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Review

Reactive oxygen species: Re-evaluation of generation, monitoring and role in stress-signaling in phototrophic organisms

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ABSTRACT

This review provides an overview about recent developments and current knowledge about monitoring, generation and the functional role of reactive oxygen species (ROS) – H_2O_2, $HO_2^{\bullet}$, $HO^{\bullet}$, OH^-, $^1O_2$ and $O_2^{-\bullet}$ – in both oxidative degradation and signal transduction in photosynthetic organisms including microscopic techniques for ROS detection and controlled generation. Reaction schemes elucidating formation, decay and signaling of ROS in cyanobacteria as well as from chloroplasts to the nuclear genome in eukaryotes during exposure of oxygen-evolving photosynthetic organisms to oxidative stress are discussed that target the rapidly growing field of regulatory effects of ROS on nuclear gene expression.

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1. Introduction

The "big bang" in the evolution of the biosphere was the "invention" of a nano-scale device that enabled the splitting of two water molecules into O_2 and metabolically-bound hydrogen by visible light as a driving source of free energy. This event, which occurred most likely – 2.3 billion years ago at the level of cyanobacteria [1,2] made the huge water pool on the earth surface available as a hydrogen source for the biosphere. The aerobic atmosphere, exerted deadly oxidative impact on the biomass on one hand, and opened the road for a more than ten-fold better exploitation of the Gibbs free energy content of nutrients through the aerobic respiration of heterotrophic organisms on the other hand [3]. This caused the reconstruction of the biosphere by the evolutionary development of machinery for light-induced water splitting. The formation of an aerobic atmosphere served as the bioenergetic prerequisite for the population of the earth's landmass by heterotrophic organisms including human beings [4].

The electronic configuration of the O_2 molecule in its ground state is characterized by a triplet spin state multiplicity described by the term symbol $^3\Sigma_g^-$. In marked contrast, the ground states of most biological molecules (proteins, lipids, carbohydrates) have a closed electron shell with singlet spin configuration. Therefore, the two-electron oxidation of a molecule in the singlet state by $^3\Sigma_g^-$ O_2 is "spin-forbidden" and occurs slowly at room temperature. The situation can change by two types of reactions transforming the inert $^3\Sigma_g^-$ O_2 into highly reactive oxygen species (ROS): i) Electronic excitation leads to the population of two forms of singlet oxygen characterized by the term symbols $^1\Delta_g$ and $^1\Sigma_g^+$ (which rapidly relaxes into $^1\Delta_g$ O_2). ii) The chemical reduction of $^3\Sigma_g^-$ O_2 by radicals with non-integer spin state (often doublet state) or a reducing agent like ascorbic acid results in the formation of $O_2^{-\bullet}$,

Abbreviations: Ala, delta-aminolevulinic acid; Asc, ascorbate; APX, ascorbate peroxidase; CALI, chromophore-activated laser inactivation; CHO, Chinese hamster ovary; COX, cytochrome c oxidase; DHA, dehydroascorbate; DHAR, DHA reductase; EF-G, elongation factor G; EPR, electron paramagnetic resonance; ETC, electron transfer chain; Fd, ferredoxin; GFP, green fluorescent protein; GR, glutathione reductase; GS, glutathione; GSH, reduced glutathione; GSSG, glutathione disulphide; H_2DCF-DA, 2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester; HOCl, hypochloric acid; Hsfs, heat stress transcription factors; ISC, inter system crossing; LHC, light harvesting complex; MAPK, mitogen-activated protein kinase; MDHA, monodehydroascorbate radical; MDHAR, MDHA reductase; MPO, myeloperoxidase; NBT, nitroblue tetrazolium; NOX, NADPH oxidase; NPQ, nonphotochemical quenching; OS, oxidative stress; PPFD, photosynthetic photon flux density; PQ, plastoquinone; Prx, peroxiredoxins; PS, photosystem; RBOHs, respiratory burst oxidase homologs; RFP, red fluorescent protein; ROS, reactive oxygen species; RS, redox-sensitive proteins; SOD, superoxide dismutase; t-APX, APX bound to the thylakoid membrane; TF, transcription factors; WOC, water-oxidizing complex; YFP, yellow fluorescent protein

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which is transferred by subsequent reactions to H_2O_2 and $HO^{\bullet}$. In plants, the electronic excitation of $^3\Sigma_g^-$ O_2 occurs due to close contact to chlorophyll triplets that are produced during the photoexcitation cycle.

Fig. 1 schematically illustrates the pattern of one-electron redox steps leading from water to $O_2 + 4H^+$ and vice versa. The inspection of this scheme readily reveals that the four-step reaction sequence comprises $HO^{\bullet}$, H_2O_2 and $HO_2/O_2^{-\bullet}$. In photosynthesis, the highly endergonic oxidative water splitting ($\Delta G° = +237.13$ kJ/mol, see [5]) is catalyzed by a unique Mn_4O_5Ca cluster of the water-oxidizing complex (WOC) of photosystem II (PSII) and is energetically driven by the strongly oxidizing cation radical $P680^{+\bullet}$ [6] formed via light-induced charge separation. Correspondingly, the highly exergonic process in the reverse direction is catalyzed by a binuclear heme iron–copper center of the cytochrome c oxidase (COX), and the Gibbs free energy is transformed into a transmembrane electrochemical potential difference for protons [7], which provides the driving force for ATP synthesis [8].

The ROS content in cells depends on the relative rates of generation and decay and is mainly controlled by antioxidant enzymes and low molecular weight antioxidants, such as ascorbic acid, glutathione, tocopherols, carotenoids, and flavonoids [9]. In spite of the highly controlled reaction sequences in photosynthetic WOC and respiratory COX and the highly effective antioxidant system in plants, the formation of ROS in living cells cannot be completely avoided.

Photosynthetic organisms growing under variable environmental conditions are often exposed to different types of stress like harmful irradiation (UV-B or high-intensity visible light), heat, cold, high salt concentration and also infection of the organisms with pathogens. Under these circumstances, the balance between oxidants and antioxidants within the cells is disturbed, thus giving rise to the development of intracellular oxidative stress enhancing the production of ROS [10,11].

As ROS, we consider H_2O_2, $HO_2^{\bullet}$, $HO^{\bullet}$, OH^-, $^1O_2$ and $O_2^{-\bullet}$. Highly reactive atomic oxygen or ozone, which plays a role only under very special physiological conditions, will not be considered here. In this sense, the term ROS is used in a restricted manner.

During evolution, defense mechanisms against environmental stress were established which not only decrease the rate of ROS generation or scavenging but also trigger delay or acceleration of the repair of damaged cell structures, protect the photosynthetic pigment–protein complexes by carotenoids and enable non-photochemical quenching (NPQ) of superfluous excitation energy. In addition, ROS also act as important signaling molecules with regulatory functions. ROS were found to play a key role in the transduction of intracellular signals and in control of gene expression and activity of antioxidant systems [9,12–16].

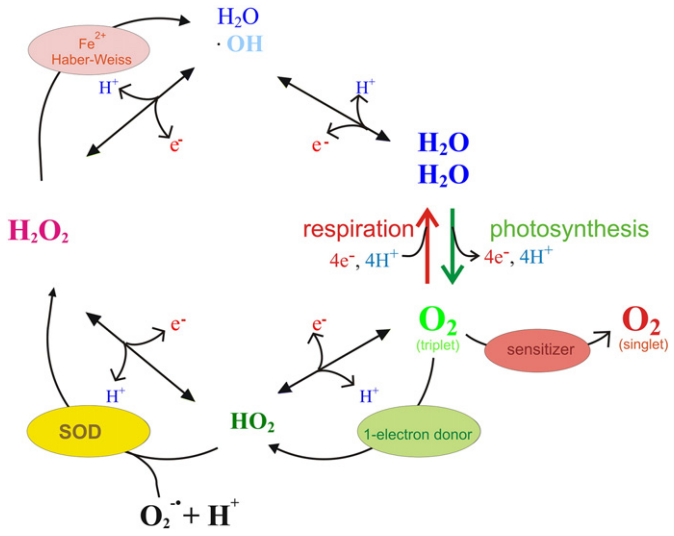

Fig. 1. Scheme of water redox chemistry and ROS formation (for details, see text). SOD denotes superoxide dismutase.

ROS also regulate processes of polar growth, stomatal activity, light-induced chloroplast movements, and plant responses to biotic and abiotic environmental factors [17,18]. In animal organisms, recent studies have established that physiological H_2O_2 signaling is essential for stem cell proliferation, as illustrated in neural stem cell models, where it can also influence subsequent neurogenesis [19].

It has to be emphasized that fundamental differences exist between prokaryotic cyanobacteria and eukaryotic plants. In cyanobacteria, photosynthetic and respiratory reactions are not as clearly separated as in eukaryotic plant cells that contain semi-autonomous organelles (chloroplasts, mitochondria, peroxisomes, and nucleus) with specific functional activities. This differentiation requires a more complex signaling system for "cross-talk" between these organelles. The main source of ROS generation in plant cells under normal light conditions is the chloroplast, but other organelles such as mitochondria and peroxisomes also contribute to ROS formation. Therefore, knowledge of the local ROS concentration in cells is a major issue, since this parameter might vary strongly.

As a consequence, the mechanisms of "handling" stress-induced ROS and the modes of protection are markedly different between cyanobacteria and plants, and even within the plant kingdom. ROS are generated by many different processes and dependent on a variety of cellular conditions (such as pH, tissue age and type), which makes it difficult to draw generalized conclusions on the lifetime of ROS in living cells. Therefore, highly selective and localized ROS reporter systems are required that enable the visualization of generation and decay of a certain ROS with high spatial precision.

2. Monitoring, generation, decay and deleterious action of ROS

Exposure to stress can lead to a drastic increase of ROS production. The so-called "oxidative burst" (rapid transient ROS generation within time periods from several minutes up to hours) is known as the rapid increase of the ROS content under stress conditions [12]. Active production of ROS as a defense strategy as well as the breakdown of the ROS depletion system can cause a steep increase of the ROS level (see Section 4.2 about the purposes of ROS production and RBOHs).

