and miR408-5p. Five of these miRNA families are upregulated by H₂O₂ (miR169, miR397, miR827, miR1425 and miR408-5p) and two are downregulated (miR528 and miR319a.2). Among the direct targets of these miRNAs fall: a HAP2-like TF for miR169; laccases for miR397 (presumably involved in lignin biosynthesis); pentatricopeptide repeat proteins for miR1425 (important for organellar biogenesis); major facilitator superfamily proteins for miR408-5p and miR827 (a group of secondary carriers); putative IAR1 proteins (IAA-alanine resistance protein 1), known to be important for auxin homeostasis, for miR528; and a putative metacaspase for miR319a.2.

**Other H₂O₂-induced factors**

The oxidative stress resulting from different environmental cues also leads to the modulation of many effectors that help to carry out adaptive changes of cellular physiology. A convenient system to study the effects of endogenous peroxisome-produced H₂O₂ is presented by cat2 knockout plants (Queval et al. 2007). This model was used to identify defence-related components that are controlled by H₂O₂. One such modulator in Arabidopsis is the early H₂O₂-responsive UDP-glucosyltransferase UGT74E2, which influences the water stress response and plant architecture by its preference for auxin indole-3-butyric acid (IBA) as a substrate for glycosylation; pentatricopeptide repeat proteins for miR1425 (important for organellar biogenesis); major facilitator superfamily proteins for miR408-5p and miR827 (a group of secondary carriers); putative IAR1 proteins (IAA-alanine resistance protein 1), known to be important for auxin homeostasis, for miR528; and a putative metacaspase for miR319a.2.

**H₂O₂ interference with signalling pathways**

Hydrogen peroxide communicates with a variety of other signalling molecules and plant hormones. Together they form an intricate network that is still not completely characterized. Here we present some details on the interaction of H₂O₂ with Ca²⁺, SA and nitric oxide (NO).
H₂O₂ and Ca²⁺

Like H₂O₂, Ca²⁺ is also a ubiquitous second messenger able to orchestrate different physiological reactions. There are numerous data in the literature indicating that H₂O₂ and Ca²⁺ homeostasis are interdependent, with some cases in which H₂O₂ regulates Ca²⁺ ion fluxes, while in others Ca²⁺ affects H₂O₂ metabolism. A classic example of H₂O₂-mediated Ca²⁺ signalling is the cascade leading to stomatal closure in response to drought. In this chain of reactions, the plant hormone abscisic acid (ABA) induces the production of H₂O₂ in guard cells, which results in the opening of Ca²⁺-permeable channels and an increase in the cytoplasmic Ca²⁺ level ([Ca²⁺]cyt) in intact guard cells (Pei et al. 2000). Moreover, if H₂O₂ production is blocked, ABA-induced closure of stomata is inhibited. In Arabidopsis seedlings, the application of H₂O₂ triggers a biphasic Ca²⁺ elevation, with the first peak located in cotyledons and the second in the root (Rentel and Knight 2004). Importantly, the short time needed before the first Ca²⁺ rise suggests that Ca²⁺ influx may be among the earliest responses to H₂O₂. The delay in the second calcium peak indicates that H₂O₂ may catalyse a [Ca²⁺]cyt rise by specific mechanisms in different tissues. More recently, it was shown that spermidine oxidase-derived H₂O₂ regulates pollen plasma membrane hyperpolarization-activated Ca²⁺ channels in order to induce pollen tube growth (Wu et al. 2010). Pollen from polyamine oxidase (PAO) mutants was unable to cause the opening of Ca²⁺-permeable channels in the presence of spermidine, which resulted in reduced pollen tube growth and seed number.

