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Review

The role of hydrogen peroxide in regulation of plant metabolism and cellular signalling in response to environmental stresses

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Hydrogen peroxide (H_2O_2) is produced predominantly in plant cells during photosynthesis and photorespiration, and to a lesser extent, in respiration processes. It is the most stable of the so–called reactive oxygen species (ROS), and therefore plays a crucial role as a signalling molecule in various physiological processes. Intra- and intercellular levels of H_2O_2 increase during environmental stresses. Hydrogen peroxide interacts with thiol-containing proteins and activates different signalling pathways as well as transcription factors, which in turn regulate gene expression and cell-cycle processes. Genetic systems controlling cellular redox homeostasis and H_2O_2 signalling are discussed. In addition to photosynthetic and respiratory metabolism, the extracellular matrix (ECM) plays an important role in the generation of H_2O_2, which regulates plant growth, development, acclimatory and defence responses. During various environmental stresses the highest levels of H_2O_2 are observed in the leaf veins. Most of our knowledge about H_2O_2 in plants has been obtained from obligate C_3 plants. The potential role of H_2O_2 in the photosynthetic mode of carbon assimilation, such as C_4 metabolism and CAM (Crassulacean acid metabolism) is discussed. We speculate that early in the evolution of oxygenic photosynthesis on Earth, H_2O_2 could have been involved in the evolution of modern photosystem II.

Keywords: acclimatory and defence responses, antioxidants, cell wall, cellular metabolism, cellular redox sensors, hormone signalling, photosynthesis, reactive oxygen species (ROS) signalling, transcription factors

INTRODUCTION

Hydrogen peroxide is well-known as an antiseptic because of its cytotoxic effects on many bacterial strains. It is often used as a disinfectant at wound sites (e.g. Halliwel & Gutteridge, 1999). The role of H_2O_2 in plant biochemistry and physiology, and various functions of H_2O_2 in plants have been described in many review papers (Kuźniak & Urbanek, 2000; Neill *et al.*, 2002a; 2002b; Apel & Hirt 2004; Hung *et al.*, 2005).

It is believed that the presence of O_2 in the Earth's atmosphere originates from photosynthetic activity. However, oxygen can be viewed as a double-edged molecular sword since it is involved in two very different roles in biological systems: it is a prerequisite for aerobic metabolism and consequent normal growth and development, but at

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Abbreviations: ABA, abscisic acid; APX, ascorbate peroxidase (EC 1.11.1.11); Asc, ascorbate; ECM, extracellular matrix; CAT, catalase (EC 1.11.1.6); EEE, excess excitation energy; GPX, glutathione peroxidase (EC 1.11.1.9); GSH, reduced glutathione; GST, glutathione-*S*-transferase (EC 2.5.1.18); OEC, oxygen-evolving complex; PET, photosynthetic electron transport; PQ, plastoquinone; PSI, photosystem I; PSII, photosystem II; ROS, reactive oxygen species; RuBisCO, ribulose-1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39); SA, salicylic acid; SOD, superoxide dismutase (EC 1.15.1.1); TF, transcription factor; TRX, thioredoxin.

the same time the reduction of molecular dioxygen in biological systems very often results in the formation of reactive oxygen species (ROS) that can cause deregulation of normal cellular processes and ultimately cause cell death. Therefore, in plants and in other aerobic organisms antioxidant systems have evolved and different ROS are used as signalling molecules in basic cellular processes. From this context it is very interesting to analyse H_2O_2 as one of the major and the most stable ROS that regulates basic acclimatory, defence and developmental processes in plants. It can be suspected that ecological niches for anaerobic organisms were limited in the presence of an increased O_2 and ROS level.

A number of different oxygen derivatives are described as ROS, also called AOS (active oxygen species). ROS include mainly the superoxide anion radical ($O_2^{-\cdot}$), H_2O_2, hydroxyl radical ($HO^{\cdot}$), perhydroxyl radical ($HO_2^{\cdot}$), and singlet oxygen ($^1O_2$) (Bartosz, 1997; Dat *et al*., 2000; Halliwell, 2006). Oxygen in the ground state is a molecule with two unpaired electrons each located in a different π^* anti-bonding orbital, and divalent reduction of O_2 is not a simple process and requires the generation of univalent intermediates. The addition of a single electron requires an energy input and reduces O_2 to the superoxide anion radical (eqn. 1):

$$O_2 + 1e \rightarrow O_2^{-\cdot} \quad (1)$$

Since the extra electron is in an unpaired state in the outer orbital, the superoxide is a free radical. It is relatively unstable, being either converted back to O_2, or in reaction with a proton, to H_2O_2, either spontaneously or in a reaction catalysed by the enzyme superoxide dismutase (SOD) (eqn. 2):

$$2O_2^{-\cdot} + 2H^+ \rightarrow H_2O_2 + O_2 \quad (2)$$

In contrast to the superoxide, H_2O_2 belongs to non-radical ROS and is a molecule that carries no net charge (Halliwell, 2006). Because of this and the longer half-life of H_2O_2 than that of the superoxide anion radical, hydrogen peroxide is more likely to be a long-distance signalling molecule than superoxide (Vranová *et al*., 2002). Membrane water channels known as aquaporins may facilitate H_2O_2 transmembrane movement together with water (Henzler & Steudel, 2000).

This paper is focused on the role of H_2O_2 in the plant cell, but it should be noted that the role of H_2O_2 in many physiological processes cannot be separated from that of other ROS and the whole antioxidant response system (Bartosz, 1997; Mittler, 2002). The common feature of ROS is that they easily react with other molecules such as lipids, nucleic acids and proteins, which can be damaging or even fatal for the cell (Inzé & Van Montagu, 1995; Matés, 2000; Mittler, 2002).

