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Review

Mechanisms of oxidative stress in plants: From classical chemistry to cell biology

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ABSTRACT

Oxidative stress is a complex chemical and physiological phenomenon that accompanies virtually all biotic and abiotic stresses in higher plants and develops as a result of overproduction and accumulation of reactive oxygen species (ROS). This review revises primary mechanisms underlying plant oxidative stress at the cellular level. Recent data have clarified the 'origins' of oxidative stress in plants, and show that apart from classical chloroplast, mitochondrial and peroxisome sources, ROS are synthesized by NADPH oxidases and peroxidases. ROS damage all major plant cell bio-polymers, resulting in their dysfunction. They activate plasma membrane Ca^{2+}-permeable and K^+-permeable cation channels as well as annexins, catalyzing Ca^{2+} signaling events, K^+ leakage and triggering programed cell death. Downstream ROS-Ca^{2+}-regulated signaling cascades probably include regulatory systems with one (ion channels and transcription factors), two (Ca^{2+}-activated NADPH oxidases and calmodulin) or multiple components (Ca^{2+}-dependent protein kinases and mitogen-activated protein kinases). Intracellular and extracellular antioxidants form sophisticated networks, protecting against oxidation and 'shaping' stress signaling. Research into plant oxidative stress has shown great potential for developing stress-tolerant crops. This can be achieved through the use of directed evolution techniques to prevent protein oxidation, bioengineering of antioxidant activities as well as modification of ROS sensing mechanisms.

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1. Introduction

Virtually all environmental and biotic stresses trigger a generalised stress response called an oxidative stress which can damage cell components and cause their dysfunction. This is induced by over-production and accumulation of molecules containing activated oxygen and called 'reactive oxygen species' (ROS). The reasons causing an oxidative stress mainly include: (i) an imbalance between ROS generation and detoxification due to disturbance of 'normal' cell physiology; (ii) ROS biosynthesis *de novo* as a constituent part of stress signaling and immunity response needed for defence and adaptation. These mechanisms co-exist, because stress factors directly producing ROS (transition metals, ultraviolet or ozone) additionally stimulate ROS generation by NADPH oxidases and peroxidases (Rao et al., 1996; Ranieri et al., 2003; Apel and Hirt, 2004; Zhang et al., 2010; Nawkar et al., 2013).

It is believed that the effect of O_2 derivatives is one of the oldest stresses on the planet (Dowling and Simmons, 2009). Plants have dealt with this for at least 2.7 billion years, i.e. since they started producing O_2 from CO_2 and H_2O. The constantly increasing level of O_2 has both directed species evolution and determined the biochemistry of modern plants and animals (Dowling and Simmons, 2009). Intriguingly, plants evolved the ability to employ an oxidative stress (or at least ROS biosynthesis) for signaling needs and sensing other stresses, regulation of growth, polarity and death (Demidchik et al., 2003, 2010; Foreman et al., 2003), sensing hormones and regulatory agents such as amino acids and purines (Murata et al., 2001; Demidchik et al., 2004, 2009; Krishnamurthy and Rathinasabapathi, 2013), generating gravitropic response (Joo et al., 2001) and a number of other processes that are not primarily related to stress or oxidation.

This review summarises and evaluates classical and some new concepts in the field of plant oxidative stress and ROS metabolism. A particular emphasis of this review is on the chemistry of individual ROS, cell and membrane mechanisms leading to ROS generation, amplification and regulation of ROS-mediated signals and programed cell death.

2. Definitions

The term ROS embraces substances containing one or more activated atoms of oxygen but are not necessarily radicals (for example H_2O_2 is not a radical). Free radicals are any chemical species that exist independently and contain unpaired electron(s). Some free radicals do not have oxygen atoms (for example, transition metals or carbon-centered radicals). Both ROS and free radicals promote oxidative stress through oxidation of cell compounds. The term 'oxidative stress' has several meanings. Firstly, it is the 'physiological state' (or conditions) when loss of electrons (oxidation) exceeds gain of electrons (reduction) leading to chemical (oxidative) damage of cell compounds. Oxidative stress is therefore associated with severe and long-term redox (reduction/oxidation) imbalance due to the lack of electrons. Secondly, it is one of 'stress factors' (similar to salinity, drought and others) damaging cells and triggering signaling and defence reactions. These definitions are related and can be combined.

In most cases oxidative stress starts from the activation of triplet oxygen (O_2). This makes O_2 more active or 'reactive'; therefore it is also often defined as the stress caused by 'reactive oxygen species', 'reactive oxygen intermediates', 'oxygen-derived species', 'free oxygen radicals' etc. Reactive nitrogen species (RNS) are another important class of substances potentially involved in oxidative stress (in this case, sometimes called 'nitrosative stress'). However experimental data are insufficient yet to understand the mechanisms of plant nitrosative stress; therefore this will not be discussed here in greater detail.

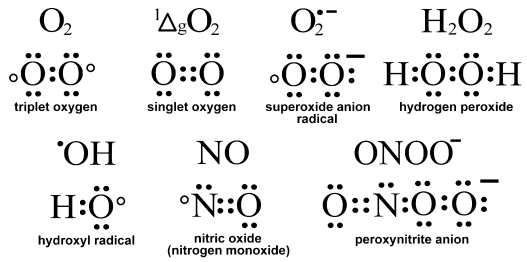

Fig. 1. Lewis dot structures of molecular oxygen (triplet oxygen) and key reactive oxygen/nitrogen species.

3. Chemistry of oxidising species

Oxygen is the most abundant element in the Earth's crust (Guido, 2001). Comprising about 89% of the mass in H_2O, oxygen is also the most abundant (by mass) element in living organisms. It is the second most powerful oxidiser known in chemistry after fluorine, which is far rarer than oxygen (Renda et al., 2004; Dowling and Simmons, 2009). Major atmospheric form of oxygen is O_2. This molecule has two unpaired electrons ($O_2^{2\bullet}$) and can exist as a free molecule; therefore it is a free radical. Both electrons in O_2 have the same spin numbers or 'parallel spins' which limit (restrict) the number of O_2 targets to those that have two similar electrons with antiparallel spins. This phenomenon is called a 'spin restriction' and decreases the reactivity of O_2. O_2 is not very chemically active and not toxic to aerobic organisms. To 'acquire' higher reactivity, O_2 requires an input of energy to remove the spin restriction. This energy comes from a number of chemical and biochemical reactions, highly energised electrons in electron transport chains (ETC), ultraviolet, ionising irradiation etc. Among many ROS found in biological systems, singlet O_2 ($^1\Delta_gO_2$), hydroxyl radical ($^{\bullet}OH$), hydrogen peroxide (H_2O_2), superoxide radical ($O_2^{\bullet-}$) and nitric oxide ($NO^{\bullet}$) (Fig. 1) are crucially important for induction of oxidative stress (Apel and Hirt, 2004). A number of other ROS could also be involved, such as peroxyl, alkoxyl and hydroperoxyl radicals, peroxinitrite, ozone and hypochlorous acid.

3.1. Superoxide radical

3.1.1. Chemistry of superoxide and measurements of superoxide production in plants

Triplet oxygen (O_2) can lose its 'spin restriction' by accepting a single electron, for example, due to the 'leak' of electrons in plant ETC or functioning of NADPH oxidase. This leads to formation of $O_2^{\bullet-}$ which is more reactive than O_2. It is called 'superoxide anion radical', 'superoxide radical anion', 'superoxide radical' or just 'superoxide'. The half-life of superoxide is typically from 1 to 1000 μs which only allows it to diffuse for few micrometers from site of the generation (Kavdia, 2006). $O_2^{\bullet-}$ participates in a

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number of reactions, but the prevalent will be the reaction with H^+ giving hydroperoxyl radical $HO_2^{\bullet}$. The latter molecule is more reactive, more stable and hypothetically permeable through biological membranes. Two molecules of $HO_2^{\bullet}$ react and give O_2 and H_2O_2 (known as 'superoxide dismutation' reaction). The ratio $O_2^{\bullet-}/HO_2^{\bullet}$ increases with pH (1:1 at pH 4.8, 10:1 at pH 5.8 and 100:1 at pH 6.8, respectively) (Sawyer and Gibian, 1979; Ross, 1985). This points to a special importance of $HO_2^{\bullet}$ at lower pHs, for example in the apoplastic space, mitochondrial intermembrane space, chloroplast thylakoid lumen, growing cell walls, vacuoles, peroxisomes and lysosomes. $O_2^{\bullet-}$ predominantly acts as a reducing agent; however $HO_2^{\bullet}$ more often functions as an oxidant. $O_2^{\bullet-}$ cannot chemically modify biological macromolecules but it is a major 'origin' of an oxidative stress. It can reduce other radicals and ROS ($Fe^{2+/3+}$, $Cu^{+/2+}$, $NO^{\bullet}$, phenoxyl radical, Fe–S clusters, etc.), leading to formation of strong oxidants (Sawyer and Gibian, 1979; Ross, 1985; Halliwell and Gutteridge, 1999). $O_2^{\bullet-}$ reduces Fe^{3+} and Cu^{2+} to Fe^{2+} and Cu^{+} respectively, which interact with H_2O_2 to produce $^{\bullet}OH$ (this ROS modifies virtually all organic molecules and is central to oxidative stress) (Pryor and Squadrito, 1995). $O_2^{\bullet-}/HO_2^{\bullet}$ pair also reacts with $NO^{\bullet}$, giving the extremely reactive RNSs, peroxynitrite ($ONOO^-$) and alkyl peroxinitrite (ROONO) respectively (Squadrito and Pryor, 1998). Peroxynitrite decomposes to hydroxyl radicals (Pryor and Squadrito, 1995). Interaction between $O_2^{\bullet-}$ and $NO^{\bullet}$ probably happens in plant cells (Delledonne et al., 2001; del Rio et al., 2002, 2003, 2006).

Depending on preparations and techniques used (Table 1), $O_2^{\bullet-}$ generation varies in kinetics, duration and intensity; but it accompanies plant responses to major stresses, such as salinity, drought, hypo- and hyperthermia, heavy metals, UV and others. $O_2^{\bullet-}$ is generated in few second after the addition of a stress factor or stress hormone (Kawano et al., 1998); but sometimes it takes hours to observe its synthesis at detectable levels (Schraudner et al., 1998). Quantitative measurements of $O_2^{\bullet-}$ do not make much sense because it is a short-living and locally produced molecule. Thousands of studies detected stress-induced increase in superoxide dismutase activity (SOD; enzyme scavenging $O_2^{\bullet-}$) but not many of them have really dealt with $O_2^{\bullet-}$ biosynthesis examination. A number of fluorescent (dihydrorhodamine 123, MitoSOX etc.), colorimetric (nitroblue tetrazolium) and luminescent (luminol, lucigenin, coelenterazine etc.) probes have been used for $O_2^{\bullet-}$ detection. However, these dyes are not "exclusively" specific to $O_2^{\bullet-}$ (Henderson and Chappell, 1993; Halliwell and Gutteridge, 1999; Kervinen et al., 2004). Lucigenin, nitroblue tetrazolium and dihydrorhodamine catalyse the redox cycling themselves and generate $O_2^{\bullet-}$ (thus leading to artefacts).