Cytosolic Ca²⁺ is in turn able to trigger changes in H₂O₂ concentration. Hydrogen peroxide synthesis requires a continuous Ca²⁺ influx which activates NADPH oxidases located at the plasma membrane (Lamb and Dixon 1997). Experiments with Arabidopsis challenged with Pseudomonas syringae demonstrated that the NADPH oxidase inhibitor diphenylene iodonium does not change the Ca²⁺ balance, but the Ca²⁺ channel blocker LaCl₃ suppresses H₂O₂ accumulation and the hypersensitive response (Grant et al. 2000). Thus, in this case it is again Ca²⁺ that acts upstream of H₂O₂. Similarly, it appears that in tobacco cells, the free sphingoid Long Chain Base (LCB) sphinganine induces transient increases in the nuclear and cytosolic Ca²⁺ concentration and the downstream production of H₂O₂ (Lachaud et al. 2011). Interestingly, according to the model of sphinganine signalling proposed by the authors, only the nuclear Ca²⁺ flow is implicated in subsequent H₂O₂-independent cell death mechanisms, while the [Ca²⁺]cyt current is necessary for H₂O₂-induced basal cell defence responses. Therefore, it seems that in this model system, cell death is uncoupled from H₂O₂ pathways.

The antioxidant system may also be a target of Ca²⁺ influence. For example, the efficiency of H₂O₂ scavenging in Arabidopsis plants depends on the peroxisomal Ca²⁺ concentration (Costa et al. 2010). It appears that the intracellular Ca²⁺ rise significantly accelerates H₂O₂ detoxification and this is achieved at least in part with the help of the Ca²⁺-sensitive catalase CAT3. Calmodulin (CaM) is a ubiquitous Ca²⁺-binding protein, which can regulate a number of different protein targets, thereby affecting many different cellular functions (Chin and Means 2000). One of its targets is the MAP kinase 8 (MPK8) which is mainly operative after mechanical wounding. Takahashi et al. (2011) recently proved that the MPK8 pathway in turn negatively regulates H₂O₂ synthesis by controlling the expression of RbohD.

Calcium-dependent protein kinases (CDPKs) sense the Ca²⁺ concentration changes in plant cells and play important roles in signalling pathways for disease resistance and a number of stress responses. In some cases, biotic and abiotic stresses are mediated by CDPKs, which are upregulated after an H₂O₂ burst. In tomato, LeCDPK1 expression is rapidly and transiently enhanced in leaves treated with H₂O₂ (Chico et al. 2002). Similarly, in wheat, eight out of 20 studied CDPKs respond to H₂O₂ treatment (Li et al. 2008).

H₂O₂ and SA

Salicylic acid is a key signalling molecule in plants exposed to biotrophic pathogens (Pieterse et al. 2009), required for the establishment of the systemic acquired resistance (SAR) response (Dempsey and Klessig 1994). Hydrogen peroxide can be linked directly to SA, since benzoic acid (the immediate precursor of SA) is converted into SA by the H₂O₂-mediated activation of benzoic acid 2-hydroxylase (León et al. 1995). At the same time, SA also has the capacity to enhance the endogenous level of H₂O₂, mainly by inducing the activation of SOD (Rao et al. 1997). Thus, according to this scenario H₂O₂ and SA can work together as a self-amplifying system.

In cat2 plants, which were mentioned earlier, the effect of oxidative stress also relies on isochorismate synthase 1 (ICS1). This conclusion was drawn after the cat2 line was crossed with sid2, which is defective in ICS1, and it was observed that lesion formation is completely absent in the cat2 sid2 double mutants (Chaouch et al. 2010). This indicates that the isochorismate pathway of SA synthesis couples intracellular oxidative stress to cell death and disease resistance responses. The cat2 mutants were also used in a study that
addresses the role of glutathione reductase 1 (GR1) in H$_2$O$_2$ responses (Mhamdi et al. 2010). The authors carried out analyses in cat2 gr1 double mutants and established that the GR1-dependent glutathione status is decisive for multiple responses to an increased H$_2$O$_2$ concentration, including the accumulation of SA and the subsequent induction of pathogenesis-related genes. In turn, sid2 plants were utilized to investigate seed germination processes under high salinity (Lee et al. 2010). While the inhibitory effect of salt stress on sid2 germination is strengthened in the presence of higher SA concentrations, treatment with <50 μM SA significantly alleviates it. This can be partially explained by the fact that the elevation of endogenous H$_2$O$_2$ levels is hindered after treatment with moderate doses of SA. The germination of sid2 seeds under such conditions of high salinity is hypersensitive to H$_2$O$_2$, but the physiological concentrations of SA modulate antioxidant activity to prevent oxidative damage (Lee et al. 2010).