CELLULAR AND EXTRACELLULAR SOURCES OF H_2O_2

Hydrogen peroxide is produced not only through the disproportionation of superoxide (eqn. 2), but also due to the reduction of $O_2^{-\cdot}$ by a reductant (X) such as ascorbate (Asc), thiols, ferredoxins and others (Asada & Takahashi, 1987) (eqn. 3):

$$O_2^{-\cdot} + H^+ + XH_2 \rightarrow H_2O_2 + XH \quad (3)$$

Thus, hydrogen peroxide cellular levels are strictly linked with the generation of superoxide ($O_2^{-\cdot}$). Nevertheless, other oxidases such as glycolate oxidases, glucose oxidases, amino-acid oxidases, and sulfite oxidases release H_2O_2 following the oxidation of their respective substrates (Asada & Takahashi, 1987; Asada, 1999). In recent years other enzymatic sources of $O_2^{-\cdot}/H_2O_2$ such as cell wall-bound peroxidases, oxalate, amine and plasma membrane NADPH oxidases have been identified (Fig. 1; Wojtaszek, 1997; Bolwell *et al*., 2002; Svedružić *et al*., 2005). Moreover, many reactions involved in photosynthesis and respiration are responsible for the production of H_2O_2. This means that H_2O_2 and other ROS are unavoidable by-products of normal aerobic metabolism (e.g. Bartosz *et al*., 1997; Dat *et al*., 2000; Mittler, 2002).

Chloroplasts

The photosynthetic electron transport (PET) chain in the chloroplast is responsible for H_2O_2 production. The PET chain includes a number of enzymes on the reducing (acceptor) side of photosystem I (PSI): Fe–S centers, reduced thioredoxin (TRX), and ferredoxin. These electron transport components are auto-oxidizable and under conditions limiting the availability of NADP, superoxide anion radical can be formed (Dat *et al*., 2000; Foyer & Noctor, 2000). Mehler (1951) described the photo-reduction of O_2 in chloroplasts *in vitro* and identified H_2O_2 as the reaction product. Later, $O_2^{-\cdot}$ was identified as the primary product of O_2 photoreduction in thylakoids (Fig. 1; Asada & Takahashi, 1987). Currently, the so-called 'Mehler reaction' is considered as the primary and the most powerful source of H_2O_2/ROS in chloroplasts, and the rate of O_2 photoreduction depends on environmental conditions (Asada & Takahashi, 1987; Mullineaux & Karpinski, 2002; Logan *et al*., 2006). It is widely accepted that the Mehler reaction acts as an alternative sink for an excess of electrons, generated during excess excitation energy (EEE) stress (Karpinski *et al*., 1999; 2003), and the whole PET chain from water to O_2 is known as 'pseudocyclic electron flow' (Asada, 1999; Foyer & Noctor, 2000). The H_2O_2 production in chloroplasts is catalyzed by SOD forms containing in the active site copper/zinc (Cu/Zn-SOD) or iron (Fe-SOD) (for

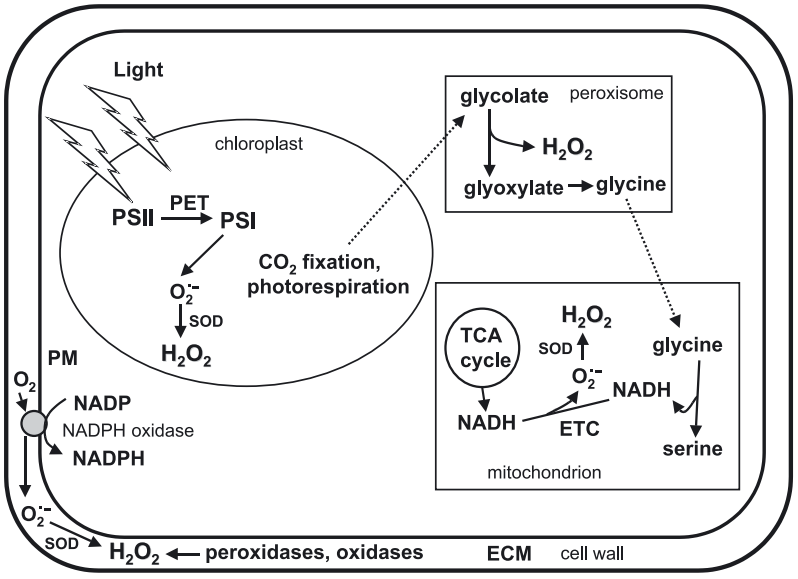

Figure 1. Main sources of H_2O_2 in different cellular compartments of mesophyll cells during C_3 photosynthesis. The ratio of H_2O_2 production between chloroplasts and peroxisomes is 1:2.5, and between chloroplasts/peroxisomes and mitochondria: 35:1. These data are estimated from the rates of H_2O_2 production according to Foyer and Noctor (2003). The contribution of ECM in production of H_2O_2 in photosynthetically active cells has not been estimated yet. ECM, extracellular matrix; ETC, electron transport chain; PET, photosynthetic electron transport chain; PM, plasma membrane; PSI and PSII, photosystem I and photosystem II; SOD, superoxide dismutase; TCA, tricarboxylic acid cycle.

a review see: Alscher *et al*., 2002). The reduction of $O_2^{\cdot-}$ by ascorbate (Asc) and reduced glutathione (GSH) does not contribute much to the production of H_2O_2 in the chloroplast (Asada, 1999). Water oxidation at photosystem II (PSII) and univalent photoreduction of O_2 (Mehler reaction) in PSI is coupled to both the production and destruction of H_2O_2 by ascorbate peroxidase (APX). The final reaction is the regeneration of ascorbate from monodehydroascorbate (MDHA) by monodehydroascorbate reductase (MDHAR). This transfer of electrons from H_2O to H_2O is often called the Mehler-peroxidase cycle (Foyer & Noctor, 2000) or the water–water cycle. The name denotes that water is the source of electrons at PSII and the final product of the cycle (Asada, 1999). Additionally, partial water oxidation by the manganese-containing, oxygen evolving complex in PSII can also lead to H_2O_2 formation (Fine & Frasch, 1992).

