REACTIVE OXYGEN SPECIES in PLANTS

BOON or BANE
REVISITING THE ROLE OF ROS

EDITED BY
VIJAY PRATAP SINGH
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Boon Or Bane - Revisiting the Role of ROS

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1

Generation Mechanisms of Reactive Oxygen Species in the Plant Cell: An Overview

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Introduction

During the course of evolution, life on the Earth started in a reducing environment and about 3.2 billion years ago, the reducing environment changed to an oxidizing one due to the appearance and proliferation of the first oxygen-evolving photosynthetic organisms, that is, cyanobacteria (Schopf *et al.*, 2007). In other words, the cyanobacteria are considered to be the first organisms to release oxygen in the environment by means of an oxygen evolving complex (OEC) (Bekker *et al.*, 2004). The outermost orbital of the dioxygen (O_2) molecule has two unpaired electrons having same spin quantum number, and this enables O_2 to accept electrons one at a time efficiently, and generate the reactive oxygen species (ROS). Out of the total O_2 utilized by plants, 1% is diverted to produce ROS in various cell organelles (del Rio *et al.*, 2002). Reactive oxygen species are essential by-products of all aerobic organisms that are produced during normal metabolic processes as well as under stress conditions. The ROS-producing subcellular organelles are mainly mitochondria, chloroplasts, peroxisomes, cytosol, and plasma membrane (Corpas *et al.*, 2015; Hasan *et al.*, 2016) (Figure 1.1). The roles of ROS are contradictory, they may have negative as well as positive roles depending upon their concentrations in the particular cell organelles. At higher concentrations, ROS cause damaging effects on proteins, DNA/RNA, and lipids by oxidative modification in plant cells (Gill and Tuteja, 2010; Hasan *et al.*, 2016). On the other hand, previous evidence clearly showed that at lower concentrations ROS act as signaling molecules in plants for regulating developmental pathways and control of redox homeostasis and defense responses against pathogens and environmental stress (Wood *et al.*, 2003; Apel and Hirt, 2004). There are well-described mechanisms in prokaryotes where the concentration of ROS directly activates transcription factors that overexpress the genes to combat oxidative stress (Kiley and Storz, 2004). There are several ROS, such as superoxide radical ($O_2^{\cdot-}$), hydroxyl radical (·OH), hydroperoxyl radical ($HO_2\cdot$), hydrogen peroxide (H_2O_2), alkoxy radical (RO·), peroxy radical (ROO·), singlet oxygen ($^1O_2$), and excited carbonyl (RO*), all of which are cytotoxic to plants at elevated concentrations (Dismukes *et al.*, 2001; Karuppanapandian *et al.*, 2011). In the cell organelles accumulation of superoxide enhances oxidative stress rather than playing a role in redox signaling. However, in some cases it damages certain proteins that activate specific signaling pathways

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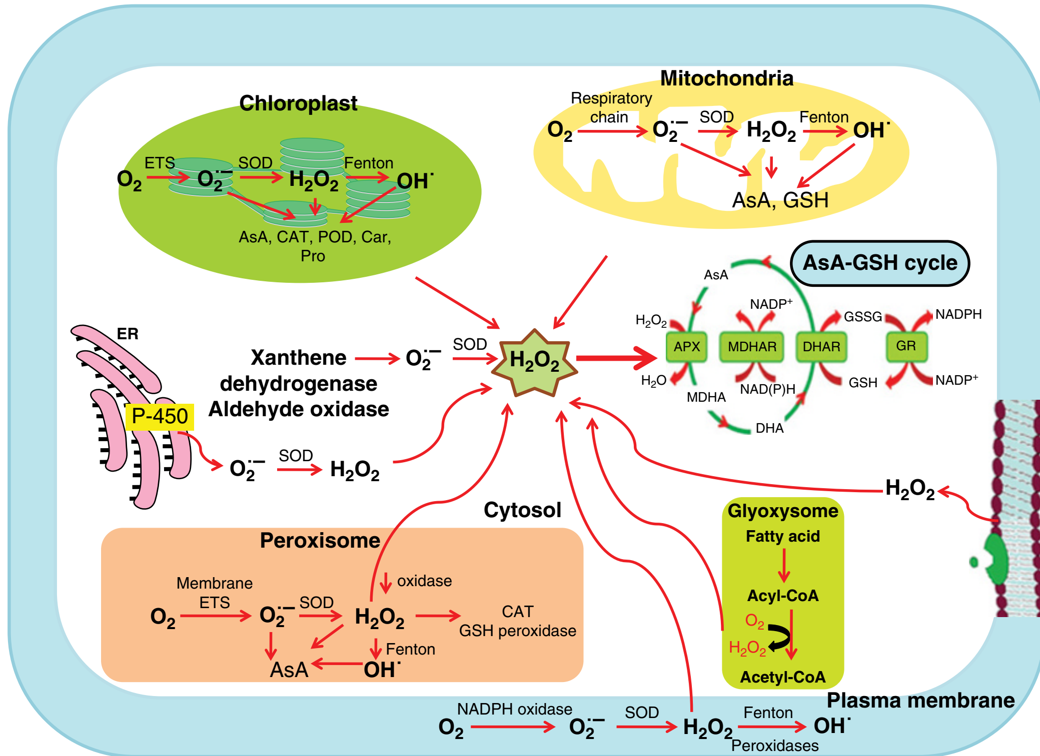


Figure 1.1 Schematic representation of major sites involved in reactive oxygen species (ROS) production and different scavenging mechanisms in plant cells. Abiotic and biotic stresses cause generation of toxic reactive oxygen species (ROS) such as the superoxide radical ($O_2^{\cdot-}$), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), and hydroxyl radical (·OH). These interact with several essential macromolecules and metabolites causing cellular damage. Moreover, the process of formation of ROS due to the spilling of electrons (e^-) from chloroplasts (ETS or Mehler's reaction), mitochondria (ETS involved in respiratory chain), peroxisomes (ETS involved in photorespiration) and plasma membranes (ETS); these electrons are taken up by molecular O_2 and quickly converted into superoxide radical (SOR). SOR produced during stress conditions is detoxified by superoxide dismutase (SOD) activity, leading to formation of H_2O_2. Detoxification mechanisms involve enzymatic as well non-enzymatic antioxidants to mitigate ROS-induced damage in plants. The ascorbate–glutathione (AsA–GSH) cycle plays an important role in H_2O_2 breakdown. H_2O_2 is reduced to H_2O with the help of ascorbate peroxidase (APX) using ascorbate (AsA) as the specific electron donor. APX is present in different organelles such as chloroplast (chlAPX), mitochondria (mitAPX), peroxisome (mAPX), and cytosol (cAPX). It protects plants from oxidative damage by delivering the electrons as well as minimizing excess production of ROS at these subcellular compartments. APX, ascorbate peroxidase; AsA, ascorbic acid; Car, carotenoids; CAT, catalase; DHA, dehydroascorbate; DHAR, dehydroascorbate reductase; ER, endoplasmic reticulum; ETS, electron transport system; Fenton, breakdown of H_2O_2 to highly reactive ·OH in the presence of iron; GSH, reduced glutathione; GSSG, oxidized glutathione; GR, glutathione reductase; H_2O_2, hydrogen peroxide; MDHA, monodehydroascorbate; MDHAR, monodehydroascorbate reductase; POD, peroxidase; Pro, proline content; SOD, superoxide dismutase. *Source:* Adapted from Jajic *et al*., 2015.