Electron Paramagnetic Resonance (EPR) spectroscopy is a key technique that can specifically measure generation of $O_2^{\bullet-}$ (Halliwell and Gutteridge, 1999; Mojovic et al., 2005). EPR spectroscopy is used in combination with spin traps, such as 'DMPO', 'DEPMPO' and others, providing characteristic spectra of stable oxygen-centered radical adducts of these traps (Mojovic et al., 2005). EPR spectroscopy has been successfully applied for measurements of extracellular $O_2^{\bullet-}$ and other radicals in intact maize (Liszkay et al., 2004) and *Arabidopsis* roots (Renew et al., 2005; Demidchik et al., 2010) and different *in vitro* preparations (Mojovic et al., 2005). However, EPR techniques have limitations. For example, recording EPR signal from thylakoids requires preparation of thylakoid-rich vesicles or isolated chloroplasts (Menconi et al., 1995). This procedure is an extreme 'wounding' stress for cells that stimulates ROS generation itself. Other intracellular tests based on the EPR spectroscopy, such as isolation of mitochondria or peroxisomes, 'suffer' the same problem.

Recently, hydroethidine was demonstrated to be a fluorescent probe with a high specificity to $O_2^{\bullet-}$ (Zhao et al., 2005). Hydroethidine reacted with $O_2^{\bullet-}$ forming two-electron oxidation product (E-oxy) while other key ROS, nitrogen and halogen species, such as H_2O_2, hydroxyl radical, peroxynitrite, and hypochlorous acid failed to oxidize hydroethidine to the same product (Zhao et al., 2005). This probe has now been successfully used for the detection of $O_2^{\bullet-}$ during metal-induced oxidative stress in plant cell cultures, roots and leaves (reviewed by Sandalio et al., 2008). Thus, hydroethidine looks promising for addressing the question on the specificity of $O_2^{\bullet-}$ generation and action in plant stress responses. The only problem of hydroethidine is its high instability (rapid decomposition in water solutions).

3.1.2. Sources of superoxide in stressed plants

It is widely believed that the major source of $O_2^{\bullet-}$ and contributor to oxidative stress in plants is an 'electron leakage' in ETCs of chloroplasts and mitochondria (Smirnoff, 1993; Apel and Hirt, 2004; Lesser, 2006; Møller et al., 2007; Møller, 2001; Rinalducci et al., 2008; Takahashi and Badger, 2011). 1–5% of electrons can be 'lost' in ETCs and some of these electrons seem to target and activate O_2 with formation of $O_2^{\bullet-}$ (Møller, 2001). $O_2^{\bullet-}$ biosynthesis is a common feature of organelles in non-stressed plants, but this can increase under stress, producing more $O_2^{\bullet-}$ than antioxidants can detoxify. Sites for of 'electron leakage' are demonstrated on the Fig. 2 and are found in photosystem I (Asada, 2006) and photosystem II (Pospíšil et al., 2004), in addition to mitochondrial complexes I and III (reviewed in Møller, 2001; Rinalducci et al., 2008; Hirst et al., 2008; they are not discussed in details here). EPR spectroscopy tests have demonstrated that pheophytin (pheo.), primary quinone acceptor (Q_A) and cytochrome *b559* are probably all able to reduce O_2 leading to the formation of $O_2^{\bullet-}$ at the electron acceptor side of photosystem II (Fig. 2) (Ananyev et al., 1994; Cleland and Grace, 1999; Pospíšil et al., 2004, 2006). $O_2^{\bullet-}$ is additionally produced in photosystem II by one-electron oxidation of H_2O_2 at the electron donor side (Chen et al., 1992, 1995). However, photosystem I is considered to be a major site for $O_2^{\bullet-}$ generation in chloroplasts (Genty and Harbinson, 1996; Asada, 2006; Foyer and Noctor, 2009). In photosystem I, $O_2^{\bullet-}$ is probably synthesised by the 4Fe–4S complex (clusters X) on psaA and psaB or A/B on psaC at the electron accepting (stromal) side (Asada, 1999).

Specialised antioxidant enzymes function in organelles (as well as everywhere in the cell) to prevent oxidative stress (Smirnoff, 2005; Foyer and Noctor, 2009). In chloroplasts, $O_2^{\bullet-}$ undergoes superoxide dismutase-catalysed disproportionation to O_2 and H_2O_2, which is then detoxified by ascorbate peroxidases (ascorbate + H_2O_2 → dehydroascorbate + H_2O) (Asada, 2006). Mitochondrial $O_2^{\bullet-}$ is also converted by superoxide dismutase (SOD) to O_2 and H_2O_2 but is mainly detoxified by glutathione peroxidases, however catalase and enzymes of so-called ascorbate–gluthatione cycle could also be involved in some tissues (Foyer and Halliwell, 1976; Møller, 2001).

Under pathogen attack, salinity, Cd^{2+}, herbicides and xenobiotics, large amount of $O_2^{\bullet-}$ is produced in peroxisomes, although normally these organelles function in photorespiration (where producing and detoxifying H_2O_2), oxidation of fatty acids, metabolism of nitrogen compounds and ROS detoxification (del Rio et al., 2006; Reumann and Weber, 2006). Two mechanisms of $O_2^{\bullet-}$ generation have been found in peroxisomes. First mechanism is driven by xanthine oxidase in the cellular matrix. The second one is catalysed by NADH/NADPH-dependent small ETC in the peroxisomal membrane comprising NADH:ferricyanide reductase, cytochrome *b*, monodehydroascorbate reductase and NADPH:cytochrome P450 reductase and producing $O_2^{\bullet-}$ in the cytosol (del Rio et al., 2002, 2006; Lopez-Huertas et al., 2000). Stresses stimulate $O_2^{\bullet-}$ generation leading to H_2O_2 accumulation in peroxisomes and cytosol by yet unknown pathway. They also decrease the activity of peroxisomal antioxidative defence systems (del Rio et al., 2006). SOD is a major $O_2^{\bullet-}$ -scavenging system in peroxisomes (del Rio et al.,

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Table 1
Production of superoxide radicals in responses to major stresses.

Stress	Object	Treatment	Organ	References
Salinity	*Pisum sativum*	Long-term	Leaf	Hernández et al. (1993)
	Nicotiana tabacum	Short-term	Suspension culture	Kawano et al. (2002)
	Arabidopsis thaliana	Short-term	Root	Demidchik et al. (2003)
Pathogens	*Hordeum vulgare*	Long-term	Leaf	Huckelhoven et al. (2000)
	Arabidopsis thaliana	Short-term	Root	Demidchik et al. (2003)
	Triticum aestivum	Long-term	Leaf	Giovanini et al. (2006)
Drought	*Triticum aestivum*	Short-term	Leaf	Menconi et al. (1995)
	Triticum aestivum	Long-term	Leaf and root	Selotea et al. (2003)
	Helianthus annuus	Long-term	Leaf	Sgherri et al. (1996)
High light	*Arabidopsis thaliana*	Short-term	Leaf	Fryer et al., 2003
Hyperthermia	*Arabidopsis thaliana*	Long-term	Leaf	Lee et al. (2002) Dong et al. (2009)
Hypothermia	*Phaseolus vulgaris*	Long-term	Leaf	Edreva et al. (1998)
Heavy metals	*Lycopersicum esculentum*	Long-term	Leaf	Wanga et al. (2008)
Herbicides	*Triticum aestivum*	Long-term	Leaf	Song et al. (2007)

Long-term and short-term are >1 h and < 1 h, respectively.

2002, 2006). H_2O_2 produced during the $HO_2^{\bullet -}$ dismutation reaction is probably detoxified through ascorbate-glutathione cycle (Foyer and Halliwell, 1976), similar to other plant cell compartments (del Rio et al., 2003; Reumann and Weber, 2006). Peroxisomes can also be involved in the control of plant antioxidant levels and enzymes repairing oxidised cell components (Reumann et al., 2007).

High light stress and ultraviolet, many xenobiotics and herbicides directly modify chloroplast, mitochondria or peroxisome ETCs, leading to $O_2^{\bullet -}$ generation. For these stresses, ETC electron leakage is definitely a prime cause of oxidative stress. However, for other stresses, such as salinity, pathogen attack, wounding, drought, hypothermia, hyperthermia, heavy metals, ozone, hypoxia, aluminum and other important stresses, disturbance of ETCs seems to be a secondary process occurring in the later stages of stress response (nevertheless, they may still play very important role). This probably develops as a result of decreased antioxidant pool that is exhausted during early (primary) oxidative burst. A central mechanism for these stresses is $O_2^{\bullet -}$ generation due to increased activities of NADPH oxidases and extra- and intracellular peroxidases (Doke, 1983; Bolwell et al., 2002; Foreman et al., 2003; Torres and Dangl, 2005; Torres et al., 1998, 2005; Bindschedler et al., 2006; Demidchik et al., 2003, 2009; Fluhr, 2009; Chang et al., 2012; Steffens et al., 2013).

NADPH oxidase or peroxidase generated ROS can be distinguished by the sensitivity to the NADPH oxidase inhibitor diphenylene iodonium (DPI) (Foreman et al., 2003; Fluhr, 2009). NADPH oxidase is blocked by 1–10 μM DPI while peroxidases are inhibited by one to two orders higher levels of DPI. Additionally, peroxidases (but not NADPH oxidases) are sensitive to azide and cyanide (Halliwell and Gutteridge, 1999; Bindschedler et al., 2006). One cautionary note should be made about the use of these pharmacological techniques. DPI is dissolved in 1–3% dimethyl sulfoxide, which is a radical scavenger affecting adequate ROS measurements; therefore a proper control tests with dimethyl sulfoxide should always be carried out. It should also be noted that a number of more specific blockers of NADPH oxidase have been developed (Williams and Griendling, 2007).