Peroxisomes

The main function of peroxisomes in the plant cell is photorespiration, which is light-dependent uptake of O_2 and the associated release of CO_2 connected with the generation of H_2O_2 (Fig. 1.; Dat *et al*., 2000; Wingler *et al*., 2000). Besides glycolate oxidation, H_2O_2 can be generated in the peroxisome e.g. *via* β-oxidation of fatty acids and oxidation of other substrates (Dat *et al*., 2000), but the significance of these processes in H_2O_2 production in comparison to glycolate oxidation in C_3 plants appears minor (Foyer & Noctor, 2003). Photorespiration results from the oxygenase activity of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), and is associated with increased H_2O_2 production in the peroxisome, and supply of glycine for the synthesis of the primary antioxidant glutathione (Noctor *et al*., 1999). Because of the consumption of reducing power and energy, this apparently wasteful process could in fact act as an electronic valve and ease the electronic burden of the photosynthetic machinery (Kozaki & Takeba, 1996; Willekens *et al*., 1997; Wingler *et al*., 2000; Cornic & Fresneau, 2002). Because they drain electrons through the PET, the Mehler reaction and photorespiration participate through the redox status of the plastoquinone (PQ) pool in the induction of the *APX1* and *APX2* genes (Karpinski *et al*., 1997; 1999). In C_3 plants exposed to conditions favouring high rates of oxygenation (warm sunny days — EEE conditions), the photorespiratory pathway would appear to be the fastest process for generating H_2O_2 (Foyer & Noctor, 2000).

Mitochondria

In plant mitochondria superoxide anion radical production occurs mainly at two sites of the electron transport chain: NAD(P)H dehydrogenases (complex I) and the cytochrome bc_1 complex (complex III) (Møller, 2001). This process results in the formation of H_2O_2, primarily through the action of a mitochondrion-specific manganese SOD (Mn-SOD) (Fig. 1; Rhoads *et al*., 2006). The amount of H_2O_2 produced in plant mitochondria is less than that of chloroplasts or peroxisomes when exposed to light (Foyer & Noctor, 2003), but in the dark or in non-green tissues, mitochondria can be a major source of ROS (Rhoads *et al*., 2006). Theoretical calculations suggest that mitochondrial H_2O_2/ROS production probably does not differ between light and dark conditions, since total O_2 consumption is less affected by light than by tricarboxylic acid (TCA) cycle activity (Foyer & Noctor, 2003). The alternative oxidase (AOX) which catalyzes the tetravalent reduction of O_2 by ubiquinone can limit H_2O_2/ROS generation (Juszczuk & Rychter, 2003). The AOX competes with complex III for electrons and thus might help to lower ROS

production (Apel & Hirt, 2004). The mitochondrial contribution in H_2O_2 generation in plant cells under light conditions requires further study, since photosynthesis and respiration in the light are strongly interdependent (Van Lis & Atteia, 2004).

Other sources of H_2O_2 in the plant cell

H_2O_2 is also produced in the cytoplasm, plasma membrane and in the extracellular matrix (ECM). In the cytoplasm, the electron transport chain associated with the endoplasmic reticulum is the main source of H_2O_2/ROS, where reduced forms of cytochrome P450 and cytochrome P450 reductase that are involved in oxidation and hydroxylation processes, as well as cytochrome b_5 and cytochrome b_5 reductase that are engaged in fatty acid desaturation, donate electrons to O_2 producing superoxide, and a cytosolic form of SOD can convert $O_2^{-\cdot}$ to H_2O_2 (e.g. Bartosz, 1997; Mittler *et al*., 2004). The cytosol cannot be regarded as a major source of H_2O_2 in plant cells, but it may act as a sink for hydrogen peroxide leaking from other cellular compartments. However, the function of cytosol-generated H_2O_2 is not understood.

NADPH oxidase at the plasma membrane in the plant cell is the most intensively studied oxidase system. The NADPH-dependent oxidase system (sometimes referred to as *rboh* (for respiratory burst oxidase homologue)), similar to that present in mammalian neutrophils, has received the most attention. It catalyzes the production of $O_2^{-\cdot}$ by one-electron reduction of oxygen using NADPH as the electron donor (Desikan *et al*., 2003; Mahalingam & Federoff, 2003; Apel & Hirt, 2004). The superoxide anion radical is most likely located in the apoplastic space and is converted either spontaneously or by extracellular SOD to H_2O_2 (Fig. 1; Karpinska *et al*., 2001; Bolwell *et al*., 2002). Genes for NADPH oxidase or *rboh* genes have been cloned from several plant species (Desikan *et al*., 2003). It should be noted that the ECM in higher plants is composed of cellulose, hemicelluloses, pectins, lignins and protein-based fiber matrices and also contains H_2O_2 producing/removing enzymes. For this reason, in addition to NADPH oxidase, many enzymes of ECM, such as: pH-dependent cell wall peroxidases, germins, germin-like oxalate oxidases, and amine oxidases have been proposed as sources of H_2O_2 in the apoplast (Lane, 1994; Bolwell *et al*., 2002; Kacperska, 2004). Therefore, the role of hydrogen peroxide and other ROS in ECM should not only be limited to defense responses, but their primary role is in the regulation of the synthesis of cell wall components, e.g. lignin (Olson & Varner, 1993; Wojtaszek, 1997; Ros Barceló, 1998).

