Chapter 2
Plant Cell Redox Homeostasis and Reactive Oxygen Species

A. Trchounian, M. Petrosyan and N. Sahakyan

Abstract Plant cell redox homeostasis is formed as a result of the balance between the accumulation of reactive oxygen species (ROS), the functioning of the antioxidant enzymes system and antioxidants with low molecular weight. Complex of different changes occurs in plants under stress conditions which often lead to a variety of the intracellular and tissue functional disorders. Under these conditions for the survival, the functioning of the systems of homeostasis maintaining is extremely important. Understanding of the molecular mechanisms of resistance formation to adverse environmental factors is one of the most urgent issues that will help to cope with the problem of increasing plant resistance to stressors. Maintenance of cellular homeostasis in plants under the influence of various external factors is provided by a number of protective systems. Organization of metabolic pathways in plants characterized by having two main separate compartments, generating ATP and reducing equivalents: chloroplasts and mitochondria. The interaction of these two cell energetic organelles with opposite types of functions in plant involves in metabolite fluxes organizing, which is an integral controlled system specific only to the plant organism. Normally, ROS are generated by metabolic activity of the plants and act as signaling molecules for activating plant metabolic pathway. However, under environmental stresses, generation of ROS increases in different compartments of the cell such as chloroplast, peroxisomes and mitochondria. Higher accumulation of ROS leads to oxidative stress in plant causing damage to the cell membranes (lipid peroxidation) and biomolecules. To combat the harmful effect of increased ROS accumulation, plants are equipped with effective ROS-scavenging mechanisms. Plants have evolved two types of scavenging tools; enzymes (superoxide dismutase (SOD), catalase (CAT), monodehydroascorbate reductase (MDAR), dihydroascorbate reductase (DHAR), glutathione reductase (GR) and glutathione peroxidase (GP)) and antioxidant molecules like ascorbic acid, α—tocopherols, glutathione, prolin, flavonoids and

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D.K. Gupta et al. (eds.), *Redox State as a Central Regulator of Plant-Cell Stress Responses*, DOI 10.1007/978-3-319-44081-1_2

carotenoids. In implementation of these reactions, vacuoles as well as cell wall and plasma membrane also play an important role.

Keywords Redox homeostasis · Oxidative stress · Reactive oxygen species · Signaling molecules · Antioxidants · Plant cell

Abbreviations

ABA	Abscisic ascid
APX	Ascorbate peroxidase
AsA (AA)	Ascorbate
CAT	Catalase
DAR (DHAR)	Dehydroascorbate reductase
DHA	Dehydroascorbate
DPPH	1,1-Diphenyl-2-picrylhydrazyl radical
DTT	DL-dithiothreitol
ER	Endoplasmic reticulum
ETC	Electron transport chain
GP	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione
GST	Glutathione *S*-transferase
HR	Hypersensitive response
LHCs	Light-harvesting complexes
MDA	Monodehydroascorbate
MDAR (MDHAR)	Monodehydroascorbate reductase
NOX	NADPH oxidases
PX	Peroxidase
PS I, PS II	Photosystem I, photosystem II
Rboh	Respiratory burst oxidase homologs
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SOD	Superoxide dismutase
V	Violaxanthin
XOD	Xanthine oxidase
Z	Zeaxanthin

2.1 The Concept of Redox Homeostasis in Plants

It is a well-known fact that plants produce oxygen (O_2) during photosynthesis; however, they require O_2 for mitochondrial energy production. In these highly complex metabolic processes, the hyperoxic environments of plant cells generate a

group of free radicals, reactive molecules and ions—reactive oxygen species (ROS). Although atmospheric oxygen is relatively non-reactive, it can give rise to ROS which include superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\cdot}OH$) and singlet oxygen ($^1O_2$). In order to keep redox homeostasis, plants have evolved complex ROS-scavenging system, in which networks of reactions are performed by different enzymes and metabolites (De Gara et al. 2010). The balance between energy generation and consumption in plants largely depends on a signaling network that coordinates three of the most central processes in plant's life: photosynthesis, respiration and photorespiration. These activities are linked in terms of electron transfer, substrates, reductants and energy (Suzuki et al. 2012). Plant electron transport cascades require the simultaneous presence of both oxidized and reduced forms of electron carriers. This involves a continuous flux of electrons to O_2 from multiple sites in the photosynthetic and respiratory electron transport chains. Further, the initial product of this flux is superoxide, from which other ROS like, H_2O_2, $^{\cdot}OH$ and $^1O_2$ are subsequently produced. ROS are generated by a number of different mechanisms and are formed in different cell compartments, such as apoplasts, mitochondria, peroxisomes, chloroplasts and endoplasmic reticulum (Moucheshi et al. 2014). $^1O_2$ is also formed during light capture by the reaction of excited chlorophyll in its triplet state with molecular oxygen (Roach and Kriger-Liszkay 2014). Different enzyme systems produce superoxide or H_2O_2

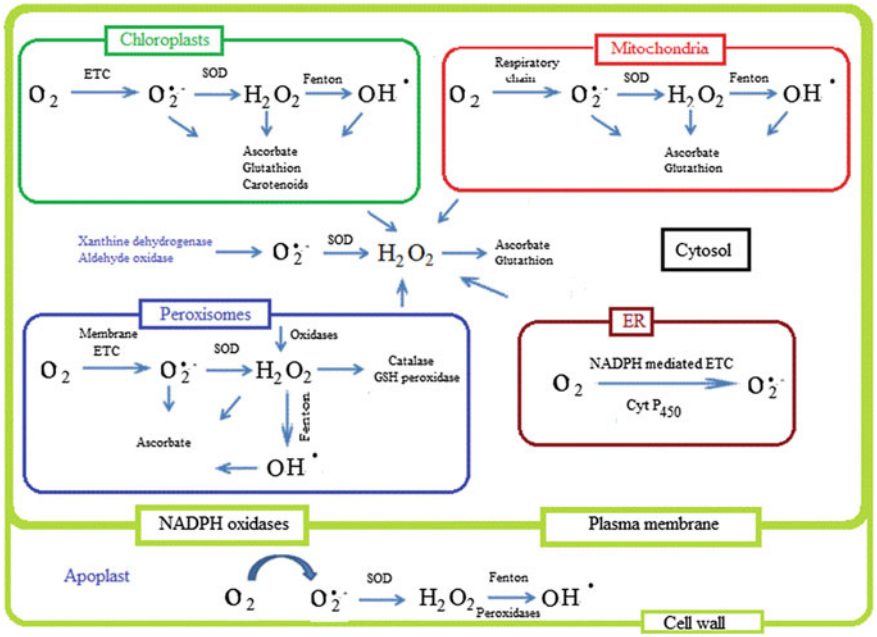


Fig. 2.1 Production and scavenging of ROS in a plant cell (superoxide dismutase (SOD); electron transport chain (ETC); Fenton (decomposition of hydrogen peroxide to highly reactive hydroxyl radical in the presence of iron

(Fig. 2.1; Jajic et al. 2015). The reactive nature of these intermediates means that their accumulation must be controlled. These ROS are also able to act as signaling molecules (Foyer 2005). In all types of cells, ROS are extremely reactive and can modify structure and function of proteins, acting on thiol groups and iron-containing clusters (Moucheshi et al. 2014; Vassilian and Trchounian 2009; Ray et al. 2012; Kreslavski et al. 2012). In addition, ROS could change the redox potential of redox-sensitive cell components (glutathione system, ascorbate system, plastoquinone pool, thioredoxin, etc.). In a consequence to the changing natural environments, different compounds are accumulated in plant cells and tissues, which include signaling intermediates (calcium ions, cAMP, nitrogen oxide), phytohormones (ethylene, abscisic acid (ABA), salicylic acid), osmolytes (amino acids, sugar alcohols, tertiary amines) and other metabolites (Kreslavski et al. 2012). In the normal conditions, the extent to which ROS are accumulated is determined by the antioxidative system, which enables organisms to maintain all cellular components in an active state for metabolism. The redox system is essential in maintaining cellular homeostasis. Under physiological conditions, cells maintain redox balance through generation and elimination of ROS/reactive nitrogen species (RNS) (Trachootham et al. 2008).