The function of NADPH oxidase in ROS promoted stress reactions, signaling, survival or death, is conserved among Kingdoms (Kawahara et al., 2007; Fluhr, 2009; Jiang et al., 2011). NADPH oxidase is encoded by the Respiratory Burst Oxidase Homologues (*RBOH*) gene family. This includes ten genes in *Arabidopsis* (*AtRBOHA-J*) and nine in rice; it also exists in all sequenced plant genomes (Groom et al., 1996; Torres et al., 1998; Torres and Dangl, 2005; Kawahara et al., 2007; Wong et al., 2007; Jiang et al., 2011). A reverse genetics approach and 'over-expression' of *RBOH* genes have demonstrated the relation between specific NADPH oxidase homologues (their activity and expression level) and reactions induced by different stresses (reviewed by Apel and Hirt, 2004; Torres and Dangl, 2005; Fluhr, 2009). For example, increase of *AtrbohD* and *AtrbohF* expression is required for oxidative burst induced by pathogenic *Pseudomonas syringae* or *Hyaloperonospora parasitica*, (Torres et al., 2002). The mechanism of this specificity is yet to be understood.

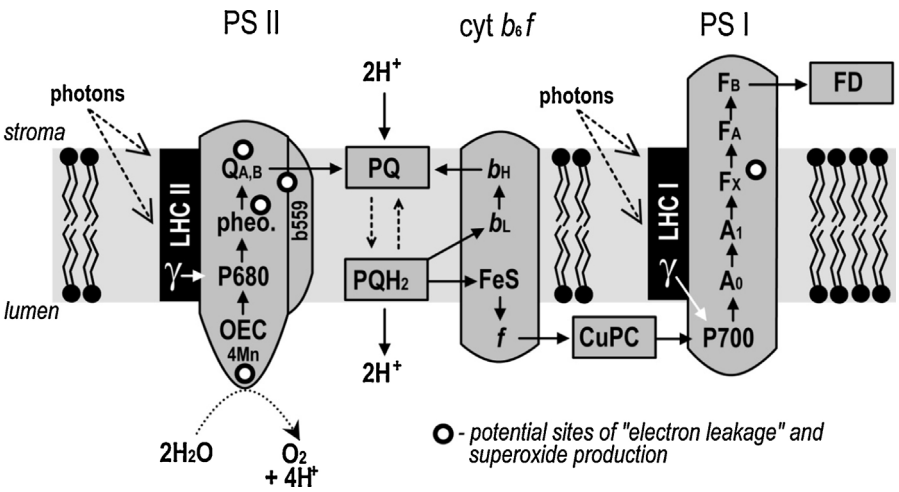

Fig. 2. Hypothetical sites of superoxide generation in photosystems I and II.

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The relationship between NADPH oxidase structure and function has recently been characterised (Kawahara et al., 2007; Sumimoto, 2008; Fluhr, 2009). The transfer of electrons in NADPH oxidase is catalysed by C-terminal cytoplasmic superdomain that is homologous to the ferredoxin reductase. It includes the NADPH-binding and FAD-binding sites which transfer the electron to the N-terminal six transmembrane segments containing the di-heme system (Kawahara et al., 2007; Sumimoto, 2008). Di-heme reacts with O_2 producing $O_2^{\bullet-}$ at the apoplastic side of the plasma membrane. NADPH oxidase is not functional in the absence of Ca^{2+}. The activation by Ca^{2+} is stucturally related to the N-terminus of plant NADPH oxidase containing two Ca^{2+}-binding helix–loop–helix structural domains (EF-hands) which are similar to Ca^{2+}-binding domains in calmodulin and troponin-C. Binding of Ca^{2+} causes a conformational change and intramolecular interaction of the N-terminal Ca^{2+}-binding domain with the C-terminal superdomain, resulting in the activation of electron transfer (Bànfi et al., 2004). Half-maximal activation of model NADPH oxidase (animal Nox5; *in vitro*) is caused by 1 μM Ca^{2+} (Bànfi et al., 2004). Maximal stress induced $[Ca^{2+}]_{cyt}$ increases are in the range from 0.3 to 3 μM, fitting well to this number (Demidchik et al., 2003, 2009; Hetherington and Brownlee, 2004; Demidchik and Maathuis, 2007).

Sensitivity to Ca^{2+} increases after calmodulin binding to the NADPH-binding domain or phosphorylation of serine/tryptophan residues in the FAD-binding domain by protein kinase C (Kobayashi et al., 2007; Tirone and Cox, 2007). Calmodulin-like domain protein kinases (CDPKs) stimulate NADPH oxidases through phosphorylation (Xing et al., 2001; Wu et al., 2010). For example, an increased CDPK expression causes elevation of plant NADPH oxidase activity, which is abolished by protein phosphatase 2A and unaffected by protein phosphatase 1 (Xing et al., 2001). Small G proteins (Rac/Rop GTPases) increase NADPH oxidase activity in Ca^{2+}-dependent manner (Baxter-Burrell et al., 2002; Wong et al., 2007).

Ca^{2+}-activated NADPH oxidase works in concert with ROS-activated Ca^{2+}-permeable cation channels to generate and amplify stress-induced Ca^{2+} and ROS signals (reviewed by Demidchik and Maathuis, 2007) (Fig. 3). Elevation of cytosolic $[Ca^{2+}]$ causes an increase in $O_2^{\bullet-}$ production and vice versa, $O_2^{\bullet-}$ activates Ca^{2+} influx through ROS-activated cation channels (Demidchik et al., 2009; Demidchik and Maathuis, 2007; Demidchik, 2010). This self-amplification mechanism, likely being an upstream component for many stresses may catalyse amplification and encode weak stimuli (transducing them into dramatic $O_2^{\bullet-}$ –Ca^{2+} alterations). For example, a marker of wounding stress, an extracellular ATP (Dark et al., 2011), acts through $O_2^{\bullet-}$ -Ca^{2+} signal amplification cycle (Demidchik et al., 2009). Initial small transient ATP-induced increase in $[Ca^{2+}]_{cyt}$ results in the production of ROS, which in turn induces massive activation of Ca^{2+}-permeable cation channels (Demidchik et al., 2009).

Systems that control $[Ca^{2+}]_{cyt}$, such as Ca^{2+}-permeable nonselective cation channels (NSCCs), depolarisation- and hyperpolarisation-activated Ca^{2+} channels, Ca^{2+}-ATPase, Na^+/Ca^{2+} and Ca^{2+}/H^+ exchangers, cytosolic Ca^{2+}-binding proteins and endomembrane Ca^{2+} transporters can potentially regulate the NADPH oxidase activity. These systems are modulated by a number of regulatory enzymes, for example specialised kinases and phosphatases, as well as physical/chemical regulators (pH, hormones etc.). This maintains fine oxidative balance and generate adequate $O_2^{\bullet-}$ – Ca^{2+} responses, which encode information about individual stress factors. The spatial distribution of the $O_2^{\bullet-}$ –Ca^{2+} system within a cell is also regulated. For example, SCN1/AtrhoGDI1 RhoGTPase GDP dissociation inhibitor is capable of "focusing" $O_2^{\bullet-}$ production by AtrbohC to specific zones of the cell (Carol et al., 2005). This explains greater ROS-Ca^{2+} responses in young elongating parts of the cell (such as tips of root hairs) in response to various stimuli (Demidchik et al., 2003, 2007, 2009, 2010; Foreman et al., 2003). Having four-dimensional system (X-Y-Z-time) $O_2^{\bullet-}$ –Ca^{2+}-mediated signals have high complexity and diversity, which is probably necessary for simultaneous interaction with a multitude of environmental cues.

Although some studies suggest that NADPH oxidase activation is not ubiquitous, virtually all stress-factors tested were able to produce $O_2^{\bullet-}$ burst through this mechanism (Fluhr, 2009). This has been shown for a number of plant species, all organs and tissues, calluses, suspensions and protoplasts (Foreman et al., 2003; Apel and Hirt, 2004; Torres and Dangl, 2005; Demidchik et al., 2009; Fluhr, 2009; Dubiella et al., 2013). The analysis of the available data suggests that NADPH oxidase activity during stress is mainly required for the following physiological functions: (a) recognition of stress factor and its intensity (to adjust gene expression and metabolism for adaptation); (b) triggering the programed cell death (to defend against pathogens and some abiotic stresses); (c) stomatal closure during drought stress response; and (d) simultaneous "processing" stress, immunity, defense and developmental chemical signals (abscisic acid, ethylene, brassinosteroids, auxin, gibberellic acid, methyl jasmonate, salicylic acid, volatiles etc.). NADPH oxidase and Ca^{2+}-permeable cation channels form a "regulatory hub", which is responsible for perception, transduction and encoding of stress stimuli (Demidchik and Maathuis, 2007; Fig. 3).

3.2. Hydrogen peroxide

3.2.1. Chemistry of H_2O_2 and its role in oxidative stress

Hydrogen peroxide (H_2O_2, HOOH; Fig. 2) is the most stable ROS with essential physiological functions (Halliwell and Gutteridge, 2007; Apel and Hirt, 2004; Foyer and Noctor, 2009). H_2O_2 is a weak acid without unpaired electrons ("non-radical"), and it is stable molecule as compared to superoxide, hydroxyl and singlet oxygen. Nevertheless, the lifetime of H_2O_2 in living tissues is not tremendously long (<1s) due to activities of catalases and peroxidases that decompose this substance (Halliwell and Gutteridge, 2007). In contrast to cytoplasm, which is a highly-reduced and antioxidant-enriched alkaline compartment, extracellular space is acidic and normally does not contain high levels of H_2O_2 scavenging enzymes, catalases and peroxidases, as well as superoxide dismutase removing its precursor (Hernández et al., 2001; Mhamdi et al., 2012). This low activity of enzymatic ROS scavengers provokes H_2O_2 accumulation in the apoplast, promoting oxidative stress.

The cytoplasm is typically a thin layer because the vacuole occupies up to 95% of cell volume; therefore endogenously produced H_2O_2 may rapidly diffuse outside, presumably crossing the plasma membrane through aquaporins (Dynowski et al., 2008). Another source of H_2O_2 is directly from the apoplast, where it is produced through dismutation of $O_2^{\bullet-}/HO_2^{\bullet-}$ synthetized by NADPH oxidases and extracellular heme-containing Class III peroxidases (Apel and Hirt, 2004; Cosio and Dunand, 2009). A number of other non-radical organic peroxides (ROOR, ROOH or RO_2OH) can be produced in the cell, such as lipid peroxides. These compounds promote oxidative stress in different ways, for example in lipid peroxidation chain reactions, and probably also involved in 'shaping' the intra- and extracellular redox signal (Møller et al., 2007; Suzuki et al., 2012).

