Chapter from the book *Cell Metabolism - Cell Homeostasis and Stress Response*

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1. Introduction

Oxygen was almost non-existent in the Earth's atmosphere before the oxygenic photosynthetic bacteria appeared. Since O$_2$ is capable of combining with most chemical elements, the stable level of O$_2$ in the atmosphere is the result of it being continuously regenerated by the oxygenic photosynthetic organisms, i.e. the cyanobacteria, algae and plants.

The molecular mechanism of water oxidation to O$_2$ is still unclear, although many structural details are known and some of the details of the charge accumulating cycle are well worked out (reviewed in Barber, 2008; Brudvig, 2008). The water-oxidizing complex, with a Mn$_4$Ca cluster as the active site, is an integral part of the Photosystem II (PSII), one of the main complexes of the photosynthetic electron transport chain (PETC). When the energy of a quantum of light absorbed by a chlorophyll molecule in this photosystem reaches the reaction center, photochemistry occurs leading to charge separation. The electron is used to reduce plastoquinone, while the electron hole is used to oxidize a Mn ion of the cluster and eventually used to oxidize water. Two sequential photochemical turnovers are required to reduce quinone to quinol, while four sequential turnovers are required to oxidize two water molecules forming O$_2$. It is important to note that the water oxidation/oxygen evolution process is the most easily damaged function of the PETC under stress conditions.

Sixty years ago, the first data were published indicating the light-induced reduction of O$_2$ in the chloroplasts (Mehler, 1951) (see 2.2). There has been much debate concerning what is the proportion of the total electron flow from water that ends up on O$_2$. It seems likely that there is no generally applicable answer to this question and it seems that the best answer is that it depends on the conditions. Under continuous illumination the proportion of electrons transferred to O$_2$ was reported to be less than 10 % in C$_3$-plants, up to 15 % in C$_4$-plants (mesophyll cells), and even 30 % in algae (Badger et al., 2000). In a recent study with leaves of Hibiscus rosa-sinensis, it was concluded that in this plant it was almost 40 % (Kuvykin et al., 2008). We believe that both the rate of oxygen reduction and its proportion of the total electron transport depends on i) the plant species, the genome of which determines the range of these values, ii) environmental factors (light, temperature, mineral nutrition, supply of water, and so on), and iii) the age of the plant.

The reduction of O$_2$ by the PETC in chloroplasts results in the formation of a series of reduced forms of O$_2$ that are termed Reactive Oxygen Species (ROS), namely, superoxide anion radical (O$_2$•$^-$), hydrogen peroxide (H$_2$O$_2$), and hydroxyl radical (OH$^-$). ROS also
include the singlet oxygen ($^{1}\text{O}_2$), which is not generated by $\text{O}_2$ reduction but by energy transfer from other molecule, mainly from excited chlorophyll triplet state (see 2.2.2).

The above ROS-generating reactions should be distinguished from ROS-mediated reactions, in which the ROS themselves interact with components of the chloroplast. The reactions of both types have “positive” and “negative” effects on chloroplast functions. The occurrence of both types of ROS reactions and to what degree their influence is positive or negative can change as conditions change during the life of the plant, being primarily determined by the level of stress encountered.

2. Oxygen metabolism in chloroplast

2.1 The properties of $\text{O}_2$ molecule and reactive oxygen species

Under usual conditions in the nature, oxygen is a gas composed of diatomic molecules $\text{O}_2$, dioxygen. Triplet is the ground state of the dioxygen since the molecule has two electrons with parallel spins in two antibonding molecular orbitals. Since these electrons are unpaired, dioxygen is a biradical. However, the reaction of this biradical with cell components has quantum-mechanical constraint because these components are in the singlet state, i.e. they have the valence electrons with antiparallel spins. Due to the above reasons the spontaneous reactions of cell metabolites with dioxygen are highly retarded despite its high oxidizing potential, $E^\prime_0 = +0.845 \text{ V}$ of the full reduction of $\text{O}_2$ to $\text{H}_2\text{O}$. Such situation is saving for organisms, and the reactions of cell metabolites with $\text{O}_2$ proceed generally with involvement of enzymes, which activate a substrate to speed up these reactions. However the oxidation of cell components can readily proceed by ROS.

Singlet oxygen, $^{1}\text{O}_2$, is formed as the result of the spin flip of one of unpaired electrons. The transformation of $^{1}\text{O}_2$ to triplet is relatively slow; its lifetime in the cell was estimated to be appr. 3 $\mu$s (Hatz et al., 2007). This estimation is higher than the previous one for cytoplasm, 0.2 $\mu$s (Matheson et al., 1975). In the apolar media this lifetime is higher, 12 $\mu$s in ethanol and 24 $\mu$s in benzene, and in the heavy water the lifetime increases almost twentyfold and reaches 68 $\mu$s (Krasnovsky, 1998). The chloroplast is a prevailing source of $^{1}\text{O}_2$ in the living organisms.

Superoxide anion radical, $\text{O}_2^{\cdot-}$, can appear if one additional electron is transferred to the antibonding orbital of $\text{O}_2$. This transfer is possible only if a donor molecule has a redox potential close or lower than the redox potential of pair $\text{O}_2/\text{O}_2^{\cdot-}$. In the aqueous solutions $E^\prime_0 (\text{O}_2/\text{O}_2^{\cdot-})$ is equal to $-0.16 \text{ V}$ vs. the normal hydrogen electrode (NHE) at 1 M $\text{O}_2$. This value should be used in all thermodynamic consideration of the reactions in the aqueous solutions, instead of $-0.33 \text{ V}$, which is the standard potential at 1 atm of $\text{O}_2$. The value of the midpoint redox-potential in aprotic media is much lower, in the region $-0.55 \div -0.6 \text{ V}$ vs. NHE (Afanas’ev, 1989). Thus in aprotic media $\text{O}_2^{\cdot-}$ is a very strong reductant.

The heavy solvation of $\text{O}_2^{\cdot-}$ in aqueous solutions evidently determines its moderate activity in deprotonation reaction in this media; p$K_a$ value of perhydroxyl radical, $\text{HO}_2^{\cdot}$, is equal to 4.8. Thus in the aqueous solutions at physiological pH 7.7 the amount of $\text{HO}_2^{\cdot}$ is near 0.25 % from total amount of $\text{HO}_2^{\cdot} + \text{O}_2^{\cdot-}$. The basicity of superoxide ion is much stronger in aprotic media; it was estimated that ‘thermodinamic’ value of p$K_a$ is close to 12. However more detailed consideration of full deprotonation process leads to a statement that in such media
O₂**− should be considered as a deprotonating agent with pKₐ of approximately 24 (Afanas’ev et al., 1987). Moreover, considering deprotonation of any substrate by O₂**− it is necessary to take into account that the basicity of proton donors can also increase in aprotic medium, and e.g. the rate constant of deprotonation of α-tocopherol by O₂**− is higher in water than in aprotic solvents (Afanas’ev et al., 1987). Being the neutral free radical, HO₂* cannot abstract a proton, but it can abstract a hydrogen atom from substrates with active C-H bonds, initiating fatty acid peroxidation (see further).

O₂**− ion is rather stable even in aqueous solution; the half-life of O₂**− was found to be close to 15 s at pH 11 (Fujiwara et al., 2006). The pH value is very important since the rate constant of spontaneous dismutation (Reaction 1) has maximum at pH 4.8 being equal to 10⁸ M⁻¹ s⁻¹ at pH 7.7.

\[
O_2^{•−} + O_2^{•−} \rightarrow H_2O_2 + O_2
\]  

The living cells contain the special enzyme superoxide dismutase (SOD), which catalyzes the dismutation of O₂**− and determines a lifetime of O₂**−, and thus the possibility of its involvement in biochemical processes (see further). In the aprotic solvents the O₂**− dismutation is prohibited, and e.g. in dimethylformamid O₂**− can persist almost one month (Wei et al., 2004).