Plants maintain most cytoplasmic thiols in the reduced (–SH) form because of the low thiol–disulfide redox potential imposed by millimolar amounts of the thiol buffer, glutathione. Unlike many animal cells, plant cells synthesize high concentrations of ascorbic acid, which serve as an additional hydrophilic redox buffer that provides robust protection against oxidative challenge. Redox homeostasis is governed by the presence of large pools of these antioxidants that absorb and buffer reductants and oxidants. Plants also synthesize tocopherols (vitamin E) that act as lipo-soluble redox buffers. Tocopherol is considered to be an effective scavenger of other ROS, especially, $^1O_2$ scavenger. Because the tocopherol redox couple has a more positive midpoint potential than that of the ascorbate pool, it increases even further the range of effective superoxide scavenging. The ability of the ascorbate, glutathione and tocopherol pools to act as redox buffers in plant cells is one of their most important attributes.

Pathways of ROS signalling are made possible by homeostatic regulation due to comprehensive antioxidant redox buffering. Because antioxidants continuously process ROS, they determine the lifetime and the specificity of the ROS signal. Usually, plant cells have the ability to cope with high rates of generation of $O_2^{\cdot -}$, H_2O_2 and even $^1O_2$ (Foyer et al. 2005). However, under the influence of different biotic and/or abiotic stress factors (drought, salinity, chilling, metal toxicity, UV-B radiation as well as pathogens attack), the rate of ROS formation may exceed the reductive ability of cells due to disruption of cellular homeostasis (Sharma et al. 2012). Efficient scavenging of ROS produced during various environmental stresses requires the action of several non-enzymatic as well as enzymatic antioxidants present in the tissues.

2.2 Production of Reactive Oxygen Species

It has been stated that about 1 % of O_2 consumed by plants is directed to ROS formation in different subcellular units (chloroplasts, mitochondria, peroxisomes, etc.) Sharma et al. (2012). Apart from typical chloroplast, mitochondrial and peroxisome sources, ROS are also synthesized by NADPH oxidases (NOX) and peroxidases (Demidchik 2015). In plants, NOX homologs have been named respiratory burst oxidase homologs (Rboh) which involved in ROS production in response to pathogen invasion (Sagi and Fluhr 2006). Plant cells, like mammalian cells, can initiate and most likely amplify ROS production for the purpose of signaling. Plant peroxidases are the proteins which are induced during the host plant defense. They are also involved in broad range of physiological processes, such as lignin and suberin formations, cross-linking of cell wall components and synthesis of phytoalexins, and importantly participate in the metabolism of ROS, RNS nitric oxide ($NO^{\cdot}$) and peroxynitrite ($ONOO^{-}$). They both switching on the hypersensitive response (HR) of programmed cell death of the infected host cells associated with pathogen development (Almagro et al. 2009). The important detoxification mechanisms in plants are catalyzed by cytochrome P450 in cytoplasm and endoplasmic reticulum (ER). ROS are also generated at plasma membrane level or in outside of cell membranes, in apoplast in plants. pH-dependent cell wall peroxidases are activated by alkaline pH, which produces H_2O_2.

As already mentioned, ROS are well recognized for playing a dual role in plant metabolism—harmful and beneficial, depending on concentration and ROS species. At high concentrations, they cause damage to biomolecules, whereas at low or moderate concentrations they act as second messengers in intracellular signaling cascades that mediate several responses in plant cells.

2.2.1 Types of ROS

ROS are produced in both unstressed and stressed cells at several locations of different cell compartments (Das and Roychoudhury 2014; Jajic et al. 2015; Fig. 2.1). ROS are usually formed by the unavoidable leakage of electrons to O_2 from the electron transport activities of chloroplasts, mitochondria and plasma membranes or as a by-product of various metabolic pathways localized in different cellular compartments.

As mentioned above, the most common ROS are $^1O_2$, $O_2^{\cdot -}$, H_2O_2 and $OH^{\cdot}$. $O^{\cdot}$ itself is a totally harmless molecule (Sharma et al. 2012; Goraya and Asthir 2016), as in its ground state it has two unpaired electrons with parallel spin which makes it paramagnetic and, hence, unlikely to participate in reactions with organic molecules (Apel and Hirt 2004; Fig. 2.2), whereas most non-radical organic molecules are diamagnetic, with pairs of electrons with opposite spins.

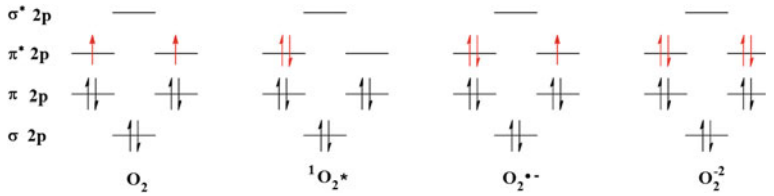


Fig. 2.2 Molecular orbital diagrams for ground-state molecular oxygen (O_2), singlet oxygen ($^1O_2$), and ROS (superoxide radical anion ($O_2^{\cdot-}$) and peroxide ion (O_2^{-2})

Triplet Oxygen	·O−O·
Singlet Oxygen	O−O:
Superoxide	·O−O:
Perhydroxyl Radical	·O−O: H
Hydrogen Peroxide	H: O−O: H
Hydroxyl Radical	H: O·
Hydroxyl Ion	H: O:
Water	H: O: H

Fig. 2.3 Order of oxygen reduction

A spin restriction applies for O_2 to participate in redox reactions with other atoms or molecules as it has to accept, from the reductant, a pair of electrons that have the same spin (i.e., non-diamagnetic) so they can fit into the vacant spaces in the π* orbitals of O_2. The latter is thus unable to efficiently oxidize biomolecules (as, for example, via addition, which is 2-electron process) (Krumova and Cosa 2016; Fig. 2.2). Its activation may occur by two different mechanisms: absorption of sufficient energy to reverse the spin on one of the unpaired electrons, or stepwise monovalent reduction. At first case $^1O_2$ if formed, in latter, O_2 is sequentially reduced to $O_2^{\cdot-}$, H_2O_2 and $OH^{\cdot}$. If triplet oxygen absorbs sufficient energy to reverse the spin of one of its unpaired electrons, it will form the singlet state, in which the two electrons have opposite spins. The oxygen reduction order is depicted in Fig. 2.3.