REMOVAL OF H_2O_2

Elimination of H_2O_2 is connected with scavenging of other ROS and the steady state level of cellular H_2O_2 depends on the redox status of the cell (e.g. Karpinski *et al*., 2003; Mateo *et al*., 2006). In plant cells both H_2O_2 production and removal processes are precisely regulated and co-ordinated in the same or in different cellular compartments. The mechanisms of H_2O_2 scavenging are regulated by both non-enzymatic and enzymatic antioxidants.

Low molecular mass antioxidants

To complement the dissipation mechanisms mentioned above and to counter-act the oxidative pressure imposed by H_2O_2/ROS formation, plants have a multi-level antioxidant system, consisting of antioxidants, such as: Asc, α-tocopherol and GSH as well as a multitude of ROS scavenging enzymes (Apel & Hirt, 2004). Ascorbate (vitamin C) has been shown to react not only with H_2O_2, but also with $O_2^{-\cdot}$, $HO^{\cdot}$, and lipid peroxides (Smirnoff, 1996; Noctor & Foyer, 1998). It is also involved as a co-factor in the reaction catalysed by violaxanthine de-epoxidase of the xanthophyll cycle (Demmig-Adams & Adams, 1996). Tocopherol (vitamin E) is a lipid-soluble antioxidant capable of bringing to an end the free radical reactions that cause lipid peroxidation. Reduced glutathione (GSH) can react directly with ROS to detoxify them or can scavenge peroxides as a cofactor of glutathione peroxidases (GPXs). Moreover, GSH is used to regenerate oxidised ascorbate and tocopherol and oxidised-SH groups of proteins (Noctor & Foyer, 1998; Mullineaux *et al*., 2000). Since glutathione is present in plant cells in millimolar concentrations, it is regarded as the key determinant of the cellular redox state (Creissen *et al*., 1999; Pastori & Foyer, 2002). Glutathione levels, redox status and biosynthesis can regulate the expression of a large number of genes, among them components of the antioxidant defence system and the pathogen related 1 (*PR1*) gene (Ball *et al*., 2004; Mateo *et al*., 2006).

Enzymatic antioxidant system

Ascorbate and glutathione are also used as cofactors in reactions catalyzed by peroxidases (APX and GPX) to reduce H_2O_2 to water. Expression of genes for the cytosolic APX1 and APX2 is controlled, at least in part, at the chloroplast level. The activity of APXs is thought to form a second barrier of defence against H_2O_2/ROS produced in the chloroplasts (Karpinski *et al*., 1997; 1999; Karpinska *et al*., 2000; Fryer *et al*., 2003; Chang *et al*., 2004). The role of GPXs as an H_2O_2 scavenger

has received modest attention (Mullineaux *et al*., 1998). Superoxide dismutases convert $O_2^{-\cdot}$ to H_2O_2 (eqn. 2) and thus form a crucial part of the cellular antioxidant response system. The various SOD forms are induced with different kinetics during sustained stress conditions (Alscher *et al*., 2002). Oxidised glutathione (GSSG) is regenerated by glutathione reductase (GR) in a NADPH-consuming reaction (Edwards *et al*., 1994; Winglse & Karpinski, 1996). Glutathione reductase completes the ascorbate–glutathione cycle by regenerating the glutathione pool with NADPH as the electron donor (Apel & Hirt, 2004). Glutathione-*S*-transferase (GST) catalyses the conjugation of GSH to a variety of molecules and thus marks them for secretion. GSTs detoxify breakdown products of lipid peroxides. They are induced *inter alia* by ROS and pathogen attack stresses (Dixon & Lamb, 1990; Dixon *et al*., 2002). Apart from the peroxidases (APX and GPX) also a group of enzymes collectively called peroxiredoxins are able to reduce H_2O_2 and many other diverse peroxides (Horling *et al*., 2002). Catalase (CAT) is another key enzyme responsible for H_2O_2 decomposition (Apel & Hirt, 2004). In *Arabidopsis*, CAT is encoded by a multi-gene family consisting of three genes *(CAT1, CAT2, CAT3)* encoding individual subunits that associate to form at least six isoenzymes (Frugoli *et al*., 1996). The steady state levels of catalase mRNA, protein synthesis and activity are tightly regulated in a number of plant species. Furthermore, peroxisomal catalase has been suggested to be photosensitive (Feierabend *et al*., 1992; Hertwig *et al*., 1992; Schmidt *et al*., 2002). Recently, a genetic system that controls H_2O_2 levels with the involvement of CAT has been identified and described in *Arabidopsis* (Mateo *et al*., 2004).

H_2O_2 IN STRESS CONDITIONS AND AS A SIGNALLING MOLECULE

It is well-documented that H_2O_2 plays a central role in responses to both abiotic and biotic stresses in plants. This molecule seems to be a "master hormone" that controls a variety of stress responses and physiological adjustments (Fig. 2), including the ROS/hormonal homeostasis in the cell. Many studies have reported increases in H_2O_2 concentration after exposure to stress (for a review see: Neill *et al*., 2002a; Kacperska, 2004). The rate of H_2O_2 production depends on the strength and duration of the imposed stress. Moreover, H_2O_2 levels differ in various cell compartments and these levels are related to the type of stress, e.g. excess light stress is responsible for the overproduction of H_2O_2 mainly in the chloroplast (Karpinski *et al*., 1999; Karpinska *et al*., 2000; Ślesak *et al*., 2003).