O₂**− can interpenetrate cell membranes; the permeability coefficient of the soybean phospholipid bilayer for O₂**− was estimated to be 20 nm s⁻¹ (Takahashi & Asada, 1983). The permeability of the egg yolk phospholipid membrane for HO₂* was estimated to be than for O₂**− by almost three orders greater (Gus’kova et al., 1984).

**Hydrogen peroxide**, H₂O₂, is the most stable ROS. Eₒ′ (O₂**−/H₂O₂) is equal to +0.94 V (Asada & Takahashi, 1987) in the aqueous solutions and in the presence of the electron donors and protons O₂**− can react as a good oxidant producing H₂O₂. Ascorbate, quinols, glutathione, and so on can be such donors. In the absence of donors, the dismutation of O₂**− is the main reaction of H₂O₂ production. In the cell, H₂O₂ can also be produced by two-electron oxidases such as glycolate, glucose, amino and sulfite oxidases, which oxidize these substrates by dioxygen directly (Byczkowski & Gessener, 1988).

The lowest pKₐ value of H₂O₂ is 11.8, and under physiological pHs H₂O₂ exists mostly in the neutral form. The properties of H₂O₂ in the aqueous solutions are determined mainly by hydrogen bonds between water and H₂O₂ molecules. These bonds can prevent transfer of H₂O₂ molecules from the aqueous solution to the hydrophobic solvent in spite of their neutral form. The value of Eₒ′ (H₂O₂/H₂O) in aqueous solutions is equal to +1.3 V vs. NHE, and in acidic solutions H₂O₂ is one of the most powerful chemical oxidizers. The reduction of H₂O₂ to water requires the breaking of O-O bond, and under physiological conditions the main target of oxidizing action of H₂O₂ are the reduced sulfhydrl groups of biomolecules.

**Hydroxyl radical**, OH•, the most destructive ROS, can be produced in cells in the reaction of H₂O₂ molecule decomposition, which is catalyzed by metal. The reaction in which the reductant of H₂O₂ is ferrous iron terms as the Fenton reaction (Reaction 2).

\[
H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH^- + OH^*
\]  

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This reaction can also be catalyzed by univalent cuprous ion, which is oxidized to divalent ion. Both the oxidized iron and cuprum can be re-reduced by O₂⁻⁻, and the total reaction of H₂O₂ reduction by O₂⁻⁻ terms as the Haber-Weiss reaction. The reduction of ferric ion to ferrous can also occur by the reduced cell components, such as ascorbate.

Hydroxyl radical is the penultimate step of dioxygen reduction to water, but this ROS is the strongest oxidant with E₀′ (OH⁺/H₂O) = +2.3 V. Because of high reactivity, OH⁺ is able to readily oxidize almost all biomolecules at nearly diffusion controlled rates. Therefore OH⁺ interacts with lipids, proteins and nucleic acids right in the place where it is generated. Since such generation depends on the location of H₂O₂ production, as well as the presence of both metals and reductants, all these circumstances determine the site specificity of the destructive effect of OH⁺ on biomolecules (Asada & Takahashi, 1987).

The peroxyl radical, ROO⁺, and hydroperoxide, ROOH, of organic molecule can be considered as long-lived ROS. Their generation usually occurs during the free radical chain reaction known as lipid peroxidation, where they are termed as LOO⁺ and LOOH. The lipid peroxidation is actually the oxidation of polyunsaturated fatty acid side chains of the membrane phospholipids, and it is initiated by the abstraction of hydrogen atom from the bis-allylic methylene of LH to produce L⁺. The abstraction can be executed by perhydroxyl radical as stated above, whereas the role of O₂⁻⁻ is usually denied (Bielski et al., 1983), as well as by hydroxyl radical, if the latter does appear in the membrane, and by other ways, e.g. by long-lived oxidized reaction center of PSII (see 2.2.2). Under physiological conditions the most possible reaction of L⁺ is the reaction with dioxygen, when one active electron from organic radical can occupy one of partially filled antibonding orbitals of dioxygen, producing LOO⁺. This radical is reactive enough to attack adjacent fatty acid side chain, abstracting hydrogen, producing LOOH and new L⁺; and thus propagating the chain oxidation of lipids. ¹O₂, reacting with fatty acid can form LOOH directly. LOOH can decompose to highly cytotoxic products, among of which the aldehydes are most dangerous.

2.2 Production of ROS in chloroplasts

Mehler observed the oxygen uptake and H₂O₂ formation under illumination in broken chloroplasts, i.e. the chloroplasts with destroyed envelope (Mehler, 1951). Later, it was shown that the primary product of O₂ reduction in the photosynthetic electron transport chain is the O₂⁻⁻ (Allen & Hall, 1973; Asada et al., 1974). The oxygen reduction rate averages 25 µmol O₂ mg Chl⁻¹ h⁻¹ in isolated thylakoids under saturating light intensity (Asada & Takahashi, 1987; Khorobrykh et al., 2004). Oxygen uptake and H₂O₂ formation under illumination of thylakoids is the result of the reactions

\[
2\text{H}_2\text{O} - 4e^- \rightarrow 4\text{H}^+ + \text{O}_2 \text{ (release)} - \text{water oxidation in PSII} \tag{3}
\]

\[
4\text{O}_2 + 4e^- \rightarrow 4 \text{O}_2^-^- \text{ - dioxygen reduction} \tag{4}
\]

and subsequent dismutation of O₂⁻⁻ (Reaction 1). Taking into account the peculiarities of this electron flow, namely that the donor and the acceptor are the forms of oxygen, and the fact that an electron does not return back to the place of its donation to PETC, this flow besides “the Mehler reaction” was termed “pseudocyclic electron transport”.

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2.2.1 Production of ROS in chloroplast stroma: mechanism and producers

Production of superoxide in stroma

Ferredoxin (Fd), a stromal protein and the electron carrier between PSI and NADP⁺, has long been regarded as the main reductant of oxygen in the Mehler reaction. The addition of Fd to the suspension of isolated thylakoids led to an increase of an oxygen consumption rate (Allen, 1975a; Furbank & Badger, 1983; Ivanov et al., 1980). $E_m$ for Fd/Fdred is $-420$ mV that enables the reduced Fd (Fdred) to reduce O₂ to O₂⁻⁻ in the water media. The pseudo-first order rate constant of this reaction was found to be in the region $0.07 - 0.19 \text{ s}^{-1}$ (Golbeck & Radmer, 1984, Hosein & Palmer, 1983, Kozuleva et al., 2007). The weak capability of Fdred to reduce O₂ is important for function of chloroplasts since Fdred is a key metabolite that is required for many metabolic reactions in chloroplasts, first of all, the reduction of NADP⁺.

Recently it was shown that oxygen reduction by Fd is only a part of the total oxygen reduction by PETC (Kozuleva & Ivanov, 2010). The share of oxygen reduction by Fd was measured to be 40-70 % in the absence and 1-5 % in the presence of NADP⁺. It means that in vivo oxygen reduction occurs mostly by the membrane-bound components of PETC rather than by Fdred, however the role of Fd can increase if the NADP⁺ supply becomes limited.

It was shown that some stromal flavoenzymes such as ferredoxin-NADP⁺ oxidoreductase, monodehydroascorbate reductase and glutathion reductase added to thylakoid suspension also can produce O₂⁻⁻ (Miyake et al., 1998). The authors have suggested that these enzymes are reduced by Photosystem I (PSI) directly. However in vivo the enzymes have to compete with Fd for electrons from terminal acceptors of PSI at the docking site that is optimized for association with Fd. So this way of oxygen reduction is unlikely under normal conditions.