2.2.1.1 Singlet Oxygen

Singlet oxygen ($^1O_2$) is the first excited electronic state of O_2. It is the highly reactive ROS that can be formed in a reaction between O_2 and the chlorophyll triplet state (Krieger-Liszkay 2005; Jajic et al. 2015). Since paired electrons are common in organic molecules, $^1O_2$ is much more reactive toward organic molecules than its triplet counterpart ($^3O_2$) (Afanas'ev 1985). This activation overcomes the spin restriction and $^1O_2$ can consequently participate in reactions involving the simultaneous transfer of two electrons (divalent deduction) (Millard et al. 1964). In the presence of light, the chlorophyll (Chl) pigments in the antenna system and in the reaction center of photosystem II (PS II) are primary sources producing highly reactive $^1O_2$ via triplet chlorophyll formation. In the antenna, insufficient energy dissipation during photosynthesis can lead to the formation of Chl triplet state, whereas in the reaction center it is formed via charge recombination of the light-induced charge pair. The Chl triplet state (^{3}Chl) can react with $^3O_2$ to give up the very highly destructive ROS $^1O_2$ (Sharma et al. 2012).

$$\text{Chl} \rightarrow {}^3\text{Chl},^3$$
$$\text{Chl} + {}^3O_2 \rightarrow \text{Chl} + {}^1O_2$$

The life time of $^1O_2$ within the cell is probably 3 μs or less. A fraction of $^1O_2$ has been shown to be able to diffuse over considerable distances of several hundred nanometers (nm). It can last for 4 μs in water and 100 μs in a nonpolar environment. $^1O_2$ reacts with most of the biological molecules at near diffusion-controlled rates (Foyer and Harbinson 1994). It directly oxidizes protein, unsaturated fatty acids and DNA. $^1O_2$ can be generated as a by-product resulting from activity of lipoxygenase as well (Moucheshi et al. 2014). The formation of $^1O_2$ during photosynthesis has a powerful damaging effect on photosystem I (PS I) and PS II as well as on the whole photosynthetic machinery (Sharma et al. 2012). Further, various abiotic stresses (salinity, drought) lead to closing of stomata and resulted low intercellular CO_2 concentration in the chloroplast favor the formation of $^1O_2$. The latter is an oxidizing agent for a wide range of biological molecules and can react with proteins, pigments, nucleic acids and lipids, and it is the most important species responsible for light-induced loss of PS II activity which can lead to cell death (Gill and Tuteja 2010).

$^1O_2$ can be quenched by β-carotene, α-tocopherol or plastoquinone and can react with the D1 protein of PS II as target (Sharma et al. 2012). It can activate the upregulation of genes, involved in molecular defense responses against photooxidative stress (Gill and Tuteja 2010). In these conditions, $^1O_2$ is generated in the plastids and is involved in activating distinct groups of early stress–response genes that are different from those activated by $O_2^{\cdot-}$ and/or H_2O_2. It was suggested that $^1O_2$ does not act primarily as a toxin but rather as a signal that activate several stress–response pathways (Gill and Tuteja 2010).

Plants trigger the production of antimicrobial secondary metabolites (phytoalexins) as a mechanism of resistance in plant–pathogen interactions (González-Lamothe et al. 2009). The occurrence of phenalenone chromophores in phytoalexins of plants originally non-phototoxic which suggests that plants respond to pathogen attacks by biosynthesizing $^1O_2$ photosensitizers. Moreover, some species constitutively produce different types of secondary metabolite with photosensitizing properties that make use of $^1O_2$ to increase their efficacy as antimicrobial agents (Flors and Nonell 2006). Due to spin restriction, molecular O_2 cannot accept four electrons simultaneously in order to produce H_2O. It accepts only one electron at one time, and hence during the one-electron (univalent) reduction of O_2, stable intermediates are formed in a stepwise fashion (Halliwell and Gutteridge 1989).

2.2.1.2 Superoxide Radical

$O_2^{\cdot -}$ is the primary ROS formed in the cell which initiates a cascade of reactions to generate other "secondary" ROS, either directly or prevalently through enzyme- or metal-catalyzed processes depending on the cell type or cellular compartment (Figs. 2.4 and 2.5). In plants, $O_2^{\cdot -}$ is generated in different cell compartments (chloroplasts, peroxisomes, apoplast, the mitochondrial electron transport chain, plasma membrane, ER). Another important source of $O_2^{\cdot -}$ in plant cells is NOX–Rbohs, which play key roles in number of physiological processes, such as ROS signaling and stress responses (Jajic et al. 2015). $O_2^{\cdot -}$ is also produced in cytosol by action of xanthine dehydrogenase and the aldehyde oxidase (Fig. 2.1). Different studies have reported an increase in the production of $O_2^{\cdot -}$ during natural and artificially induced senescence (McRae and Thomson 1983; Pastori and del Rio 1997). However, attributing a specific signaling role to this increase is extremely difficult since the increase in the most cases is accompanied by the production of

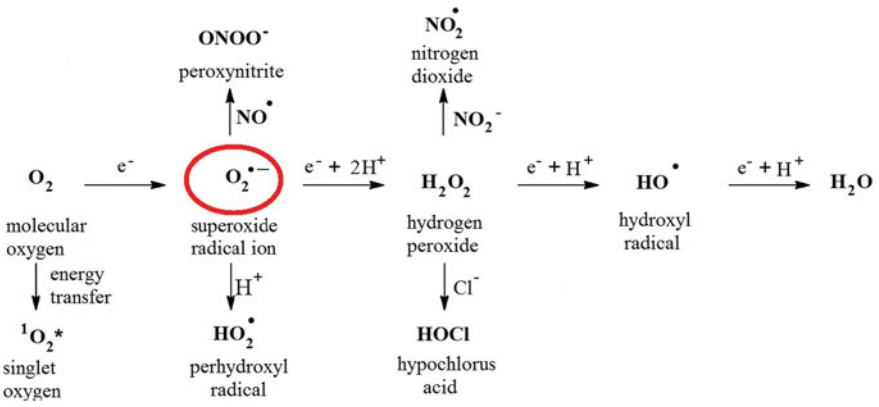


Fig. 2.4 Pathways in the univalent reduction of O_2 to water leading to the formation of various intermediate ROS

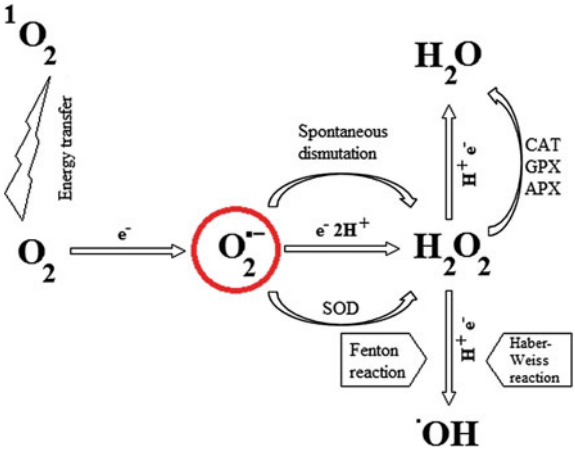


Fig. 2.5 Schematic representation of ROS generation in plants by two different mechanisms: stepwise monovalent reduction of O_2 and energy transfer to O_2

other ROS and the quick conversion of $O_2^{\cdot-}$ to H_2O_2 (see Fig. 2.1). $O_2^{\cdot-}$ is a moderately reactive, short-lived ROS with a half-life of approximately 1 μs. It is a nucleophilic reactant with both oxidizing and reducing properties. Anionic charge of $O_2^{\cdot-}$ inhibits its electrophilic activity toward electron-rich molecules. $O_2^{\cdot-}$ has been shown to oxidize enzymes containing the [4Fe–4S] clusters (aconitase or dehydratase as examples) and reduce cytochrome C (Sharma et al. 2012). $O_2^{\cdot-}$ can accept one electron and two protons to form H_2O_2 (Figs. 2.4 and 2.5). It is easily dismutated to H_2O_2 either non-enzymatically or by SOD catalyzed reaction to hydrogen peroxide:

$$2O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2,$$
$$2O_2^{\cdot-} + 2H^+ + \text{SOD} \rightarrow H_2O_2 + O_2.$$

Among these defenses is the antioxidant enzyme catalase, which converts H_20_2 to oxygen and water.