and consequently leads to death of the particular cell (Chen *et al*., 2009). Hydrogen peroxide (H_2O_2) acts as a signaling molecule that diffuses across membranes and triggers specific signal transduction pathways (Veal and Day, 2011). The balance between production and elimination of ROS is dependent upon various biotic and abiotic factors such as temperature, heavy metal concentration, drought, salinity, UV radiation, light, nutrient deficiency, and excessive use of pesticides and/or herbicides as well as pathogen attacks. This disturbance ultimately leads to

increased concentration of ROS in particular cell organelles. The damaging effects of ROS are ameliorated by different antioxidative defense systems. The antioxidant system consists of enzymatic antioxidants, namely superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and glutathione *S*-transferase (GST), ascorbate peroxidase (APX) and non-enzymatic antioxidants, including non-protein thiols (NP-SH) ascorbate, tocopherol, caretenoid and cysteine. These act together as a machine to detoxify ROS (Kumar *et al.*, 2015; Singh *et al.*, 2016; Tripathi *et al.*, 2012a,b, 2016a,b) (see Figure 1.1). Among these, SOD is considered as first line of defense; it dismutates the superoxide and subsequently H_2O_2 is generated. Further, H_2O_2 detoxification involves different enzyme systems in distinct cellular compartments. The peroxidase family includes ascorbate peroxidase (APX), glutathione peroxidase (GPX), and peroxidase (POD) for the elimination of H_2O_2. Another enzyme, CAT, is important in the removal of H_2O_2 generated in peroxisomes. GPX also reduces H_2O_2 as well as organic and lipid hydroperoxides by using glutathione (GSH) as substrate. Among the various H_2O_2-detoxifying enzymes, APX plays the most essential role in scavenging ROS. APX is present in thylakoid, glyoxisome, chloroplast stroma, and cytosol, and is involved in the scavenging of H_2O_2 through water-water and ascorbate-glutathione (AsA-GSH) cycles, utilizing AsA as the electron donor. One of the antioxidant enzymes, GST, participates in herbicide detoxification, hormone homeostasis, and regulation of apoptosis and also is involved in plant responses to biotic and abiotic stresses. Non-enzymatic antioxidants include the major cellular redox buffers ascorbate and glutathione, as well as tocopherol, flavonoids, alkaloids, and carotenoids.

With the above context, the present chapter gives an overview of reactive oxygen species, their production sites, and biochemistry as well as the mechanism for their amelioration, particularly in the plant system.

ROS Biochemistry and their Effects

Oxygen is necessary for every aerobic organism. In normal conditions it is involved in several biochemical reactions. The reduction of O_2 to H_2O provides the energy that allows the impressive complexity of higher organisms. However, incomplete reduction of O_2 leads to the production of ROS, which are extremely reactive and can oxidize almost every biological molecule. All ROS can react with DNA, proteins, and lipids (Gill and Tuteja, 2010; Singh *et al.*, 2016). Under these conditions, firstly $^1O_2$ is produced; then H_2O_2 is synthesized via the disproportionation of superoxide catalyzed by SOD, or non-enzymatically in the process of superoxide diffusion with a low yield of the reaction (Quinlan *et al.*, 2013; Singh *et al.*, 2015). H_2O_2 is reduced to water with the involvement of ascorbate peroxidase and ascorbate. Ascorbate is oxidized and then regenerated by the reduced glutathione at the expense of NADPH. Reduction of molecular O_2 proceeds through four steps, thus generating several O_2 radical species (Kalyanaraman *et al.*, 2016). The reaction chain requires initiation at the first step whereas subsequent steps are exothermic and can occur spontaneously, either catalyzed or not. The first step in O_2 reduction produces relatively short-lived ROS that are not readily diffusible: hydroperoxyl ($H_2O_2^-$) and peroxide ($O_2^{\cdot-}$). The second O_2 reduction generates hydrogen peroxide (H_2O_2), which can diffuse upto some distance from its site of production (Quinlan *et al.*, 2013).

Singlet Oxygen Species

Environmental stresses that impact CO_2 fixation (Gul *et al.*, 2016), such as drought and salt stress, ozone, and high or low temperatures, reduce $NADP^+$ regeneration during C-3 cycle, so the photosynthetic electron transport chain is over-reduced, by which singlet oxygen species

are produced in the chloroplasts (Wu and Tang, 2004; Bechtold *et al.*, 2005). The chlorophyll pigments associated with the electron transport system (ETS) are the prime source of singlet oxygen ($^1O_2$). The latter may also arise as a by-product of lipoxygenase activity. Like other reactive oxygen species, $^1O_2$ is also highly destructive, and reacts with most biological molecules at near diffusion-controlled rates. This mainly occurs due to the excitement of chlorophyll molecules; although the lifetime of excited chlorophyll is short within these aggregates, its duration varies according to physiological conditions. The excited singlet state of chlorophyll is used for the transfer of energy or electrons. However, there are two other possible routes of de-excitation, radioactive decay (fluorescence) and conversion of the singlet chlorophyll state to the triplet chlorophyll state. The latter interacts with oxygen to produce $^1O_2$.

Superoxide Radical

The half-life for $O_2^{\cdot-}$ is approximately 2–4 ms (Saxena *et al.*, 2016). It is produced at different sites in the cell but the mechanism of its production is almost similar at all sites. Reduction of dioxygen by light in the chloroplasts was first shown by the production of acetaldehyde in the presence of ethanol and catalase, and the photo-reduced product was assumed to be hydrogen peroxide. Under most circumstances, the control of electron flow between photosystems II (PSII) and I (PSI) regulates the reduction state of the acceptor side of PSI. The regulated activation of the C-3 cycle and control of the rate of electron flow are important factors determining the redox state of the ferredoxin pool (Tóth, *et al.* 2007). This is important because ferredoxin and the electron carriers on the reducing side of PSI have sufficiently negative electrochemical potentials to donate electrons to oxygen resulting in the formation of superoxide radical $O_2^{\cdot-}$. The majority of O_2 reduction *in vivo* is thought to proceed via reduced ferredoxin (Fd_{red}), which reduces molecular oxygen to the superoxide radical (Reaction 1). Hydrogen peroxide is then formed through dismutation of O_2^{-} (Reaction 2). The latter occurs spontaneously, but the velocity of the reaction is greatly increased by SOD (Reaction 3):

$$2O_2 + 2Fd_{red} \rightarrow 2O_2^{\cdot-} + 2Fd_{ox} \quad \text{(Reaction 1)}$$