Production of hydrogen peroxide in stroma

It is considered that the dismutation of O₂⁻⁻ with involvement of SOD is the main producer of H₂O₂ in chloroplasts stroma. The production of H₂O₂ in stroma through the reduction of O₂⁻⁻ by ascorbic acid or by reduced glutathione (GSH) is also possible. However the rate constants for these reactions are $3.3 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$ (Gotoh & Niki, 1992) and $10^2-10^3 \text{ M}^{-1}\text{s}^{-1}$ (Winterbourn & Metodiewa, 1994), respectively, i.e. they are considerably less than that for SOD-catalyzed dismutation, $2 \times 10^9 \text{ M}^{-1}\text{s}^{-1}$. Fdred was also proposed to produce H₂O₂ in the reaction with O₂⁻⁻ generated in course of the Mehler reaction (Allen, 1975b). However in vivo Fdred is involved in a number of reactions and its steady-state concentration is not high, and this way of H₂O₂ production in stroma should be unlikely in the case of effective operation of SOD.

Production of hydroxyl radical in stroma

The main way of OH⁺ generation is the Fenton reaction (Reaction 2). In chloroplasts stroma there are pools of iron deposited in a redox inactive form. Iron is bound with chelators such as ferritin, the iron storage protein (Theil, 2004), as well as low molecular mass chelators, e.g. nicotianamine (Anderegg & Ripperger, 1989). The concentration of free iron ions can be increased when the accumulation of the iron either exceeds the chelating ability of chloroplasts or the iron is released from its complex with chelators (Thomas et al., 1985). The authors have suggested that O₂⁻⁻ can cause the releasing of iron from ferritin.
The reduced ferredoxin can catalyze the Fenton reaction probably due to it has Fe in its structure (Hosein & Palmer, 1983; Snyrychova et al., 2006). However as it was noted above, the reduced ferredoxin in chloroplast is effectively used for various metabolic pathways, and its level is not high. So, this way of OH• generation can be significant only under stress conditions. The production of OH• also can occur during sulfite oxidation in chloroplasts, and both sulfite radical and hydroxyl radical can initiate oxidative damage of unsaturated lipids and chlorophyll molecules (Pieser et al., 1982).

2.2.2 Production of ROS in thylakoid membrane: mechanism and producers

Production of singlet oxygen in thylakoid membrane

The main route of 1O2 generation in thylakoids is the transfer of energy from the chlorophyll in triplet state to molecular oxygen (Neverov & Krasnovsky Jr., 2004; Rutherford & Krieger-Liszkay, 2001). The main place of the chlorophyll triplet state formation in thylakoids is PSII, presumably a chlorophyll a molecule located on the surface of the pigment-protein complexes and a chlorophyll a molecule of the special pair (P680) (Neverov & Krasnovsky Jr., 2004). The chlorophyll triplet state and hence 1O2 are usually formed under conditions that are favourable for the charge recombination in P680+Pheo− when forward electron transport is very limited (for review see Krieger-Liszkay, 2005), for example when the plastoquinone pool (PQ-pool) becomes over-reduced. This leads to the full reduction of QA and results in a low yield of charge separation due to the electrostatic effect of QA− on the P680+Pheo− radical pair. This is known as closed PSII however still around 15 % of charge separation occurs at such conditions leading to the formation of the chlorophyll triplet state.

The chlorophyll triplet state formation can occur by a true back reaction through P680+Pheo− or by a direct (tunneling) recombination (Keren et al., 1995). These processes can happen under normal functional conditions but with a very low rate. The distribution of these two routes is determined by the energy gap between the P680+Pheo− radical pair and the P680+QA− radical pair. It was shown that true back reactions with the electron coming back from Qb− leads to deactivation of some steps in water-oxidizing cycle giving rise to the chlorophyll triplet state formation and 1O2 generation (Rutherford & Inoue, 1984).

It was found that the treatment of plants by some herbicides that are known to bind to Qb site in PSII and to block photosynthetic electron transport results in formation of the chlorophyll triplet state and 1O2 that finally leads to death of plants (Krieger-Liszkay & Rutherford, 1998).

Production of superoxide in thylakoid membrane

As had been repeatedly proposed O2•− can be generated within thylakoid membrane (Kruk et al., 2003; Mubarakshina et al., 2006; Takahashi & Asada, 1988) and the first direct evidence was recently obtained using detectors of O2•− with different lipophilicity (Kozuleva et al., 2011).

PSI. Traditionally it was supposed that the components of acceptor side of PSI, which have highly negative Em values are the main reductants of oxygen. O2•− production can possibly occur under oxidation by oxygen of the FeS centers FA and FB, which are located in Psal subunit of PSI exposed to stroma. This O2•− production would occur outside the thylakoid membrane. The media within thylakoid membrane has low permittivity where Em of O2/O2•− pair could be approximately −600 mV (see 2.1). The components of PSI that are
situated below the surface of the membrane, phylloquinone A₁ and the FeS cluster FX, have Em values ~820 mV and ~730 mV, respectively (Brettel & Leibl, 2001). Thus the reduction of O₂ by these centers is thermodynamically allowed.

**PSII.** The O₂⁻⁻ generation in PSII has been also shown (Ananyev et al., 1994). However oxygen reduction in this photosystem can achieve only about 1–1.5 μmol O₂ mg Chl⁻¹ h⁻¹ at physiological pHs (Khorobrykh et al., 2002). In PSII thermodynamically only Pheo⁻⁻ (Em of Pheo/Pheo⁻⁻ ~−610 mV) is able to reduce O₂ to O₂⁻⁻. However under normal functional conditions fast electron transfer from Pheo⁻⁻ to QA⁻⁻ (300–500 ps (Dekker & Grondelle, 2000)) prevents the electron transfer from Pheo⁻⁻ to O₂. If QA⁻⁻ is fully reduced (e.g. under strong stress conditions) this process likely can occur. It is discussed in the literature (Bondarava et al., 2010; Pospíšil, 2011) that other components of PSII such as QA⁻⁻ (Em of QA⁻⁻/QA⁻⁻ ~−80 mV (Krieger et al., 1995)) and low-potential form of cytochrome b₅₅₉ (Em is 0–80 mV (Stewart & Brudvig, 1998)) can reduce molecular oxygen. However these processes are less favorable thermodynamically and probably do not occur under normal functional conditions.

The plastoquinone pool. Plastoquinone (PQ) is the mobile electron carrier between PS II and cytochrome b₆/f complexes in the thylakoid lipid bilayer phase and it simultaneously transfers the protons across the thylakoid membrane. TKhorobrykh & Ivanov (2002) provided the evidences of the involvement of the PQ-pool in the process of oxygen reduction. Using the inhibitor of the plastoquinol oxidation by cytochrome b₆/f complexes, dinitrophenylether of 2-iodo-4-nitrothymol (DNP-INT), the rate of oxygen uptake was measured to be 9-10 μmol O₂ mg Chl⁻¹ h⁻¹ at pHs higher than 6.5. It was shown that in the course of oxygen reduction in the PQ-pool, O₂⁻⁻ was produced. Thermodynamical analysis of the data revealed that only plastosemiquinone (PQ⁻⁻) (Em of PQ/PQ⁻⁻ is ~−170 mV) in the PQ-pool could reduce O₂ to O₂⁻⁻ (Reaction 5).

\[
PQ⁻⁻ + O₂ → PQ + O₂⁻⁻
\]  

(5)

It was proposed that the Q-cycle operation eliminates an appearance of long-lived PQ⁻⁻ in the plastoquinol-oxidizing site (Oscyczka et al., 2004). However the free PQ⁻⁻ can be produced in the reaction of plastoquinone/plastoquinol disproportionation (Rich, 1985) and thus the PQ⁻⁻ can reduce oxygen to O₂⁻⁻ under normal functional conditions. It was estimated that the product of the free PQ⁻⁻ concentration and the rate constant of the reaction between semiquinone and O₂ for quinones with Em values close to those of PQ/PQ⁻⁻, is very similar to the experimentally observed rates of oxygen reduction in the presence of DNP-INT (Mubarakshina & Ivanov, 2010). Moreover the detailed consideration of this process leads to a conclusion that the reaction between PQ⁻⁻ and O₂ proceeds at the membrane-water interface.