The relative content of different ROS depends on the mode of stress, e.g. high-light stress primarily leads to $^1\Delta_gO_2$ which is mainly formed by energy transfer from the triplet chlorophyll to triplet oxygen (see below). The inhibition of electron transport leads to over-reduction of many components of the electron transfer chain (ETC), first, at the acceptor side of PS II [20]. This might be caused by decreasing the activity of Rubisco, a key enzyme in the Calvin–Benson cycle, under abiotic stress conditions such as chilling or drought [21]. In plant cells, ROS are produced in different organelles, predominantly in chloroplasts and peroxisomes, while the contribution from mitochondria is smaller [20]. Imaging of oxidative stress in the leaves of *Arabidopsis thaliana* (*A. thaliana*) revealed that $^1\Delta_gO_2$ and $O_2^{-\bullet}$ are primarily located in mesophyll tissues, while H_2O_2 was predominantly detected in vascular tissues [22]. However, the real amount of ROS production has to be carefully evaluated since the non-invasive detection techniques like the signal of most ROS markers, for example quenching of dansyl-2,2,5,5,-tetramethyl-2,5-dihydro-1H-pyrrole (DanePy), respond in a nonlinear fashion to the ROS level. Therefore, it is difficult to compare the absolute amount of different ROS monitored with different techniques. This is a general problem that arises from the fact that most ROS sensors are not perfectly linear and completely specific to a certain reactive oxygen species.

The simultaneous formation of several ROS complicates analyses on the formation, decay and degradation of individual species, e.g. $^1\Delta_gO_2$ versus $H_2O_2/O_2^{-\bullet}$. Therefore, suitable species-specific probes are required to monitor the different ROS molecules simultaneously.

Another important approach to study effects of different ROS is the selection of special assay conditions and the use of mutant strains that differ in the generation of individual ROS and/or the content of protection systems/enzymes. This point is of high relevance for studies on

specific signaling pathways and will be discussed in more detail in Section 3.

2.1. Monitoring of ROS

Detailed analyses of different ROS species in plants require the use of suitable probe molecules, which specifically change their properties due to a species-selective reaction. Since exogenous dyes typically respond in a certain oxidative potential range, appropriate mixtures can deliver assays that are selective in a certain restricted range of oxidative potentials (e.g. when both dyes show fluorescence or only one dye shows fluorescence, but the other does not). These assays are more selective than a single dye.

In principle, $^1\Delta_gO_2$ can be directly monitored via its characteristic phosphorescence with a maximum around 1270 nm [23], and even $^1\Delta_g$ O_2 microscopy within the visible spectrum has been reported [24]. However, the detection of $^1\Delta_gO_2$ and its time-dependent profile in biological systems is difficult [25] because of the very low quantum yield of the phosphorescence emission, which ranges from 10^{-7} to 10^{-4} depending on the solvent [26], and the interference with Chl emission. The approach of direct monitoring of the $^1\Delta_g$ O_2 phorphorescence, therefore, can only be applied in highly purified PS II core complexes.

Next to $^1\Delta_gO_2$, the detection of other ROS ($H_2O_2/O_2^{-\bullet}$) also requires the use of appropriate indicators. Spin traps (see Table 3) are suitable to interact with ROS and give rise to EPR-detectable species via characteristic electron paramagnetic resonance (EPR) signals [27,28]. Fluorophores (see Tables 1, 2) typically change their emission properties due to the interaction with ROS, which permits the application of recently developed, advanced techniques of time- and space-resolved fluorescence microscopy for in vivo studies [29,30].

In the case of fluorophores, two different approaches exist: i) the addition of exogenous fluorescence probes, which penetrate into the cell and change their fluorescence properties due to reaction with ROS (Table 1), and ii) the expression of ROS-sensitive fluorescent proteins, mostly variants of the green fluorescent protein (GFP), which act as real-time redox reporters for the use in intact cyanobacteria, algae and higher plants (Table 2).

In principle, the permeability across membranes and the ROS specificity are necessary for the applicability of the exogenous ROS-sensing fluorophores (Table 1). However, for a quantitative analysis, it is necessary to know the reaction mechanism in detail, as well as possible interfering side effects and the cellular localization of these dyes. Generally, the water/octanol partition coefficient could be utilized to quantify membrane permeability of the probes.

Some of these ROS probes can be tuned regarding their properties inside the cell by enzymatic reactions. For instance, the commercially available 2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (H_2DCF-DA), a fluorescein-based dye, which is virtually non-fluorescent in the reduced state, becomes fluorescent after oxidation and concomitant splitting of the acetate groups by cellular esterases. H_2DCF-DA is widely used in (nonphotosynthetic) animal cells.

One approach to overcome problems regarding the specificity of localization of the applied dyes is the application of genetically-encoded fluorescence proteins, in particular the green-fluorescent protein (GFP) and its variants. These proteins can be selectively expressed as fluorescence markers, fused to specific target proteins or to organelle-specific targeting sequences, thus enabling a specific and localized monitoring (and manipulation) of ROS at a molecular level [37]. The GFP-based ROS sensor variants contain pairs of redox-active cysteines forming a disulfide bridge as redox switch. One example of these GFP derivatives is rxYFP based on the yellow fluorescent protein (YFP) [38] rxYFP is one of the first reported redox switches used to monitor the formation of disulfide bonds in living cells. The disulfide bridge in the oxidized rxYFP leads to a distortion of the typical beta-barrel structure of GFP derivatives, thus changing the fluorescence properties of rxYFP [38]. The mitochondrially-targeted redox sensitive GFP termed roGFP-mito does not specifically react in response to a certain species of ROS, but it is used to selectively label mitochondria in plants [39].

In an alternative approach, the H_2O_2-sensitive probe termed HyPer was constructed by fusing the regulatory domain of the H_2O_2-sensitive transcription factor OxyR from *Escherichia coli* (see Section 3.2) to a cyclically permuted YFP [40]. In a similar manner, a fluorescent biosensor responding to the NAD^+/NADH ratio termed Peredox has been constructed by combining the circularly permuted GFP derivative T-Sapphire with the bacterial NADH-binding repressor protein T-Rex after elimination of the pH sensitivity by mutagenesis [41]. Chromophore transformations in red-fluorescent proteins offer tools for designing suitable red-shifted probes, which are advantageous for imaging studies due to the strong absorption in the green spectral range, in which chlorophylls exhibit only very low absorption. Excitation with longer wavelengths also leads to reduced autofluorescence [42]. For applications in studies on ROS effects, see [43–45].

The application of fluorescence markers for ROS sensing is generally complicated by photobleaching. In addition, fluorophores often act as $^1\Delta_gO_2$ sensitizers themselves (see chapter 2.3). This problem is especially important for GFP derivates as ROS sensors. However, the generation of new GFP mutants is a promising approach to overcome this problem, which votes for the importance of developing improved genetically encoded fluorescence proteins for ROS sensing for future studies. Table 2 gives an overview about genetically encoded fluorescence proteins and their basic properties of selectivity and applicability in plants.

While fluorescent ROS-sensing dyes respond to their target molecules without further spectroscopic signal structure, which impedes the selectivity of the otherwise highly sensitive fluorescence technique, the detection of the electron paramagnetic resonance (EPR) with spin traps enables a more selective technique for ROS monitoring. Since some ROS species are radicals, the application of spin traps appears sound to focus on spin-carrying ROS. Therefore, spin traps are widely applied to EPR-detectable ROS species like superoxide and hydroxyl radicals [28,47–49]. Fluorescent spin traps for ROS detection like DanePy which is quenched in presence of $^1\Delta_g$ O_2 are suitable for an optical measurement of the interaction between ROS and spin trap molecules [47,49,50].

The detection of ROS in cyanobacteria faces additional difficulties because their accessibility to EPR and fluorescent spin traps is limited. An alternative technique is chemical trapping by ROS scavengers like histidine. Recently, it was shown that chemical trapping by histidine is suitable to monitor singlet oxygen generation in *Synechocystis* sp. PCC 6803 [54].

Fig. 2 (upper and middle panels) illustrates the use of this type of probes in imaging ROS production in *A. thaliana* leaves. The top panel of Fig. 2 shows the fluorescence emission from an *A. thaliana* leaf which was infiltrated with 40 mM DanePy [22]. Subsequently, an area of the leaf tip was exposed to a photosynthetic photon flux density (PPFD) of 600 μmol m^{-2} s^{-1} for 60 min. The white oval in frame A shows the illuminated area, frames B and C show the images of fluorescence emission from the DanePy before and after high light treatment, respectively. The fluorescence quenching occurs within the area of the leaf tip exposed to the high light. This fluorescence quenching in frame C reflects the formation of non-fluorescent DanePyO due to the reaction with $^1\Delta_gO_2$. The middle panel shows an image of an *A. thaliana* leaf infiltrated with 6 mM nitroblue tetrazolium (NBT). The top half of the leaf was exposed to 600 μmol m^{-2} s^{-1} for 60 min. The purple coloration indicates the formation of insoluble formazan deposits due to reaction of NBT with superoxide.

Fig. 2, lower panel, illustrates the increase of CM-H_2DCFDA (5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester) fluorescence due to ROS production upon exposure of mammalian Chinese hamster ovary (CHO) cells to 440–480 nm light with an intensity of about 1000 W/m^2. Three cells can be seen in the green observation regime (emission filter FF01-520/35-25, AHF Analysentechnik, Tübingen, Germany). 6 s after switching on

Table 1
Compilation of ROS-sensitive exogenous fluorescence probes.