In contrast to animal cells, plants seem to be much more resistant to high concentrations of H_2O_2. Hydrogen peroxide is toxic for most animal cells at levels of about 10–10^2 μM (Halliwel & Gutteridge, 1999). Experiments with plant material have demonstrated that plant tissues can tolerate high concentrations of H_2O_2 in the range 10^2–2×10^5 μM. Moreover, plants pre-treated with H_2O_2 were more resistant to excess light and chilling stresses (Prasad *et al*., 1994; Karpinski *et al*., 1999; Karpinska *et al*., 2000; Yu *et al*., 2003). The results of these experiments appear to be largely in disagreement with *in vitro* experiments concerning inhibition of activity of some enzymes involved in the Calvin cycle at relatively low (10 μM) concentrations of H_2O_2 (Kaiser, 1979). The problem with the interpretation of these data relates to the actual intracellular H_2O_2 steady state concentra-

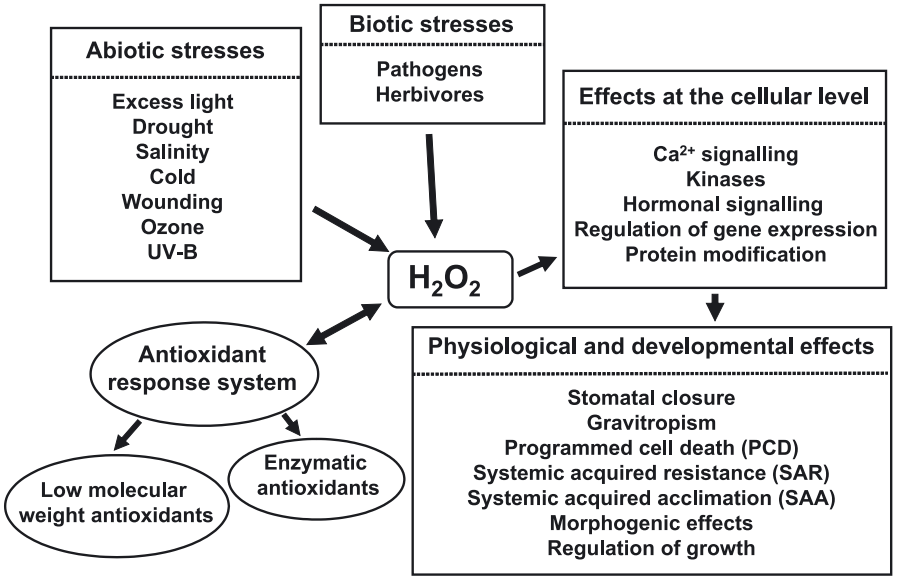

Figure 2. The central role of H_2O_2 in plant responses to various environmental stresses.
The increased H_2O_2 level stays high in relation to stress situations and the efficiency of the antioxidant response system. Changes in H_2O_2 content lead to various effects at the cellular and organism level (physiological and developmental effects); modified after Desikan *et al*. (2003).

tion in selected plant tissues. The methods used for H_2O_2 determination in plant cells or plant extracts are imperfect and non-specific (Shulaev & Oliver, 2006). In the literature, the endogenous concentration of H_2O_2 is reported to lie in a wide range, ranging from nanomoles to several hundred micromoles of H_2O_2 per gram fresh mass (Asada & Takahashi, 1987; Willekens *et al*., 1997; Karpinski *et al*., 1999; Veljovic-Jovanovic *et al*., 2002). Veljovic-Jovanovic *et al*. (2002) have shown that substances such as Asc and phenolic compounds present in plant extracts strongly affect the concentration of H_2O_2. Nevertheless, it is possible to calculate rates of H_2O_2 production in different cell compartments using some experimental data and theoretical estimations (Asada & Takahashi, 1987; Polle, 2001; Foyer & Noctor, 2003). It is suggested that the tolerance of plants to high H_2O_2 levels is due to the fact that plant antioxidant response systems are designed more for the control of the cellular redox state than for complete elimination of H_2O_2. Photo-active chloroplasts are the main suppliers of H_2O_2 and it is estimated that the chloroplast/peroxisome system generates about 90% of the total H_2O_2 in the photosynthetically active plant cell (Foyer & Noctor, 2003). The relative stability and higher concentrations of H_2O_2 in plant cells could point to the fact that H_2O_2 plays a key role as a signal transduction factor. However, signal molecules are usually present in cells in very low concentrations; the relatively high level of H_2O_2 in plant tissues supports the assumption that H_2O_2 is not only a signalling molecule, but also plays a key role in primary plant metabolism. Moreover, H_2O_2 regulates the expression of various genes, including those encoding antioxidant enzymes and modulators of H_2O_2 production (Neill *et al*., 2002a; 2002b; Geisler *et al*., 2006). A micro-array study showed that the expression of several hundreds genes was altered in H_2O_2-treated *Arabidopsis* cell cultures, and among them several genes associated with PET chain were repressed (Desikan *et al*., 2001). H_2O_2/ROS-responsive *cis*-regulatory elements in gene promoter regions have been identified recently (Geisler *et al*., 2006). Although H_2O_2 is a signal molecule affecting the transcriptome, it is not clear whether H_2O_2 is actually the signal *per se*, or whether oxidation of other molecules by H_2O_2 is needed to generate an intracellular signal (Desikan *et al*., 2003). To date, no H_2O_2 receptor has been definitively identified in plants (Foyer & Noctor, 2003; Geisler *et al*., 2006). However, such plant genetic systems like *NPR1* (*non-expresser of pathogen responsive gene 1*) and *LSD1* (*lesion simulating disease response 1*), analogous to the NF-κB system in animals, could play a role in the H_2O_2 sensing mechanism (Mateo *et al*., 2004; 2006). Specific H_2O_2-regulated kinase signalling cascades and transcription factors (*trans*-elements) have not been identified and characterized yet. It is speculated that soluble thiol-containing low molecular mass compounds (e.g. GSH) or some proteins possessing active cysteine thiols (e.g. GPX) can activate H_2O_2-responsive transcription factors (TFs) directly or indirectly. H_2O_2 might also activate signalling protein kinases (e.g. mitogen activated protein kinases, MAPK); a protein kinase can phosphorylate a TF, and then the TF interacting with H_2O_2-responsive elements regulates the expression of a gene (for a review see: Neill *et al*., 2002a; Foyer & Noctor, 2003; Apel & Hirt, 2004). Candidates for H_2O_2-dependent redox regulation are thiol group-containing TRXs. Especially, it could be that the oxidation/reduction cycles of the ferredoxin/TRX system in chloroplasts can regulate the activity of many different enzymes, including those involved in the Calvin cycle (Buchanan & Balmer, 2005).