**PTOX.** Plastid terminal oxidase (PTOX) is the enzyme that oxidizes plastoquinol and reduces oxygen to water thus it is involved in chlororespiratory and play important role in many processes under stress conditions (for review see Nixon & Rich, 2006). Using Tobacco plants with over-expressing of PTOX it was proposed that PTOX also can reduce dioxygen to O₂⁻⁻ (Heyno et al., 2009). However under normal functional conditions this process (even if occurs) should not give the essential contribution to the overall generation of O₂⁻⁻ in PETC taking into account that the quantity of PTOX per PSII is ~1 % only (Andersson & Nordlund, 1999; Lennon et al., 2003).
Production of hydrogen peroxide in thylakoid membrane

Spontaneous dismutation of $\text{O}_2^{\cdot-}$ in the thylakoid membrane should be very low owing to a strong electrostatic repulsion in the membrane interior with low permittivity. However it has been found that $\text{H}_2\text{O}_2$ is produced within the membrane with significant rate and the production increases with an increase of light intensity (Mubarakshina et al., 2006). On the basis of the data presented in (Ivanov et al., 2007; Khorobrykh et al., 2004; Mubarakshina et al., 2006) it was proposed that $\text{H}_2\text{O}_2$ within thylakoid membrane is produced due to the reduction of $\text{O}_2^{\cdot-}$ by plastoquinol (Reaction 6) (for review see Mubarakshina & Ivanov, 2010).

$$\text{PQH}_2 + \text{O}_2^{\cdot-} \rightarrow \text{PQ}^{\cdot-} + \text{H}_2\text{O}_2$$ (6)

$\text{H}_2\text{O}_2$ can also be produced at PSII donor and acceptor sides. At the acceptor side, $\text{H}_2\text{O}_2$ can be formed outside thylakoids by the dismutation of $\text{O}_2^{\cdot-}$ produced within membrane (Arato et al., 2004; Khorobrykh et al., 2002; Klimov et al., 1993) or inside the membrane by the interaction of $\text{O}_2^{\cdot-}$ with non-heme iron of PSII (Pospíšil et al., 2004). At the donor side $\text{H}_2\text{O}_2$ can be formed as an intermediate during water oxidizing cycle operation if this cycle is seriously disrupted (Ananyev et al., 1992; Hillier & Wydrzynski, 1993). Thus $\text{H}_2\text{O}_2$ production at PSII donor and acceptor sides should be largely neglected under normal conditions.

Production of hydroxyl radical in thylakoid membrane

The various treatments of isolated PSII particles can lead to hydroxyl radical generation (Arato et al., 2004; Pospíšil et al., 2004). Production of hydroxyl radical by PSII is limited under normal functional conditions unlike under the strong stress conditions. It was suggested that in PSI the reduced $\text{F}_A$ and $\text{F}_B$ can catalyze the Fenton's reaction and form $\text{OH}^\cdot$ (Snyrychova et al., 2006). The presence of effective electron acceptors from PSI such as methylviologen (Snyrychova et al., 2006) and probably Fd and NADP$^+$, results in a decrease of $\text{OH}^\cdot$ generation. So in vivo the production of $\text{OH}^\cdot$ by PSI would be minor.

Production of organic peroxides (ROOH) in thylakoid membrane

It was shown that oxygen uptake in the PSII particles at pH above 8 and after the Tris treatment was not the result of oxygen reduction to $\text{O}_2^{\cdot-}$ only (Khorobrykh et al., 2002). These conditions can lead to destruction of the water-oxidizing complex and it was proposed that this can result in the formation of long-lived P680$^+$, which can oxidize the close lipids. These lipids can react with oxygen producing the lipid peroxides and thus increasing the oxygen uptake. Using the fluorescent probe Spy-HP it has been recently shown that organic peroxides (ROOH) are produced in PSII membranes when the function of the water-oxidizing complex is disrupted (Khorobrykh et al., accepted).

2.3 Negative effects of ROS in chloroplasts. ROS scavenging systems as the part of chloroplast metabolism

2.3.1 Destructive action of ROS in chloroplasts

The destructive action of ROS in chloroplasts as well as in other parts of the cell is targeted on proteins, nucleic acids and lipids, which can lose their specific functions even due to small changes in their structure after interaction with ROS. Chloroplasts contain own
genome represented by the DNA with 110-120 genes, accompanied by own system of the protein biosynthesis, including RNA and ribosomes (Cui et al., 2006). It is interesting that in every chloroplast there are a few tens of genome copies, and this may be an adaptation to the existence under conditions of continuous ROS production by PETC. OH• is considered as the main ROS injuring DNA. It preferably attacks the thimines and cytosines, and in a less extent, adenines, guanines, and the rest of deoxyribose (Cadet et al., 1999). O2•− has weaker effects on the DNA, and attacks preferably guanines. Since chloroplast genome contains the genes coding some components of PETC, the breakdown of the operation of such genes can affect the normal electron transfer, and the modified PETC in its turn can increase the production of O2•−. In stroma, a toxic O2•− action is aimed mostly at heme-containing enzymes, such as peroxidases (Asada, 1994). In the thylakoid membrane, perhydroxyl radical, can initiate lipid peroxidation that leads to disturbing the membrane structure and its functions, such as barrier, transport, maintenance of the membrane proteins, and so on.

The damaging effect of H2O2 on the genome is determined by the production of OH• in the vicinity of DNA. More specific effect of H2O2 in chloroplasts is the inhibition of photosynthesis. It was found that H2O2 inhibits the photosynthesis in intact chloroplasts with a half-inhibition at 10 µM (Kaiser, 1976). Electron transfer through PETC is rather resistant to H2O2, and the photosynthesis inhibition in the presence of H2O2 occurs due to oxidation of thiol groups of enzymes involved in carbon fixation cycle (Charles & Halliwell, 1980; Kaiser, 1979). It can be calculated that in chloroplasts 10 µM H2O2 can arise during less than for 1 min under usual photosynthesis rates even if only 1% of electrons are transferred to O2. The survival of the chloroplast is provided by the protective (antioxidant) system, which is very active in chloroplasts (see further).

1O2 being produced in PSII interacts mainly with D1 protein of the core complex of PSII reaction center (Aro et al., 1993; Trebst et al., 2002). This process possibly explains the very high rate of the replacement of D1 by newly synthesized proteins at high light intensity. It may be noted that the PSII activity can also be destroyed not only by 1O2 produced in PSII but also by ROS produced in PSI (Krieger-Liszkay et al., 2011; Tjus et al., 2001).