Compound/reference	Specificity Further information/localizability
CM-H2DCFDA [29]	Unspecific Permeates into animal cells, requires the presence of cellular esterases. Not easily applicable in plants
Singlet oxygen sensor green (SOSG) [31]	Highly specific to singlet oxygen Successfully used for detection of $^1\Delta_gO_2$ in *A. thaliana* leaves
3,3′-diaminobenzidine (DAB) [22,32]	Specific to H_2O_2 in presence of peroxidase (and other haem-containing proteins) Generates a dark brown precipitate which reports the presence and distribution of hydrogen peroxide in plant cells. Permeates into plant cells.
Aminophenyl fluorescein (APF)	APF is a cell permeable indicator that can be used to detect hydroxyl radicals ($HO^•$), peroxynitrite ($ONOO^-$) and hypochlorite (OCl^-) production in cells. Shows limited photoxidation (see section "reactive oxygen species" in http://www.interchim.fr/cat/ApoptosisAssays)
hydroxyphenyl fluorescein (HPF)	Specific to hydroxyl radical and peroxynitrite. Minor sensitivity to other ROS. HPF is cell permeable (see section "reactive oxygen species" in http://www.interchim.fr/cat/ApoptosisAssays)
nitroblue tetrazolium (NBT) [32,33]	Specific to superoxide and with slightly reduced reactivity to hydrogen peroxide
Proxyl fluorescamine [34]	Specific to hydroxyl radicals and superoxide Complementary use as spin trap
Hydroethidine (dihydroethidium) [35]	Unspecific Binds specifically to DNA, marking the nucleus
DPPP (diphenyl-1-pyrenylphosphine) [35]	Unspecific Lipophilic, detects ROS in lipids, blood plasma, tissues and food
MCLA (2-methyl-6-(4-methoxyphenyl)-3,7-dihydroimidazo[1, 2-a] pyrazin-3-one, hydrochloride) [36]	Specific to superoxide or singlet oxygen (see section "reactive oxygen species" in http://www.interchim.fr/cat/ApoptosisAssays)
Trans-1-(2′-methoxyvinyl)pyrene	Highly specific to singlet oxygen (see section "reactive oxygen species" in http://www.interchim.fr/cat/ApoptosisAssays)

the illumination, the lower cell has developed a higher cytosolic ROS concentration (higher fluorescence yield) than the upper two cells that show a less intense luminescence but some white dots indicating "hot spots" of accumulated CM-H_2DCFDA and/or higher local ROS formation.

2.2. Generation, decay and deleterious action of different ROS

2.2.1. Singlet oxygen ($^1\Delta_g$ O_2)

The interaction between chlorophyll triplets (^{3}Chl) and ground state triplets of molecular oxygen ($^3\Sigma_g^-$ O_2): $^3Chl + {}^3\Sigma_g^- O_2 \rightarrow {}^1Chl + {}^1\Delta_gO_2$ is the predominant reaction forming singlet oxygen ($^1\Delta_gO_2$) in photosynthetic organisms. ^{3}Chl is populated either via intersystem crossing (ISC) of antenna Chls or via radical pair recombination in the reaction centers of photosystem II (PSII) (for reviews, see [55,56]). Alternatively, $^1\Delta_gO_2$ can also be formed by controlled chemical reactions, which play an essential role in programmed cell death upon pathogenic (e.g. viral) infections.

The lifetime of $^1\Delta_gO_2$ in aqueous solution is about 3.5 μs [57]. Due to its high reactivity, which rapidly attacks all relevant biomolecules (pigments, proteins, lipids, DNA), thus leading to serious deleterious effects [58,59], the lifetime of $^1\Delta_gO_2$ can be shortened down to 200 ns in cells [60]. Within this time span, the species can diffuse over a range of 10 nm or more under physiological conditions [61], thus permitting penetration through membranes. Larger distances up to 25–50 nm have been reported [62], which suggest that $^1\Delta_gO_2$ can permeate through the cell walls of *E. coli*. Higher values of up to 14 μs lifetime and 400 nm diffusion distance in lipid membranes suggest that $^1\Delta_gO_2$ can indeed diffuse across membranes of cell organelles and cell walls [63]. The values show that the possibility for $^1\Delta_gO_2$ alone to work as a direct messenger is rather limited.

Proteins are prominent targets with reaction rate constants in the range of 10^8–10^9 M^{-1} s^{-1} [64]. Among the natural amino acids, only five (Tyr, His, Trp, Met and Cys) are primarily attacked by a chemical reaction with $^1\Delta_gO_2$, from which Trp is unique by additionally exhibiting a significant physical deactivation channel that leads to the ground state $^3\Sigma_g^-$ O_2 in a similar way as quenching by carotenoids.

The reaction of $^1\Delta_gO_2$ with Trp primarily leads to the formation of peroxides, which subsequently degrade into different stable products such as N-formylkynurenine [65]. This compound exhibits optical and Raman spectroscopic characteristics that might be useful for the identification of ROS generation sites [66]. The reactivity of Trp in proteins was shown to markedly depend on the local environment of the target [67]. Detailed mass spectrometric studies revealed that a large number of oxidative modifications of amino acids are caused by ROS and reactive nitrogen species [68]. Such high reactivity leads to an extensive oxidation of fundamental structures of PS II where oxygen is formed in the water-oxidizing complex. $^1\Delta_gO_2$ is directly involved in the direct damage of PS II [58,59,69], most probably by destroying predominantly the D1 protein, which plays a central role in the primary processes of charge separation and stabilization in PS II. The resulting photoinhibition of PS II [70] leads to dysfunction of D1 and high turnover rates during the so called D1-repair cycle. D1 by far exhibits the highest turnover rate of all thylakoid proteins and underlies complex regulatory mechanisms [71].

Carotenoids play a pivotal role in ^{3}Chl suppression and quenching [72,73]. In addition, NPQ developed under light stress also reduces the population of ^{3}Chl in antenna systems as well as PSII of plants [74–76]. The interaction between $^1\Delta_gO_2$ and singlet ground state carotenoids does lead not only to photophysical quenching, but also to oxidation of carotenoids by formation of species that can act as signal molecules for stress response [77]. Likewise, lipid (hydro)peroxides generated upon oxidation of polyunsaturated fatty acids by $^1\Delta_gO_2$ can act as triggers to initiate signal pathways, and propagation of cellular damage [14,78]. Detailed studies of the damage of the photosystems by $^1\Delta_gO_2$ are additionally found in [25,79–83].

2.2.2. Superoxide and hydrogen peroxide

Among all ROS, the $O_2^{-•}$/H_2O_2 system is one of the key elements in cell signaling and other plant functions (see Fig. 1). $O_2^{-•}$ and H_2O_2 are assumed to initiate reaction cascades for the generation of "secondary" ROS as necessary for long-distance signaling from the chloroplasts to or between other cell organelles [84,85].

The one-electron reduction of O_2 to $O_2^{-•}$ is the initial step in the formation of many ROS species in all cells (see Fig. 1). Therefore, special emphasis is given to the formation and decay of superoxide and hydrogen peroxide.

$O_2^{-•}$ and H_2O_2 are mainly formed in chloroplasts, peroxisomes, mitochondria and cell walls [85]. Enzymatic sources of $O_2^{-•}$/H_2O_2 generation have been identified such as cell wall-bound peroxidases, aminooxidases, flavin-containing oxidases, oxalate and plasma membrane NADPH

Table 2
Genetically encoded fluorescence proteins applicable for ROS monitoring.

Compound/reference	Specificity Further information/localizability
rxYFP [38]	Unspecific
roGFP [39]	Unspecific, applied to label plant mitochondria
HyPer [40]	H_2O_2 sensitive by fusing the regulatory domain of the H_2O_2-sensitive transcription factor OxyR to YFP, not yet expressed in plant cells
Peredox [41]	Selective NADH reporter by combining a circularly permuted GFP T-Sapphire with a bacterial NADH-binding repressor protein T-Rex, not yet expressed in plant cells
GFP redox sensor [46]	Specific to H_2O_2, successfully applied in Zebrafish larvae to detect H_2O_2 patterns after wounding

oxidases [86–88]. In particular, sources of ROS in the apoplast are oxidases bound to the cell wall, peroxidases, and polyamino oxidases [89,90].

It was reported that one source of $O_2^{-\bullet}$/H_2O_2 production in chloroplasts is the acceptor side of photosystem I (PS I) mainly by the transfer of electrons from reduced ferredoxin to O_2 and then via ferredoxin–thioredoxin reductase [91]. NADPH oxidase (NOX, see Section 4.2) is considered to be the main producer of ROS both in animal and plant cells [92,93] according to the reaction $NADPH + 2O_2 \rightarrow NADPH^+ + 2O_2^{-\bullet} + H^+$.

Under conditions of limited NADPH consumption due to impaired CO_2 fixation rates during the Calvin–Benson cycle in photosynthetic organisms, some components of the ETC will stay reduced and can also perform $^3\Sigma_g^-$ O_2 reduction to $O_2^{-\bullet}$ [94]. It is suggested that H_2O_2 formation takes place in the plastoquinone (PQ) pool, but with a low rate [95].

Recent literature suggests very short lifetimes for $O_2^{-\bullet}$ radicals in the μs regime (1 μs half-life is published in [84], while 2–4 μs is found in [91]). It is assumed that some $O_2^{-\bullet}$ radicals are rapidly transformed into H_2O_2 via the one-electron steps of the dismutation reaction catalyzed by superoxide dismutase (SOD) (see Fig. 1) [94]. Three forms of SODs exist in plants containing different metal centers, such as manganese (Mn-SOD), iron (Fe-SOD), and copper–zinc (Cu/Zn-SOD) [96,97].

Earlier literature suggests a low reactivity of $O_2^{-\bullet}$ radicals indicating that the exact mechanisms of the $O_2^{-\bullet}$ reaction pathways in living cells might need further elucidation (see [98] and references therein). In earlier studies, Halliwell [99] pointed out that $O_2^{-\bullet}$ is a moderately reactive nucleophilic reactant with both oxidizing and reducing properties. The negative charge of the $O_2^{-\bullet}$ radical leads to an inhibition of its electrophilic properties towards molecules with many electrons, while molecules with a low electron number might be oxidized. $O_2^{-\bullet}$ oxidizes enzymes containing the [4Fe–4S] clusters [100], while cytochrome C is reduced [101]. Among the amino acids, mainly histidine, methionine, and tryptophan can be oxidized by $O_2^{-\bullet}$ [102].