3.2.2. Measurements of H_2O_2 production in plants

Although quantitative test of unstable oxygen-centered radicals is meaningless, $[H_2O_2]$ measurement provides useful information on the development of oxidative stress. Standard techniques to test $[H_2O_2]$ include application of colorimetric, fluorescent and luminescent probes, which, if oxidised by H_2O_2, change their spectral characteristics or emit photons (Rhee et al., 2010). These probes can be membrane-permeant or -impermeant; hence they allow differentiate with H_2O_2 content in the cell and apoplast. They are

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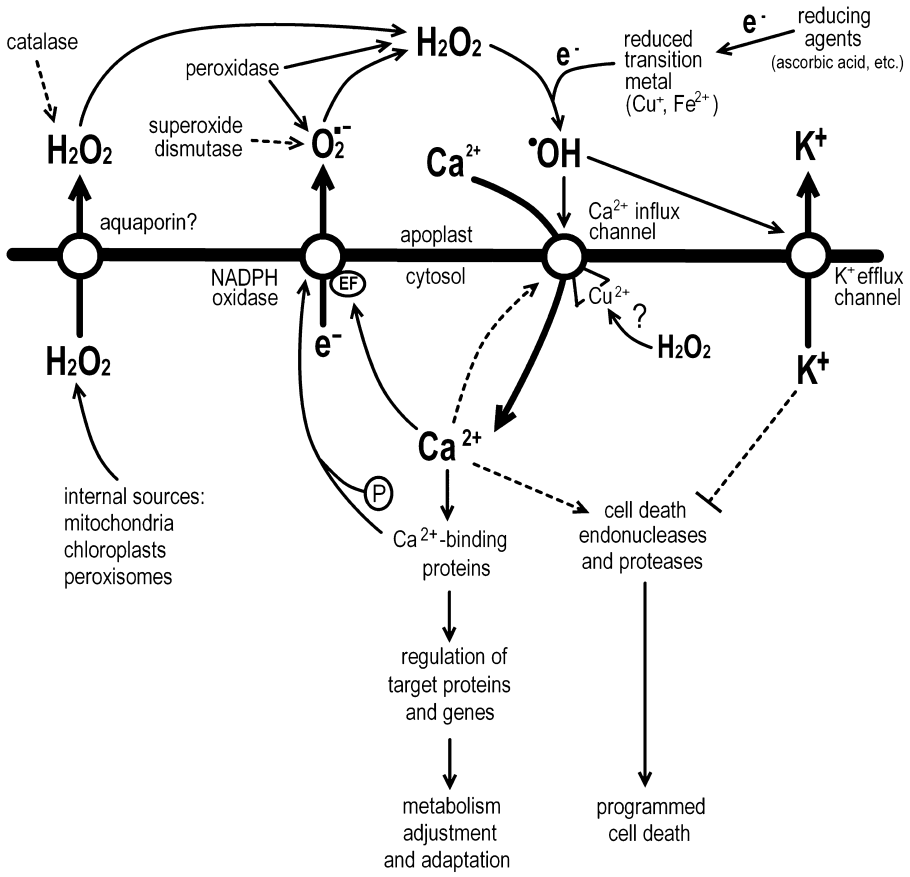

Fig. 3. Involvement of plasma membrane in the regulation of oxidative stress in higher plants.

not 100% specific to H_2O_2 and, in fact, interact with other ROS (Rhee et al., 2010). The analysis of reports dealing with H_2O_2 shows that H_2O_2 concentration increases from 0.03–1 μM ("at rest") to 0.1–10 mM ("under stress") depending on preparation, stress factor, technique and other variables. Huge variation in the kinetics and amplitude of H_2O_2 accumulation has also been reported. Detectable [H_2O_2] can be found from a few seconds to several days after the application of stress factor. This difference in results can be caused by a number of reasons. H_2O_2 is probably produced in specific areas (hot spots), for example in tips of growing root hairs (Foreman et al., 2003); meaning total tissue/organism H_2O_2 activity can be very low. Most techniques used for H_2O_2 measurements show the cumulative effect of H_2O_2 production and cannot monitor H_2O_2 dynamics. Chemiluminescent probes report H_2O_2 faster and they are more sensitive than fluorescent probes. Physical and chemical conditions used for H_2O_2 detection, such as high pH values (pH 8–9), can alter the cell potency to generate ROS. H_2O_2 measurements can also be affected by the structure of plant tissues. For example, leaf cuticula is virtually impermeable for H_2O_2 probes. Finally, the nature, exposure time and intensity of the imposed stress vary dramatically across studies.

More efficient methods for measuring H_2O_2 have been recently developed (Rhee et al., 2010; Michelet et al., 2013). Boronate-based fluorescent probes ('peroxysensor family' of H_2O_2 probes) fluoresce after the H_2O_2-induced removal of a boronate group (Miller et al., 2005). They are membrane-permeant and can be combined with SNAP-tag, which targets them to different cell compartments to assess [H_2O_2] spatially. Although these probes do not interact with other ROS, they react with H_2O_2 irreversibly and cannot test [H_2O_2] dynamics (Dickinson et al., 2010). Another group of probes utilizes specially modified green fluorescent proteins (GFP) which include two redox-sensitive cysteine residues (Rhee et al., 2010). H_2O_2 induces formation of di-sulfide bond and modifies spectral characteristics of protein fluorescence. However, this probe lacks specificity to H_2O_2. The latest addition to fluorescent H_2O_2 probes is a genetically encoded and reversible "hydrogen peroxide sensor" (HyPer) (Malinouski et al., 2011). The design of HyPer is based on bacterial OxyR transcription factor (Kim et al., 2002) with a yellow fluorescent protein inserted into the regulatory domain of this molecule (Belousov et al., 2006). H_2O_2 acts on OxyR regulatory domain and causes the formation of a di-sulfide bond between C199 and C208 which changes the spectral properties of YFP (Kim et al., 2002; Belousov et al., 2006). This di-sulfide bond can be repaired by thiol-disulfide oxidoreductase *in vivo*; therefore HyPer can react with H_2O_2 again (Belousov et al., 2006). Formation of oxidised HyPer shows the dynamics of H_2O_2 in individual cell compartments (Malinouski et al., 2011). Another approach for detection of [H_2O_2] involves peroxalate nanoparticles, which undergo a three-component chemiluminescent reaction between H_2O_2, peroxalate esters and fluorescent dyes inside the cell (Lee et al., 2007a,b). This technique is probably sensitive and specific to H_2O_2, however the delivery of nanoparticles and their potential intracellular redox activity may cause problems. Michelet et al. (2013) have recently developed novel EPR spectroscopy-based techniques to measure extracellular H_2O_2 content in cells of *Chlamydomonas reinhardtii*. These authors have used a spin-trapping assay containing 4-POBN/ethanol/Fe-EDTA. Fe-EDTA reacts with H_2O_2 forming $^{\bullet}OH$ which is trapped by 4-POBN/ethanol (giving stable detectable adduct).

3.2.3. *Sites of H_2O_2 generation and "targets" of H_2O_2 in plant cells*

Sites of superoxide productions are widely considered as "origins" of H_2O_2 generation. Peroxidases were first proposed to be involved in the generation of H_2O_2 during biotic stress (Bolwell

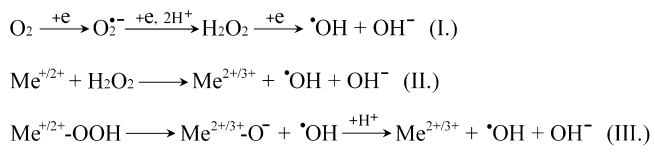

Fig. 4. Reactions synthesising hydroxyl radical in plants. I.—reduction of oxygen leads to formation of hydroxyl radicals. II.—"outer-sphere electron transfer" in classical Fenton-like reaction (when transition metals do not bind covalently to H_2O_2). III.—"inner-sphere electron transfer" that involves strong peroxide binding to a transition metal.

and Wojtaszek, 1997; Bolwell et al., 1998; Bindschedler et al., 2006). Now it is widely accepted that some class III peroxidases, for example Cu-containing amino oxidases and polyamine oxidases, gluthatione and ascorbate oxidases are also important for ROS production induced by salinity, high light, heavy metal and other abiotic stresses (Rodríguez et al., 2002, 2007; Chang et al., 2009). Interestingly, peroxidases are probably regulated by a negative feedback mechanism; some of them are inhibited by H_2O_2 (Kitajima, 2008).

Oxidative stress is widely believed to be induced by H_2O_2. It is not exactly true because •OH biosynthesis is required to accomplish H_2O_2-mediated oxidation. The biosynthesis of H_2O_2 is difficult to relate directly with distinct physiological functions; hence why, in a number of studies, H_2O_2 (0.01–10 mM) is added to plant cells exogenously and the induced reactions are investigated. H_2O_2 is a weak oxidant and cannot chemically modify DNA, amino acids or lipids. It may directly interact with SH- groups but its main 'target' is transition metal binding sites converting it to OH• (Fry et al., 2002; Fry, 2004). H_2O_2 has been reported to deactivate some enzymes, for example fructose biphosphate; however, this may be caused by residues of transition metals in experimental solutions (Halliwell and Gutteridge, 1999). Most proteins withstand 100 mM H_2O_2 in transition metal-free solutions (Halliwell and Gutteridge, 1999). A number of H_2O_2 'sensors' have been proposed to exist in plant cells (Apel and Hirt, 2004), however only a few of them have been proven by experiments.

3.3. Hydroxyl radical

The hydroxyl radical (•OH; Fig. 1) is a prime cause of oxidative damage to proteins and nucleic acids as well as lipid peroxidation during oxidative stress. It is directly involved in oxidative stress signaling and PCD (Demidchik et al., 2003,2010). For example, this ROS induces the greatest activation of Ca^{2+} and K^+ channels amongst all known ROS and free radicals, leading to Ca^{2+} influx and K^+ efflux immediately after application of a stress factor (Demidchik, 2010). The estimated '*in vivo*' half-life of •OH is approximately 1 ns, which allows •OH diffusion over very short distances (<1 nm) (Sies, 1993). Second-order rate constants for reactions of •OH with most organic molecules are extremely high. •OH interactions with cell biopolymers are limited by diffusion-controlled rate (Anbar and Neta, 1967; Sies, 1993). Specific •OH scavengers and antioxidants do not exist and the widely reported effects of mannitol, sorbitol, dimethyl sulfoxide, thiourea or others are, in fact, not due to •OH scavenging. These substances probably interact with •OH 'longer-living' precursors or chelate transition metals.