2.3.2 Mechanisms and components of ROS scavenging reactions in stroma and in the thylakoid membrane

Chloroplasts of the leaf cells are the building sites of the plant. Since potentially harmful ROS are continuously produced in chloroplasts in the light, these organelles are supplied with an efficient system of ROS scavenging. This system may be divided into stromal and membrane parts, however, these parts are connected by common metabolites and operate jointly to maintain chloroplast function. Averaged O2 concentration in chloroplasts under illumination does not estimably differ from the one in the dark owing to a fast equilibration of new O2 molecules in the water phase (Ligeza et al., 1998). The quasi-stationary O2 concentration in the thylakoid membrane in the light can be higher than in other compartments of a chloroplast, due to the production of O2 molecules in water-oxidizing complex. Futhemore the O2 concentration in hydrophobic media is approximately ten times higher than in water. Taking into account the primary generation of ROS by the membrane components, the thylakoid membrane requires particularly strong protection.
2.3.2.1 Stromal defense system

Superoxide dismutase. SODs are the water-soluble proteins. The main chloroplast isoform of SOD in all plants is CuZn-SOD, and some plants also contain Fe-SOD in stroma (Kurepa et al., 1997). Immunogold labeling of the chloroplastic CuZn-SOD revealed that the enzyme is mostly concentrated, almost 70% of its total amount, in 5-nm layer in the vicinity of the thylakoid membrane surface (Ogawa et al., 1995). Authors stated its local concentration in this layer as about 1 mM. Thus SOD prevents the incoming of $O_2^{•-}$ from the membrane to stroma. SOD scavenges $O_2^{•-}$ also in the bulk of stroma, where $O_2^{•-}$ can emerge due to oxidation of Fd$^{\text{red}}$ or some other enzymes by oxygen.

Ascorbate and ascorbate peroxidase. The concentration of ascorbate in chloroplasts is very high, achieving 10–50 mM (for review see Smirnoff, 2000), and even about 300 mM in alpine plants (Streb et al., 1997). Ascorbate can act as an effective quencher of $O_2^{•-}$ with a high rate constant. Moreover ascorbate is involved in regeneration of the α-tocopherol radicals formed during detoxification of lipid peroxide radicals. The scavenging of $H_2O_2$ in chloroplasts is performed by ascorbate peroxidase (APX), which catalyzes the reaction of $H_2O_2$ with ascorbate. Catalase was not found in chloroplasts, although the low catalase activity of thylakoids and some stromal components is not ruled out. Having more low value of $K_m(H_2O_2)$ as compared with catalase, 80 μM vs. 25 mM, APX can provide more low $H_2O_2$ concentration; and this is important, taking into account the inhibitory effect of $H_2O_2$ on the Calvin cycle enzymes (see 2.3.1). The reaction, which is catalyzed by APX has a high rate constant, $10^7 M^{-1}s^{-1}$. Chloroplasts contain APX in two isoforms, thylakoid-bound and soluble stromal ones (Miyake & Asada, 1992). Both APXs are highly specific to ascorbate as the electron donor, and they are promptly inactivated, during 10 s, in its absence (Nakano & Asada, 1987). These peroxidases form two defending lines to protect stromal components from $H_2O_2$.

Glutathione and glutathione peroxidase. The reduced form of glutathione (GSH) plays an important role in the stabilization of many stromal enzymes. For the antioxidant function it is important that it serves as a substrate for dehydroascorbate reductase. GSH is able to react directly with ROS including $H_2O_2$ (Dalton et al., 1986), hydroxyl radical (Smirnoff & Cumbes, 1989) and even $^{1}O_2$ (Devasagayam et al., 1991). Chloroplasts also contain phospholipid hydroperoxide-scavenging glutathione peroxidase (Eshdat et al., 1997) that may be involved in the reduction of lipid peroxide of thylakoid membranes to its alcohol, suppressing the chain oxidation of thylakoid phospholipids. This glutathione peroxidase may be considered as the part of the membrane defense system.

Osmolytes. Osmolytes are the group of metabolites that decrease water potential inside the cell and prevent intracellular water loss. This group includes soluble sugars, glycine, betaine, proline etc. The antioxidant capacity of proline is the result of its ability to quench $^{1}O_2$ and scavenge OH$^•$ (Matysik et al., 2002). Recently it was shown that synthesis of proline occurs, at least partly, in chloroplasts (Székely et al., 2008) where proline can execute the antioxidant function and protect both the membranes against lipid peroxidation and the stromal enzymes against desactivation.

Some soluble sugars were recently recognized as antioxidants (for review see Bolouri-Moghaddam et al., 2010). Addition of mannitol to thylakoid suspension resulted in decrease of OH$^•$ production, and the transgenic tobacco plants with enhanced mannitol production
targeted to the chloroplast had the increased OH• scavenging capacity (Shen et al., 1997). The mutants of Arabidopsis with overexpressed enzymes providing the elevated concentration of galactinol and raffinose in leaves were more resistant to oxidative stress caused by methylviologen treatment than wild-type plants (Nishizawa et al., 2008). The authors concluded that antioxidant capacity of these sugars could be explained by their reaction with OH• (the rate constants were measured as 7.8×10^9 M⁻¹s⁻¹ and 8.4×10^9 M⁻¹s⁻¹ for galactinol and raffinose, respectively).

**Peroxiredoxins.** Peroxiredoxins (PRXs) are identified as antioxidant enzymes for detoxification of H₂O₂ (for review see Dietz et al., 2006). Furthermore it was found that PRXs can also detoxify alkyl hydroperoxides and peroxinitrite, and probably can modulate oxilipid-dependent and NO-related signalling (Baier and Dietz, 2005; Rhee et al., 2005; Sakamoto et al., 2003). PRXs are 17-22 kDa enzymes that possess N-terminal cysteine residue(s) responsible for peroxidase activity. Four PRXs that are targeted to chloroplasts were identified in Arabidopsis: 2-cysteine (2-Cys) PRXs dimeric and oligomeric forms, PRX Q and PRX II E (Dietz et al., 2006). 2-Cys PRXs and PRX Q are associated with thylakoid membrane components while PRX II E has been identified as stromal enzyme. PRXs become oxidized after reaction with H₂O₂. Re-activation of oxidized PRXs in chloroplasts occurs via action of thioredoxin and thioredoxin-like proteins (Broin et al., 2002).

**Flavonoids.** Flavonoids were found to perform an antioxidant function in tissues exposed to a wide range of environmental stressors (Babu et al., 2003; Reuber et al., 1996). It has been recently assumed that antioxidant activity of flavonoids outperforms that of well-known antioxidants, such as ascorbate and α-tocopherol (Hernández et al., 2008). Flavonoids effectively scavenge the free radicals (for review see Rice-Evans et al., 1996). This can occur due to their ability to quench unpaired electrons of radicals, e.g. O₂•− (Sichel et al., 1991). It was also shown that flavonoids situated in chloroplasts can scavenge ¹⁰O₂ (Agati et al., 2007). Flavonoids include the substances with different lipophilicity, and thus perform their antioxidant functions in stroma as well as in the membrane.

### 2.3.2.2 Membrane defense system

**Vitamin E.** Vitamin E is the class of lipophilic compounds (α-, β-, γ- and δ-tocopherols (Tocs); α-, β-, γ- and δ- tocotrienols and their derivatives). Vitamin E is synthesized in the plastid envelope and is stored in plastoglobuli (for review see Lichtenthaler, 2007). The greatest amount of Tocs is found in the membranes of chloroplasts (including thylakoid membranes) where they execute the antioxidant function.