It should be noted that these radicals have to interact with other radicals due to the spin selection rules. For example, superoxide interacts with radicals like nitric oxide and with transition metals or with other superoxide radicals (dismutation). As an example, Fe(III) is reduced by $O_2^{-\bullet}$, then H_2O_2 interacts with Fe^{2+} (Fenton reaction), in effect forming $HO^\bullet$, which is the most reactive species among all ROS (see also Fig. 1). This reaction is particularly mentioned due to its importance for the generation of highly reactive $HO^\bullet$ from long-lived H_2O_2 which might act as long distance messenger.

According to early studies of Rigo et al. [103], the rate of the uncatalyzed reaction is negligible, while the spontaneous dismutation of $O_2^{-\bullet}$ is a rapid process with a rate constant of $8 \cdot 10^4\ M^{-1}\ s^{-1}$ at pH 7.8 [104]. However, this reaction is of second order in $O_2^{-\bullet}$ and its first half-lifetime strongly depends on the initial concentration of $O_2^{-\bullet}$. According to Fridovich [104], the half-lifetime in the absence of scavengers will be approximately 0.05 s. The rate constant for the SOD-catalyzed reaction is close to the diffusion limit and equal to $2 \cdot 10^9\ M^{-1}\ s^{-1}$ [105]. The reaction between $O_2^{-\bullet}$ and the SOD is, in contrast to the spontaneous reaction, of first order in $O_2^{-\bullet}$, and the half-lifetime is independent from the $O_2^{-\bullet}$ concentration. At concentrations of approximately $1 \cdot 10^{-5}$ M for SOD and a reasonable steady state concentration of $1 \cdot 10^{-11}$ M $O_2^{-\bullet}$, the enzymatic dismutation reaction is therefore about 5 orders of magnitude faster than the uncatalyzed one. The reaction of $O_2^{-\bullet}$ with protons either in aqueous solution or catalyzed by SOD: $2\ O_2^{-\bullet} + 2\ H^+ \rightarrow H_2O_2 + O_2$ comprises the oxidation and reduction of this radical [85].

Within the chloroplasts, H_2O_2 is rapidly reduced to H_2O by ascorbate (Asc) via a reaction catalyzed by soluble stromal ascorbate peroxidase (APX) [94,106] or APX bound to the thylakoid membrane (t-APX). As shown in Fig. 3, the Asc oxidized to the monodehydroascorbate radical (MDHA) is regenerated by reduction of MDHA either directly by ferredoxin (Fd) or by NAD(P)H catalyzed by MDHA reductase (MDHAR). The MDHA radical always decays partially into dehydroascorbate (DHA), which is reduced by DHA reductase (DHAR). In that step, reduced glutathione (GSH) is oxidized to glutathione disulphide (GSSG). The reduction of GSSG to GSH occurs from NAD(P)H by glutathione reductase (GR) [94,107].

The reaction sequence of O_2 reduction to $O_2^{-\bullet}$ at the acceptor side of PS I, followed by dismutation of $O_2^{-\bullet}$ by SOD, and the reduction of H_2O_2 at t-APX finally results in the reduction of one O_2 molecule to two H_2O molecules. This four-electron reduction process counterbalances the oxidation of two H_2O molecules to one O_2 molecule at the donor side of PS II so that no net change in the overall turnover of O_2 is obtained, as is schematically illustrated in Fig. 3. Therefore, this "water–water cycle" is referred to as pseudocyclic electron transport [94] which can be coupled to the formation of a transmembrane pH difference.

In contrast to superoxide, H_2O_2 is neutral. It also is less toxic compared to other ROS [108] and has longer lifetime of about 1 ms [85,109]. Therefore, it can principally diffuse across great distances

Table 3
Spin traps suitable for imaging ROS.

Compound	Specificity Further information/localizability
DMPO [51]	Spin trapping of $^1O_2$, superoxide and hydroxyl radicals, Transient EPR spectra specific for trapped radicals but spontaneous decay of DMPO-superoxide adduct with 45 s. half lifetime
Alpha-phenyl N-tertiary-butyl nitrone (PBN) [51]	EPR spectra rather unspecific for trapped radicals.
3,5-Dibromo-4-nitrosobenzenesulfonic acid (DBNBS) [51]	Used for H_2O_2 sensing, specific EPR spectra
5-Diisopropoxyphosphoryl-5-methyl-1-pyrroline-N-oxide (DIPPMPO) [52]	Used in mitochondria, strongly applied for detecting superoxide
TEMPO-9-AC [34]	Fluorogenic spin trap specific for hydroxyl radicals and superoxide
BODIPY® 665/676 [53]	Sensitive fluorescent reporter for lipid peroxidation
DanePy [47,49]	Specific to $^1O_2$ Fluorescent spin trap — fluorescence is quenched in the presence of $^1O_2$

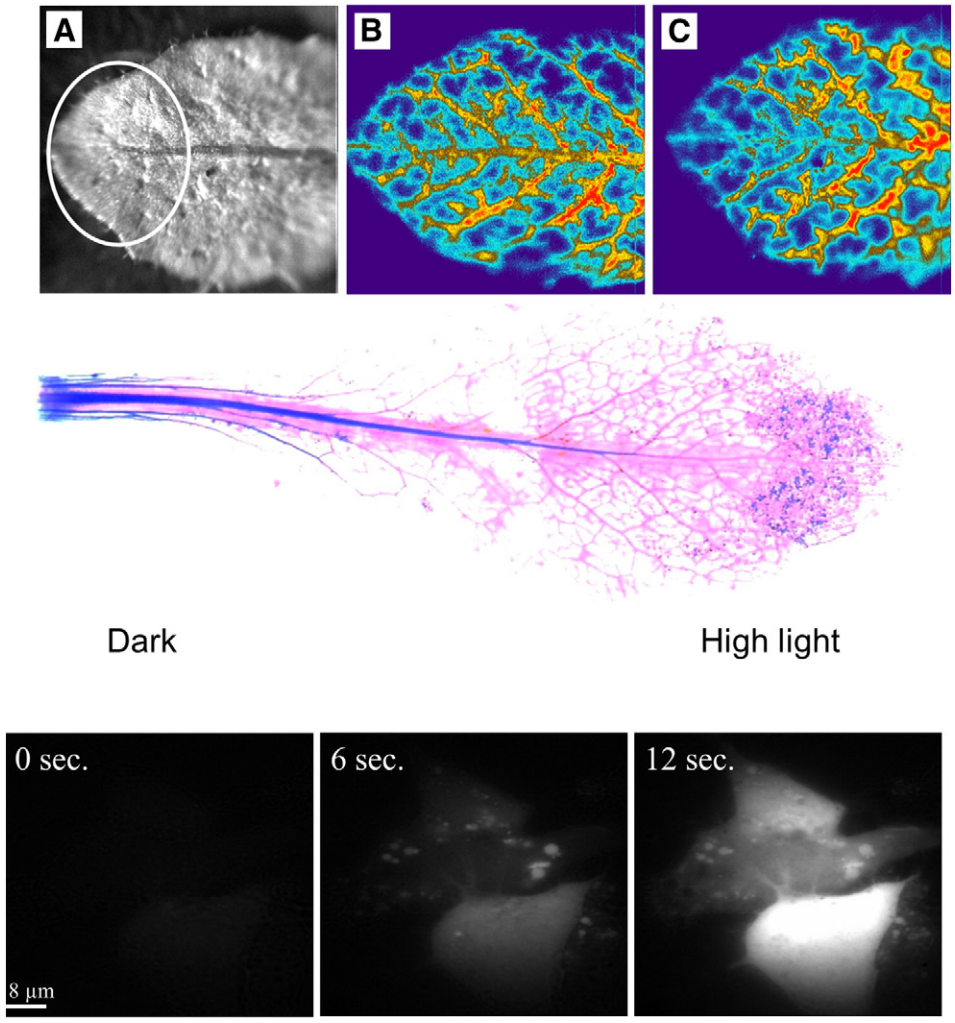

Fig. 2. Top panel: fluorescence emission from an *Arabidopsis thaliana* leaf, frame A: illuminated area, frames B and C: fluorescence emission from DanePy before and after high light treatment. Middle panel: image of the formation of insoluble formazan deposits due to the reaction of NBT with superoxide (adapted from [22]). Lower panel: Increase of CM-H_2DCFDA fluorescence due to ROS production upon exposure of Chinese hamster ovary (CHO) cells to 440–480 nm light, for details see text.

and eventually penetrate through membranes, possibly through water channels like aquaporins [110]. It is assumed that H_2O_2 can travel at least some µm while interacting with H_2O_2-metabolizing enzymes and, therefore, can play an important role in signaling (see Section 3).

Elimination of H_2O_2 is tightly associated with scavenging of other ROS in plant cells. Both, H_2O_2 production and removal are precisely regulated and coordinated in different cellular compartments [20,111–113]. The mechanisms of H_2O_2 scavenging are regulated by both, non-enzymatic and enzymatic antioxidants preventing the generation, primarily by chelating transition metals which catalyze $HO^{\bullet}$ radical formation or by radical scavenging by antioxidant enzymes and low-molecular weight antioxidants, such as carotenoids, anthocyanins, ascorbic acid and GS [17].

Detailed studies revealed that Asc and GSH, which are the most widespread and abundant water-soluble antioxidants, can be accumulated in chloroplasts at millimolar concentrations with a widespread protective effect [114]. On the other hand, the photosynthesis efficiency reduces significantly, if Asc is exhausted and chloroplast peroxidases are inactivated [115].

A central element of the antioxidant defense systems in plant and cyanobacterial cells is represented by peroxiredoxins (Prx), which are part of the antioxidant defense system and the dithiol–disulfide redox regulatory network of plant and cyanobacterial cells. Via a thiol-based catalytic mechanism, different kinds of hyperoxides (R-OOH) are reduced. In higher plants, at least two types of nucleo-cytoplasmic and one chloroplast Prx are found in addition to three so-called "type II" Prx species of cytosolic, mitochondrial and plastidic type. Cyanobacteria express variable sets of three or more Prxs [116].