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

$$2O_2^{\cdot-} + 2H \xrightarrow{SOD} H_2O_2 + O_2 \quad \text{(Reaction 3)}$$

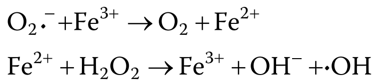
The major site of superoxide formation lies in the electron transfer chain (ETC), of mitochondria especially at the level of Complex I and Complex III. It was shown in animal mitochondria that the flavin mononucleotide (FMN)-containing subunit and an iron-sulfur cluster of the nicotinamide adenine dinucleotide (NADH) dehydrogenase of Complex I are the sites of O_2 generation (Chen *et al.*, 2009), especially when this complex is glutathionylated after oxidative stress (Taylor *et al.*, 2003). This complex could amplify ROS production and participate in the regulation of ROS concentrations in the whole cell. The over-reduction of the ubiquinone pool by Complex I can also lead to a reverse functioning of the chain, and to the formation of large amounts of ROS. In Complex III, the over-reduction state of the ubiquinone pool can lead to a direct electron transfer to molecular oxygen, and to the formation of superoxide anions. Superoxides are known to be produced during NADPH-dependent microsomal electron transport. Two possible loci of $O_2^{\cdot-}$ production in microsomes are auto-oxidation of the oxycytochrome-P450 complex that forms during microsomal mixed function oxidase (MFO) reactions, and/or auto-oxidation of cytochrome P450 reductase, a flavoprotein that contains both FAD and FMN.

Hydrogen Peroxide

Hydrogen peroxide is produced by the dismutation of superoxide radicals in a reaction mostly catalyzed by superoxide dismutase (Tripathi *et al.*, 2016). In leaf cells, catalase is exclusively localized in peroxisomes and has not been found in chloroplasts. The hydrogen peroxide in chloroplasts is scavenged by a peroxidase reaction using the photo-reductant produced in the thylakoid as the electron donor. Thus, diffusion of hydrogen peroxide from chloroplasts to peroxisomes and its scavenging by catalase are very unlikely to occur. The electron donor for the peroxidase reaction has been identified as ascorbate.

H_2O_2 is moderately reactive, has a relatively long half-life (1 ms), and can diffuse upto some distance from its site of production. H_2O_2 may inactivate the enzymes by oxidizing their thiol groups. Dismutation and oxidation reactions of superoxide yield hydrogen peroxide. Hydrogen peroxide, although more oxidizing than superoxide, is biologically less toxic: picomolar intracellular levels of superoxide are lethal, whereas micromolar levels of H_2O_2 can be tolerated. H_2O_2 is a potent oxidizer (although not always a fast oxidizer), and is much more diffusible than superoxide, because it is less reactive and is membrane permeable: $O_2^{\cdot-}$ is generally considered membrane impermeable except in its $HO_2\cdot$ form, which is in low abundance at physiological pH. The biological toxicity of H_2O_2 through oxidation of -SH groups has long been known, and it can be enhanced in the presence of metal catalysts through Haber–Weiss or Fenton-type reactions (see Figure 1.1). Fenton showed that the formation of toxic hydroxyl radicals (·OH) from hydrogen peroxide (H_2O_2) is catalyzed by iron ions, called the "Fenton reaction."

The Fenton or Haber–Weiss reactions are:



$$O_2\cdot^- + Fe^{3+} \rightarrow O_2 + Fe^{2+}$$
$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + \cdot OH$$

Iron ions are required to form toxic·OH radicals. Joseph Weiss and Fritz Haber discovered that $O_2^{\cdot-}$ can be converted into H_2O_2 and further to·OH, called the Haber–Weiss reaction. The last species generated by this series of reductions is the hydroxyl radical (·OH). It has high reactivity and has half-life of less than 1 ms. As a result, it has a very high affinity for biological molecules to react at its site of production.

Hydroxyl Radical

The generation of·OH from H_2O_2 and $O_2^{\cdot-}$ by the Haber–Weiss process is well known. In this process catalysis is necessary in the presence of a metal since the rate of uncatalyzed reaction is negligible. The hydroxyl radical is highly reactive among ROS. It has a single unpaired electron, and thus can easily bind with oxygen in the triplet ground state. Because cells have no enzymatic mechanism to eliminate·OH, its excess production can ultimately lead to cell death (Tripathi *et al.*, 2016). The oxidation of organic substrates by·OH may proceed by two possible reactions: either by addition of·OH to organic molecules or by abstraction of a hydrogen atom. In this context, organic oxygen radicals such as alkoxy, peroxy, semiquinones, reduced hydrogen peroxide, and hydrogen peroxide-electron donor complexes, as well as metallo-oxygen complexes, have been proposed as the ultimate active species besides destructive free·OH. These·OH are thought to be largely responsible for mediating oxygen toxicity *in vivo*. The hydroxyl radical can potentially react with all biological molecules, including DNA, proteins, and lipids, and almost any constituent of cells, and due to the absence of any enzymatic mechanism for the elimination of this highly reactive ROS, excess production of·OH ultimately leads to cell death (Table 1.1; see also Figure 1.1).

Table 1.1 Production of reactive oxygen species (ROS) and membrane damage under different stress conditions and their effect on plants.

Serial number	Reactive oxygen species & membrane damage	Stress	Plant	Damaging effect	Antioxidants and other defense systems	Reference
1.	$O_2^{\cdot-}$, H_2O_2, MDA equivalents contents and RNS	Salt and drought	*Ailanthus altissima*	Reduce chlorophyll fluorescence, stomatal conductance, and NR activity	SOD, CAT activity, and Pro content increased for detoxification	Filippou *et al*., 2014
2.	$O_2^{\cdot-}$, H_2O_2, and MDA equivalents contents	Temperature	*Cucumis sativus* and *Cucurbita ficifolia*	Loss of root cell viability; low root zone changes the mitochondrial electron distribution between the COX and AOX pathway in cucumber root	APX, GPOD, and CAT activity; temperature-mediated production of ROS in cucurbit species may act as signaling molecules, which activate MRR, and subsequently induce expression of genes encoding AOX protein of mitochondria and maintain ROS levels and redox homeostasis	Zhang *et al*., 2012
3.	Electrolyte leakage (EL)	Salt and zinc	*Vigna radiata*	Reduced plant growth, gas exchange parameters, carbonic anhydrase and nitrate reductase activity	Exogenous epibrassinolide and spermidine application enhances SOD, POD, and CAT activity, which reduces/detoxifies the damaging effect of stress It increases uptake of nutrients, stabilizes the photosynthetic enzyme and pH and enhances carboxylase activity that consequently increases growth of *Vigna* plant	Mir *et al*., 2015
4.	H_2O_2 and TBARS	Salt	*Brassica juncea*	Reduced photosynthetic rate, stomatal conductance, intercellular CO_2 concentration, quantum yield efficiency of PSII, rubisco activity, and total nitrogen content, and enhanced accumulation of Na^+ and Cl^- ions	Provide salinity tolerance by decreasing Na^+ and Cl^- accumulation and also regulate Pro and ethylene production	Iqbal *et al*., 2015
5.	$O_2^{\cdot-}$, H_2O_2, MDA equivalents contents, and EL	Arsenic stress	*Solanum melongena*	Reduced growth, photosynthetic pigment, and chlorophyll fluorescence; seedlings accumulate more arsenic (As) content	Reduce the damaging effect of As by upregulating the synthesis of SOD, POD, CAT, GST activity, and Pro as well as Pro synthetic enzyme	Singh *et al*., 2015