Vitamin E can react with almost all ROS. It can reduce O₂•− with the rate constant of 10⁶ M⁻¹s⁻¹ (Polle & Rennenberg, 1994). It was shown that vitamin E has scavenging activity against OH• (Wang & Jiao, 2000) and can decompose H₂O₂ (Srivastava et al., 1983). α-Toc protects PSII from oxidative damage. Trebst et al. (2002) showed that inhibition of Toc biosynthesis in Chlamydomonas resulted in a stimulation of light-induced loss of PSII activity and D1 protein degradation. This implies that Toc can come close to the site of ¹⁰O₂ generation in the reaction center of PSII. The rate constants for ¹⁰O₂ quenching by different Tocs are appr. 0.13 - 3.13×10⁸ M⁻¹ s⁻¹ in organic solvents (Gruszka et al., 2008), so Tocs can be the effective scavengers of ¹⁰O₂ within the membrane. It is also possible that α-Tocs can protect the β-carotene molecules in PSII, thereby preventing the PSII damage (Havaux et al., 2005). Tocs can reduce fatty acyl peroxy radicals, thus terminating lipid peroxidation chain
reactions (Polle & Rennenberg, 1994). The regeneration of Tocs occurs with involving of water-soluble antioxidants. For example, formation of α-Toc from α-Toc quinone has been reported to take place in vitro in the presence of ascorbate (Gruszka et al., 2008).

**Carotenoids.** There are two major types of carotenoids: the hydrocarbon class, or carotenes, and the oxygenated (alcoholic) class, or xanthophylls. Carotenoids can efficiently quench the dangerous triplet state of chlorophylls that is the origin of the \(^1\)O\(_2\) (Cogdell et al., 2000). This mostly occurs in the antenna system (Mozzo et al., 2008) but not in the reaction center. It is also known, that carotenoids, namely β-carotene, can quench \(^1\)O\(_2\) directly (Foote and Denny, 1968). It was shown that a lack of such carotenoids as zeaxanthin and lutein leads to \(^1\)O\(_2\) accumulation in thylakoids (Alboresi et al., 2011).

**Plastoquinone.** Plastoquinone (PQ-9), which as the chemical substance is the isoprenoid prenyllipid, is present in thylakoid membranes, chloroplast envelope and osmiophilic plastoglobuli of the stroma (Lichtenthaler, 2007). Plastoglobuli represent the storage compartments for plastoquinone, mainly in its reduced state. In the thylakoid membrane, PQ-pool maintained in the reduced state can execute antioxidant function, preventing membrane lipid peroxidation and pigment bleaching (Hundal et al., 1995). Furthermore it was shown that in vitro plastoquinol has an antioxidant activity similar or even higher than that of tocopherols (Kruk et al., 1994, 1997). It was also found that the added quinones can quench the excited states of chlorophyll molecules (Rajagopal et al., 2003), thus inhibiting the \(^1\)O\(_2\) generation. Moreover plastoquinone can also directly scavenge \(^1\)O\(_2\) that is produced by the reaction center triplet chlorophyll of PSII (Kruk & Trebst, 2008; Yadav et al., 2010). It is very possible that plastoquinol effectively scavenges O\(_2\)•• and perhydroxyls in thylakoid membrane (Reaction 6) (for review see Mubarakshina & Ivanov, 2010). These reactions are the mechanisms by which the PQ-pool can prevent membrane lipid peroxidation. It is known that even in the dark the PQ-pool can be in the reduced state owing to operation of the Ndh complex. This can provide the protective function of the PQ-pool in the membrane if ROS are produced under stress in the dark. It was found that the extent of the PQ-pool reduction in the dark increased upon heat stress, and this was considered as involvement of the Ndh complex in the defense system (Sazanov et al., 1998). Thus it is possible to assume that the higher amount of plastoquinone than of other components of PETC is needed in order to execute the antioxidant function rather than the electron carrier function.

### 2.4 Role of reaction of oxygen with chloroplast components in the constructive metabolism

**2.4.1 Photorespiration**

Photorespiration is a pathway of oxidative carbon metabolism which resulted from oxygenase activity of ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) (for review see Maurino & Peterhansel, 2010). Photorespiration cycle starts in chloroplasts from the reaction of ribulose-1,5-bisphosphate with O\(_2\) molecule. As the result 3-phosphoglycerate and 2-phosphoglycolate are produced. The latter is dephosphorylated to glycolate, a toxic molecule. The following reactions of glycolate metabolism lead to a recovery of 3-phosphoglycerate and occur in peroxisomes, mitochondria, cytosol and, finally, in chloroplasts again. At current atmospheric levels of CO\(_2\) and O\(_2\), photorespiration in C\(_3\)-plants dissipates 25 % of the carbon fixed during CO\(_2\) assimilation (Sharkey, 1988).
Photorespiration is required for all photosynthetic organisms, including cyanobacteria (Eisenhut et al., 2008) and even higher plants with C₄-type of photosynthesis (Zelitch et al., 2008). As a part of chloroplast metabolism, photorespiration can be considered as one of the sinks for the excess of the energetic equivalents such as ATP and NAD(P)H under different stresses (for review see Wingler et al., 2000). Photorespiration operates also as a safety valve protecting PETC from over-reduction, and thus preventing the generation of ROS by components of PETC. Furthermore it can prevent the ROS formation in stroma by using NAD(P)H itself, since NAD(P)H can to some extent spontaneously reduce oxygen to $\text{O}_2^{•−}$ giving H₂O₂.

### 2.4.2 The electron transport to oxygen in PETC and ATP production for chloroplast metabolism

Oxygen reduction in PETC was mainly considered as an important part of the chloroplasts metabolism due to possibility of additional ATP synthesis coupled with this process (Badger, 1985; Heber, 1973). There were calculations of ATP synthesis coupled with O₂ reduction from the total ATP synthesis during simultaneous reductions of both NADP⁺ and O₂ (Furbank & Badger, 1983; Ivanov et al., 1980; Robinson & Gibbs, 1982). Observed in some studies lower efficiency for ATP production of the pseudocyclic electron transport as compare with the efficiency of the non-cyclic electron transport in C₃- (Woo, 1983) as well as in C₄-plants (Ivanov & Edwards, 2000) can be probably explained now as a result of the use of the protons bound by PQH₂ not only for the $\Delta$pH formation across the thylakoid membrane but also for the reduction of O₂•− to H₂O₂ in the membrane (see 2.2.2).

The Mehler reaction is now considered as a part of so called water-water cycle (WWC). The last term was proposed by Asada, who described the reactions involved in WWC in the fullest detail (Asada, 1999). Briefly, WWC includes the transport of the electrons from water to oxygen, the O₂•− dismutation catalyzed by SOD, the reduction of H₂O₂ by ascorbate with involvement of APX (see 2.3.2.1), followed by the reduction of monodehydroascorbate (MDHA) appearing in the latter reaction to ascorbate by the electrons from PETC (i.e. ultimately from water). The reduction of O₂ in PETC is the slowest reaction in WWC, while other reactions proceed with almost diffusion-controlled rates. The latter ensures the minimal accumulation of O₂•− and H₂O₂, preventing their interaction with the target molecules in stroma. The equal amounts of electrons reduce O₂ and MDHA in WWC. Thus WWC constitutes in the total photosynthetic electron flow the double of what is the electron transport to O₂ itself. The reduction of MDHA can deprive the Calvin cycle of electrons, i.e. can cease the photosynthesis. The photosynthesis of the intact chloroplasts was completely suppressed at H₂O₂ addition in the light and the CO₂ fixation restarted after the H₂O₂ was exhausted (Nakano & Asada, 1980). This result demonstrated that the system of H₂O₂ scavenging has priority over the system of CO₂ fixation in receiving electrons from PETC; this conclusion was supported in (Backhausen et al., 2000).

It was shown that WWC does play the important role in chloroplasts, and the breakdown of its normal operation negatively influences on the metabolism of these organelles (Rizhsky et al., 2003). This may mainly result from the destructive effects of ROS, but possibly also from the decrease of ATP/NADPH ratio. It is accepted now, that the electron flow to NADP⁺ provides, the ATP/NADPH of 1.5 owing to Q-cycle operation (Ivanov, 1993). Exactly such ratio is required in order the Calvin cycle reactions to proceed, but ATP is necessary in
chloroplasts not only for the cycle, but also for biosynthesis of protein and numerous transport processes. The cyclic electron transport around PSI, which can be essential producer of ATP also cannot operate under anaerobic conditions when PETC is overreduced, and the necessary redox poising for this transport is provided by the electron transfer to O$_2$ (Ziem-Hanck & Heber, 1980).