Similar results were obtained in tomato, where the effect of SA on root viability during NaCl treatments is also concentration dependent (Gémes et al. 2011). Again, the lower quantities of SA reduce the accumulation of ROS and NO, and help the plant withstand the salt stress, while the higher ones provoke an oxidative burst and disorganization of the root meristem. Salicylic acid provokes ameliorative effects during heavy metal stress as well. In a similar manner, the exogenous application of SA relieves Cd toxicity by reducing H$_2$O$_2$ accumulation in root apoplasts of the legumes Phaseolus aureus and Vicia sativa (Zhang et al. 2011). An example of H$_2$O$_2$ antagonizing SA signalling is the nuclear translocation of the SAR-associated transcription activator NPR1. In this case, cytoplasmic H$_2$O$_2$ was shown to inhibit the SA-induced transport of NPR1 to the nucleus and thus block the activation of pathogenesis-related (PR) genes (Peleg-Grossman et al. 2010).

These results demonstrate that the interactions between H$_2$O$_2$ and SA are multi-faceted, and can vary from cooperation to mutual inhibition in different contexts, concentrations and conditions.

**H$_2$O$_2$ and NO**

Nitric oxide is a second messenger in plants which displays both pro-oxidant and antioxidant properties. On the one hand, NO is able to induce scavenging of excess H$_2$O$_2$ and thus inhibit peroxide signalling pathways; on the other hand, NO may also collaborate with H$_2$O$_2$ to switch on SAR, or stress tolerance (Małolepsza and Różyńska 2005). For example, NO and H$_2$O$_2$ have overlapping roles in the acclimation to salinity. Proteomic analysis revealed that 85 proteins vary quantitatively in citrus plants exposed to salt stress. However, pre-treatment with either H$_2$O$_2$ or sodium nitroprusside (a NO-releasing chemical) prevented changes in the accumulation levels of 45 of them and alleviated the detrimental effects of high salinity (Tanou et al. 2009). Another example of H$_2$O$_2$ and NO exerting similar functions is their influence on the stimulation or inhibition of root hair growth induced by different concentrations of extracellular nucleotides in Arabidopsis (Clark et al. 2010). In contrast, NO mediates tolerance to copper toxicity in tomatoes, partly by antagonizing the H$_2$O$_2$ effect, as it efficiently leads to a boost of the antioxidant system activity (Wang et al. 2010c). Similarly, in maize leaves ABA-induced H$_2$O$_2$ production leads in turn to NO generation, which activates MAPK pathways, to finally result in the upregulation of the expression and activities of antioxidant enzymes (Zhang et al. 2007).

It was demonstrated that synergistic interactions between NO and H$_2$O$_2$ can be necessary for induction of PCD. For example, in tobacco Bright Yellow-2 (TBY-2) cells, both molecules are required for the activation of PCD and the addition of an NO scavenger restores the cell viability after treatment with H$_2$O$_2$ (de Pinto et al. 2006). Interestingly, the NO synthesis is stimulated only after direct application of H$_2$O$_2$, but not after prolonged exposure to H$_2$O$_2$, supporting the view that distinct signalling mechanisms are activated under different combinations of conditions. Similarly, in soybean cells, PCD can be triggered by high levels of NO, but only when supplemented with sublethal amounts of H$_2$O$_2$. Moreover, the SOD inhibitor sodium diethylthiocarbamate blocks PCD progression in these cells (Delledonne et al. 2001). This proves that during HR, NO does not require the presence of O$_2$, but needs H$_2$O$_2$ (produced after the dismutation of O$_2$) catalysed by SOD in order to activate the PCD machinery. However, in other cases, NO and H$_2$O$_2$ do not seem to cooperate and only one of them is enough to cause PCD. For instance, in Arabidopsis suspension cultures, high concentrations of NO are sufficient to induce PCD independently of H$_2$O$_2$ (Clarke et al. 2000).