6.	$O_2^{\cdot-}$, H_2O_2, MDA equivalents contents, and EL	Salt	*Solanum melongena*	Reduced growth, K^+ content, photosynthetic pigment, and chlorophyll fluorescence; enhanced accumulation of Na^+	The damaging effect of NaCl is reduced by increased synthesis of enzymatic and non-enzymatic antioxidants	Singh *et al.*, 2016
7.	H_2O_2 and LPO	UV-B radiation	*Ginkgo biloba*	Reduced growth, photosynthetic pigment, and total protein content observed in *Ginkgo biloba*	Increased PAL activity as well as NO enhance accumulation of UV-B filters such as flavonoids	Hao *et al.*, 2009
8.	LPO and MDA equivalent content	UV-B radiation	*Phaseolus vulgaris*	Reduced biomass accumulation, biomass allocation pattern, and physiological and biochemical responses of *P. vulgaris*	UV-B induced enhancement in enzymatic and non-enzymatic antioxidants, ascorbic acid, Pro and TPC, which provide tolerance against UV-B damage. UV-B radiation induces two enzymes, namely chalcone synthase and PAL of the phenylpropanoid pathway, that are essential for synthesis of flavonoids, which act as selective UV-B filters	Raghuvanshi and Sharma, 2016

AOX, alternative oxidase; APX, ascorbate peroxidase; As, arsenic; CAT, catalase; COX, cytochrome *c* oxidase; EL, electrolyte leakage; ER, endoplasmic reticulum; GPOD, guaiacol peroxidase; GSH, reduced glutathione; GST, glutathione *S*-transferase; H_2O_2 hydrogen peroxide; LPO, lipid peroxidation; MDA equivalents contents, malondialdehyde; MRR, mitochondrial retrograde regulation; NR, nitrate reductase activity; $O_2^{\cdot-}$, superoxide radical; PAL, phenylalanine ammonium-lyase activity; POD, peroxidase; Pro, proline content; PSII, photosystem II; RNS, reactive nitrogen species; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances; TPC, total phenolic content; UV-B, ultraviolet B.

In response to all these ROS, peroxidation of lipids is considered as the most harmful process known to occur in every living organism. Membrane injury is sometimes taken as a single parameter to determine the level of lipid demolition under various stresses. ROS are very reactive and damage membranes and various cell components; this results in mobilization of various defense systems to reduce ROS generation and enhance ROS scavenging. This response entails *de novo* synthesis of antioxidant enzymes (i.e., superoxide dismutase, catalase, ascorbate peroxidase, glutathione reductase) and/or activation of their low molecular weight antioxidant precursors (i.e., ascorbate, glutathione, tocopherols, flavonoids) (Minibaeva and Gordon, 2003; Foyer and Noctor, 2005; Hung *et al.*, 2005). Production and removal of ROS must be strictly controlled in order to avoid oxidative stress. When the level of ROS overwhelms the defense mechanisms, a cell is said to be in a state of "oxidative stress." However, the balance between production and scavenging of ROS is disturbed under a number of stressful conditions such as salinity, drought, high light levels, toxicity due to metals, pathogens, and so forth (Table 1.1). The damage caused to biomolecules by enhanced levels of ROS can alter core membrane properties like fluidity and ion transport, lead to loss of enzyme activity, affect protein cross-linking, inhibit protein synthesis, damage DNA, and so forth, ultimately resulting in cell death. When ROS levels exceed a certain threshold, enhanced lipid peroxidation takes place in both the cellular as well as organellar membranes, which, in turn, affects normal cellular functioning. Lipid peroxidation intensifies the oxidative stress through production of lipid-derived radicals that themselves can react with and damage proteins and DNA. The level of lipid peroxidation has been widely used as an indicator of ROS-mediated damage to cell membranes under stressful conditions. Two common sites of ROS attack on the phospholipid molecules are the unsaturated (double) bond between two carbon atoms and the ester linkage between glycerol and the fatty acid. The polyunsaturated fatty acids (PUFAs) present in membrane phospholipids are particularly sensitive to attack by ROS. A single $\cdot OH$ can result in peroxidation of many polyunsaturated fatty acids because the reactions involved in this process are part of a cyclic reaction.

Attack by ROS can modify proteins in a variety of ways, some direct and others indirect. Direct modification involves modulation of a protein's activity through nitrosylation, carbonylation, disulfide bond formation, and glutathionylation. Proteins can be modified indirectly by conjugation with breakdown products of fatty acid peroxidation (Yamauchi *et al.*, 2005). Tissues injured by oxidative stress generally contain increased concentrations of carbonylated proteins, the degree of which is widely used as a marker of protein oxidation (Møller and Kristensen, 2004). Greater alteration of proteins has been reported in plants under various stresses (Romero-Puertas *et al.*, 2002; Sharma and Dubey, 2005; Maheshwari and Dubey, 2009; Tanou *et al.*, 2009).

Production Sites of ROS

Oxidative stress occurs when there is a serious imbalance in any cell compartment between production of ROS and antioxidant defense, which leads to cellular damage (Halliwell and Gutteridge, 1999). ROS are a group of free radicals, reactive molecules, and ions that are derived from O_2. They are produced in unstressed and stressed cells in several cell organelles, chiefly chloroplasts, mitochondria, and peroxisomes (see Figure 1.1). There is little contribution from the apoplast, cell wall, or endoplasmic reticulum. ROS are continuously formed by the leakage of electrons to O_2 from the electron transport in different cell organelles. They are also formed in various metabolic pathways as by-products, which are localized in different cellular organelles. Photosynthesizing plants are under threat of oxidative damage, because of

their oxygenic conditions and the abundance of the photosensitizer in the chloroplast envelope. It has been reported that two primary processes are mainly involved in the formation of ROS during photosynthesis: (i) direct photoreduction of O_2 to the superoxide radical by reduced electron transport components associated with PSI; and (ii) reactions linked to the photorespiratory cycle, including rubisco (in chloroplasts) and glycolate-oxidase and CAT-peroxidase reactions (in peroxisomes). Of the three major cell organelles, chloroplasts and peroxisomes produce ROS in the presence of light (Foyer and Noctor, 2003) while mitochondria generate ROS in the absence of light. Because ROS can cause damage to proteins, lipids, and DNA, their generation and scavenging must be strictly controlled. To manage this problem, the cell has a survival strategy including mechanisms for scavenging ROS and repairing damage caused by ROS. Excessive ROS reduce the rate of electron transport in the photosynthetic ETC; this leads to activation of alternative pseudocyclic electron transport and photorespiration. Under these conditions, first $^1O_2$ is produced; then H_2O_2 is synthesized in the reaction of disproportionation of superoxide catalyzed by SOD or non-enzymatically in the process of superoxide diffusion with a low yield of the reaction.