Hydrogen peroxide has also been shown to be an intercellular signal mediating systemic acquired resistance (SAR) (e.g. Alvarez *et al*., 1998) and systemic acquired acclimation (SAA; Karpinski *et al*., 1999); a role for H_2O_2 has also been described in response to wounding (Orozco-Cárdenas & Ryan, 1999). An important question can be raised here: if H_2O_2 is involved in so many diverse responses, how can it be specific? It has been discussed that depending on the site of generation and interaction with specific hormonal compounds, such as salicylic acid (SA), nitric oxide (NO), ethylene, auxins, abscisic acid (ABA) and others, H_2O_2 is able to act in a specific manner (Van Breusegem *et al*., 2001; Neill *et al*., 2002b; Pastori & Foyer, 2002; Torres *et al*., 2006). Especially the interconnections between H_2O_2/ROS and NO/reactive nitrogen species (RNS) in the context of plant metabolism are intensively studied (e.g. Yamasaki, 2005).

Hydrogen peroxide under non-stress and stress responses shows tissue-specific localization. The tissue-specific localization of H_2O_2 is associated mainly with vascular tissues of leaf veins (Orozco-Cárdenas & Ryan, 1999; Fryer *et al*., 2003; Mullineaux *et al*., 2006). Numerous studies demonstrate that H_2O_2 is produced in the xylem and phloem (Hérouart *et al*., 1994; Ogawa *et al*., 1997; Ros Barceló, 1998; Karpinska *et al*., 2001; Fryer *et al*., 2003). Responses of *Arabidopsis* leaves to light stress have been observed as the accumulation of H_2O_2, especially in the vascular bundles (Fryer *et al*., 2003). Apart from the various enzymes producing H_2O_2, such as the oxidases and peroxidases, the localization of Cu/Zn-SOD isoforms in the ECM has been described (Karpinska *et al*., 2001; Alscher *et al*., 2002). It has been shown that gene expression and enzyme activity of Cu/Zn-SOD is linked with both xylem and phloem tissues (Ogawa *et al*., 1997; Karpinska *et al*., 2001; Walz *et al*., 2002). It has also been proposed by Karpinska *et*

al. (2001) that Cu/Zn-SOD isoforms in vascular tissues might act as regulators of H_2O_2 pulses, being involved in the transmission of systemic signals in wounding or pathogen responses. Localized cellular responses were also observed by Orozco-Cárdenas and Ryan (1999), Fryer *et al*. (2003), Chang *et al*. (2004), and Mateo *et al*. (2004). On this basis it has been suggested that photo-produced H_2O_2 under high light stress is predominantly derived from photosynthetically active bundle sheath cell chloroplasts and that H_2O_2 is transported into the transpiration stream in the xylem. A weak point of this hypothesis is the requirement for H_2O_2 transport from the chloroplast through the cytosol to the apoplast (Mullineaux *et al*., 2006). Another offered explanation of the high level of H_2O_2 in the leaf venation system is based on the fact that the ECM of the xylem/phloem cell walls is the main donor of H_2O_2 required for lignification in the cell wall (Olson & Varner, 1993; Ros Barceló, 1998). Continuous production and removal of H_2O_2 during cell wall synthesis is most likely the reason for its very high steady state concentration in the leaf venation system.

It should be emphasised that the spatial distribution of H_2O_2 is not uniform within the whole leaf, but its concentration strongly depends on the type of leaf tissue and environmental conditions. Most studies related to H_2O_2 production in plant cells have focused on the photosynthetic activity of the chloroplast, and peroxisomes as the main donors of H_2O_2, but much evidence indicates that the ECM also becomes an important source of H_2O_2 in plants, especially during plant responses to environmental stress.

H_2O_2 signalling during growth and development

Recently, information on the role of H_2O_2/ROS as signal molecules regulating growth and morphogenesis has emerged, suggesting that H_2O_2 is not only a stress signal molecule, but may also be an intrinsic signal in plant growth and development. H_2O_2 has also been shown to be involved in differentiation of the cellulose-rich cell wall (Potikha *et al*., 1999). Recently, it has been demonstrated that diminished extracellular Cu/Zn-SOD expression in poplar trees regulates extracellular H_2O_2 level and plant development (Srivastava *et al*., 2006).