Fenton reaction(s) is central to •OH bio-synthesis (Fig. 4). Study conducted by Fenton in 19th century aimed to establish effect of Fe^{2+} on tartaric acid (Fenton, 1894). However his findings were so important for natural sciences that they have been used for more than a century. Nowadays, "Fenton chemistry" and "Fenton-like reagents" are referred to reactions taking place in the presence of H_2O_2 and transition metals and producing •OH, water and superoxide (Goldstein et al., 1993). Although many intermediates are formed, the net "Fenton reaction" is as follows: (I) metal reduced + H_2O_2 → metal oxidised + •OH + OH^-; (II) metal oxidised + H_2O_2 → metal reduced + $HO_2^{\bullet-}$ + H^+ (Koppenol, 2001). These equations were proposed by Fritz Haber (Nobel Prize winner in 1918) and his student Joseph Weiss in the 1930s and since called the Haber-Weiss cycle (Haber and Weiss, 1932). Importantly, ascorbic acid is likely to serve as a pro-oxidant reductant for iron and copper in Haber-Weiss cycle in plants, because its concentration in plants (including the apoplast) is very high (1–20 mM) (Fry et al., 2002; Foyer and Noctor, 2011). This pro-oxidant activity of the ascorbate is poorly studied and sometimes ignored.

•OH can also be generated by homolytic bond fission of H_2O. In this case, electrons in covalent bonds are equally distributed to atoms. This requires significant input of energy by ultraviolet quanta, freezing–drying cycle, heat or ionizing radiation (Halliwell and Gutteridge, 1999). The generation of •OH from H_2O_2 though has a lower energy threshold therefore, under some natural conditions, •OH can probably be directly generated from H_2O_2 (HOOH → •OH + •OH) and hydroperoxides (ROOH → •RH + •OH) by sunlight (Downes and Blunt, 1879). This process is called "photo Fenton". The physiological importance of •OH produced by homolytic fission is still debated, apart from studies on UV stress where it is directly involved in oxidative destruction of cell components (Jain et al., 2004; Kataria et al., 2005).

In classical Fenton-like reactions metals do not bind covalently to H_2O_2 (outer-sphere electron transfer; Fig. 4; reaction II). Another mechanism of •OH generation that involves strong peroxide binding to a metal center has been shown in biological systems over the last two decades, (Fig. 4; reaction III) (Sawyer et al., 1993, 1996; Fridovich, 1998; Pospíšil et al., 2004). This is the so-called "inner-sphere electron transfer" process forming a temporary covalent bond between peroxide and metal ion. Heme oxygenases (Ortiz de Montellano, 1998), cytochrome P450 (Sono et al., 1996), bleomycin (Burger, 2000), superoxide reductases (Mathe et al., 2002) and some PSII proteins (Pospíšil et al., 2004) are examples of systems, which 'use' this mechanism of radical generation.

Measurement of •OH is a problem due the extreme reactivity of •OH and its very short lifetime. Nevertheless, several studies have successfully undertaken such measurements. A number of techniques for •OH detection has been developed, including colorimetric, fluorescent, luminescent and radioactively-labeled probes (Halliwell and Gutteridge 1999). However, it seems that EPR spectroscopy is only the technique providing specificity of •OH measurement (Liszkay et al., 2003, 2004; Jain et al., 2004; Renew et al., 2005; Demidchik et al., 2010; Michelet et al., 2013; Šeršeň and Král'ová, 2013). EPR spectrometry is also not an 'ideal' method because •OH spin traps (DMPO, EMPO, DEPMPO or POBN) decompose at room temperature (so, the signal intensity drops) and react with other radicals, for example with superoxide (Pou et al., 1989). Moreover EPR spectroscopy cannot be used efficiently for studying inner tissues. Nevertheless, EPR-based techniques are still far more sensitive and accurate than widely used imaging approaches.

EPR analysis allowed the assessment of •OH production in a single root of *Arabidopsis thaliana* (Renew et al., 2005). This study has clearly demonstrated that the root produces •OH without stress that is probably required for ROS-Ca^{2+}-driven cell elongation (Demidchik et al., 2003; Foreman et al., 2003) and cell wall remodeling (Liszkay et al., 2004). This production depends on the activity of NADPH oxidase RbohC and the presence of transition metals in the cell wall. Nevertheless, over-production of •OH, for example resulting from UV-B irradiation, has been shown to block elongation growth in plants (Jain et al., 2004; Kataria et al., 2005). Salt-induced •OH production in intact plants has been demonstrated in *Arabidopsis thaliana* roots (Demidchik et al., 2010). This production results in K^+ loss through •OH-activated K^+ efflux channels and

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leads to PCD via K^+-dependent cell death proteases and endonucleases (Demidchik et al., 2010) (Fig. 3). Thus •OH over-production can be the source of severe stresses and plant cell death.

The importance of •OH over-production for destructive changes in photosynthetic apparatus is widely acknowledged (Møller et al., 2007; Šeršeň and Král'ová, 2013), but this has only recently been confirmed by direct EPR spectroscopy tests (Pospíšil et al., 2004; Šnyrychová et al., 2006; Pospíšil, 2009). Under stress conditions, •OH is generated by both photosystems. In PSI, leakage of electrons leads to biosynthesis of superoxide (Fig. 2) that dismutes to H_2O_2, which in turn accumulates in stroma. H_2O_2 can be reduced to •OH by free transition metals in stroma (classical Fenton-like reaction; Fig. 4) or by formation of H_2O_2-Fe complexes of ferredoxin (inner-sphere electron transfer) (Šnyrychová et al., 2006; Fig. 4). In PSII, three transition metal binding sites are probably involved in •OH production from H_2O_2 (Pospíšil et al., 2004; Pospíšil, 2009). Firstly, Fenton-like reaction of H_2O_2 with free transition metals can occur in stroma; secondly, non-heme iron (the ligand is unknown) could be involved through inner-sphere electron transfer; and, thirdly, heme-iron of cyt b_{559} could also participate in similar reaction and form Fe–H_2O_2 complexes. Overall, this can result in an oxidative damage of chloroplasts and their dysfunction.

The production of •OH for signaling needs could be related to the presence in the cell of the specific chemical sites (metal pockets), which bind catalytically active transition metal cations, such as $Cu^{+/2+}$, $Fe^{2+/3+}$ or $Mn^{2+/3+}$ (Demidchik et al., 2014) (Fig. 3). These sites usually contain pairs of cysteine and histidine forming a complex with transition metal (Demidchik et al., 2014). Intriguingly, Demidchik et al. (2007) have demonstrated that the activation of single-channel conductances by H_2O_2 requires the delivery of H_2O_2 directly to the channel macromolecule at the extracellular side of the plasma membrane. Supporting this hypothesis, Rodrigo-Moreno et al. (2013) have recently found that Cu^{2+} acts on K^+ efflux at the cytosolic side of the plasma membrane.

3.4. Singlet oxygen

The activation of O_2 in chloroplasts and mitochondria, for example through absorption of light quanta in P680 (Asada, 2006), can lead to the formation of two types of extremely reactive O_2-derived species: non-radical $^1\Delta_gO_2$ (22.4 kcal) and the more reactive free radical $^1\Sigma g^+O_2$ (37.5 kcal) (Schweitzer and Schmidt, 2003). The term 'singlet O_2' traditionally covers both species. $^1\Sigma g^+O_2$ can decay into $^1\Delta_gO_2$, but the significance of this process in the cell is not proven. Singlet O_2 is detoxified rapidly by beta-carotene in the PSII reaction centre, water, tocopherol, reduced plastoquinone or flavonoids (Trebst and Depka, 1997; Schweitzer and Schmidt, 2003; Asada, 2006; Kruk and Trebst, 2008; Fischer et al., 2013). It is believed that over-production of singlet oxygen takes place under stress conditions (in particular, under photo-oxidative stress) (Apel and Hirt, 2004; Asada, 2006; Fischer et al., 2013). This results in oxidative injuries, induction of programed cell death and retrograde signaling (Møller et al., 2007; Fornazari et al., 2008; Przybyla et al., 2008; Fischer et al., 2013).

The classical view claims that the lifetime and diffusion distance of singlet oxygen is short (3.1 to 3.9 μs and 190 nm, respectively, accordingly to Asada, 2006). Some researchers suggest that the diffusion limit for singlet oxygen in chloroplasts is just few nanometers due to high viscosity and complexity of cell solution (Krasnovsky, 1998). However, recent measurements of singlet O_2 with a highly selective Singlet Oxygen Sensor Green (SOSG) have shown that this substance is more stable in plant cells than previously thought and can diffuse outside the chloroplast and even reach the cell wall (Flors et al., 2006; Driever et al., 2009). Therefore singlet oxygen probably has a broader spectrum of targets in cells, including cytosolic signaling cascades, plasma membrane and tonoplast ion channels (Fischer et al., 2013).

3.5. Transition metals

Transition metals and their complexes with S-containing amino acids and some other organic ligands, are major redox switches in living systems (Outten and Theil, 2009; Robinson and Winge, 2010). IUPAC defines transition metal as any element with an incomplete d sub-shell, or which can give rise to cations with an incomplete d sub-shell (http://goldbook.iupac.org/). Forty elements (21 to 30, 39 to 48, 71 to 80, and 103 to 112) fit in this definition and can be considered as transition metals (McCleverty, 1999). However quite few transition metals have demonstrated significance for biological systems. From the physiological point of view, the most important transition metals are Cu, Fe and Mn. Although Ni, Hg, Cr and Co can also be involved in some metabolic reactions, they are clearly less important for cell physiology. Cu and Fe are the most abundant transition metals in living systems (Outten and Theil, 2009; Robinson and Winge, 2010). Their role as electron transport components in most redox enzymes is based on their ability to change their valence more easily as compared to other metals found in cells (Bergmann, 1992; Robinson and Winge, 2010). Mn and Ni show very similar properties to Cu and Fe, when coordinated by some organic ligands (for example by His residues); however they almost completely lack electron transfer capacity in free ionic form.

The catalytic activity of Cu and Fe may increase several times during stress conditions (Becana and Klucas, 1992; Moran et al., 1994, 1997; Becana et al., 1998). The toxic and regulatory effects of Cu and Fe are mainly related to •OH generation. Cu is well-known eco-toxicant (Bergmann, 1992). After application of Cu-containing fungicides or in Cu mining areas, Cu directly affects plants, causing an oxidative stress, lesions, inhibition of metabolic reactions, suppression of photosynthesis, mineral disorders, loss of membrane integrity and cell death (Bergmann, 1992; Demidchik et al., 1997, 2001).