Chloroplast

Chloroplasts are considered the most powerful source of ROS generation in plants (Foyer *et al.*, 1994). It is the cellular site of photosynthesis, which proceeds through successive redox reactions during which light energy is transferred to different reaction centers of the two photosystems with the help of the light-harvesting complexes. Oxygen is continuously produced during light-driven photosynthetic electron transport and simultaneously removed from chloroplasts by reduction and assimilation. There are three types of oxygen-consuming processes closely associated with photosynthesis: (i) direct reduction of molecular oxygen by photosystem I (PSI) electron transport; (ii) the oxygenase reaction of ribulose 1,5-bisphosphate carboxylase-oxygenase (RuBisCO); and (iii) chlororespiration within thylakoid membrane (Alric *et al.*, 2010). The electrons released during the process are transferred to an ultimate acceptor via a path called the photosynthetic electron transport chain. The cyclic electron transport chain includes a number of enzymes on the reducing (acceptor) side of PSI: Fe-S centers, reduced thioredoxin, and ferredoxin (Noctor and Foyer, 1998). These electron transport components are auto-oxidizable (Biehler and Fock, 1996). Further generation of ROS is elaborated by the Mehler peroxidase reaction, which explains the transfer of electrons from H_2O to O_2, resulting in production of O_2^- at PSII, O_2^- at PSI, and the trans-thylakoid proton gradient necessary to drive phosphorylation and photochemistry of PSII. It has been suggested that photoreduction of O_2 to water by the Mehler peroxidase pathway in intense light may involve up to 30% of the total electron transport (Oukarroum, 2016). Oxygen reduction sustains significant levels of photosynthetic electron flux, not only through its role in photorespiration but also by its direct reduction through PSI (Asada, 1999). This would suggest that O_2 plays an important role as an alternative electron acceptor in photo-protection. Producing large amounts of ROS is an unavoidable consequence of the photosynthetic reduction of oxygen, and plants have to evolve efficient strategies to deal with the accumulation of these potentially toxic compounds that are integral components of oxygenic photosynthesis.

Mitochondria

Mitochondria, which are considered the cell's "energy hub," are believed to be the foremost sites of ROS production. It has been shown that ROS generated in mitochondria and protein oxidation are contributing factors to the "oxidative stress" syndrome in plants (Sweetlove *et al.*, 2002; Kristensen *et al.*, 2004; Møller and Kristensen 2004). It has also been proven that in the dark or

in non-green tissues of plants, mitochondria are a major source of ROS. Firstly, in 1966 it was reported that the respiratory electron transport system produces ROS, and their production can be enhanced in response to various biotic and abiotic stresses. Subsequent innovative work of (Belt *et al.*, 2017) showed that isolated mitochondria produce H_2O_2. The mitochondrial inner membrane is where respiratory electron transport occurs. This mitochondrial ETS harbors electrons with sufficient free energy to directly reduce O_2, which is considered a primary source of ROS generation. It was confirmed later that H_2O_2 arose from the dismutation of superoxide ($O_2^{\cdot-}$) generated within mitochondria. There are two pathways of O_2 consumption, namely: (i) O_2 consumption via cytochrome oxidase to produce H_2O, a process that accounts for more than 95% of O_2 consumption under normal conditions; and (ii) direct reduction of O_2 to $O_2^{\cdot-}$ in the flavoprotein region of the NADH dehydrogenase segment of the respiratory chain (Jezek and Hlavata, 2005). During mitochondrial electron transport, the oxygen radical is markedly enhanced in the presence of antimycin A, which blocks electron flow after ubiquinone. This results in the accumulation of reduced ubiquinone, which may undergo auto-oxidation, resulting in the production of $O_2^{\cdot-}$ (Li *et al.*, 2016). Several observations reveal ubiquinone as a major H_2O_2-generating location of the mitochondrial electron transport chain *in vitro*, and it would appear that $O_2^{\cdot-}$ is a major precursor of H_2O_2 (Winston, 1990). The mitochondrial electron transport chain is comprised of several dehydrogenase complexes that reduce a common pool of ubiquinone (Møller, 1997). The ubiquinone pool is then oxidized by either the cytochrome or the alternative pathway. In general, the main $O_2^{\cdot-}$ generators in the mitochondria are the ubiquinone radical and NADH dehydrogenases (Richter and Schweizer, 1997). Because the ETC harbors electrons with sufficient free energy to directly reduce molecular oxygen, it is considered the unavoidable primary source of mitochondrial ROS production, a necessary accompaniment to aerobic respiration. Production of ROS will increase if the rate of electrons leaving the ETC through the terminal oxidases is slowed and/or the rate of electron input increases in excess of the ability of the two respiratory pathways to process the electrons, leading to an over-reduced ubiquinone pool.

Peroxisomes

Peroxisomes are single membrane-bounded subcellular organelles with an essentially oxidative type of metabolism and a simple morphology that does not reflect the complexity of their enzymatic composition. At the beginning of the 1960s, when peroxisomes were first isolated and characterized from mammalian tissues, their main function was perceived to be the removal of toxic H_2O_2 by catalase. H_2O_2 is typically generated in the peroxisomal respiratory pathway by different flavin oxidases (see Figure 1.1). However, it has become increasingly clear that peroxisomes are involved in a range of important cellular functions in almost all eukaryotic cells. An important property of peroxisomes is their metabolic plasticity, because their enzymatic content can vary depending on the organism, cell or tissue type, and environmental conditions (Jezek and Hlavata, 2005). ROS are also generated by major metabolic pathways, especially those in the peroxisomes, and are used as a weapon against invading pathogens in the oxidative burst. There is another route by which H_2O_2 can be produced during photosynthesis (Elstner, 1982). During carbon assimilation, ribulose 1,5-bisphosphate carboxylase uses CO_2 to carboxylate ribulose 1,5-bisphosphate. However, ribulose 1,5-bisphosphate carboxylase can also use O_2 to oxygenate ribulose 1,5-bisphosphate. Oxygenation yields two glycolates, which are then transported from the chloroplasts to the peroxisomes. Therefore, glycolate oxidation is catalyzed by glycolate oxidase yielding H_2O_2. In addition, the microbodies contain fatty acid beta-oxidase and xanthine oxidase as $H_2O_2^{-}$ and $O_2^{\cdot-}$-producing enzymes, respectively. Peroxisomes are small, usually spherical microbodies

bounded by a single lipid bilayer membrane. They are subcellular organelles with an essentially oxidative type of metabolism and are probably the major sites of intracellular ROS production. Like mitochondria and chloroplasts, peroxisomes produce $O_2^{\cdot-}$ radicals as a consequence of their normal metabolism. Two sites of $O_2^{\cdot-}$ generation are established in peroxisomes (del Río *et al.*, 2002). The first is in the organelle matrix, where xanthine oxidase (XOD) catalyzes the oxidation of xanthine and hypoxanthine to uric acid (Corpas *et al.*, 2001). The second site, in the peroxisome membranes, is dependent on NAD(P)H where a small ETC is composed of a flavoprotein NADH and cytochrome *b*; here $O_2^{\cdot-}$ is produced by the peroxisome ETC. Monodehydroascorbate reductase (MDHAR) participates in $O_2^{\cdot-}$ production by peroxisome membranes.