In an intriguing new study, the map-based positional cloning of rice NOE1, a gene whose knockout leads to an excess of NO, revealed that this is actually the rice catalase OsCATC (Lin et al. 2011). Mutant noe1 plants are characterized by increased leaf H$_2$O$_2$ concentrations, which in turn promote the synthesis of NO by the activation of the enzyme NR. This demonstrates that in some scenarios NO is produced downstream of H$_2$O$_2$ as well. Since the removal of NO alleviates the symptoms of cell death in the noe1 mutants, in this case NO seems to serve as an endogenous mediator of H$_2$O$_2$-induced cell death.
As described earlier, stomatal closure initiated by ABA is realized through an intracellular signalling pathway that includes H$_2$O$_2$ and Ca$^{2+}$-permeable channels. However, another major component in this chain of effectors appears to be NO. Indeed, exogenous NO is actually able to induce stomatal closure. Increased H$_2$O$_2$ concentrations provoked by ABA may in turn trigger NO generation by NR and nitrogen oxide synthase (NOS)-like enzymes (Neill et al. 2008). This presumably occurs through the action of the protein kinase OX11 and involves Ca$^{2+}$ (Rentel et al. 2004). Another elicitor of stomatal closure is extracellular calmodulin (ExtCaM), which plays an important physiological role in the regulation of stomatal diurnal rhythm. Extracellular calmodulin and ABA can induce some parallel changes in second messenger levels in guard cells, including H$_2$O$_2$ and NO. In the ExtCaM-mediated stomatal closure scenario, H$_2$O$_2$ first accumulates after activation of GPA1—the α subunit of a heterotrimeric G protein, further downstream follows the AtN0A1-dependent NO accumulation, which ultimately leads to stomatal closure (Li et al. 2009).

In a recent study, genetic screening for mutants defective in H$_2$O$_2$-induced NO accumulation has identified the prohibitin gene PHB3 as a player in the NO homeostasis system (Wang et al. 2010b). Remarkably, phb3 mutations do not appear to affect H$_2$O$_2$ metabolism or signalling, positioning prohibitin only in the NO pathway. These findings prove that although H$_2$O$_2$ and NO influence and partly complement each other, their downstream networks diverge at some points.

Conclusions and forward look

In the course of evolution, plants developed mechanisms to utilize potentially toxic ROS for signalling purposes. This turns ROS, and mainly H$_2$O$_2$, into signalling agents with tremendous impact on plant growth and behaviour. Since accumulation of higher concentrations of H$_2$O$_2$ is a potent inducer of cell death, a major scientific challenge is to reveal the regulatory processes that govern H$_2$O$_2$ metabolism and find a way to prevent excessive H$_2$O$_2$ synthesis in the field. Although the progress made in the last few years contributed to the overall knowledge concerning H$_2$O$_2$ physiology and unveiled many components in its signalling network, our knowledge is only scratching the surface. Their answers will have not only fundamental, but also practical importance, since crop yield depends on the capacity of plants to tolerate elevations in ROS. Perhaps the future deciphering of the subtle mechanisms of plants to fine-tune the H$_2$O$_2$ concentration holds the key for improving agriculture and responding to the demands of the ever-growing world population.

Sources of funding

This work was financially supported by EC FP7, 245588 project ‘Biosupport’ and by the Multidisciplinary Research Partnership ‘Ghent Bio-economy’.

Contributions by the authors

V.P. and F.B. wrote the paper.

Acknowledgements

We thank Annick Bleys for help in preparing the manuscript and Viktor Ivanov for designing the figures.

Conflict of interest statement

None declared.

References


on 24 August 2018

Petrov and Van Breusegem — Hydrogen peroxide is a hub for information flow in plants


mitogen-activated protein kinase kinases MKK1 and MKK2 have overlapping functions in defense signalling mediated by MEKK1, MPK4, and MKS1. *Plant Physiology* **148**: 212–222.