Under specific conditions, certain somatic plant cells are capable of forming embryos following the resumption of cell division activity and totipotency through the developmental pathway of somatic embryogenesis. One possible link between oxidative stress and plant regeneration in tissue cultures could be H_2O_2. The specific induction of GPX and GST by 2 mM H_2O_2 in cell suspension cultures of soybean and the cross-membrane trafficking of H_2O_2 were the first demonstration of H_2O_2-inducible gene expression in plants (Levine *et al*., 1994). Whereas cell cycle progression is under negative control of ROS, somatic embryogenesis is stimulated by H_2O_2 in *Lycium barbarum* (Cui *et al*., 1999). A modification in endogenous H_2O_2 content was also noted in *Mesembryanthemum crystallinum* plant tissue culture and was related to the ability of callus tissue to regenerate. The highest H_2O_2 concentration was found in callus with regeneration potential (Libik *et al*., 2005a). Differences in H_2O_2 content between rhizogenic and embryogenic calli of *M. crystallinum* were also evident. Root formation was performed by heterotrophic callus with low activity of PSII, while somatic embryos appeared on calli with an autotrophic nature and high activity of PSII (Libik *et al*., 2005a). These results suggest that differences in oxidative events during morphogenic processes might be linked to photosynthesis accompanying the formation of roots or somatic embryos.

Light and H_2O_2 in response to pathogens

Following pathogen infection, transient H_2O_2/ROS overproduction and accumulation can promote a local defence response connected with NADPH oxidase activation (Keller *et al*., 1998). The increase of H_2O_2/ROS levels causes the hypersensitive response (HR) as well, leading to rapid localized cell death at infection sites (lesions), which is a form of programmed cell death (PCD), or can induce SAR (Alvarez *et al*., 1998; Kuźniak & Urbanek, 2000; Mateo *et al*., 2004; Talarczyk & Hennig, 2001). Recent studies indicated that H_2O_2/ROS-triggered HR restricted the spread of biotrophs (Hückelhoven & Kogel, 2003), whereas necrotrophic plant pathogen growth was facilitated (Govrin & Levine, 2000). In response to pathogens, H_2O_2 is able to stimulate cross-linking of cell wall proteins, which reinforces the cell wall, thereby blocking the pathogen spread that occurs upon infection (Brisson *et al*., 1994). Moreover, it has been found that feruloyl-arabinoxylan trisaccharide in the cell wall can be oxidatively coupled by H_2O_2, creating a barrier against pathogens (Encina & Fry, 2005). It has been shown that CAT activity and hydrogen peroxide level strongly influence local responses of the plant to infection (Talarczyk *et al*., 2002). The crosstalk between all H_2O_2 producing cell compartments that adapts the metabolism to stress conditions is strongly connected to light-dependent photosynthetic processes in the cell (Mateo *et al*., 2004). According to a traditional point of view, during pathogen resistance responses the primary source of H_2O_2/ROS is not the chloroplast, but a plasma membrane NADPH oxidase (Apel & Hirt, 2004), and it has been suggested by others that the photosynthesis and photorespira-

tion are not likely to be important sources of H_2O_2 in defence against pathogens and the hypersensitive disease response. Recent evidence shows that the development of plant resistance to microbial pathogens requires illumination during infection (Mateo *et al*., 2004; Roberts & Paul, 2006). In most cases in both resistant and susceptible plant–pathogen interactions, perturbations in photosynthesis, such as the depression of PET chain, reduced CO_2 assimilation and decreased stomatal conductance have been observed (for a review see: Bechtold *et al*., 2005). Moreover, experiments with lesion mimic mutants, which have lesions similar to those formed during HR, showed a link between H_2O_2/ROS produced in chloroplasts and plant stress responses, including pathogen resistance (Karpinski *et al*., 2003; Mateo *et al*., 2004; 2006; Bechtold *et al*., 2005). Additionally, at least part of the pathways involved in the biosynthesis of major defence-related hormones: jasmonic acid (JA), SA and ABA are located in chloroplasts (Karpinski *et al*., 2003; Roberts & Paul, 2006). This fact also indicates a role for photosynthesis in both abiotic and biotic stress responses. We know now of a co-ordination of the processes that regulate light acclimation and the establishment of immunity to some pathogens (Bechtold *et al*., 2005). The above considerations might lead to the suggestion that light acclimatory responses or those to other abiotic stress factors (temperature, relative humidity) will lead to immunity against infection by a pathogen. This was recently confirmed by an analysis of different SA mutants (with deregulated high or low SA levels), which demonstrated that an optimal SA level is required for optimal photosynthesis and that cellular levels of SA, GSH and H_2O_2 are physiologically and genetically linked (Mateo *et al*., 2006).