General Mechanisms to Ameliorate the Toxic Effects of ROS

In general the ROS play dual roles, that is, positive as well as negative roles, depending upon their concentrations. In positive ways they can act as signaling molecules to activate the different signaling pathways that participate in development and growth of the plant as well as being involved in defense mechanisms; levels of ROS are maintained via the production of antioxidants in different organelles. However, when the concentration of ROS exceeds than the capacity of antioxidant system, damaging effects of ROS occur. Thus, cells had to evolve sophisticated strategies to keep the concentrations of superoxide radical, hydrogen peroxide, and other reactive oxygen species under tight control (Apel and Hirt, 2004).

Enzymatic ROS Scavenging Mechanisms

Various enzymatic antioxidants are present in certain plant cell organelles to detoxify the ROS; these include SOD, POD, CAT, APX, GST and ascorbate-glutathione cycle enzymes.

Superoxide Dismutase

Superoxide dismutase (SOD) (EC.1.15.1.1) is considered as a first line of defense and is a key enzyme in the plant's defense against oxidative damage induced by various environmental factors (Shi and Zhu, 2008; Mora *et al.*, 2009; Srivastava and Dubey, 2011) (see Table 1.1 and Figure 1.1). The SODs participate in removing the superoxide anion ($O_2^{\cdot-}$) by dismutation whereby one $O_2^{\cdot-}$ is reduced to form H_2O_2 and O_2 is formed by oxidation of another $O_2^{\cdot-}$. It decreases the formation of OH^- due to the absence of O_2^- via a metal-catalyzed Haber–Weiss-type reaction (Abouzari and Fakheri, 2015). In general, several isoforms of SOD are classified according to their subcellular localization on the basis of various metal prosthetic groups. In vascular plants there are three isoforms: Fe-SOD, conserved in chloroplast and cytosol; Mn-SODs, mainly localized in the matrix of mitochondria; and Cu/Zn-SODs, which occur in cytosol, peroxisomes, and plastids (Bowler *et al.*, 1992; Perry *et al.*, 2010). Isomers of SOD show variation in their structure: the prokaryotic Mn-SOD and Fe-SOD, and the eukaryotic Cu/Zn-SOD enzymes are dimers, whereas Mn-SOD of mitochondria is a tetramer. Among these three isozymes, in eukaryotic cells Cu/Zn-SOD comprises approximately 90% of total SOD activity (Liu, 2004). All isoforms of SOD are nuclear encoded, and targeted to their respective subcellular compartments by an amino-terminal targeting sequence. SOD activity under various abiotic stress conditions, such as drought, salinity, extreme temperature, waterlogging, and the presence of heavy metals, suggests that different mechanisms may be involved in various oxidative stress injuries (Babu and Devaraj, 2008; Karuppanapandian *et al.*, 2009; Singh *et al.*, 2011; Singh *et al.*, 2015, 2017; Tripathi *et al.*, 2017a–c).

Catalase

Catalase (CAT) (EC 1.11.1.6) is a tetrameric, heme-containing enzyme found in all aerobic organisms. Catalase activity is largely located in subcellular organelles known as peroxisomes. It converts hydrogen peroxide into water and oxygen (Weydert and Cullen, 2010). Among all antioxidative enzymes, CAT has one of the highest turnover rates: one molecule of CAT can convert around 6 million H_2O_2 molecules to H_2O and O_2 per minute, and stress conditions reduce the rate of protein turnover (Hojati *et al.*, 2010). In general, generation of H_2O_2 occurs through β-oxidation of fatty acids, photorespiration, and purine catabolism during oxidative stress in peroxisomes (Vellosillo *et al.*, 2010).

Among all H_2O_2-degrading enzymes, catalase has the unique property of degrading H_2O_2 without consuming cellular reducing equivalents. Hence, catalase provides the cell with a very energy-efficient mechanism to remove hydrogen peroxide. Therefore, when cells are stressed for energy, cells start to produce H_2O_2 through catabolic processes, and catalase degrades H_2O_2 in an energy-efficient manner (Mallick and Mohn, 2000). This should result in a net gain of reducing equivalents and therefore cellular energy. Like the D1 protein of PSII, the CAT molecule is also highly sensitive to light due to the presence of a heme group that absorbs light quanta. Various researchers have investigated the role of CAT in pathogen defense, by either overexpressing or suppressing CAT in transgenic plants (Vandenabeele *et al.*, 2004). Increase in CAT activity is supposed to be an adaptive trait possibly helping to overcome the damage to tissue metabolism by reducing toxic levels of H_2O_2.

Glutathione Peroxidase (GPX)

Peroxidases are enzymes involved in many physiological and developmental processes of plants along with pathogen infection and countering abiotic stresses. Many other functions like auxin catabolism and biosynthesis of secondary metabolites and ethylene are regulated by peroxidases (Cosio and Dunand, 2009). Glutathione peroxidase (GPX) (EC 1.11.1.9) is the general name for an enzyme family with peroxidase activity whose main biological role is to protect the organism from oxidative damage. The plant glutathione peroxidase (GPX) family consists of multiple isozymes with distinct subcellular locations and different tissue-specific expression patterns and environmental stress responses; they are named AtGPX1 to AtGPX8 in *Arabidopsis* (Passia *et al.*, 2014). The plant GPXs have lower efficiency compared to mammalian GPXs due to the presence of solenocysteine instead of cysteine (Bela *et al.*, 2015), and generally use thioredoxin as a reducing agent rather than glutathione (Navrot *et al.*, 2006). The biochemical function of glutathione peroxidase is to reduce lipid hydroperoxides to their corresponding alcohols and to reduce free hydrogen peroxide to water (Noctor *et al.*, 2002). In the stress response, they actively participate in the maintenance of H_2O_2 homeostasis by the elimination of H_2O_2 and organic hydroperoxides. This enzyme also participates in protein complexes involved in stress defense. Several reports have demonstrated that GPX genes are also regulated by the level of phytohormones in plants (Zhai *et al.*, 2013), indicating their role in plant development. During differentiation of roots the AtGPX2, 3, and 8 are overexpressed while others are repressed. AtGPX4 and 5 are involved in pollen tube growth, as evidenced by the high level of expression of these genes in stamens and pollens. The exact mechanism of plant GPXs is not yet known, but they can be regarded as more than simple antioxidant enzymes. The different expression patterns and intracellular locations of plant GPXs indicate that individual isoforms have particular functions.