2.2.1.3 Hydrogen Peroxide

H_2O_2 plays an important role in plant organism under stress conditions as a signaling molecule that mediates between different physiological processes. It is involved in the regulation of the senescence process, protection against pathogens, the reduction of stress intensity at low light and the alleviation of drought stress, and it can influence on the expression of different genes (Jajic et al. 2015). Unlike the oxygen radicals, H_2O_2 can diffuse across biological membranes; hence, it can cause oxidative stress far from the site of formation. It will accept an electron and proton to form H_2O and $OH^{\cdot}$ (Figs. 2.4 and 2.5).

H_2O_2 itself is a relatively stable oxidant and not highly reactive, and some biomolecules are directly sensitive to it. For example, many proteins are known to be sensitive to physiologically relevant levels of H_2O_2 (about 100 nM) (Scandalios et al. 1997). H_2O_2 is generated in the cells under normal and wide range of stressful conditions such as drought, chilling, UV irradiation, intense light, wounding and invasion by pathogens. It is produced in significant quantities in various subcellular organelles. Each organelle also has potential targets for H_2O_2 oxidative stress as well as mechanisms for eliminating H_2O_2. Furthermore, H_2O_2 can readily diffuse through intra- and inter-cellular membranes, allowing the interaction of organelles or even cell types (Sharma et al. 2012). Because H_2O_2 is the only ROS that can diffuse through aquaporins in the membranes and over larger distances within the cell (Bienert et al. 2007) and is relatively stable compared to other ROS at low concentrations, it has received particular attention as a signal molecule involved in the regulation of specific biological processes and triggering tolerance against various environmental stresses, such as plant–pathogen interactions. At high concentrations, H_2O_2 can oxidize the cysteine (–SH) or methionine residues ($–SCH_3$) and inactivate enzymes by oxidizing their thiol groups, such as enzymes of Calvin cycle, Cu/Zn-SOD and Fe-SOD (Halliwell and Gutteridge1989). When H_2O_2 accumulates at the level of 10 μM, the enzymes in the Calvin cycle, such as fructose-1,6-bisphosphatase, sedoheptulose-1,7-bisphosphatase and phosphoribulokinase, lose 50 % of their activity (Kaiser 1979; Leegood and Walker 1982). It also oxidizes protein kinases, phosphatases and transcription factors containing thiolate residues.

H_2O_2 is produced in plants via two possible pathways: dismutation of $O_2^{\cdot -}$ with the help of SOD (Asada 2006) and via oxidases, such as amino and oxalate oxidases (Hu et al. 2003; see Fig. 2.1).

ETC of chloroplast, mitochondria, endoplasmic reticulum and plasma membrane, β-oxidation of fatty acid and photorespiration are major sources of H_2O_2 generation in plant cells. Photooxidation reactions (Asada 2006), NADPH oxidase (Sagi and Fluhr 2006) as well as xanthine oxidase (XOD) also contribute to H_2O_2 production in plants (Jajic et al. 2015; see Fig. 2.1). It is also produced in tissues as being a substrate for biosynthesis during lignification and suberization processes (Wang et al. 2013).

H_2O_2 has no unpaired electrons, unlike other oxygen radicals (Fig. 2.3); it can readily cross biological membranes and consequently can cause oxidative damage far from the site of its formation. Both $O_2^{\cdot -}$ and H_2O_2 are only moderately reactive. In comparison with other ROS, H_2O_2 is the most stable and last reactive and, as already been mentioned, can easily cross the membrane. This ability makes it a good signaling molecule and involved in the regulation of different abiotic and biotic stresses (Perez and Brown 2014). At high concentrations, H_2O_2 plays an important role in cell death and during the final stages of senescence, by contributing to cell degradation.

During the senescence process, H_2O_2 acts as a promoter and is a part of a complex regulatory network. It works as a signal molecule for the induction of senescence and in the degradation of molecules at later stages of senescence

(Jajic et al. 2015; Dat et al. 2000). Several studies have indicated that H_2O_2 can interact with other signal molecules that are important for plant development and during senescence such as abscisic acid (ABA) and ethylene (Jubany-Mari et al. 2009; Chen et al. 2012). It was shown that H_2O_2 could be involved also in the signaling of plant growth regulators such as ethephon (Chen et al. 2012). The application of ethephon results in an elevation in H_2O_2 levels, which is accompanied by the increased expression of sweet potato catalase. The elimination of H_2O_2 influence by exogenous-reduced glutathione alleviates ethephon-mediated effects. Drought stress–ABA–H_2O_2 interaction can induce an increase in ascorbic acid, maintaining and even decreasing the ascorbate oxidative status under summer drought conditions, thereby protecting plants from oxidative damage (Jajic et al. 2015; Nuruzzaman et al. 2013). So, the number of investigations shows that H_2O_2 is important in the formation of plant tolerance to different biotic and abiotic stresses.

It was also shown that pre-treatment with H_2O_2 provides protection against heat stress and low-light-induced oxidative stress by modulating the activity of antioxidant enzymes. The exogenous application of H_2O_2 can induce tolerance to heat stress in seedlings of some cultivated plants (Gao et al. 2010). The pre-treatment of cucumber leaves with H_2O_2 and heat increased antioxidant enzyme activities, decreased lipid peroxidation, and thus protected the ultrastructure of chloroplasts under heat stress. Similarly, exogenous H_2O_2 can have a beneficial effect on low-light-induced oxidative stress (Zhang et al. 2011). Low light induces an oxidative stress (Sielewiesiuk 2002), which increases ROS and causes lipid peroxidation. H_2O_2 pre-treatment of cucumber leaves resulted in decreased levels of $O_2^{\cdot -}$, endogenous H_2O_2 and malonaldehyde by moderating the activities of antioxidant enzymes and therefore reducing lipid peroxidation and stress intensity at low light. Pre-treatment with H_2O_2 can also increase drought stress tolerance in soybean leaves by promoting the expression of stress–response genes (Desikan et al. 2001). Exogenous application of H_2O_2 caused an increase in the mRNA levels of key enzymes for the biosynthesis of oligosaccharides, which are known to help plants tolerate drought stress. This enabled the soybean plant to avoid drought stress through the maintenance of leaf water content and thus to delay foliar wilting. Hydrogen peroxide contributes also to defense responses against pathogens. It was demonstrated that H_2O_2 is important for the greater tolerance of kumquat leaves infected with *Xanthomonas axonopodis* than that of grapefruit (Kumar et al. 2011). Infected kumquat leaves have a high accumulation of H_2O_2, which is promoted by the suppression of ascorbate peroxidase activity and later by the suppression of catalase activity, both involved in maintaining H_2O_2 at low levels. H_2O_2 can then be used as a substrate for the higher activity of class III peroxidase in the apoplast, which is known to be involved in plant defense against pathogens (Jajic et al. 2015). Some investigations confirm the dual role of H_2O_2 for plant organism. Liao et al. (2012) showed that treatment with 600 μM H_2O_2 caused an increase in the life of a cut lily "Manissa," while concentrations of 800 and 1200 μM gave the opposite effects. The other investigations also confirm the fact of dose-dependent effect of H_2O_2 (Khandaker et al. 2012).

2.2.1.4 Hydroxyl Radical

The cellular damage by ROS appears to be due to their conversion into more reactive species. The formation of ·OH is dependent on both H_2O_2 and $O_2^{\cdot -}$, and thus, its formation is subject to inhibition by both SOD and CAT. The hydroxyl radical is the most reactive of the oxygen species. It is an extremely potent oxidant and reacts with organic molecules at nearly diffusion rates. Activation of O_2 occurs by two different mechanisms. Stepwise monovalent reduction of O_2 leads to the formation of $O_2^{\cdot -}$, H_2O_2 and ·OH, whereas energy transfer to O_2 leads to the formation of $^1O_2$. $O_2^{\cdot -}$ is easily dismutated to H_2O_2 either non-enzymatically or by superoxide dismutase (SOD) catalyzed reaction to H_2O_2. H_2O_2 is converted to H_2O by catalase (CAT), glutathione peroxidase (GPX) and ascorbate peroxidase (APX).