2.4.3 Role of electron transfer to oxygen in the protection of PETC from photoinhibition

The electron flows involved in WWC besides the ATP production play an important role in the protection of photosynthetic apparatus from photoinhibition under illumination. The excess of photon energy beyond the necessary one to fulfil CO$_2$ assimilation can arise not only in strong light, but also in moderate and even low light when the environmental conditions (the improper temperature, insufficient water supply, high salt concentration, the presence of pollutants, and so on) lead to suppression of the capacity of the photosynthetic apparatus for CO$_2$ assimilation.

WWC and cyclic electron transport are primarily coupled with the proton pumping into the thylakoid lumen, and these protons in the lumen initiate conformational and biochemical changes, which accelerate thermal energy dissipation in antenna and reaction centers (Horton et al., 1996). The pigment apparatus state providing the dissipation of the photon energy into a heat originates due to violaxanthin de-epoxidation to antheraxanthin and zeaxanthin. This reaction, which requires ascorbate, is catalyzed by violaxanthin deepoxidase (VDE) situated at the lumen side of thylakoid membrane; VDE is activated by a decrease of lumen pH to 5-6 (for review see Demmig-Adams, 1990). Since WWC does not consume ATP, the accumulation of protons in lumen is very fast in the absence of inorganic carbon fixation in C$_3$- and in C$_4$-plants (Ivanov et al., 1998; Ivanov & Edwards, 2000). This allows WWC to respond to such changes in light intensity as sunflecks.

The overreduction of the acceptor side of PSII is one of the pre-conditions of photoinhibition initiation, and the electron withdrawal from PETC to O$_2$ and MDHA within WWC also can effectively use the excess of light energy for the electron transfer. This is especially important under limiting supply of CO$_2$. It is known that photoinhibition of PSII owing to overreduction occurs under anaerobic conditions even at rather low light intensity (Park et al., 1996) and is prevented even by low dioxygen concentrations when WWC begins to function. The drain of electrons directly from the PQ-pool to oxygen (see 2.2.2) can be a very important mechanism in protection of PETC from photoinhibiton since the PQ-pool operation is known to be a limiting step of photosynthesis.

2.5 Signalling pathways under stress conditions.

The system of signal transfer in plants during stress conditions is a very complicated regulatory mechanism (for recent reviews see Foyer & Noctor, 2009; Li et al., 2009; Mullineaux, 2009; Suzuki et al., 2011). The biosynthesis of chloroplast proteins are coordinated by both chloroplast and nuclear genomes. Thus the tight cooperation between these two systems is obligatory for the assembly of functionally active chloroplasts. This can only happen due to well-co-ordinatated work of regulatory signals coming from nucleus to chloroplasts and from chloroplasts to nucleus. The latter is called the retrograde signalling (Beck, 2005; Chan et al., 2010; Mullineaux & Karpinski, 2002).
2.5.1 ROS as important signalling agents

Enhanced ROS production under stress conditions is considered to be a signal in order to regulate the cell redox homeostasis. ROS play a key role in the regulation of plant development, programmed cell death and also in biotic and abiotic stress responses (Apel & Hirt, 2004; Desikan et al., 2001; Mittler et al., 2004). Many of the $^{1}\text{O}_2$-responsive genes are different from those activated by $\text{O}_2^\cdot \cdot$ or $\text{H}_2\text{O}_2$, assuming that the signalling by different ROS occurs via distinct pathways (Laloi et al., 2006; op den Camp et al., 2003) however some $^{1}\text{O}_2$-responsive nuclear genes can be activated by other ROS (Anthony et al., 2005; Gadjev et al., 2006). Moreover it has been recently shown that $\text{H}_2\text{O}_2$ may also either directly or indirectly antagonize $^{1}\text{O}_2$-mediated signalling (Baruah et al., 2009; Ledford et al., 2007).

Production of $^{1}\text{O}_2$ in Arabidopsis thaliana under stress conditions was shown using a specific fluorescent dye (Hideg et al., 2001; op den Camp et al., 2003). Generation of $^{1}\text{O}_2$ leads to a rapid change in nuclear gene expression that reveals the transfer of $^{1}\text{O}_2$-derived signals from the plastid to the nucleus (Laloi et al. 2006; op den Camp et al. 2003). Using the flu mutant with disturbed chlorophyll biosynthesis it was shown that $^{1}\text{O}_2$ forms an integral part of a signalling network that is important not only for stress responses but also for the plant development (Baruah et al., 2009). It was proposed that $^{1}\text{O}_2$ accumulation in thylakoids represents a signalling pathway in the early stages of stress acclimation (Alboresi et al., 2011).

A role for $\text{O}_2^\cdot \cdot$ in retrograde signalling was suggested using gene expression arrays (Scarpeci et al., 2008) and mutations in chloroplastic CuZn-SOD (Rizhsky et al., 2003). The generation of $\text{O}_2^\cdot \cdot$ in the absence of $\text{H}_2\text{O}_2$ accumulation revealed a subset of nuclear encoded genes that are likely to be specific for an $\text{O}_2^\cdot \cdot$-mediated signalling pathway (Scarpeci et al., 2008). In CuZn-SOD mutants, the accumulation of $\text{O}_2^\cdot \cdot$ results in activation of chloroplast-encoded genes that is not stimulated by other ROS (Rizhsky et al., 2003).

$\text{H}_2\text{O}_2$ has been recognized as the ROS causing the largest changes in the levels of gene expression in plants in retrograde signalling (Bechtold et al., 2008; Dat et al., 2000; Fahnenstich et al., 2008; Foyer and Noctor, 2009; Li et al., 2009). $\text{H}_2\text{O}_2$ possibly induces protein phosphorylation by mitogen-activated protein kinases (MAPKs) (Desikan et al., 1999) which are involved in signalling pathways regulating gene expression (Grant et al., 2000). The reversible redox modulation of Cys residues of the proteins is perhaps the most obvious mechanism for the $\text{H}_2\text{O}_2$-mediated activation of MAPK pathways. Transcriptomic analyses of Arabidopsis plants have revealed hundreds of $\text{H}_2\text{O}_2$-responsive genes (Ding et al., 2010; Yun et al., 2010). It was shown that $\text{H}_2\text{O}_2$ produced inside the chloroplasts can leave the chloroplasts thus escaping the effective antioxidant systems located inside the chloroplast (Mubarakshina et al., 2010). It is possible that the appearance of $\text{H}_2\text{O}_2$ in cytoplasm can be important for the executing of retrograde signalling mediated by $\text{H}_2\text{O}_2$.

2.5.2 Other signalling agents and crosstalk between different signalling pathways and ROS

Antioxidants can also be the sensors of the stress conditions by the regulation of the level of ROS. It was proposed that the ascorbate content could influence the expression of antioxidative genes in Arabidopsis (Noctor et al., 2000), and APXs located in chloroplasts are
crucial for photoprotection and signalling (Danna et al., 2003; Kangasjärvi et al., 2008). It was shown that the level of Tocs is also elevated in response to a variety of abiotic stresses, including high-intensity light, drought, toxic metals, and high and low temperatures (Maeda & DellaPenna, 2007). Furthermore it was suggested that α-Toc may affect intracellular signalling in plant cells by interacting with key components of the signalling cascade (Munné-Bosch et al., 2007).