The Ascorbate-glutathione Cycle Enzymes

The AsA-GSH cycle of mitochondria, chloroplasts, cytosol, and other cell organelles is one of the major antioxidant protection systems for detoxifying H_2O_2 to water at the expense of AsA. The AsA-GSH cycle comprises enzymatic as well as non-enzymatic antioxidants (Bashri and Prasad, 2016). Enzymatic antioxidants include APX, MDHAR, dehydroascorbate reductase

(DHAR), and glutathione reductase (GR), while non-enzymatic antioxidants include ascorbate and glutathione, which act as substrates for AsA-GSH cycle enzymes (Foyer and Noctor, 2011) (see Figure 1.1). APX is involved in the primary reaction, reducing H_2O_2 to water using ascorbate as the electron donor. Several isoforms of APX are present including thylakoid (tAPX) and glyoxisome membrane forms (gmAPX), as well as a chloroplast stromal soluble form (sAPX) and a cytosolic form (cAPX) (Noctor and Foyer, 1998). This enzyme has a greater affinity towards H_2O_2 detoxification than CAT and POD, hence it plays a crucial role in maintaining the ROS level inside the cell. The ascorbate has to be regenerated to maintain the activity of APX, and this is performed by MDHAR (Locato *et al.*, 2008), which yields oxidized ascorbate (monodehydroascorbate). MDHAR is present in two isoforms, chloroplastic and cytosolic, and has FAD as a cofactor. MDHAR exhibits a high specificity for monodehydroascorbate (MDHA) as the electron acceptor, preferring NADH rather than NADPH as the electron donor. Along with APX, MDHAR also scavenges H_2O_2 in mitochondria and peroxisomes (del Rio *et al.*, 2002). Being a radical, if monodehydroascorbate is not rapidly reduced, it disproportionates into ascorbate and dehydroascorbate. After this, due to involvement of DHAR, ascorbate is regenerated via the reduction of dehydroascorbate at the expense of GSH yielding oxidized glutathione (GSSG), which is crucial for tolerance to various abiotic stresses that cause the production of ROS. At the end of the AsA-GSH cycle, reduced glutathione (GSH) is formed from oxidized glutathione (GSSG) via involvement of the enzyme glutathione reductase (GR) using NADPH as electron donor. GSH is the most abundant non-protein -SH-containing metabolite and takes part in the regeneration of AsA (Foyer and Noctor, 2005). It is present in both prokaryotes and eukaryotes (Romero-Puertas *et al.*, 2006) and is a flavoprotein oxidoreductase localized in mitochondria and cytosol. It is a potential enzyme of the AsA-GSH cycle and plays an essential role in defense against ROS by sustaining the reduced status of GSH. It is predominantly localized. Thus it is concluded that non-enzymatic antioxidants: ascorbate and glutathione, are not consumed and net electron flow is from NADPH to H_2O_2. Recent studies have reported that reduction of dehydroascorbate (DHA) may be non-enzymatic or catalyzed by proteins with dehydroascorbate reductase (DHAR) activity, such as GST omega 1 or glutaredoxins (Wood *et al.*, 2003). Moreover, ascorbate and glutathione are associated with the cellular redox balance, and the ratios of AsA:DHA and GSH:GSSG may function as signals for the regulation of antioxidant mechanisms (Mittler, 2002).

Glutathione *S*-transferase

Detoxification of the xenobiotic compounds that are produced from oxidative stress, like secondary metabolites, as well as human-derived chemicals such as herbicides involve a three-phase detoxification system (Neuefeind *et al.*, 1997). The first phase reactions (oxidation, reduction, or hydrolysis) are catalyzed by cytochrome P450 monooxygenases and result in the exposure of a functional group. After this, with the help of sugars or tripeptide glutathione (GSH), these metabolites are conjugated via glutathione *S*-transferases (GSTs). GSTs are dimeric multifunctional enzymes that catalyze the conjugation of GSH to a variety of electrophilic, hydrophobic, and often toxic substrates thereby reducing their toxicity; they are present in both plant and animal cells (Dixon *et al.*, 1998). In addition to this, GSTs may also exhibit glutathione peroxidase (GPX) or isomerase activities, or function as binding proteins known as ligandins (Edwards *et al.*, 2000). It has also been found that GST overexpression also enhances plant tolerance to various abiotic stresses.

Non-enzymatic Antioxidants

Non-enzymatic antioxidative defense systems include the major cellular redox buffers like ascorbate (AsA) and glutathione (γ-glutamyl-cysteinyl-glycine, GSH) as well as tocopherols

and carotenoids. They interact with many cellular components and in addition to their essential roles in defense and as enzyme cofactors, these antioxidants affect plant growth and development by controlling processes ranging from mitosis and cell elongation to senescence and ultimately to cell death (Pinto and Gara, 2004) (see Figure 1.1). Mutants with decreased non-enzymatic antioxidants have been shown to be hypersensitive to stress (Gao and Zhang, 2008; Semchuk *et al.*, 2009).

Ascorbic Acid

Ascorbic acid is the most abundant, influential, and water-soluble antioxidant acting to prevent or reduce the damage caused by ROS in plants (Yabuta *et al.*, 2002; Wang *et al.*, 2005). It is present in almost all plant cell types, organelles, and the apoplast (Horemans *et al.*, 2000; Smirnoff, 2000). Under physiological conditions, it exists mostly in the reduced form (90% of the ascorbate pool) in chloroplasts (Smirnoff, 2000). Although ubiquitous in plant tissues, it is usually most abundant in photosynthetic cells and meristems (and some fruits). Its concentration is reported to be highest in mature leaves with fully developed chloroplasts and highest chlorophyll concentrations. It has also been reported that ascorbic acid is mostly available in reduced form in leaves and chloroplasts under normal physiological conditions. The ability of ascorbic acid to donate electrons in a wide range of enzymatic and non-enzymatic reactions makes it the main ROS-detoxifying compound in the aqueous phase. It can directly scavenge $O_2^{\cdot-}$, ·OH, and $^1O_2$, and can reduce H_2O_2 to H_2O via the APX reaction. The majority of the AsA pool in plants is contributed by D-mannose/L-galactose through what is commonly called the Smirnoff–Wheeler pathway, which proceeds via GDP-D-mannose, GDP-L-galactose, L-galactose, and L-galactono-1,4-lactone. It is also synthesized via uronic acid intermediates, such as D-galacturonic acid. In this pathway D-galacturonic acid is reduced to L-galactonic acid by galacturonic acid reductase, which is subsequently converted to L-galactono-1,4-lactone. The L-galactono-1,4-lactone is further oxidized to ascorbic acid by L-galactono-1,4-lactone dehydrogenase (GALDH). In mitochondria it is synthesized by L-galactono-γ-lactone dehydrogenase. Then it is transported to the other cell components by a proton-electrochemical gradient or through facilitated diffusion. The level of ascorbic acid under various environmental stresses depends on the balance between the rate and capacity of ascorbic acid biosynthesis and turnover related to antioxidant demand (Chaves *et al.*, 2002). Overexpression of enzymes involved in AsA biosynthesis confers abiotic stress tolerance in plants. GDP-mannose 3,5-epimerase (GME) catalyzes the conversion of GDP-D-mannose to GDP-L-galactose, an important step in the Smirnoff–Wheeler pathway of AsA biosynthesis in higher plants. Overexpression of two members of the *GME* gene family resulted in increased accumulation of ascorbate and improved tolerance to abiotic stresses in tomato plants (Zhang *et al.*, 2011). Ascorbic acid present in apoplast is believed to represent the first line of defense against external oxidants, protecting critical macromolecules from oxidative damage. It regenerates tocoperoxyl (TOC) from its radical (TOC·), which provides protection to membranes (Horemans *et al.*, 2000; Smirnoff, 2000). Thus, elevated levels of endogenous ascorbic acid in plants are necessary to combat oxidative stress in addition to regulate other plant metabolic process (Smirnoff, 2000). Plant mitochondria not only synthesize AsA by L-galactono-γ-lactone dehydrogenase but also take part in the regeneration of AsA from its oxidized forms (Szarka *et al.*, 2007). The regeneration of AsA is extremely important because fully oxidized dehydroascorbic acid has a short half-life and would be lost unless it is reduced back. In addition to the importance of AsA in the AsA-GSH cycle, it also plays an important role in preserving the activities of enzymes that contain prosthetic transition metal ions (Noctor *et al.*, 1998). The AsA redox system consists of L-ascorbic acid, MDHA, and DHA. Both oxidized forms of AsA are relatively unstable in aqueous environments, while DHA can be chemically reduced by GSH to AsA (Foyer and Halliwell, 1976).