The Harber–Weiss reaction generates ·OH from H_2O_2 and $O_2^{\cdot -}$. It consists of the following reactions:

$$Fe^{3+} + O_2^{\cdot -} \rightarrow Fe^{2+} + O_2,$$

Fe^{3+} is reduced by $O_2^{\cdot -}$, followed by oxidation by H_2O_2 (Fenton reaction)

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\cdot} + {}^{\cdot}OH,$$

and the following reaction (Fig. 2.5):

$$O_2^{\cdot -} + H_2O_2 \rightarrow {\cdot}OH + OH^{\cdot} + O_2.$$

Metal catalysis is necessary for this reaction as the rate of unanalyzed reaction is almost negligible (Sharma et al. 2012). Hydroxyl radical has a single unpaired electron (Fig. 2.3) and can react even with oxygen in triplet ground state. ·OH is interacts with all biological molecules and causes subsequent cellular damages such as lipid peroxidation, DNA destruction, protein damage and membrane destruction (Foyer et al. 1997). Because of the absence of any enzymatic mechanism of ·OH elimination, its excess production can eventually lead to cell death (Gill and Tuteja 2010). The oxidation of organic substrates by ·OH may occur by two possible reactions, either by addition of ·OH to organic molecules or due to abstraction of a hydrogen atom from it. Because of short lifetime and the strongly positive redox potential (close to +2 V) of "free" ·OH, its sites of reaction are close to its point of formation (Sharma et al. 2012).

2.3 ROS Detoxification in Plants

Photoinduced ROS generation depends mainly on conditions of the ambient medium and on the physiological state of the photosynthetic apparatus (Foyer 2005; Moucheshi et al. 2014). Even at intensive light, when the flow of electrons through

$$M^{(n+1)+}\text{-SOD} + O_2^- \rightarrow M^{n+}\text{-SOD} + O_2$$
$$M^{n+}\text{-SOD} + O_2^- + 2H^+ \rightarrow M^{(n+1)+}\text{-SOD} + H_2O_2$$

Fig. 2.6 Functional mechanism of superoxide dismutase for detoxifying $O_2^{\cdot-}$. M = Cu^{+1}; Mn^{+2}; Fe^{+2}; Ni^{+2}

the water–water cycle (O_2 reduction to H_2O in PS I at expense of electrons generated in PS II due to photodegradation of H_2O) increases, no substantial amounts of $^1O_2$ and H_2O_2 are accumulated, as sufficient amounts of $NADP^+$ are present in the cell.

At high light intensity and CO_2 availability limit (for instance the stomatal closure), the electron flow rate increases, which leads to the redistribution of electrons. The rate of electron flow to $NADP^+$ reduces, and the rate of electron transfer to O_2 (pseudo cyclic electron transport) increases. This results in generation of $O_2^{\cdot-}$ in PS I, which is produced mainly on its acceptor side (Moucheshi et al. 2014).

ROS are generated during mitochondrial respiration, photorespiration and from the photosynthetic counterparts. Moreover, biotic or abiotic stresses can cause production of ROS by NADPH oxidases (Gupta et al. 2016). Plants can scavenge ROS by producing antioxidants. Antioxidants are commonly grouped into two types: enzymatic and non-enzymatic. Enzymatic antioxidants contain peroxidase (PX), catalase, superoxide dismutase (SOD) and some other enzymatic antioxidants that are in charge in the ascorbate–glutathione cycle, such as ascorbate peroxidase, monodehydroascorbate reductase (MDHAR or MDAR), dehydroascorbate reductase (DHAR or DAR) and glutathione reductase (GR). The known non-enzymatic antioxidants are glutathione (GSH), ascorbate, carotenoids, tocopherols, flavones and anthocyanins (Gupta et al. 2005).

Ascorbate and glutathione are the most important non-enzymatic antioxidant molecules and are involved in the ascorbate–glutathione cycle as well.

Enzymatic antioxidants containing SOD, catalase (CAT), ascorbate peroxidase (APX), peroxidase (PX), glutathione reductase (GR) and MDAR decrease the levels of $O_2^{\cdot-}$ and H_2O_2 in plants. SOD catalyzes the dismutation of $O_2^{\cdot-}$ to O_2 and H_2O_2 (Moucheshi et al. 2014; Fig. 2.6).

2.3.1 Enzymatic Antioxidants

SOD is one of the most important enzymatic antioxidants that plants are using against oxidative stresses, and it exists in every plant cell. SODs are multimeric metalloproteins based on the metal classes existing at their active sites. The best-known isoforms of SODs occurring in plants are copper–zinc (Cu/Zn-SOD), manganese (Mn-SOD), iron (Fe-SOD) and nickel (Ni-SOD) containing superoxide dismutase. Inducing of SOD in plant cells in response to altered stressful conditions

$$\text{Ascorbate} + H_2O_2 + \text{APX} \rightarrow \text{Monodehydroascorbate} + H_2O$$

$$C_6H_8O_6 + H_2O_2 + \text{APX} \rightarrow C_6H_6O_6 + 2H_2O$$

Fig. 2.7 Functional mechanism of ascorbate peroxidase for detoxifying H_2O_2

shows its great role in the plant's defense system. Under the stress conditions, usually SOD activity increases to detoxifying $O_2^{\cdot -}$ in plant cells (Zare and Pakniyat 2012).

Similar to SODs, catalases have an important role in plant defense under oxidative stresses (Scandalios et al. 1997). CATs in plants are transcript by nuclear genes and are tetrameric iron porphyrins. They are principally produced in peroxisomes and glyoxysomes in plants. CAT catalyzes a redox reaction in which dismuting of H_2O_2 changes its form to O_2 and H_2O. However, CAT is specific to detoxification of H_2O_2. It can also react with various organic hydroperoxides such as methyl hydrogen peroxide (MeOOH) (Moucheshi et al. 2014).

Ascorbate peroxidase is also an important enzyme for detoxification of H_2O_2 in plants (Asada 1999; Rasool et al. 2013). H_2O_2 reacts with ascorbate to form monodehydroascorbate and H_2O, and the reaction is catalyzed by APX (Fig. 2.7). Four classes of APX are recognized in the plant cells: glyoxysome membrane (gmAPX), chloroplast thylakoid bound (tAPX), chloroplast stromal soluble (sAPX) and cytosolic (cAPX). Investigations showed increasing APX expression in plants in response to diverse abiotic stress conditions. APX overexpressing in chloroplasts of tobacco plants made them more tolerant to salinity stress and drought conditions (Zare and Pakniyat 2012). The enzymatic antioxidants working in the ascorbate–glutathione cycle also play a central role in stabilizing oxidative stresses in plants. Ascorbate peroxidase catalyzes conversion of ascorbate to monodehydroascorbate (MDA). Revival of ascorbate from monodehydroascorbate for scavenging of H_2O_2 in chloroplasts is required. Monodehydroascorbate reduction in stroma is catalyzed by monodehydroascorbate reductase. If reduced ferredoxin or MDAR could not transform MDA to ascorbate, dehydroascorbate would be produced. Thiol enzyme DHAR causes ascorbate renewal from dehydroascorbate, although dehydroascorbate cannot produce as much ascorbate as MDAR (Asada and Takahashi 1987). Glutathione reductase is another enzyme working in the ascorbate–glutathione system. It is localized primarily in chloroplasts, while a small amount can be found in cytosol and mitochondria (Creissen et al. 1994). GR causes glutathione reduction and antioxidative processes in plants.