The biological toxicity of H_2O_2 appears through oxidation of SH groups and can be enhanced, if metal catalysts like Fe^{2+} and Cu^{2+} take part in this process (Fenton reaction) (see above and Fig. 1). The enzyme myeloperoxidase (MPO) can transform H_2O_2 to hypochloric acid (HOCl), which has high reactivity and can oxidize cysteine residues by forming sulfenic acids [19]:

$$\text{HOCl (enzyme MPO)} \leftarrow H_2O_2 \rightarrow H_2O \text{ (catalase, peroxidase)}$$
$$\downarrow Fe^{2+}, Cu^{2+}$$
$$[HO]^{\bullet}.$$

Thus, H_2O_2 takes part in the formation of reactive species like $HO^{\bullet}$ via several pathways.

Both $O_2^{-\bullet}$ and H_2O_2 are capable to initiate the peroxidation of lipids, but since $HO^{\bullet}$ is more reactive than H_2O_2, the initiation of lipid peroxidation is mainly mediated by $HO^{\bullet}$ [85,117].

2.2.3. Hydroxyl radical

The $HO^{\bullet}$ radical is the most reactive species known to biology. $HO^{\bullet}$ is isoelectronic with the fluorine atom and characterized by a midpoint potential of +2.33 V at pH 7 (for comparison, the normal reduction potential of fluorine is +2.85 V). In cells, the extremely dangerous $HO^{\bullet}$

radical can be formed by the reduction of H_2O_2 via the Haber–Weiss reaction catalyzed by Fe^{2+} (see Section 2.2.2 and Fig. 1).

$HO^{\bullet}$ radicals instantaneously attack proteins and lipids in the immediate environment of the site of production, thus giving rise to oxidative degradation [118]. Cells cannot detoxify $HO^{\bullet}$ radicals, but the suppression of H_2O_2 formation in the presence of Fe^{2+} using metal binding proteins like ferritins or metallothioneins also inhibits $HO^{\bullet}$ production (see Fig. 1, Section 2.2.2) [119]. On the other hand, $HO^{\bullet}$ radicals are produced in programmed cell death as part of defense mechanisms to pathogenic infections.

It has to be mentioned that the $HO^{\bullet}$ radical is not the only possible product of the reaction between H_2O_2 and Fe^{2+}. New calculations on the electronic structure and ab initio molecular dynamics simulations have shown that the formation of the ferri-oxo species $[Fe^{IV}(O^{2-})(H_2O)_5]^{2+}$ is energetically favored by about 100 kJ/mol compared to the generation of the $HO^{\bullet}$ radical [120]. Therefore, in future mechanistic studies, the species $[Fe^{IV}(O^{2-})(H_2O)_5]^{2+}$ should be taken into account for mechanistic considerations on the oxidative reactions of H_2O_2 in the presence of Fe^{2+}.

2.3. Perspectives offered by the CALI technique

A novel approach that applies targeted production of ROS for selective inactivation of certain proteins by spatially-confined ROS generation is chromophore-activated laser inactivation (CALI).

For investigations on ROS production and ROS effects on cells, CALI enables species-specific ROS production with high spatial precision at distinct protein targets. This procedure utilizes the property of fluorescent dyes to serve as specific ROS sensitizers.

First applications of the CALI technique used malachite-green-conjugated antibodies directed against purified proteins in solution or in cell membranes. The inactivating effect was initially traced to localized heat generation near the chromophore [121], but later reports attributed the effect to generation of hydroxyl radicals within a damage radius of 15 Å [122]. More widespread cellular applications of CALI became possible with the availability of genetically-encoded chromophores such as the GFP and its spectral variants, which can act as ROS sensitizers. $^1\Delta_g$ O_2 production occurs from eGFP [123] and TagRFP [124], albeit with a low quantum yield. In contrast, KillerRed derived from a non-fluorescent jellyfish red chromoprotein [125], exhibited more than 1000-fold enhanced phototoxicity compared to eGFP and enabled successful CALI applications [126]. The potential of CALI for plant cell research has not been explored so far.

To study the role of $^1\Delta_gO_2$ in selective PS II damage [58,59], CALI offers the framework for experiments that use artificial $^1\Delta_gO_2$ sensor dyes. The efficiency and selectivity of the $^1\Delta_gO_2$ production have to be well defined in such a case to understand the selective destruction of the D1 protein. Detailed studies of different exogenous dyes used as $^1\Delta_gO_2$ elicitors were provided by Hideg [127]. The generation of ROS as a side effect of dyes applied for ROS monitoring is now a major focus of sensing applications [128].

3. Signaling role of ROS

ROS take part in many different processes as signal molecules. The diversity of these processes is determined by the site of ROS production and their interaction with a variable set of hormonal signaling compounds such as salicylic acid, abscisic acid and others including the PQ pool and GSH [17,94,107]. Among the recent reviews elucidating the signaling role of ROS, the detailed work of Mitler et al. [129] and Krieger-Liszkay [130] should be mentioned. The mode of signaling under the participation of ROS depends on the nature of stress. ROS can activate physiological responses leading to the development of adaptive mechanisms and improving the stress tolerance (acclimation), or it can trigger a signal cascade causing programmed cell death [14,108,129,131]. In both cases, ROS function as signal molecules, which induce molecular, biochemical and physiological responses.

ROS control programmed cell death [14,108,129,131], cell wall formation, salicylic acid-induced stomatal closure [132], and responses to pathogens just to mention a selection [84,133,134].

If different environmental stresses can be perceived by only one molecular sensor, a generalized signaling scheme based on ROS might be involved [131]. For example, the histidine kinase Hik33 in *Synechocystis* was recently found to be a multisensory protein, which perceives cold, salt, and oxidative stresses [135].

A helpful classification scheme might be based on the response to abiotic (light, drought, cold, heat etc.) and biotic (infection by viruses and bacteria) stress.

The whole concept on signaling effects of ROS was firstly introduced from the research of hormonal signaling and gene expression during the

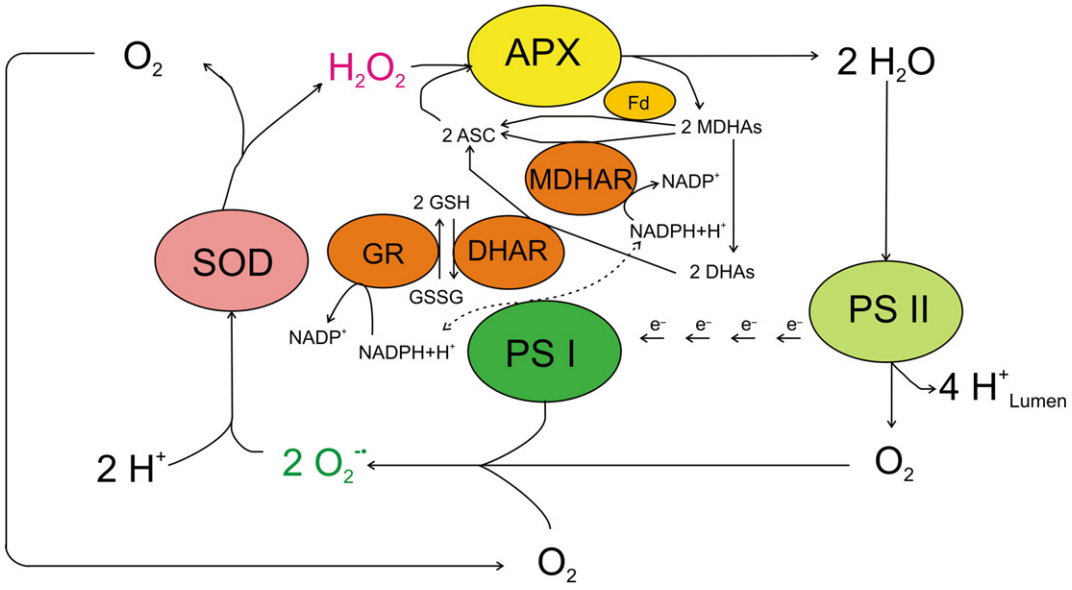

Fig. 3. Scheme of pseudocyclic "H_2O–H_2O" electron transport (for details, see text).

development of plant protection against pathogen infection [136], where ROS bursts can induce local cell death in infected sites as well as cross-linking of cell wall proteins (see Section 4.2) [113,129,134,137].

Further studies of the formation of ROS in the apoplastic space of leaves during treatment with the tropospheric pollutant ozone also elucidated the complex network of ROS signaling. The similarity of plant responses to ozone and pathogens demonstrated that ozone-induced programmed cell death takes place at millimolar concentrations. In this case, the apoplastic salicylic acid likely can be considered as a key element of cell defense against ozone [138].

In the case of abiotic stress, $^1\Delta_gO_2$ is often formed in addition to $O_2^{-\bullet}$ and H_2O_2, while biotic stress mainly leads to enzymatic generation of $O_2^{-\bullet}$ and H_2O_2 [139]. Different types of ROS give rise to specific signaling, as has been shown in animal cells [140]. Typically, animal cells are more thoroughly studied systems for ROS signaling.

It is assumed that particular properties of ROS lead to their utilization as signal molecules by evolution [117,129]: i) Cells are able to rapidly generate and scavenge different forms of ROS in a simultaneous manner, thereby permitting rapid response to stress. ii) The subcellular localization of ROS signals can be strongly controlled within cells, i.e. a spatial control of ROS accumulation exists in a highly specific manner. iii) ROS can be used as rapid long-distance auto-propagating signals to be transferred throughout the plant, as recently reported for *A. thaliana*, in which ROS signals propagate at rates of up to 8.4 cm/min [117]. In the latter case, propagating ROS waves were observed as autocatalytic and/or autoinhibited reaction patterns of plant cells leading to wave-like transport patterns of the ROS signal [117,129], see also Section 4.1.