Thioredoxin, glutathione, peroxiredoxins and glutaredoxins, which contain sulfhydryl groups (-SH), represent a part of the reduction–oxidation signalling network (for more detailed review see Scheibe & Dietz, 2011, see also Coupe et al., 2006; Fey et al., 2005; Geigenberger et al., 2005; Lindahl & Kieselbach, 2009). The oxidation of (-SH) groups can occur not only by the interaction between the components having -SH groups in their structure but also with involvement of H$_2$O$_2$ since H$_2$O$_2$ can directly oxidizes -SH groups (Quesada et al., 1996). In plants two chloroplastic thioredoxins, named thioredoxin $f$ and thioredoxin $m$, were originally identified as light dependent regulators of several carbon metabolism enzymes including Calvin cycle enzymes (Lemaire et al., 2007). It was found that glutathione is involved in the control of gene expression (Dron et al., 1988; Wingate et al., 1988). H$_2$O$_2$ can be sensed by glutathione peroxidase 3 of Arabidopsis, which modulates activities of phosphatases, protein kinases transcription factors and ion channels involved in abscisic acid signalling pathways (Wang & Song, 2008).

The PQ-pool can also be considered as both the component of PETC, the redox state of which is an important factor for the redox signalling and the antioxidant, the level of which increases under stress conditions. The treatment of plants by the pathogen-derived elicitor resulted in elevated ROS production, lipid peroxidation and lipoxygenase followed by a significant increase in total plastoquinone level (Maciejewska et al., 2002). High light conditions also lead to a massive accumulation of plastoquinone, preferentially plastoquinol in leaves (Lichtenthaler, 2007; Szymańska & Kruk, 2010). The changing of the antenna size is one of the main mechanisms of plants acclimate to changes in environmental light conditions. It was revealed that the redox state of the PQ-pool regulates the antenna size of PSII under different light conditions (Escoubas et al., 1995; Fey et al., 2005; Lindahl et al., 1995; Pfannschmidt et al., 1999; Yang et al., 2001). In the works (Frigerio et al., 2007; Morosinotto et al., 2006) it was found that the regulation of the antenna size is carried out by the changing of the antenna proteins quantity at the post-transcriptional level. However the signal from the PQ-pool for the light acclimation has still remained largely unsolved. It was shown that H$_2$O$_2$ production in the PQ-pool increases with an increase of light intensity (Khorobrykh et al., 2004; Mubarakshina et al., 2006) that is correlated with an increase of the redox state of the PQ-pool. Possibly, H$_2$O$_2$ can be the best candidate in order to be a signal of the PQ-pool over reduction.

Phytohormones are also the components of cell signalling. The first steps of synthesis of such stress hormones as abscisic, salicylic and jasmonic asids occur in plastids (Vernooij et al., 1994; Wasilewska et al., 2008; Wasternack, 2007) and their influences on the photosynthesis have been demonstrated (Filella et al., 2006; Mateo et al., 2006). However the involvement of phytohormones in chloroplast-to-nucleus signalling is still under debate.

Another component that was shown to be important for the regulation of nuclear genes by chloroplasts is Mg-protoporphyrin (Mg-Proto), the precursor of chlorophyll (Gray, 2003). It was suggested that after illumination, Mg-protoporphyrins can move from chloroplast to
cytoplasm where they can interact with a signal conductor (von Gromoff et al., 2006). Another possibility is that Mg-protoporphyrins can change the activity of regulatory proteins directly in chloroplasts (for review see Yurina & Odintsova, 2007). There are no clear evidences about the crosstalk between Mg-protoporphyrin-derived signalling and ROS. It was found that it is unlikely that the accumulation of Mg-Proto is linked to the production of $^{1}O_2$ (Mullineaux, 2009). However it was suggested that light is needed to promote the export of Mg-Proto to the cytosol from the chloroplast (Kropat et al., 1997). Probably this export may involve the co-operation with ROS.

Photosynthesis-derived metabolites can also act as a signal. One of these metabolites is sugar. The regulatory effect of sugars on the expression of nuclear photosynthetic genes and on plant metabolism was established (for review see Gupta & Kaur, 2005). Both glucose and sucrose were shown to be a signal molecule, and other sugars produced in a number of plant metabolic pathways are also potential signalling agents. The most regulatory effects can be ascribed to glucose (Ramon et al., 2009). Hexokinase, the first enzyme in glucose catabolism can reduce the intracellular ROS levels (Sun et al., 2008). It was shown that mitochondrial hexokinases play a key role as a regulator of ROS levels (Camacho-Pereira et al., 2009). In the work (Bolouri-Moghaddam et al., 2010) it was postulated that chloroplastic hexokinases also can carry out this function. It was shown that the plant with higher levels of hexokinase was more resistant to oxidative stress induced by methylviologen (Sarowar et al., 2008).

3. Conclusion

The ways of ROS production and ROS scavenging in chloroplasts are very important for the metabolisms of cell and whole organism. These ways can differ under normal functional conditions and under stress conditions as shown in Figure 1.

As stated here, ROS production can be divided into the stromal part and the membrane part. Probably, the ways of ROS production in different parts of chloroplasts initiate different signalling pathways. The signalling network in chloroplast stroma is widely studied, and many of its characteristics become clear. We also pay the attention to the signalling pathways that may begin in the thylakoid membrane. H$_2$O$_2$ that is produced in the thylakoid membrane by the reaction of superoxide with plastoquinol can diffuse out of the thylakoid membrane not only to stroma but also to lumen, thus avoiding the antioxidant systems of chloroplast stroma. Some of signal sensors such as thylakoid protein kinases are associated with the thylakoid membrane and the signalling pathway initiated by such kinases may involve H$_2$O$_2$ produced in the membrane.

The interesting fact is that during stress conditions even such components as osmolytes, flavonoids etc. that have their own functions, also are involved in ROS scavenging. Since the control of ROS level is intrinsically involved in executing their signal roles, the cooperation of the whole system of chloroplast metabolites obviously provides sustaining the chloroplast function under stress conditions.

Although the role of ROS in signalling is commonly accepted now and a lot of studies are focused on the changes in both ROS production under stress conditions and the abundance of the antioxidant and other stress enzymes, the molecular mechanisms of the signalling pathways remain unclear. For example, it is not totally clear, how ROS fulfill signalling
functions, being inside chloroplasts and how after leaving for cytoplasm. ROS produced inside chloroplast can be the signal for the inside-organelle signalling to regulate the chloroplast genes. If ROS leave the chloroplast and appear in cytoplasm, it can affect the integrated signalling network of the whole cell.

Fig. 1. The ways of ROS production and ROS scavenging in chloroplasts under normal functional conditions (A) and stress conditions (B). The scheme represents the putative ways of ROS production and scavenging that play the most important role in oxygen metabolism. APX, ascorbate peroxidase; Asc, ascorbate; Car, carotenoids; cyt b$_{6}$/f, cytochrome b$_{6}$/f complex; Fd, ferredoxin; GSH, reduced glutathione; PC, plastocyanin; PQ, plastoquinone; PQ•$, plastosemiquinone; PQH$_{2}$, plastoquinol; PRXs, peroxiredoxins; PSI and PSII, photosystem I and II, respectively; SOD, superoxide dismutase; Toc, tocopherols. For details see text.
4. References


Oxygen Metabolism in Chloroplast


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A global research community of scientists is teasing out the biochemical mechanisms that regulate normal cellular physiology in a variety of organisms. Much of current research aims to understand the network of molecular reactions that regulate cellular homeostasis, and to learn what allows cells to sense stress and activate appropriate biochemical responses. Advanced molecular tools and state-of-the-art imaging techniques discussed in this book continue to provide novel insights into how environmental changes impact organisms, as well as to develop therapeutic interventions for correcting aberrant pathways in human disease.

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