Tocopherols

Tocopherols are lipid-soluble antioxidants and are considered as potential scavengers of ROS and lipid radicals (Holländer-Czytko, 2005). Tocopherols are considered to be major antioxidants in biomembranes, where they have both antioxidant and non-antioxidant functions. Tocopherols are considered as general antioxidants that help on the protection of membrane stability, including quenching or scavenging of ROS like $^1O_2$. In plants tocopherols are localized in the thylakoid membrane of plant chloroplasts. Relative antioxidant activity of the tocopherol isomers *in vivo* is $\alpha > \beta > \gamma > \delta$, which is due to the methylation pattern and the amount of methyl groups attached to the phenolic ring of the polar head structure. Hence, *α*-tocopherol with its three methyl substituents has the highest antioxidant activity. Tocopherols (TOCs) are synthesized only by photosynthetic organisms and are present only in green parts of plants. In higher plants, chloroplast membranes containing TOCs were also known to protect lipids and other membrane components by physically quenching and chemically reacting with O_2 in chloroplasts, thus protecting the PSII structure and function (Igamberdiev *et al.*, 2004). α-TOC is a chain-breaking antioxidant, that is, it is able to repair oxidizing radicals directly and thereby prevent the chain propagation step during lipid auto-oxidation. α-TOC reacts with RO·, ROO·, and RO* derived from polyunsaturated fatty acid (PUFA) oxidation. The reaction between α-TOC and lipid radicals occurs at the membrane-water interface, where α-TOC donates hydrogen atoms to lipid radicals, with the consequent formation of TOH·, which can be recycled back to the corresponding α-TOC by reacting with AsA or other antioxidants (Igamberdiev *et al.*, 2004). The tocopherol biosynthetic pathway utilizes two compounds as precursors, homogentisic acid (HGA) and phytyl diphosphate (PDP). At least five enzymes, 4-hydroxyphenylpyruvate dioxygenase (HPPD), homogentisate phytyl transferases (VTE2), 2-methyl-6-phytylbenzoquinol methyltransferase (VTE3), tocopherol cyclase (VTE1), and *γ*-tocopherol methyltransferase (VTE4), are involved in the biosynthesis of tocopherols, exclusive of the bypass pathway of phytyl-tail synthesis and utilization (Ahmad *et al.*, 2008). A high level of α-tocopherol has been found in the leaves of many plant species, including *Arabidopsis*. Nitration of α-tocopherol is considered to be an important mechanism for the regulation and detoxification of NOx in animal tissues. In plants also *in vivo* 5-nitro-γ-tocopherol (5-NgT) was identified in leaves of an *Arabidopsis* mutant line (vte4). Germinating seeds of *Brassica napus, Nicotiana tabacum* and *A. thaliana* also showed the presence of 5-NgT. It can be said that γ-tocopherol or 5-NgT prolongs early development by reducing NOx concentration (Desel *et al.*, 2007). Tocopherol has been shown to prevent the chain propagation step in lipid auto-oxidation, which makes it an effective free radical trap. Additionally, it has been estimated that one molecule of α-tocopherol can scavenge up to 120 $^1O_2$ molecules by resonance energy transfer (Munné-Bosch, 2005). Recently, it has been found that oxidative stress activates the expression of genes responsible for the synthesis of tocopherols in higher plants. Regeneration of the oxidized tocopherol back to its reduced form can be achieved by AsA, GSH, or coenzyme Q (Kagan, 2000). Accumulation of α-tocopherol has been shown to induce tolerance to chilling, water deficit, and salinity in different plant species (Guo *et al.*, 2004).

Carotenoids (CARs)

Plants have evolved several mechanisms to get rid of excess energy present in photosynthetic membranes. In all photosynthetic organisms, the carotenoids β-carotene and zeaxanthin and tocopherols play an important photoprotective role, either by dissipating excess excitation energy as heat or by scavenging ROS and suppressing lipid peroxidation (LPO). Carotenoids are pigments found in plants and microorganisms, and they exhibit different forms in nature. CARs are lipophilic organic compounds located in the plastids of both

photosynthetic and non-photosynthetic plant tissues. CARs have a multitude of functions in plant metabolism including a role in oxidative stress tolerance. They are also referred as antenna molecules because they absorb light in the region 450–570 nm of the visible spectrum and transfer the captured energy to the chlorophyll. In chloroplasts, CARs function as accessory pigments in light harvesting; however, perhaps a more important role is their ability to detoxify various forms of ROS. CARs can exist in a ground state or in one of two excited states after the absorption of light energy. In terms of their antioxidant properties, CARs can protect photosystems in one of four ways: (i) by reacting with lipid peroxidation (LP) products to terminate chain reactions; (ii) by scavenging $^1O_2$ and dissipating the energy as heat; (iii) by reacting with triplet chlorophyll (3Chl*) or excited chlorophyll (Chl*) molecules to prevent the formation of $^1O_2$; and (iv) by dissipating excess excitation energy through the xanthophyll cycle. The main protective role of β-carotene in photosynthetic tissue may be accomplished via direct quenching of 3Chl*, which prevents $^1O_2$ generation and thereby inhibits oxidative damage (Collins, 2001). During quenching of 3Chl*, energy is transferred from Chl to CAR, which subsequently dissipates the energy in a non-radiative form (i.e., heat). Thus, CARs act as competitive inhibitors of $^1O_2$ formation, and this is aided by their proximity to Chl in the light-harvesting complex. This method of protection is especially critical when light intensity increases above saturating levels (Collins, 2001). Another form of CAR, zeaxanthin, has been implicated in the dissipation of thermal energy, but the precise mechanism underlying this dissipation has not been resolved. Zeaxanthin appears to facilitate the conversion of 3Chl* to 1Chl* more efficiently than does β-carotene (Mortensen *et al.*, 2001).

Conclusions and Future Perspectives

Environmental stresses such as heavy metals, salinity, drought, radiation, temperature, pathogens, and so forth are the major factors that limit plant growth, development, and productivity. A consequence of the generation of ROS in plants is the loss of agricultural productivity due to impairment in the functioning of the plant's metabolism. ROS are produced due to leakage of electrons during general metabolic processes and lead to oxidative modification in nucleic acids, lipids, and proteins. On the other hand, cells can deploy several counteracting mechanisms involving enzymatic and non-enzymatic antioxidant defense systems to combat the damaging effects of ROS. Several plant cell organelles, such as chloroplasts, mitochondria, peroxisomes, and glyoxysomes, have antioxidant defense systems to protect themselves against ROS. Thus, plants have the capability to scavenge or reduce the level of ROS, and hence tolerate harsh environmental stresses/conditions.

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