ROS in general and H_2O_2 in particular are involved in the regulation of gene expression, an important regulatory control in acclimation of organisms to different stress factors [9,141]. Studies using DNA microarrays [142] revealed that an increase of the ROS concentration affects the expression of a rather large number of genes. This response can sometimes comprise up to one third of the entire genome. Experiments performed with the unicellular green alga *Chlamydomonas* (*C.*) *reinhardtii* showed that H_2O_2 and $^1\Delta_gO_2$ interact with different targets leading to the activation of specific promoters [143].

Depending on the lifetime, different types of ROS molecules can either directly act as signal molecules or generate signal chains by the formation of oxidation products (e.g. lipoperoxides). Such species involved in signaling chains and generated by primary ROS are typically denoted as “secondary messengers”. Unraveling the role of ROS involved in various signal pathways and modulation of gene expression requires the use of suitable analytical tools for selective ROS monitoring with sufficient resolution in time and space (see Section 2) and the availability of appropriate sample material. Genetic engineering is the most powerful approach in offering mutants with specifically changed properties in the expression of genes for enzymes that are involved in both generation and decay of ROS. Characteristic examples describing the signaling action of $^1\Delta_gO_2$, H_2O_2 and $O_2^{-\bullet}$ particularly in plant cells are given in [17].

3.1. Signaling and gene regulation by singlet oxygen ($^1\Delta_g$ O_2)

Regarding the role of $^1\Delta_gO_2$ in signaling especially by gene regulation, in-depth investigations have been published [130,144,145].

In plants cells, $^1\Delta_gO_2$ is known to function predominantly as a plastid ROS signal which activates nuclear gene expression [25]. Because of its high reactivity, $^1\Delta_gO_2$ has a very short lifetime and secondary messengers are required for signal transfer from the site of formation within the chloroplast through the cytosol to the nucleus (see Fig. 4).

A $^1\Delta_gO_2$ signaling pathway in *C. reinhardtii* was shown to give rise to gene expression that leads to increased tolerance to ROS (acclimation). This phenomenon comprises enhanced expression of genes for ROS protection and detoxification, e.g. of a glutathione peroxidase-homologous gene and the σ-class glutathione-S-transferase gene [141]. The effect on components participating in signaling was analyzed in a $^1\Delta_gO_2$-resistant mutant (SOR1). The results obtained revealed the involvement of reactive electrophilic species that are formed by $^1\Delta_gO_2$-induced lipid peroxidation [146]. It was found that the *SOR1* gene encodes a leucine zipper transcription factor, which controls the expression of numerous genes of stress response and detoxification. It was inferred from these results that ROS play a key signaling role in acclimation of *C. reinhardtii* cells to $^1\Delta_gO_2$ stress.

$^1\Delta_gO_2$ signaling induces programmed cell death, in particular under biotic stress. Information on the genetic control of this phenomenon has been gathered from investigations on the *A. thaliana* mutant *flu1*, which is defective in the feedback control of the Chl biosynthesis pathway. This mutant, which accumulates the photosensitizer protochlorophyllide in the dark, generates $^1\Delta_gO_2$ within the first minute of illumination after a dark-to-light shift [147]. The $^1\Delta_gO_2$ formation taking place in the vicinity of the thylakoid membrane [148] can be manipulated by altering the degree of light exposure and the preceding dark period. In contrast to wild-type plants, the $^1\Delta_gO_2$ production in *flu1* is not associated with excess excitation of PSII [149]. The studies on the *flu1* mutant revealed that $^1\Delta_gO_2$ can trigger the activation of programmed cell death and that two chloroplast-located proteins, EXECUTER1 and 2 (EXE1 and EXE2), control this process (see Fig. 4). EXE1 and EXE2 act as suppressors [148,150,151], but their mode of function in signaling of the $^1\Delta_gO_2$-induced programmed cell death is not yet resolved.

As a consequence of the special mode of $^1\Delta_gO_2$ formation in the *flu1* mutant, a cell death in their leaves can be induced either due to direct oxidative destruction (necrosis) under a large excess of ROS or, at a slower rate of $^1\Delta_gO_2$ formation, via the activation of a programmed cell death pathway. By autocatalytic cascades, lipoperoxide radicals can result in the generation of $^1\Delta_gO_2$ in the cytosol [31] and trigger the EXE1/EXE2-mediated pathway of programmed cell death [151]. Treatment with delta-aminolevulinic acid (Ala) leads to singlet oxygen-induced photodamage in PS II due to its induction of protochlorophyllide synthesis [152–154]. A negative feedback exists between the chlorophyllide concentration and Ala synthesis for example in angiosperms [153], and it is also known that the FLU protein, downregulates Ala [155] and, therefore, the tetrapyrrole biosynthesis. Most probably, the tetratricopeptide repeat domain of FLU interacts with glutamyl-tRNA reductase in this process [155]. However, in contrast to apoptosis, the role of $^1\Delta_gO_2$ in the direct chlorophyllide regulation has not been resolved yet. To analyze these effects, further experiments might be helpful, in which plants are externally treated with Ala [152,154] in the $^1\Delta_gO_2$-resistant SOR1 mutant.

The enzymatic peroxidation of lipids is catalyzed by lipoxygenases. These enzymes play an essential role in the response to pathogen infection and wounding [156–158]. Specific lipoxygenase pathways lead to the formation of lipoxide species, which are likely to be different when induced by chemically different ROS like $^1\Delta_gO_2$ versus $O_2^{-\bullet}$/H_2O_2. Studies on the *flu1* mutant of *A. thaliana* revealed that 70 genes are up-regulated by $^1\Delta_gO_2$ but not by $O_2^{-\bullet}$/H_2O_2, the latter being formed at PS I via the methylviologen mediation reaction [147]. Further pathways of $^1\Delta_gO_2$ signaling are described in [17].

A general problem in identifying different $^1\Delta_gO_2$-induced signal pathways has to be mentioned. The effect on the gene expression pattern is expected to depend on the nature of the nearest neighborhood of $^1\Delta_gO_2$ formation, if one accepts that direct signaling by $^1\Delta_gO_2$ can take place only at a site very close to its generation. This would also imply that the signal pathway involves the participation of oxidation products of carotenoids, lipids and other molecules acting as second messengers, which can induce different genetic responses. The $^1\Delta_gO_2$ site differs in *Arabidopsis* WT plants and in mutants like *flu1* and, also, if $^1\Delta gO_2$ is generated by using exogenous sensitizers [48,130]. Therefore, different types of second messenger species are likely to be formed. Thus, it is difficult to reach straightforward conclusions on the mechanism of $^1\Delta_gO_2$ signaling from studies performed under different assay conditions with different sample material. This important problem needs to be clarified in forthcoming studies.

3.2. Signaling by superoxide and hydrogen peroxide

In cyanobacteria and plants, the $O_2^{-\bullet}$ radical is predominantly produced at the acceptor side of PS I [159]. The lifetime of $O_2^{-\bullet}$ is mainly determined by the presence of SOD and does not exceed a few microseconds in cells [91], see Section 2.2.2. The signaling function of $O_2^{-\bullet}$ has been investigated by the analyses of gene expression using DNA microarrays [160] and studies on $O_2^{-\bullet}$ accumulation in plants deficient in Cu/Zn-SOD [161]. The results favor a signaling role of this radical, but details of the pathway(s) are still elusive.

Due to its charge, the superoxide anion itself cannot pass the membrane. However, after conversion to H_2O_2, uptake can occur through