Cu is about 60 times more potent as a catalyst of the Haber-Weiss cycle and several billion times more soluble than Fe under biological pH range, but Fe is more abundant in the cell (Bergmann, 1992; Halliwell and Gutteridge 1999; Fry et al., 2002). Taking this into account, Cu as a major catalyst of •OH generation seems to be much more probable (Fry et al., 2002; Demidchik et al., 2003). Cu and Fe are typically bound in organic complexes with carbohydrates and proteins; hence their catalytic activity should be considered instead as a concentration of free ionic forms. Moran et al. (1997) discovered that specific Fe-chelating phenolic compounds can be synthesised in response to stresses that increase Fe catalytic activity, promote •OH generation, triggering DNA damage and lipid peroxidation. Another potential mechanism of Cu and Fe "mobilization" and increase of their catalytic activities during stresses is through formation of complexes with polyamines such as spermine, spermidine and putrescine. These substances have been shown to be synthesised in plant cells in response to stresses (Alcázar et al., 2006; Moschou et al., 2009; Shi and Chan, 2014) and proven to form redox-active complexes with both Cu (Guskos et al., 2007) and Fe (Tadolini, 1988). Thus, H_2O_2-induced damage and signaling are focused on Cu- and Fe-binding centres that generate •OH (Demidchik et al., 2007, 2014). Cu and Fe may also bind to specific targets in proteins, such as His-pockets or Cys-pockets and cause their oxidation, leading to damages (Demidchik et al., 2014). There is accumulating evidence that oxidative stress would not be possible without Fe and Cu as removal of these metals (using specific chelators or decreasing accumulation) results in termination of oxidative burst (Sayre et al., 2008; Demidchik et al., 2014).

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4. ROS-mediated signaling and regulation of plant cell physiology

A number of models for ROS sensing and signaling in plant cells have been proposed (Apel and Hirt, 2004; Asada, 2006; Demidchik and Maathuis, 2007; Foyer and Noctor, 2009; Sierla et al., 2013; Demidchik et al., 2014). Potential ROS receptors include the following: (A) two-component histidine kinases; (B) redox-sensitive transcription factors, such as NPR1 or Heat Shock Factors; (C) ROS-sensitive phosphatases; (D) redox-regulated ion channels (reviewed by Apel and Hirt, 2004; Demidchik and Maathuis, 2007; Sierla et al., 2013; Demidchik et al., 2014). The role of first three mechanisms requires investigation while the direct involvement of ion channels in ROS sensing has recently been studied in details.

The changes of ion activities through modulation of ion channel/receptor conductance are the fastest and most important switch of physiological and biochemical parameters in animal cells responding to external and internal factors including oxidative stress. It involves at least two mechanisms: (A) a change in ionic composition modifying metabolic interactions, as ions are direct regulators of enzymes of osmotic pressure; (B) changing the electric potential difference across the membrane which can modulate activities of active transporters, such as H^+-ATPase, and functioning of vesicular transport and cytoskeleton. Evidence obtained *in vitro* using recombinant protein techniques show that plant G-proteins (modulators of signaling cascades and ion channels) are directly regulated by ROS (20 μM H_2O_2) via stimulation of alpha subunit dissociation (Wang et al., 2008). Nevertheless, this observation requires confirmation by tests performed *in vivo*. The activity of several plant cation channels have been shown to be modified in the presence of high [H_2O_2] showing that ion channels can be involved in the perception of H_2O_2-encoded messages. Exogenous H_2O_2 activates Ca^{2+}-permeable non-selective cation channels in protoplasts isolated from *Arabidopsis thaliana* guard cells (Pei et al., 2000). These channels are probably involved in ABA- and jasmonate-induced stomata closure (Munemasa et al., 2011). Exogenous H_2O_2 inhibits K^+ outwardly rectifying channels in guard cells and root epidermis (Demidchik et al., 2003; Kohler et al. 2003), but it does not change the activity of Ca^{2+}-, K^+-, Cl^--selective channels and NSCCs in green algae *Nitella flexilis* or *Arabidopsis thaliana* root plasma membrane NSCCs when applied in the whole cell configuration (Demidchik et al., 1997,2001,2003,2007). Exogenous H_2O_2 stimulates anion efflux in cultured *Arabidopsis thaliana* cells, mimicking the ABA effect (Trouverie et al., 2008). However, this action seems to be related to the activation of Ca^{2+} conductance, which in turn activates Cl^- currents. Endogenously applied H_2O_2 activates *Arabidopsis thaliana* root Ca^{2+}-permeable NSCCs (Demidchik et al., 2003, 2007). This activation is observed only in outside-out mode when H_2O_2 was delivered closely to the plasma membrane cytoplasmic side. The direct voltage-dependent activation of *Arabidopsis thaliana* plasma membrane K^+ channel SKOR (heterologously expressed in HEK cells) by H_2O_2 has been recently discovered (Garcia-Mata et al., 2010). Cys-168 residing in S3 alpha-helix of SKOR voltage sensor complex is responsible for the sensitivity to H_2O_2.

•OH action on ion channel mediated activities has been investigated by the addition of a •OH-generating mixture (Cu^{2+}, L-ascorbic acid and H_2O_2) to protoplasts and intact cells (Demidchik et al., 2003, 2007, 2010; Foreman et al., 2003). These experiments have shown that •OH activates Ca^{2+}-permeable NSCCs (Ca^{2+} influx channels) and K^+ outwardly rectifying channels (catalysing K^+ efflux) in mature root epidermal cells, elongation zone, pericycle cells, cortex and root hairs of *Arabidopsis thaliana*. •OH-induced activation of Ca^{2+} influx and K^+ efflux has also been observed in mature and young root epidermis of maize, clover, pea, wheat and spinach (Demidchik et al., 2003). Gork-1 probably encodes •OH-activated K^+ efflux conductance while genes encoding •OH-activated Ca^{2+}-permeable channels have not been yet identified. Both •OH-induced Ca^{2+} and K^+ conductances could also be related with activities of annexins, as they are decreased in KO line lacking these systems (Laohavisit et al., 2012). Moreover, Ca^{2+}/K^+ transporting annexins might include functional peroxidase domain producing ROS (Laohavisit et al., 2009).

Activation of K^+ efflux channels by •OH (for example, during response to salinity) leads to massive loss of K^+ from root cells (Demidchik et al., 2003, 2010, 2014). Animal endonucleases and caspases are directly inhibited by cytosolic K^+; therefore cation channel mediated K^+ loss stimulates these enzymes and induces apoptosis (Seon and Ja-Eun, 2002). Similar mechanism of programed cell death induction has been discovered in plants (Demidchik et al., 2010; Fig. 3). Root cell endonucleases and proteases activate in K^+-dependent mode. This causes symptoms typical of PCD. K^+ channel antagonists or radical scavengers stop or delay K^+ loss and the appearance of PCD symptoms (Demidchik et al., 2010). Interestingly, expression of animal anti-apoptotic CED-9 gene can decrease the H_2O_2-induced K^+ efflux from leaf segments (Shabala et al., 2007). This suggests that plant and animals share similar K^+-mediated mechanisms of apoptosis-like PCD.

In animal cells, singlet O_2 generation leads to inhibition of the mitochondrial inner membrane K^+ influx channels which regulate mitochondrial volume (Duprat et al., 1995; Fornazari et al., 2008). This results in cytochrome c release and transporting superoxide anion (produced in mitochondria during stresses) to the cytosol. Whether similar reactions take place in plants is unknown. The involvement of singlet O_2 in ion channel activation could be through H_2O_2, which can accumulate after singlet oxygen detoxification in organelles and diffuse to the cytosol where it activates Ca^{2+} channels and triggers signaling cascades, for example sending ROS/Ca^{2+} messages to the nucleus. The latter is called retrograde signaling and might play an essential role in the regulation of organelle protein biosynthesis under high light and probably other environmental stresses (Fernándeza and Stranda, 2008; Chang et al., 2009; Karpiński et al., 2013; Kim and Apel, 2013).

Major physiological effect induced by ROS-induced activation of Ca^{2+}-permeable cation channels is a transient elevation of cytosolic free Ca^{2+} (Demidchik and Maathuis, 2007; McAinsh and Pittman, 2009; Fig. 4). Elevation of $[Ca^{2+}]_{cyt}$ links H_2O_2 accumulation and intracellular signaling and gene expression (Demidchik, 2010; Fig. 3). It activates downstream Ca^{2+}-dependent regulatory cascades through Ca^{2+}-binding proteins (CBPs). Approximately 250 CBP genes exist in *Arabidopsis thaliana* genome (Day et al., 2002). CBPs undergo reversible interaction with Ca^{2+} that leads to their conformational change and facilitates interactions with a number of cell targets (Gifford et al., 2007; Dubiella et al., 2013). Classical CBPs contain 'EF-hand' motifs providing high-affinity binding of Ca^{2+}. Five classes of CBPs include: calmodulins, calmodulin-like proteins, Ca^{2+}-dependent protein kinases (CDPKs), calcineurin B-like proteins and NADPH oxidases. CDPKs directly transduce ROS-Ca^{2+} signals to catalytic activity while the calmodulins, calmodulin-like proteins, and calcineurin B-like proteins play are intermediate sensors regulating downstream systems, which in turn react with final target or other regulators. NADPH oxidases, as mentioned above, enhance weak Ca^{2+} signals, amplifying these signals using ROS biosynthesis *de novo* and ROS-activated Ca^{2+} influx channels. Apart from calmodulins and NADPH oxidases that exist in animals, the other three CBP classes are only found in plants and some bacteria. These three classes of CBPs are found in all plant cell organelles and involved in a multitude of functions (DeFalco et al., 2010).

The activity and expression level of certain CBPs increase in the presence of elevated ROS concentrations, and in response to biotic and abiotic stresses which generate ROS (DeFalco et al.,

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2010). For example, H_2O_2 causes fast induction of CDPK3 in *Arabidopsis* root suspension culture protoplasts, leading to the change of the expression of some stress-responsive genes and activity of 28 target proteins (Mehlmer et al., 2010). Stress hormones (abscisic and jasmonic acids) also stimulate CDPKs (Munemasa et al., 2011). This effect probably relies on ROS-Ca^{2+} signaling induced by these hormones. CDPKs are involved in the regulation of specific NADPH oxidases (such as AtRBOHD, StRBOHB and others) via Ca^{2+}-dependent phosphorylation of these proteins (Kobayashi et al., 2007; Dubiella et al., 2013). This is another amplification mechanism of ROS-Ca^{2+} signaling.