2.3.2 Non-enzymatic Antioxidants

Apart from enzymatic antioxidants, non-enzymatic antioxidants also have significant role in neutralizing oxidative stresses (Sharma et al. 2012). They include the several groups of biologically active substances: tocopherols, ascorbic acid,

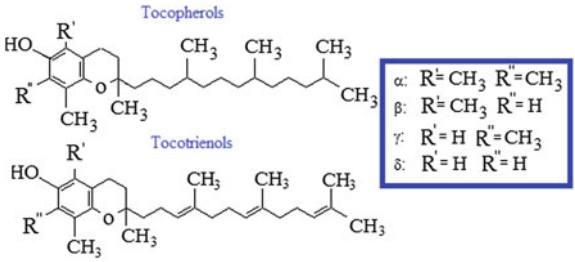


Fig. 2.8 Chemical structures of tocopherols and tocotrienols

glutathione, phenolics, carotenoids, etc. Tocopherols and tocotrienols, collectively known as tocochromanols, are lipid-soluble molecules that belong to the group of vitamin E (Falk and Munné-Bosch 2010; Kamal-Eldin and Appelqvist 1996). Tocopherols also can detoxify ROS and lipid radicals and are available in all parts of the plant. Tocopherols play the great role not only in biological membranes, but they can have both roles as antioxidant and as non-antioxidant components. Four isomers (α-, β-, γ-, δ-) of tocopherols are recognized in plants according to the number and position of methyl groups at the chromanol ring system (Fig. 2.8). α-Tocopherol which is also referred to as vitamin E is a membrane-bound compound having the highest antioxidative activity among the above-mentioned tocopherols, because of having three methyl groups in its molecular construction (Sharma et al. 2012). It is well documented that chloroplast membranes belonging to the higher plants contain a considerable quantity of α-tocopherols, so that they are well secured against photooxidative destructions. Similar to carotenoids, tocopherols protect the thylakoid membranes and avoid the chain propagation step during lipid auto-oxidation (Moucheshi et al. 2014).

Ascorbic acid (vitamin C; AsA; AA) is one of the most important antioxidants among non-enzymatic antioxidants. Similar to glutathione, it is a water-soluble metabolite and can be found in different organelles of the plant cell. Ascorbic acid is obtained frequently in its reduced form in plant leaves and chloroplasts under normal physiological conditions. It is also available regularly in apoplast. Its concentration in plant cells can rise to millimolar range and is generally greater than glutathione concentration. Ascorbic acid is the most powerful ROS detoxification compound because of its capacity to provide electrons in many non-enzymatic or enzymatic reactions. Ascorbic acid can directly quench $O_2^{\cdot -}$, $^1O_2$ and also ·OH. It can reduce H_2O_2 to water via the ascorbate peroxidase reaction:

$$C_6H_8O_6 + H_2O_2 \rightarrow C_6H_6O_6 + 2\ H_2O.$$

Furthermore, ascorbic acid can revive tocopherols from tocopheroxyl radical and thus provide membrane protection. Therefore, raised endogenous ascorbic acid levels in plants are essential to balance damaging effects of oxidative stress (Moucheshi et al. 2014).

Glutathione (or a functionally homologous thiol) is an essential metabolite with multiple functions in plants (Noctor et al. 2012; Fig. 2.9). Glutathione is a

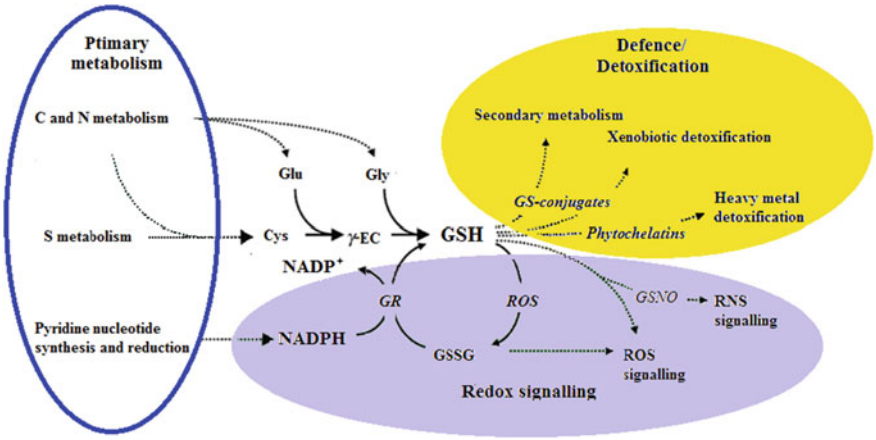


Fig. 2.9 General overview of some of the most important glutathione functions (synthesis, redox turnover, metabolism, signaling). Cys, cysteine; γ-EC, γ-glutamylcysteine; GS-conjugates, glutathione S-conjugates; GSNO, *S*-nitrosoglutathione; Glu, glutamate; Gly, glycine; RNS, reactive nitrogen species; ROS, reactive oxygen species

cysteine-containing tripeptide having important roles. It appears in reduced form in plant tissues and is localized in almost all compartments of plant cells such as chloroplasts, apoplast, mitochondria, cytosol, vacuole, peroxisomes and endoplasmic reticulum (Moucheshi et al. 2014). The fundamental and earliest recognized function of glutathione is in thiol–disulfide interactions, in which reduced glutathione (GSH) is continuously oxidized to a disulfide form (GSSG) that is recycled to GSH by NADPH-dependent glutathione reductase (GR).

Glutathione is vital for sustaining plant cells as it provides protection from all deleterious effects of oxidative stresses (Moucheshi et al. 2014). GSH (in both reduced and disulfide form) participates in the activation of secondary metabolism, xenobiotic and heavy metal detoxification process as well as formation of RNS and ROS signaling. It plays a key role in the antioxidative defense system by regenerating another potential water-soluble antioxidant, ascorbic acid, via the ascorbate–glutathione cycle (Fig. 2.10).

Glutathione is the substrate of glutathione *S*-transferase (GST), which includes in detoxifying of dehydroascorbate reductase and xenobiotics. Glutathione conserves redox equilibrium in the cellular compartments by combining with its oxidized form (GSSG). This form of glutathione has a significant biological role for conserving the normal cellular redox system under stressful or normal situations (Moucheshi et al. 2014).

Among all secondary metabolites, phenolic antioxidants appear to be the most important since they have shown expressed antioxidant activity in both in vivo and in vitro investigations. Plant phenolics are mainly classified into five major groups: phenolic acids, flavonoids, lignans, stilbenes and tannins. Flavonoids and phenolic

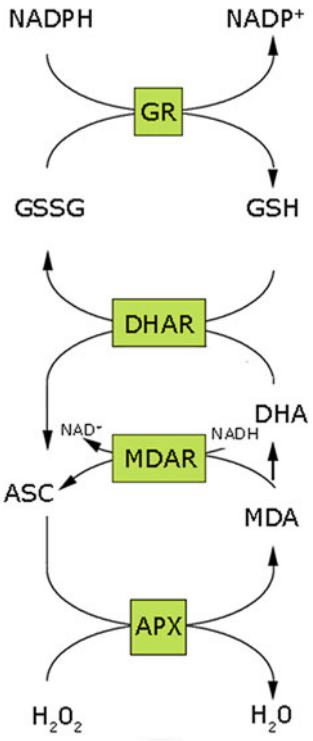


Fig. 2.10 Gutathione-ascorbate cycle

acids are the largest classes of plant phenolics biosynthetically derived from the acetate and shikimate pathways.