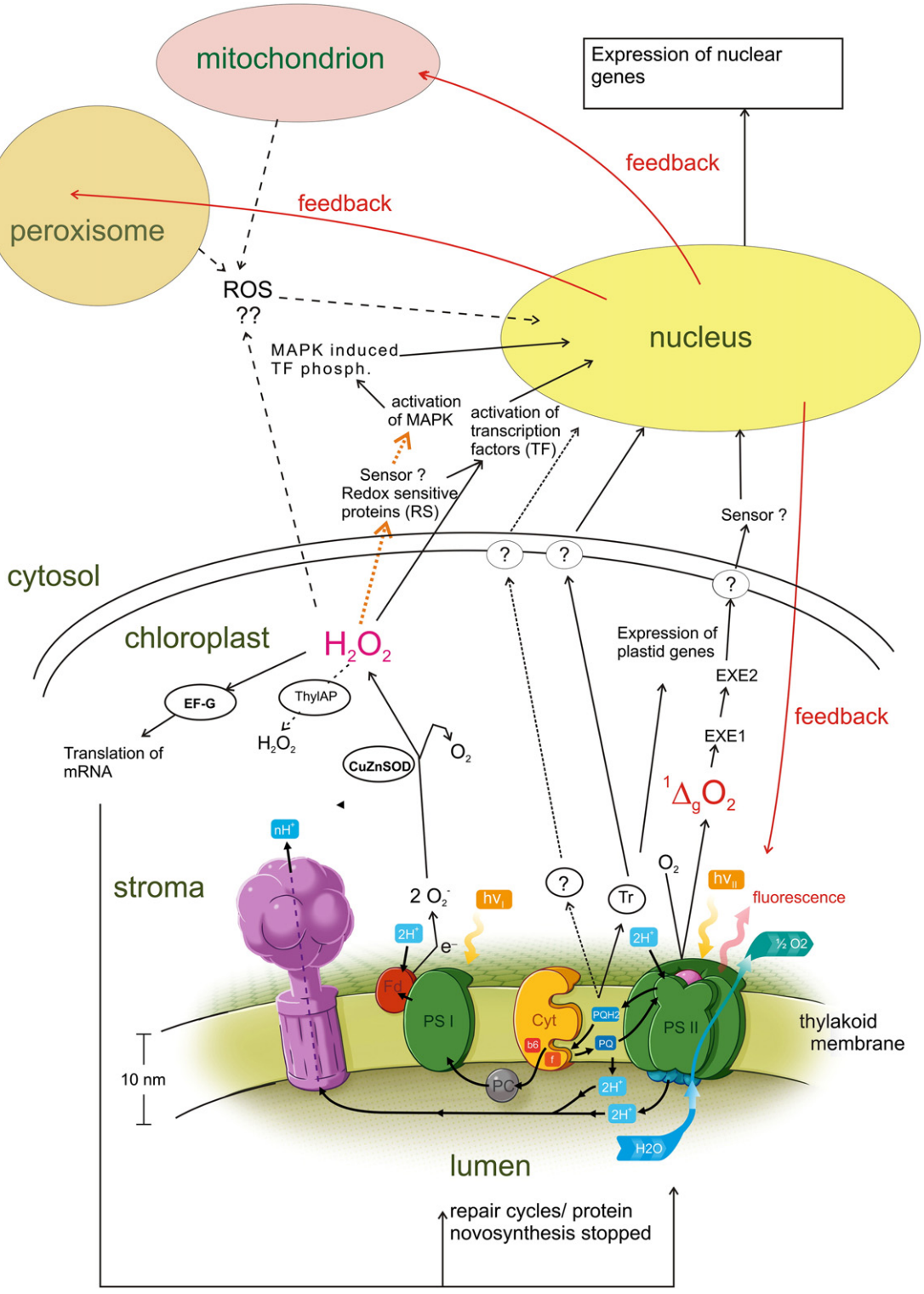

Fig. 4. Hypothetical scheme of pathways of photosynthetic redox signal transduction in plants. For the sake of simplicity, other cell organelles (nucleus, mitochondrion, peroxisome) are symbolized by colored ovals. Interrupted arrows designate hypothetic pathways of signal transduction. The question marks designate unknown components of signal transduction pathways. Solid lines designate signal transduction pathways with some experimental confirmation. Dotted lines designate experimentally established signal transduction pathways in chloroplast ETC and in the stroma. The abbreviations RS, MAPK and TF denote redox-sensitive protein(s), MAP-kinase and transcription factor(s), respectively. See text for details.

dedicated aquaporins. Alternatively, due to its pK_a of 4.8 [93], $O_2^{-\bullet}$ can get protonated at sufficiently acidic pH and pass membranes in the electrically neutral form [162], whereupon it may deprotonate in the cytoplasm and become converted to H_2O_2. According to Slovacek and Hind [163], the luminal pH can reach values down to 4.5, while other publications report values between 5.0 and 5.5 [164,165]. At pH 5.0, at least some $O_2^{-\bullet}$ molecules are protonated to $HO_2^{\bullet}$ and can cross the membrane. Especially under stress conditions, the pH in the thylakoid lumen can reach values down to 4.5, which accelerates the light-induced PS II damage [166]. However, such low pH values might lead to enhanced migration of $HO_2^{\bullet}$ across the thylakoid membrane. In this way, stress not only leads to the formation of $O_2^{-\bullet}$, but also to a change of the environmental conditions that allow for further spreading in the form of $HO_2^{\bullet}$ molecules, thus functioning as signal transducer. Moreover, the pH close to polyanionic membrane surfaces is lower than in the medium of the lumen and the appearance of $O_2^{-\bullet}$ in this restricted space near the membrane surface can lead to the protonation of $O_2^{-\bullet}$ [104], hence, to the appearance of uncharged, but stronger toxicants such as $HO_2^{\bullet}$. $O_2^{-\bullet}$ can react with NO under formation of peroxynitrite. This species is likely to be synthesized in chloroplasts, where it can fulfill signaling functions [13].

H_2O_2 is markedly less reactive than $^1\Delta_gO_2$ [98] and $O_2^{-\bullet}$ (see above) and characterized by a much longer lifetime in the order of 1 ms [109,167]. Therefore, H_2O_2 is the most straightforward candidate to serve as an intra- and intercellular messenger [13,108,129,168]. H_2O_2-sensitive proteins and signaling systems were extensively studied in bacteria and animal cells (see [129] and references therein). However, also comparable studies on the signaling role of H_2O_2 in plant cells exist [169]. More information concerning H_2O_2 and other ROS participating in plant cell signaling has been published recently [19,85,129,170]. H_2O_2 oxidizes redox-sensitive compounds like GSH, which contain thiol groups that can interact with ROS [170,171]. The accumulation of redox-active compounds within the chloroplast is associated with the rate of photosynthetic electron transport. For example, the redox-sensitive thioredoxin or PQ may act as sensors of changes in redox properties under stress conditions [170].

H_2O_2 and other ROS produced under stress conditions can also induce physical separation of PSII from its light-harvesting complex (LHC II) via degradation of D1 proteins, hence, reducing the absorption of light energy [85]. The oxidation of the primary Q_A acceptor of PS II under strong light stress leads to an increase of rate of ET that reduces the probability of $^1O_2$ formation [141].

The production of H_2O_2 and its ability to diffuse from the chloroplast into the cytoplasm has been studied by spin-trapping EPR spectroscopy and H_2O_2-sensitive fluorescence dyes such as Amplex Red and H_2DCFDA. It was shown that a part of the H_2O_2 produced inside the chloroplast can diffuse into the cell cytoplasm [172]. Based on these data, it was suggested that H_2O_2 plays an important signaling role inside the cell. Due to the discussed properties, it is likely that H_2O_2 works inside the cell organelles, including chloroplasts and contributes to long-distance signaling in the form of the $O_2^{-\bullet}/H_2O_2$ system [117,129].

Due to the high capacity of plants to scavenge ROS, the long-distance aspect of ROS signaling is strongly supported by the continuous production of ROS in individual cells leading to auto-propagating ROS waves [129] (see Section 4.1).

In conclusion, $O_2^{-\bullet}$ and H_2O_2 are moderately reactive, and, from a more general point of view, the main damage caused by these species is likely due their transformation into more reactive species. H_2O_2 is likely a very suitable molecule to function in intra- and probably, also inter-organelle messaging [129]. $O_2^{-\bullet}$ can also provide signaling functions independently from H_2O_2, especially when "activated" (i.e. converted to $HO_2^{\bullet}$) at low pH.

To elucidate the signaling role of H_2O_2 in more detail, a few examples will be discussed, which shed light on characteristic mechanistic features. The defense of bacteria against oxidative stress and adaptive regulation mechanisms has been thoroughly analyzed in *E. coli* and *Bacillus subtilis*. In eubacteria (heterotrophic, autotrophic, and chemotrophic), two global regulators, OxyR and PerR, are involved in the control of gene transcription induced by H_2O_2 addition [173,174]. Both regulators have active thiol groups and react to changes in the redox state of the cytoplasm (see below). The ferric uptake repressor (Fur)-type protein PerR was found to be the central regulator of inducible stress response [175,176]. In *Synechocystis* sp. PCC 6803, OxyR is absent, but a gene (*srl*1738) encoding a protein similar to PerR in *B. subtilis* was identified as being induced by H_2O_2 [175] in methylviologen-treated cells upon illumination [177]. It was concluded that the Fur-type protein Slr1738 induces the potent antioxidant gene *sll1621* (encoding putative type-2 peroxiredoxin) upon oxidative stress.

As an example in plants, the growth of *A. thaliana* under short-day illumination was reported to give rise to a diminished expression of several genes, which are involved in sensing and hormone synthesis [178]. It was found that the level of ROS production is about two-fold higher in leaves from short-day (8 h light) tobacco than in leaves from long-day (16 h light) plants. Based on these results, an unknown regulatory protein was hypothesized, which changes the relative extent of cyclic and pseudo-cyclic photosynthetic electron transport, thereby affecting the ROS content in chloroplasts [179]. These findings suggest the participation of light sensor(s). These findings point at a possible functional link between ROS signaling and circadian output, which provides a mechanistic link for plant response to oxidative stress [180]. The components involved and the underlying mechanism(s) of these mutual interactions of signal networks are not yet resolved and represent challenging topics for future research.

A new concept for the mechanism of photoinhibition was recently developed suggesting that an excess of ROS such as H_2O_2 or singlet oxygen leads to the inhibition of the de novo synthesis of the D1 protein at the stage of translational elongation [83,181,182].

This effect has been analyzed in *Synechocystis* sp. PCC 6803. It was shown that the translation machinery is inactivated in a quite universal way, with elongation factor G (EF-G) being the primary target [17,83,182,183]. As pointed out in [181], EF-G catalyzes the translocation of peptidyl-tRNA. EF-G is sensitive to oxidation with H_2O_2 [184,185]. In *Synechocystis* it was found that the disulfide bonds of EF-G can be oxidized by ROS and reduced by thioredoxin [186]. The fact that EF-G in *Synechocystis* [187] and spinach chloroplasts [188] is purified by affinity chromatography using thioredoxin as ligand suggests that EF-G may serve as a target for thioredoxin. H_2O_2 oxidizes two cysteine residues in EF-G. As a result, the disulfide bridge formed between Cys-105 and Cys-242 represses protein translation [181]. EF-G is a signal receptor for ROS action and a key regulator of the translation efficiency that stops the repair mechanisms when oxidized under unphysiological conditions to avoid the waste of energy. The oxidation of EF-G might lead to a complete stop of PS II repair and eventually even to the disappearance of PS II. Studies on the effect of other stress factors (heat, drought, salinity) on photoinhibition have shown that the suppression of PS II repair determines the PS II sensitivity of cyanobacteria to environmental conditions [79,182,183,189].

4. ROS as long distance signal, ROS waves and respiratory bursts

4.1. Long distance signaling and ROS waves

Studies using advanced imaging techniques (e.g. a luciferase reporter gene expressed under the control of a rapid ROS response promoter in plants [117], or a new H_2O_2/redox state GFP sensor in zebrafish [46], see Section 2), revealed that ROS bursts trigger a cascade of cell-to-cell communication events that result in the formation of a ROS wave as intercellular auto-propagating signals over long distances in tissues [129]. Miller et al. [117] showed by the local application of catalase or an NADPH oxidase inhibitor that a ROS wave triggered by different stimuli can be blocked at distances of up to 5–8 cm away from the site of signal origin.