ROS have been shown to change the activity of a number of other regulatory enzymes, in particular specific kinases and phosphatases such as MAP kinases and other Ser/Thr kinases, MAPK phosphatases etc. (reviewed by van Breusegem et al., 2008; Pitzschke and Hirt, 2009; Rodriguez et al., 2010). The mechanism by which this regulation occurs is unclear as well as not much being known about the downstream targets. In some cases, researchers do not know the nature of reactions catalysed by proteins involved in the redox regulation. For example it was demonstrated that EXECUTER 1 and 2 control the singlet oxygen induced retrograde signaling from chloroplasts to nucleus. They regulate stress-specific gene expression and this process is dependent upon enzymatic lipid peroxidation. However the mechanism by which these proteins function has yet to be indentified (Lee et al., 2007a,b; Przybyla et al., 2008; Kim and Apel, 2013). It is unlikely that many kinase/phosphatase-triggered cascades form primary components/sensors of ROS-mediated signaling. They probably serve as long-term downstream metabolic and genetic adjustment elements (switches) integrating ROS signaling into the cellular context and helping plants to adapt or make a decision for PCD. Some kinase/phosphatase systems lie upstream of ROS production providing stimulation feedback loop (van Breusegem et al., 2008).

ROS-Ca^{2+} signaling is not restricted to cytoplasm and may exist in the nucleus (Mazars et al., 2010), chloroplasts (Johnson et al., 1995) and mitochondria (Logan and Knight, 2003), where stress induced Ca^{2+} and ROS transients have been measured. These reactions could play the role of regulators of genomes in the nucleus and the organelles, although the exact their functions have not yet been identified (Mazars et al., 2010; Kim and Apel, 2013).

Some plant transcription factors and their regulators can probably play the role of ROS sensors (Despres et al., 2003; Hong et al., 2013). For example, TGA1 transcription has two specific Cys residues (Cys-260 and Cys-266) which enable its binding to NPR1 regulator (Despres et al., 2003). Oxidation of these Cys groups leads to loss of this interaction and so regulates transcription factor binding to DNA. Heat shock transcription factors may also be involved in direct ROS sensing (Miller and Mittler, 2006; Hong et al., 2013). They regulate transcription of various defense-related genes and are active during stresses including oxidative stress (Miller and Mittler, 2006). Similar systems in fungi (Hahn and Thiele, 2004; Hong et al., 2013) and animals (Ahn and Thiele, 2003) sense H_2O_2 by two Cys residues; but this mechanism has yet to be confirmed in plants.

5. Mechanisms of oxidative damage

5.1. Oxidation of lipids

Oxidative stress causes reversible or irreversible (causing complete loss of some physiological activities) modifications of bio-molecules such as proteins, polynucleic acids, carbohydrates and lipids (Sies and Cadenas, 1985; Møller et al., 2007; Farmer and Mueller, 2013). Among these, oxidation of lipids is particularly dangerous because it propogates free radicals through so-called 'chain reactions'. Lipid oxidation (also known as lipid peroxidation) is widely considered as a "hallmark" of oxidative stress (Farmer and Mueller, 2013).

The lipid peroxidation usually includes three sequential stages: initiation, propagation, and termination (Catalá, 2006; Farmer and Mueller, 2013). Initiation of lipid peroxidation (initiation stage) is triggered by hydrogen atom abstraction form the lipid molecule. This can be caused by hydroxyl, alkoxyl, peroxyl radicals as well as peroxynitrite but not by hydrogen peroxide or superoxide (Halliwell and Gutteridge, 1999). H^+ is abstracted from methylene group (-CH_2—) giving —•CH— or lipid radical (L•), which is a carbon centered radical. Phospholipids (most abundant membrane lipids) are susceptible to radicals and peroxidation because the double bond in the fatty acid weakens the C—H bond and facilitates H^+ subtraction. L• can activate O_2 and form an oxygen centered 'lipid peroxyl radical' (LOO•), which in turn is capable of abstracting H^+ from a neighboring fatty acid to produce a lipid hydroperoxide (LOOH) and a second lipid radical (L•) (Catalá, 2006). This gives a rise to the propagation phase. LOOH can undergo 'reductive cleavage' by reduced transition metals (mainly Fe^{2+} or Cu^+) and form lipid alkoxyl radical (LO•), which is also reactive and induces further abstraction of H^+ from neighboring fatty acid. Another important mechanism of lipid peroxidation is via direct reaction of double bonds with singlet oxygen from PSII reaction centre, which gives LOOH (Krieger-Liszkay et al., 2008; Przybyla et al., 2008; Farmer and Mueller, 2013). Singlet oxygen is also formed in reaction of two LOO• molecules.

Severe lipid peroxidation leads to the damage of membranes, collapse of their barrier function, followed by disintegration of organelles, oxidation and dysfunction of proteins, DNA and RNA (Halliwell and Gutteridge, 1999; Farmer and Mueller, 2013). Terminal products of lipid peroxidation are 'aggressive' substances, such as aldehydic secondary products (malondialdehyde, 4-hydroxy-2-nonenal, 4-hydroxy-2-hexenal and acrolein), which are markers of oxidative stress (Del et al., 2005; Farmer and Mueller, 2013). They are easy to measure, for example using classical thiobarbituric acid assay for malondialdehyde (Hodges et al., 1999) or more modern sensitive mass spectrometry-based techniques that can identify individual lipid species targeted by peroxidation and to study the chemical complexity of oxidative products formed due to this process (reviewed by Shulaev and Oliver, 2006; Farmer and Mueller, 2013).

5.2. Modification of proteins

ROS can oxidise any proteinogenic amino acid (Halliwell and Gutteridge, 1999; Møller et al., 2007; Avery, 2011). Such modification to this key organic component of life results in loss of a given protein-mediated function, such as specific metabolic, structural, transport or regulatory activities. Protein oxidation also results in accumulation of toxic protein aggregates, and in the case of severe damage, induces PCD (Demidchik et al., 2010; Avery, 2011). Major ROS-induced modifications to amino acids are summarised in Table 2.

Products of lipid peroxidation, such as 4-hydroxynonenal and malondialdehyde have been shown to react and oxidise a number of amino acids (such as lysine or histidine) (Table 2). Møller et al. (2007) have recently reviewed mechanisms of oxidative damages to the most important plant protein complexes, such as photosystem I, D1 protein of photosystem II, ribulose-1,5-bisphosphate carboxylase/oxygenase and SOD. With an exception of D1 protein, which is probably affected by singlet oxygen, these damages require the presence or increase of activity of catalytically-active transition metals (catalysing biosynthesis of •OH).

Reversibility of protein oxidation reactions *in vivo* is not well understood due to a lack of analytical tools for studying protein

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Table 2
Most important ROS-induced modifications to amino acids (accordingly to Berlett and Stadtman, 1997; Shacter, 2000; Stadtman and Levine, 2000; Cecarini et al., 2007; Avery, 2011).

Amino acid	Oxidised form
Cysteine	Cysteine → cystine → cysteine sulfenic acid → cysteine sulfinic acid → cysteic acid
Methionine	R- and S-stereoisomers of methionine sulfoxide → methionine sulfone
Histidine	2-Oxohistidine, asparagine, aspartate, 4-hydroxynonenal-histidine (HNE-His)
Glutamyl (glutamine, glutathione, glutamate)	Oxalic acid, pyruvic acid
Lysine	α-Aminoadipic semialdehyde, chloramines, malondialdehyde-lysine (MDA-Lys), 4-hydroxynonenal-lysine (HNE-Lys), acrolein-lysine, carboxymethyllysine, p-hydroxyphenylacetaldehyde-lysine (pHA-Lys)
Tyrosine	p-Hydroxyphenylacetaldehyde, dityrosine, nitrotyrosine, chlorotyrosines, L-3,4-dihydroxyphenylalanine (L-DOPA),
Threonine	2-Amino-3-ketobutyric acid
Tryptophan	Hydroxy- and nitro-tryptophans, kynurenines
Phenylalanine	Hydroxyphenylalanines
Valine, Leucine	Hydroperoxides and hydroxides
Proline	Hydroxyproline, pyrrolidone, glutamic semialdehyde
Arginine	Glutamic semialdehyde, chloramines

structure inside the cell and hence the existing data is mainly based on *in vitro* analyses. It is believed that most types of protein oxidation damage are irreversible with an exception of S-containing amino acids, such as Met and Cys (Shacter, 2000; Bechtold et al., 2004; Møller et al., 2007; Hawkins et al., 2009; Onda, 2013). The oxidation of most amino acids is widely considered to be a pathophysiological phenomenon, while the oxidation of S-containing amino acids is thought to play a regulatory role (for example through protein folding).

Reversibility of Cys reaction with ROS is based on incomplete oxidation of this amino acid. •OH and singlet oxygen induce the sequential formation of more oxidised derivatives of this amino acid: cysteine → cystine → cysteine sulfenic acid → cysteine sulfinic acid → cysteic acid, all of which apart from the last one, are enzymatically reversible by glutaredoxin or thioredoxin systems (Biteau et al., 2003; Tang et al., 2004; Buchanan and Balmer, 2005; Møller et al., 2007; Onda, 2013). Formation of a disulphide bond between two oxidised SH-groups is a key mechanism of protein regulation. Cys–Cys dimer can be then reduced by several electron donors in the cell.

Oxidation of different amino acids in the protein have different functional consequences (Shacter, 2000; Stadtman and Levine, 2000; Cecarini et al., 2007; Avery, 2011; Onda, 2013). For example, Met is extremely sensitive to ROS: it is easily and reversibly oxidized; however its modification typically does not affect the entire protein's function, although it sometimes prevents oxidation of other amino acids. At the same time, oxidation of Cys, results in significant regulatory consequences, although induced dysfunction has also been recorded.

Formation (insertion) of carbonyl group called carbonylation is the most commonly occurring oxidative protein modification after modifications to Cys and Met (Lounifi et al., 2013). It requires higher 'inputs of energy' than oxidation of Cys and Met, involves most amino acids and results in severe changes in protein structure/function and pathophysiological effects (Berlett and Stadtman, 1997; Shacter, 2000; Tanou et al., 2009; Lounifi et al., 2013). Carbonylation usually refers to a process that forms reactive ketones or aldehydes which can be detected by 'Brady's test' with 2,4-dinitrophenylhydrazine (leading to formation of hydrazones). The oxidation of side chains of lysine, arginine, proline and threonine is considered to be a primary protein carbonylation reaction, which produces 2,4-dinitrophenylhydrazine detectable products. 'Secondary protein carbonylation' reaction occurs via the addition of aldehydes which are produced during lipid peroxidation (usually they are aggressive carbonyl species having three to nine carbons in length). Carbonylation leads to the addition of a large and reactive group into the protein chain. It has a number of dangerous effects on protein characteristics, including covalent intermolecular cross-linking, cleavage, or changing the rate of protein degradation. All these significantly modify (usually inhibit) protein enzymatic and physiological activities. Elevated protein carbonylation has been found for a number of plant stresses such as salinity (Tanou et al., 2009), drought (Bartoli et al., 2004) and cadmium toxicity (Romero-Puertas et al., 2002). Protein carbonylation has been always considered as irreversible (Berlett and Stadtman, 1997) but evidence has now appeared that it can be enzymatically reversed for some transcription factors (Wong et al., 2008). Therefore this reaction can potentially be a novel ROS signaling mechanism (Wong et al., 2008; Lounifi et al., 2013).