Flavonoids are the nitrogen-deficient group of plant pigments with antioxidant properties against a range of oxidizing compounds. Moreover, they are known to interact with other physiological antioxidants such as ascorbate or tocopherol and synergistically amplify their biological effect (Kasote et al. 2015). Flavonoids are commonly found in plants and are generally found in floral parts, pollens and plant leaves. These pigments are regularly accumulated in the vacuole of the plant as glycosides, but they also can be observed on the leaf surfaces, and other aerial parts of the plants as exudates. A number of flavonoids act as the potential inhibitor of the lipoxygenase enzyme, which converts polyunsaturated fatty acids to oxygen-containing derivatives (Moucheshi et al. 2014). Flavonoids and phenylpropanoids are also oxidized by peroxidase and act as H_2O_2 scavengers. Under experimental conditions, the antioxidant potential of plant phenolics is always linked to their electron donation, reducing power and metal ion chelating ability (Kasote et al. 2015). This ability is clearly expressed by the reaction of phenolics with DPPH (1,1-diphenyl-2-picrylhydrazyl radical). The latter is composed of stable free radical molecules (Fig. 2.11).

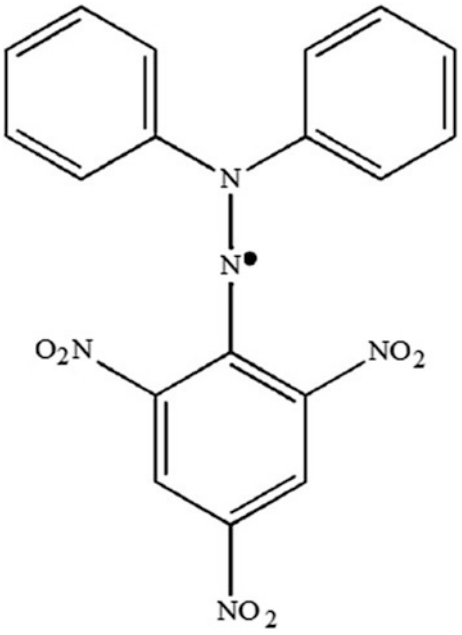
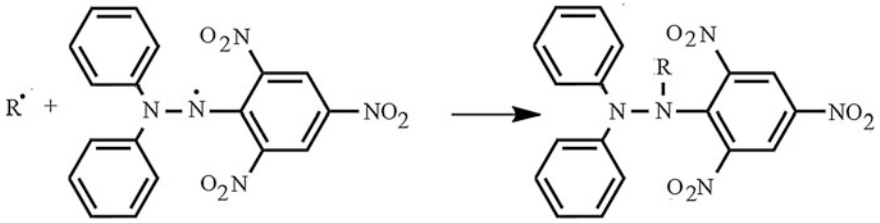


Fig. 2.11 1,1-Diphenyl-2-picrylhydrazyl radical structure

The DPPH assay method is based on the reduction of DPPH. The stable free radical DPPH with an odd electron gives a maximum absorption at 517 nm (purple color). When antioxidants react with DPPH, it becomes paired off in the presence of a hydrogen donor (e.g., a free radical scavenging antioxidant) and is reduced to the DPPH-H and as consequence the absorbance's decreased from the DPPH:



Radical to the DPPH-H form, results in discoloration (yellow color) with respect to the number of electrons captured. More the discoloration more is the reducing ability. This test has been the most accepted model for evaluating the free radical scavenging capacity of any new drug. When a solution of DPPH is mixed with that of a substance that can donate a hydrogen atom, then this gives rise to the reduced form (diphenylpicrylhydrazine—non-radical) with the loss of this violet color

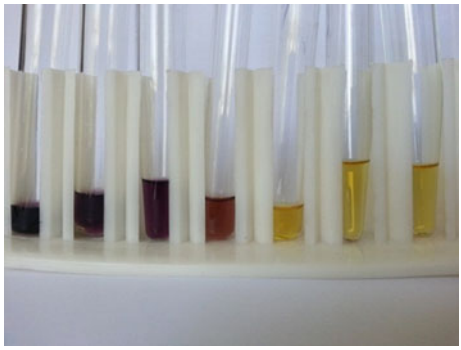


Fig. 2.12 Discoloration of DPPH under the influence of rose (*Rosa* × *damascene* Mill.) sepal extract at different concentrations—0.25–0.75 $mgml^{-1}$

(although there would be expected to be a residual pale yellow color from the picryl group still present) (Fig. 2.12). Terpenoids (or isoprenoids) are the large family of secondary metabolites, consisting of over 40,000 different compounds. Monoterpenes, sesquiterpenes and diterpenes possess notable antioxidant activity in different in vitro assays (Kasote et al. 2015).

Carotenoids, also called tetraterpenoids (Fig. 2.13), are organic pigments that are found in the chloroplasts and chromoplasts of plants and some other photosynthetic organisms, including some bacteria and some fungi. Carotenoids have multiple functions in metabolism of the plants, including tolerance to oxidative stress. They are lipophilic organic compounds. These kinds of plant pigments are generally mentioned as antenna molecules, which can capture photon light of the sun in the visible spectrum ranging between 450 and 570 nm and transport it to the plant chlorophylls. Moreover, carotenoids have a responsibility for providing photoprotection to the photosynthetic structures and apparatus. Different forms of carotenoids are available in plant cells, but β-carotenes are the most important and principal carotenoids in higher plants. By means of quenching the triplet state, β-carotenes can efficiently avoid singlet oxygen being produced in chlorophyll molecules (Moucheshi et al. 2014). These functions have been demonstrated in vitro in PS II complexes (Hager and Holocher 1994). Under excess light, there is a rapid change in the carotenoid composition of the light-harvesting complexes (LHCs): the diepoxide xanthophyll violaxanthin (V) is rapidly and reversibly converted via the intermediate antheraxanthin (A) to the epoxide-free zeaxanthin (Z) under the action of the enzyme V deepoxidase (Jahns et al. 2009; Fig. 2.14). Although this xanthophyll interconversion (V cycle) has been studied extensively in the recent years, its physiological role is not yet completely understood. The phototransformation of V is involved in the conversion of PS II to a state of high thermal energy dissipation and low Chl fluorescence emission. It has been suggested that Z could quench directly the singlet excited state of chlorophylls (^{1}Chl) or could favor proton-induced aggregation of LHCs of PS II leading to energy dissipation (Marin et al. 1996).