The activation of systemic signals by local application of high light stress was recently shown to be accompanied by plasma membrane electrical signals in a light wavelength-specific manner [190]. Interestingly the membrane potential was directly affected by ROS and the velocity of certain electric signals in plants matches the speed of the ROS wave, as previously reported [117]. Further research could address a possible modulation of electric signals by ROS waves.

A comparison with ROS signaling in animal cells revealed that the communication of mitochondria in heart cells occurs via ROS-induced ROS waves. An abrupt collapse or oscillation of the mitochondrial energy state is assumed to be synchronized across the mitochondrial network by local ROS-mediated interactions [191]. This model is based on the idea that a depolarization of the electrical potential difference across the coupling membrane is specifically mediated by $O_2^{-\bullet}$ via its diffusion and the $O_2^{-\bullet}$-dependent activation of an inner membrane anion channel, in agreement with experimental data. This mode of a ROS-induced ROS release in animal cells can also be used in plants for propagation of cell-to-cell ROS signaling over long distances [117,129]. The concept of a transient ROS burst occurring in selected cells can be further extended to the more general concept of a ROS wave propagating in time and space as response to different types of stress.

4.2. Respiratory bursts and the role of respiratory burst oxidase homologs (RBOHs) in ROS signaling

The oxidative burst is an important defense strategy of plants to cope with pathogen infections [27,134,137]. Oxidative bursts occur as a response to pathogenic microorganisms as observed in suspension-cultured plant cells that are treated with elicitor preparations or pathogenic microorganisms like fungi, bacteria and viruses. Oxidative bursts are also observed in response to mechanical stress [134].

It was suggested that the majority of ROS generating oxidative bursts are related to H_2O_2 and superoxide. As sources of the oxidative burst, the NADPH oxidase complex generating superoxide or a cell wall peroxidase generating superoxide is considered [134,192]. The participation of these enzymatic systems is confirmed by studies involving different plant mutants showing deficits in the activity of the aforementioned systems [193].

The ROS wave can serve not only as a direct defense against the pathogens by oxidation, but also as a signal inducing the activation of defense genes. The signaling role of ROS in defense occurs in combination with other known signaling molecules, such as salicylic acid and NO [194]. Recent results from research using bioinformatics tools highlighted several stress-inducible gene families of the ROS signaling pathways that trigger rapid pathogen attack perception and disease resistance in plants [195].

Intriguing similarities exist between plants and animals regarding the utilization of the so-called “respiratory burst” for defense or other purposes [196]. In spite of the potentially deleterious action of ROS, most organisms utilize purposeful ROS-generating enzymes for a variety of processes such as defense. Homologs of the catalytic subunit of NADPH oxidases (NOX) are found in most eukaryotic species [197]. In humans, for example, the NADPH oxidase NOX2 is essential for pathogen killing by the respiratory burst in blood phagocytotic cells (neutrophils). NOX proteins homologous to the catalytic subunit gp91phox of mammalian NOXs of phagocytes exist in plants as well. These enzymes have been named “respiratory burst oxidase homologs” (RBOHs) accordingly, and were found to participate in pathogen response as well, although it is evident that the systemic challenges of spatially constrained plant cells and the highly mobile immune cells of animals are strikingly different. RBOHs contain two N-terminal Ca^{2+}-binding motifs (EF-hand motif) and it was suggested for NOX activities from tomato and tobacco to be activated by Ca^{2+} [92,197]. Finally, recent research suggests that RBOHs are synergistically activated by binding of Ca^{2+} to the EF-hand motifs and Ca^{2+}-dependent phosphorylation [197]. Recent years have seen the discovery of a rapidly increasing number of different processes in plants that include RBOHs as important molecular pivots, such as cell growth (cell wall stiffening and loosening), plant development, root hair cell growth, stomatal closure, wounding, as well as responses to biotic and abiotic constraints, either of pathogenic nature, or even for the purpose of symbiosis between legumes and nitrogen-fixing bacteria [138].

As integral plasma membrane proteins, RBOHs produce extracellular ROS by the abstraction of electrons from intracellular NADPH, which are transferred to the extracellular acceptor O_2, thereby producing $O_2^{-\bullet}$, which may subsequently be converted to H_2O_2 by extracellular SOD. Besides serving as an extracellular signal, the ROS products of RBOHs can be coupled to intracellular ROS signaling.

By this intriguing transport route, ROS generated by RBOHs can contribute to intracellular amplification loops, and eventually mediate crosstalk to other signaling cascades. Positive feedback amplification can occur via Ca^{2+}, which has been shown to stimulate plant RBOHs [92,198]. This in turn can lead to an even larger Ca^{2+} influx via RBOH-produced ROS, thus serving as intracellular messengers by activating redox-controlled Ca^{2+} channels [87].

In general, chemical waves and stably propagating distortions (soliton-like structures) can result from an oscillatory behavior of a redox-system. The reaction pattern needs to include at least two nonlinearly interacting components comprising autocatalytic or autoinhibitory reactions, which is the case for the respiratory burst and programmed cell death. Autoinhibition via the ROS concentration is a general feature of the typical protective response of ROS-depleting enzymes initiated by high ROS concentration. Wave-like propagation of the chemical compounds occurs consequently at certain concentration patterns of the reactants. Therefore, RBOHs open the way to “encode” chemical information with characteristic spatio-temporal distributions.

5. Networking of ROS with other signaling pathways

The role of ROS reaches far beyond toxicity and the rather direct regulation of transcription or translation, like e.g. the well-known effect of H_2O_2 on EF-G in chloroplasts, which blocks translation as protection mechanism during ROS stress [182,183]. It is well established that the ambient redox conditions control a variety of cellular processes [20,199]. In this sense, ROS are second messengers that activate or inactivate compounds regulating cell metabolism. This complex interaction profile makes ROS or, more generally, redox signals, the most fundamental forms of information monitored by plants [200].

ROS-sensitive enzymes can serve as reversible redox-“switches” responding to redox changes within the cells. Switching can occur due to direct interaction with ROS, or under the participation of redox compounds like GS or thioredoxin [20,201]. The redox-sensitive signaling proteins function in combination with other components like mitogen-activated protein kinases (MAPKs) and transcription factors [201,202]. Most likely, MAPKs are involved in transducing signals derived from ROS generated in chloroplasts [203].

A schematic overview on ROS sources in the thylakoid membrane is given in Fig. 4, which summarizes material described in [168,202,204] together with the effects of H_2O_2 on transcription and translation (see also [182,183,205] for further information). Fig. 4 shows how gene expression in the nucleus of plant cells is affected by ROS sources in chloroplasts [14,199] including the direct production of $^1\Delta_gO_2$ and $O_2^{-\bullet}$ as well as tetrapyrrole compounds serving as secondary messengers. The components of the ETC change their redox state under the influence of ROS. This leads to an overall coupling of the different signal pathways in chloroplasts, and from there to the whole cell [201,202,204,206,207]. Fig. 4 also indicates how redox-sensitive protein(s) (RS) might act as primary sensors of H_2O_2 signal transduction. The signal can be transmitted directly from H_2O_2 or via RS to the MAPK cascade and/or to transcription factors [202,204,205].

By generation of secondary messengers (e.g. oxidation products of carotenoids and lipids, see above), ROS pathways are established in plants from chloroplasts through the cytosol to the nucleus within 30 min [208], while even faster processes involving the nuclear genes encoding cytosolic ascorbate peroxidases APX1 and APX2 in *A. thaliana* seem to show a response within 15–20 min [209,210].

Regulatory proteins called heat stress transcription factors (Hsfs) could also be involved as ROS-dependent redox-sensors. Hsfs proteins contain a DNA binding domain, control the transcription of heat stress protein-encoding genes [211] and are distributed both in the cytosol (mainly in the inactive form) and in the nucleus. Once Hsfs enter the nucleus, they bind to the heat shock element of the promoter of ROS-sensitive genes such as the gene encoding APX. There is evidence that certain Hsfs directly sense ROS and control the gene expression during oxidative stress [210]. For example, transcription activity of genes of cytosolic peroxidases APX1 and APX2 can be regulated by Hsfs, which in turn can be modulated by ROS [209].

The overall ROS "networking", especially the crosstalk between ROS and other signaling pathways, is still largely unknown. There is now increasing evidence for the existence of signal networks due to close functional connections between signaling pathways triggered by ROS and those, which are induced by other stimuli like Ca^{2+} and hormones [209,212]. Although our current knowledge is still fragmentary, the general conclusion on signal networks is illustrated by the proven interference between various pathways [17,170,209,213]. New concepts about ROS as signaling intermediates first appeared in publications on hormonal signaling and regulation of expression of genes participating in plant pathogen protection [85,136]. In this case, interaction of ROS with NO plays an important role [10].

6. Concluding remarks

The enormous work performed during the last decades has clarified the deleterious effects of ROS on photosynthetic organisms as one aspect of ROS functions. Current research mainly focusses on the other side, the important signaling role in the response of cyanobacteria, alga and higher plants to different conditions of stress. In spite of significant progress achieved during the last decade, our knowledge on this topic is still rather fragmentary. To reach substantial progress, several key questions need to be answered:

1. How is the transcription of the chloroplast genome affected by ROS?
2. Can ROS leave the chloroplasts and directly induce nuclear gene expression without second messengers?
3. What are the nature and the function of second messengers formed by reaction of ROS with specific molecules like lipids and carotenoids?
4. By which mechanism do ROS waves propagate in plant cells?
5. What is the identity of the primary ROS sensor(s) (transcription factors or protein kinases) and the primary genes responding to oxidative stress?
6. Can ROS themselves act as second messengers?
7. How does a complete picture of ROS networking in different organisms look like?

Tremendous progress is expected from the development of new spectroscopic methods for ROS monitoring with high spatial resolution and the specific introduction of a certain oxidative species exactly at the desired protein target by CALI in genetically engineered organisms. The elucidation of ROS regulation pathways will provide the framework for new strategies, e.g. to improve stress tolerance of agricultural and industrial crops and might also be of high interest for the specific design of chemical agents.

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