The proteomic analyses of *Arabidopsis thaliana* have demonstrated that nitrosative stress (NO• donors) results in protein modification called S-nitrosylation (Lindermayr et al., 2005; Lounifi et al., 2013). It is likely that this reaction is induced by peroxynitrite (the product of NO• reaction with $O_2^{\bullet-}$) and leads to severe protein function disturbance or signaling events by the analogy with animal cells (Halliwell and Gutteridge, 1999; Lounifi et al., 2013).

5.3. Effect on carbohydrates

Carbohydrates are the most abundant group of organic molecules in plants (and on the planet), and at the same time, are less studied in terms of oxidative damage and role in stress signaling. They mechanically support and shape plant cells (cellulose, pectin, etc.), store reduced carbon (starch, sucrose etc.), regulate enzyme activities and osmotic pressure (low molecular weight sugars), provide non-enzymatic antioxidant defence (flavonoids, mannitol etc.) and play other key roles. Oxidation of carbohydrates is potentially harmful for plants.

Cu^{2+}-generated •OH react non-enzymatically with xyloglucans and pectins, breaking down them into the parts and causing cell wall loosening (Fry et al., 2002). This may facilitate cell expansive growth and promote fruit ripening (Fry et al., 2002; 2004). This reaction is beneficial, but in stress conditions, when catalytic Cu and Fe activities increase several-fold, it could have pathophysiological consequences (Becana and Klucas, 1992; Moran et al., 1994).

Mono and disaccharides probably act as ROS scavengers (Couée et al., 2006). Their ability to scavenge •OH is as follows (EPR and HPLC tests): maltose > sucrose > fructose > glucose > deoxyribose > sorbitol (Morelli et al., 2003). Nevertheless the metabolism of products (apart from formate) synthetised in these reactions is unclear. Accumulation of some carbohydrates (for example mannitol) has been shown to correlate with increased resistance to oxidative stress in a number of species (Shen et al., 1997; Couée et al., 2006). Nevertheless, the direct link between oxidative stress induced modifications to carbohydrates and plant

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physiology is still missing. They probably act as structural, osmotic, nutrient and signaling agents rather than redox switches or major targets for ROS (Couée et al., 2006).

5.4. Effect on polynucleic acids

Oxidative damage of DNA is a subject of extensive research in animal physiology, because it is a major reason of cancerogenesis. This problem is not relevant to agronomically important plants; therefore it is insufficiently studied in plant physiology. Nevertheless, oxidative DNA damage can be reason of aging of seeds stocks and, sometimes, death of crop plants (Britt, 1996). This is classically subdivided into three types of lesions: mismatched bases, double-strand breaks, and chemically modified bases (Cooke et al., 2003; Yoshiyama et al., 2013). Hydroxyl radicals are a main damaging factor for polynucleic acids, reacting with them by addition to double bonds of nucleotide bases and by abstraction of H^+ from each of the C-H bonds of 2′-deoxyribose and methyl group of thymine. 8-oxo-7,8-dihydroguanine (8-oxoG) and 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyG) are common detected products of $^{\bullet}OH$ induced DNA/RNA oxidation (Cooke et al., 2003; Wang et al., 2010; Yoshiyama et al., 2013).

Reparation systems preventing DNA damage exist in plants, including direct reparation of damaged part of the molecule, in addition to base and nucleotide replacement (Britt, 1996; Tuteja et al., 2001; Yoshiyama et al., 2013). Protection also includes enforcement of antioxidant defence in both cytosol and organelles. Vanderauwera et al. (2011) have shown that nuclear ROS scavenging enzymes (peroxiredoxin and glutathione) are insufficient to protect DNA during oxidative stress. They have demonstrated that catalase and cytosolic ascorbate peroxidase are important for protection of nuclear DNA in stress conditions. Moreover, ROS-induced DNA damage triggers signaling phenomena and activates specific transcription factors, inducing DNA reparation (Yoshiyama et al., 2013).

6. Defense against oxidative stress

The systems of ROS scavenging and cell reparation, which are involved in plant defense against oxidative stress has been the subject of extensive research, and has been the focus of a number of very good recent reviews (some most recent reviews: Smirnoff, 2005; Dietz et al., 2006; Pitzschke et al., 2006; Santosa and Reya, 2006; Møller et al., 2007; Foyer and Noctor, 2009, 2011; Gill and Tuteja, 2010; Asensi-Fabado and Munne-Bosch, 2010; Farmer and Mueller, 2013; Zagorchev et al., 2013). Therefore these will not be discussed in detail here.

Plants have several strategies to withstand an oxidative stress (reviewed by Alscher et al., 2002; Dietz et al., 2003, 2006; Mittler et al., 2004; Gill and Tuteja, 2010). Firstly, is an activation and de novo synthesis of antioxidants - enzymes and non-enzymatic substances directly scavenging (removing) ROS and free radicals. Major enzymatic antioxidants, which show high affinity to specific ROS, include cytosolic Cu–Zn-SOD, mitochondrial Mn-SOD, chloroplastal Fe-SOD (all SODs: superoxide + $2H^+ \rightarrow H_2O_2 + O_2$), catalases ($2H_2O_2 \rightarrow 2H_2O + O_2$), peroxidases ($R/HOOH + R\text{-}H_2 \rightarrow R + 2H_2O/ROH$), peroxiredoxins ($ROOH \rightarrow ROH$), thioredoxins and glutaredoxins (both: R–S–S–R → 2R–SH). Non-enzymatic antioxidants are non-specific to different ROS and comprise ascorbic acid, glutathione, proline, polyamines, betaine, carotenes, some flavonoids and α-tocopherol. These are most important, but a number of other molecules act as antioxidants. There is also a group of enzymes maintaining ROS scavenger function, such as monodehydroascorbate reductase, dehydroascorbate reductase, thioredoxin reductase, glutathione reductase, glutathione S-transferases and others, which reduce oxidised antioxidants, such as ascorbic acid and glutathione. The first line of defence also includes substances that decrease catalytic activity of transition metals, such as metollothioneines (small Cys-rich proteins), phytochelatines (oligomers of glutathione), pectins and other cell wall polysaccharides and structural proteins (reviewed by Fry et al., 2002; Cobbett and Goldsbrough, 2002; Hassinen et al., 2011; Zagorchev et al., 2013).

Secondly, plants probably synthetise protein isoforms and lipids which are less sensitive to oxidation. This requires upstream signaling step to activate specific genetic/metabolic pathways (Wang et al., 2004; Myouga et al., 2008). This mechanism is well established for animals but it is still debated whether it is important for plants.

Thirdly, most plants probably protect living tissues from stresses by the layers of dead cells, which die rapidly by programed ROS-induced mechanisms (Ca^{2+} influx/K^+ efflux; Demidchik and Maathuis, 2007; Demidchik et al., 2010, 2014). These provide a shield against infection or aggressive agents. The enhanced mycorrhization could be another mechanism of this kind (fungal hyphae shields root from aggressive environment), which is critically important for plant defense against heavy metals (Schutzendubel and Polle, 2002). And finally, plants activate biosynthesis systems for reparation of damaged components (reviewed by Møller et al., 2007; Yoshiyama et al., 2013).

7. Conclusions

The analysis of research into the mechanisms of oxidative stress in plants has shows that ROS with proven importance for oxidative damage and signaling include hydroxyl radical, singlet oxygen, hydrogen peroxide, superoxide radical and nitric oxide (forming peroxynitrine). In most cases, oxidative damage occurs through $^{\bullet}OH$ generation, mainly from H_2O_2 in the presence of transition metals, such as $Cu^{+/2+}$ or $Fe^{2+/3+}$. Apart from exogenous sources (UV, O_3, Cu etc.) ROS are generated by photosystems, mitochondrial complexes, electron transport chains of peroxisomal membrane, xanthine oxidase of the peroxisome matrix, NADPH oxidases of the plasma membrane and peroxidases expressed in all cell compartments including cell walls. Accumulating evidence demonstrates that all major stresses trigger the NADPH oxidase mediated oxidative burst. Many stresses also stimulate ROS generation by Class III peroxidases. Novel genetically encoded and reversible H_2O_2 probes such as HyPer provide advanced tools for testing H_2O_2 dynamics *in planta* and can shed light on unknown mechanisms of plants oxidative stress and redox signaling. It has become clear that ROS signals are sensed by cation channels which convert them into physiological responses such as Ca^{2+} influx and cytosolic Ca^{2+} elevation we well as K^+ efflux. Ca^{2+} elevation activates various two-component and multi-component signaling systems leading to induction of genetic and metabolic adjustments. K^+ efflux through ROS-activated K^+ channels results in stimulation of cytosolic cell death proteases and endonucleases, leading to programed cell death. Other potential ROS sensors include histidine kinases, redox-sensitive transcription factors and ROS-sensitive phosphatases. Oxidative stress can damage all cell components. Lipid peroxidation starts from the interaction of hydroxyl radical or singlet oxygen with double bonds of polyunsaturated fatty acids and results in a chain reaction converting functional lipids into toxic aldehydes and ketones. Reversible oxidation of S-containing amino acids plays the role of regulatory redox switches in plant cells. Major mechanisms of irreversible modification of amino acids include ‘primary’ and ‘secondary’ carbonylation. Plant carbohydrates and nucleic acids are very sensitive to $^{\bullet}OH$ but the role of their oxidative damage in physiology is still debated. Networks of enzymatic (specific) and

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non-enzymatic (non-specific) antioxidants together with reparation systems form a complex defence against oxidative stress and regulate stress signaling. Future frontiers for research into mechanisms of oxidative stress include characterisation of enzymes and messengers of ROS-Ca^{2+} signaling, breaking the ROS-Ca^{2+} code for individual stress factors, identification and genetic improvement of structural moieties related to oxidation induced protein dysfunction (for example by the directed evolution approach; Cherry et al., 1999), deeper analysis of regulation of ROS-producing systems, defining significance of carbohydrate and polynucleotide damages, roles of transition metal metabolism, antioxidant networks and establishing precise protein reparation mechanisms.

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