H_2O_2 AND PHOTOSYNTHETIC MODE OF CARBON ASSIMILATION

As it has been shown above, the PET chain is a driving force leading to H_2O_2 production in green plants. Surprisingly, almost no detailed studies focusing on a comparison between H_2O_2 production in plants representing the three main modes of CO_2 assimilation, i.e. C_3, C_4 and Crassulacean acid metabolism (CAM) have been done. Most of the available results describe studies in C_3 plants (Foyer & Noctor, 2000; 2003). Li *et al*. (2001) compared the concentration of H_2O_2 in leaves in several species of C_3, C_4 and CAM plants, and they showed that the average concentration of H_2O_2 in CAM plants is about two-fold higher than in C_3 and C_4 plants, and H_2O_2 concentration does not differ between C_3 and C_4 plants (Li *et al*., 2001), nevertheless, more research is required. A lack of other experimental data allows one only to speculate on how much H_2O_2 might be produced in C_4 and CAM plants in comparison to obligate C_3 plants. The general assumption is that photosynthetic O_2 uptake, i.e. photoreduction of O_2 (the Mehler reaction) and photorespiration, are the main suppliers of H_2O_2. According to this assumption, in C_4 plants, where photorespiration is strongly reduced, the Mehler reaction should be the more important sink for electrons. A few studies with C_4 plants showed that the level of light-dependent O_2 uptake is much lower than for C_3 plants (Badger *et al*., 2000), but the question of whether these observations indicate that the H_2O_2 concentration is lower in leaves of C_4 plants than in C_3 plants, needs a more detailed study. In CAM plants CO_2 from the atmosphere enters the mesophyll cells at night (Phase I of CAM), and in the cytosol CO_2 is fixed to form malic acid in the reaction catalysed by phospho*enol*pyruvate carboxylase (PEPC). At the beginning of the day (Phase II of CAM) malate effluxes from the vacuole to the cytosol. In the next step malate is decarboxylated. The decarboxylation takes place predominantly in the middle of the day (Phase III of CAM). During Phase III of CAM the stomata are closed, and this can lead to a parallel increase in both the intercellular partial pressure of O_2 (p_iO_2) and CO_2 (p_iCO_2) *in planta*. An increased O_2 level in Phase III behind closed stomata can promote overproduction of H_2O_2/ROS in CAM plants (Miszalski *et al*., 1998; Lüttge, 2002; 2004). In recent years, the intermediate C_3-CAM plant *M. crystallinum* has become a very useful model for studying antioxidant response systems in both modes of photosynthesis: C_3 and CAM (Miszalski *et al*., 1998; 2001; Niewiadomska *et al*., 1999; 2004; Broetto *et al*., 2002; Ślesak *et al*., 2002; 2003; Libik *et al*., 2005b; Borland *et al*., 2006). In previous work we have demonstrated that H_2O_2 is photo-produced in *M. crystallinum* leaves as a result of the PET chain activity in chloroplasts. Our data suggested that the redox status of the PQ pool and photo-produced H_2O_2 are involved in the C_3-CAM transition in *M. crystallinum* (Ślesak *et al*., 2003). Some oxidative stress factors different from H_2O_2, such as ozone (Niewiadomska *et al*., 2002; Hurst *et al*., 2004; Borland *et al*., 2006) and sulphur dioxide (Surówka *et al*., 2007), did not evoke CAM in *M. crystallinum* leaves. However, they induced some of the CAM-related enzymes. Moreover, experiments with *M. crystallinum* suggest that CAM appears to lower ROS production (oxidative stress) in plants exposed to extended periods of salinity (Borland *et al*., 2006). Nevertheless, the question of how H_2O_2/ROS are involved in the CAM mode of photosynthesis needs more detailed studies.

H_2O_2 AND THE EVOLUTION OF PSII

It has been suggested that H_2O_2 could be an early electron donor to PSII (for a review see: Olson & Blankenship, 2004). Blankenship and Hartman (1998) proposed that H_2O_2 may have been a transitional donor and that the present oxygen-evolving complex (OEC) may be structurally related to Mn-containing catalases. The oxidation of H_2O_2 in PSII might be expected to be similar to the oxidation reaction catalyzed by CAT, which decomposes H_2O_2 to water and oxygen. Mn-catalases have a binuclear Mn center that is structurally similar to half of the proposed tetranuclear center of the OEC (Dismukes, 1996; Pace & Åhrling, 2004) and that under certain conditions the OEC can act as a catalase. It cannot be excluded that the PSII reaction centre-like complex associated with a Mn-catalase might have been able to evolve O_2 using H_2O_2 as electron donor (Olson & Blankenship, 2004). When Blankenship and Hartman (1998) made their proposal, there was no evidence for a significant concentration of H_2O_2 ever existing on the Earth, but recently it has been shown that pyrite-induced H_2O_2 formation from H_2O is possible without molecular oxygen, thus it could have taken place in the absence of oxygen on the young Earth (Borda *et al*., 2001). However, experiments with H_2O_2-treated cyanobacteria cells have shown inhibition of OEC, suggesting that H_2O_2 could not be a physiological electron donor for PSII (Samuilov *et al*., 2001). The hypothesis about a role of H_2O_2 as a driving force of OEC evolution requires changes, in our opinion, concerning the state of the Earth surface many million years ago; whether the surface was more oxidised or reduced is still under debate (Lane, 2002). It is known that H_2O_2 is present in huge amounts on the surface of Mars (Hartman & McKay, 1995; Encrenaz *et al*., 2004). It might not be excluded that the surface of the Earth was also loaded with an adequate amount of H_2O_2 derived from photochemical processes about 3.5 billion years ago.

CONCLUSIONS

The main sources of H_2O_2 in the plant cell under steady-state conditions are the light-driven processes of photosynthesis and photorespiration. Hydrogen peroxide is a crucial component involved in the regulation of plant metabolism, defence and acclimatory processes, and gene expression. It also appears to coordinate plant development and the cell cycle. H_2O_2 levels increase in stress situations and its concentration in plant tissues is usually higher than in animal tissues. Specific mechanisms of H_2O_2 generation play an important role in diversification of H_2O_2 signalling. Hydrogen peroxide regulates other hormonal responses in plants, therefore it can play an imperative role as a 'master hormone'. Genetic systems controlling H_2O_2 signalling in plants have been suggested (Mateo *et al*., 2004; 2006).

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