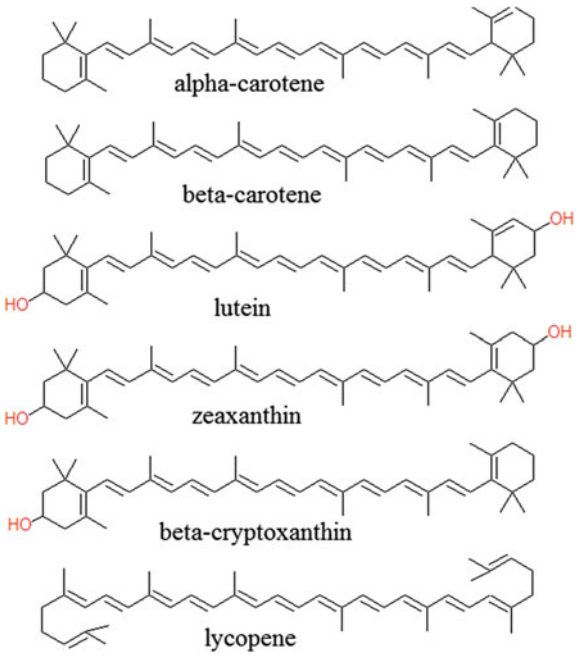


Fig. 2.13 Chemical structure of carotenoids

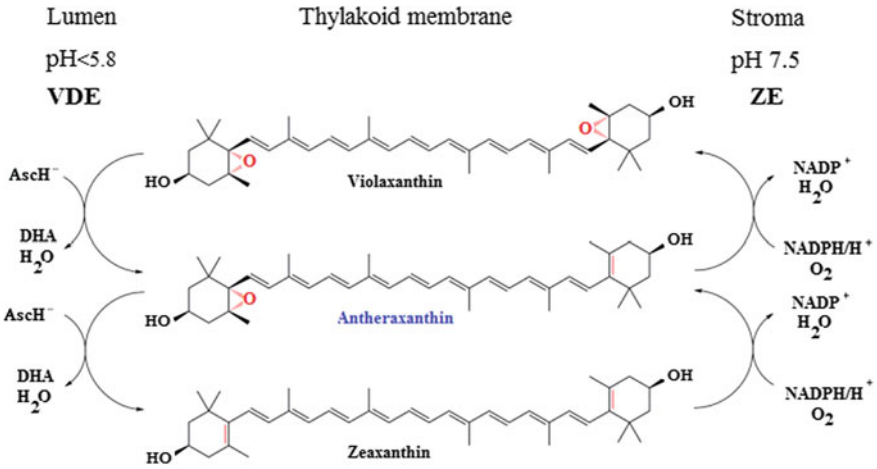


Fig. 2.14 Violaxanthine cycle (VDE—violaxanthine de-epoxidation; ZE—zeaxanthin epoxidation)

An increase in thermal deactivation of ^{1}Chl is potentially beneficial because it can protect the PS II reaction centers from over excitation and subsequent photoinhibition and it also can reduce the probability of ^{3}Chl and $^{1}O_2$ formation in LHCs. It must be noted, however, that the protective function of the V cycle is

probably not restricted to PS II, because the cycle takes place in both PS II and PS I. Inhibition of the V cycle by DL-dithiothreitol (DTT), a potent (though non-specific) inhibitor of the V deepoxidase, was associated with only a slight increase in the level of PS I photoinhibition in strong light, whereas, concomitantly, pronounced lipid peroxidation monitored by the production of ethane was induced. This suggests that the V cycle could be involved in a general protection of the photosynthetic apparatus against photooxidation (Havaux and Niyogi 1999; see Fig. 2.14).

There are various other metabolites having antioxidizing activity, such as alkaloids, phenolic acid, diterpenes, polyamines, proline and other amino acids and amines. Antioxidative activities of enzymatic and non-enzymatic antioxidants are upregulated under stress conditions, but their activities are divers in different plant species (Smirnoff 2005). It can be observed that the effectiveness of plant antioxidant systems for detoxification of ROS and keeping homeostasis depends on the plant species and genus, together with plant genetic background, stress intensity levels and also the growth stage of exposed plants. On the other hand, plant biologists have focused more on understanding the functions of different antioxidants in plant response to stresses (Ashraf and Harris 2004). Because of significant differences in the protection process against ROS in plants, a general validity for effectiveness of antioxidants in plants' tolerance to stresses cannot be clearly established, but it is well known that they have a significant effect on plant metabolism pathways under normal or stress conditions.

2.4 Conclusion

For each organism, an unavoidable consequence of living in oxygen-containing environment is the constant formation of ROS as by-products of different metabolic pathways. And for the adaptation in these conditions, there are numerous mechanisms acting in both animal and plant cells. ROS formation process is more active in plant cells than in animal cells, as plants differ by the immobility and the presence of three pivotal processes in their life: photosynthesis, respiration (especially existence of alternative oxidases) and photorespiration. Thus, plants have the most sophisticated complex of ROS-scavenging system to keep homeostatic balance in cells. The plant ROS-scavenging system includes different enzymes as well as different antioxidant metabolites.

Under the normal conditions, ROS production intensity in different cell compartments is low. ROS play multiple role in plants among which is important a signaling role controlling processes such as growth, development, response to biotic and abiotic environmental stimuli, senescence and programmed cell death. To utilize ROS as signaling molecules, non-toxic levels must be maintained in a delicate balancing act between ROS production, involving ROS-producing enzymes and the unavoidable production of ROS during basic cellular processes, and the metabolic counter-process involving various ROS-scavenging pathways. The recent identification of ROS-generating enzymes has led to the demonstration that plant

cells, like mammalian cells, can initiate and most likely amplify ROS production for the purpose of signaling. Localized ROS production in organelles such as plastids, mitochondria and peroxisomes may also initiate signaling cascades.

Generally, ROS affect stress responses in two different ways. They react with a large variety of biomolecules and may thus cause irreversible damage that can lead to tissue necrosis and may ultimately kill the plants. On the other hand, ROS influence the expression of a number of genes and signal transduction pathways. So, cells have evolved strategies to utilize ROS as environmental indicators and biological signals that activate and control various genetic stress response programs. ROS would be ideally suited to act as such signaling molecules. ROS are small and can diffuse short distances, there are several mechanisms for ROS production, and there are numerous mechanisms for rapid removal of ROS.

The effect of ROS on gene expression is variable. Depending on the character of the environmental stress, plants differentially enhance the release of ROS that are either chemically distinct or are generated within different cellular compartments. For instance, during an incompatible plant–pathogen interaction, superoxide anions are produced enzymatically outside the cell and are rapidly converted to H_2O_2 that can cross the plasma membrane. The same ROS are also produced in chloroplasts exposed to high light stress, albeit by a different mechanism. The stress reactions of plants induced by pathogens differ from those induced by high light intensities. If ROS act as signals that evoke these different stress responses, their biological activities should exhibit a high degree of selectivity and specificity that could be derived from their chemical identity and/or the intracellular locations where they were generated.

The number of H_2O_2-responsive genes in cell culture was in contrast to that found in whole plants treated with low concentrations of paraquat (Op den Camp et al. 2003). In the absence of visible necrotic lesions, very few genes were initially upregulated, some of which are involved in the detoxification of H_2O_2, like ascorbate peroxidases or ferritin (Karpinski et al. 1999). These genes were different from those activated by $^1O_2$, which suggests that chemical differences between the two ROS might have contributed to the selectivity of the induced stress responses (Op den Camp et al. 2003). However, some experiments show that either $^1O_2$, superoxide and H_2O_2 may replace each other in triggering pathogen defense reactions or the constitutive accumulation of photodynamically active tetrapyrrole intermediates and catabolites throughout the entire life cycle of these genetically modified plants may lead to photooxidative damage and injury (Mock et al. 1999). These may promote a multifactorial induction of several overlapping secondary effects, some of which may mimic responses to pathogens. This latter interpretation agrees with in vivo measurements of ROS production in leaves under photooxidative stress, showing that $^1O_2$, superoxide and H_2O_2 were produced simultaneously in the same leaf (Fryer et al. 2002).

There are several lines of evidence suggesting that ·OHs may not only be a noxious side product of O_2 metabolism but may play a more significant role not only during oxidative stress but also during extension growth of roots, coleoptiles and hypocotyls, or during seed germination. $^1O_2$ induces a specific set of stress

responses (Op den Camp et al. 2003). Its biological activity exhibits a high degree of selectivity that is derived from the chemical identity of this ROS and/or the intracellular location at which it is generated (Apel and Hirt 2004). The numerous studies indicate that the biological activities of ROS may significantly differ from each other.

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http://www.springer.com/978-3-319-44080-4

Redox State as a Central Regulator of Plant-Cell Stress Responses

Gupta, D.K.; Palma, J.M.; Corpas, F.J. (Eds.)
2016, XI, 386 p. 60 illus., 48 illus. in color., Hardcover
ISBN: 978-3-319-44080-4