Oxidative stress and antioxidative system in plants

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Free radicals and other active derivatives of oxygen are inevitable by-products of biological redox reactions. Reactive oxygen species inactivate enzymes and damage important cellular components. The increased production of toxic oxygen derivatives is considered to be a universal or common feature of stress conditions. Plant and other organisms have evolved a wide range of mechanisms to contend with this problem. The antioxidant defence system of the plant comprises a variety of antioxidant molecules and enzymes. The effects of the action of free radicals on membranes include the induction of lipid peroxidation and fatty acid de-esterification. Both ethylene biosynthesis and membrane breakdown, which appear to be closely linked, seem to involve free radicals, although the sequence of events generating these free radicals is still poorly understood. It is clear that the capacity and activity of the antioxidative defence system are important in limiting oxidative damage and in destroying active oxygen species that are produced in excess of those normally required for metabolism. Transgenic plants offered us a means by which to achieve complete understanding of the roles of the enzymes involved in protection against stress of many types, environmental and induced. Studies on transformed plants expressing increased activities of single enzymes of the antioxidative defence system indicate that it is possible to confer a degree of tolerance to stress by these means. The advent of plant transformation has placed within our grasp the possibility of engineering greater stress tolerance in plants by enhancements of the antioxidative defence system.

LIVING organisms are exposed to different kinds of stresses, which may originate from human activities or natural causes such as air pollution, drought, temperature, light intensities and nutritional limitation. Since plants have limited mechanisms of stress avoidance, they require flexible means of adaptation to changing environmental conditions. A common feature of different stress factors is their potential to increase the production of reactive oxygen species in plant tissues. Reactive oxygen species are also generated in plant cells during normal metabolic processes[1,2]. The photosynthetic electron transport system is the major source of active oxygen in plant tissues[3], having potential to generate singlet oxygen, $^1O_2$ and superoxide, O_2^-. The production of active oxygen is an unavoidable consequence of the operation of the photosynthetic electron transport chain in an oxygen atmosphere.

The major oxygen-consuming processes associated with photosynthesis are: (a) the oxygenase reaction of ribulose-1,5-bisphosphate carboxylase (Rubisco), which is the initiating reaction of the photorespiratory pathway, and (b) direct reduction of molecular oxygen by the photosystem I (PSI) electron transport chain. In addition, certain photosystem II (PSII) components are also capable of converting molecular O_2 to high-energy singlet oxygen ($^1O_2$). A cyanide-insensitive respiratory pathway in chloroplasts that competes for electrons with photosynthetic electron transport[4] may also reduce oxygen.

Oxidative stress is essentially a regulated process, the equilibrium between the oxidative and antioxidative capacities determining the fate of the plant. Under non-stressful conditions the antioxidant defence system provides adequate protection against active oxygen and free radicals[5]. Both natural and man-made stress situations provoke increased production of toxic oxygen derivatives. In response, the capacity of the antioxidative defence system is increased[6]. But in most situations the response is moderate[7]. Furthermore, some important sites such as the reaction centre protein of PSII (DI) and the apoplastic space, appear to have very little protection against oxidative damage[8,9].

Generation of toxic reactive oxygen species and associated regulatory mechanisms

Molecular oxygen is produced as a result of the oxidation of water by the photosynthetic electron transport chain. The latter, however, can also use oxygen as an electron acceptor (Figure 1). In addition, molecular oxygen is assimilated during photorespiration producing phosphoglycollate. Both of these reactions have positive and negative effects. Superoxide, produced by the transport of electrons to oxygen, is not compatible with metabolism and must be eliminated by the antioxidative defence system while recycling of phosphoglycollate to phosphoglycerate (in order to re-enter the Benson–Calvin cycle) results in a considerable loss of assimilated carbon. In addition, large amounts of H_2O_2 are

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produced during the oxidation of the glycollate in the peroxisomes. Although much of this H_2O_2 is destroyed by catalase, some chemical decarboxylation of keto acids by H_2O_2 still occurs[10,11]. Nevertheless, photosynthesis benefits since photorespiration protects the photosynthetic membrane against light-induced damage at times when carbon assimilation is limited[12,13]. This may indeed be regarded as the principal function of photorespiration, which is far more effective than electron transport to oxygen (termed pseudocyclic electron flow or the Mehler reaction) in protecting against photoinhibition[14].

Formation of singlet oxygen

The chlorophyll pigments associated with the electron transport system are the primary source of singlet oxygen ($^1O_2$; Figure 1). Singlet oxygen may also arise as a by-product of lipoxygenase activity. Like the hydroxyl radical, $OH^{\cdot}$, $^1O_2$ is highly destructive, reacting with most biological molecules at near diffusion-controlled rates[15,16]. The lifetime of excited chlorophyll singlet state is short within these aggregates, but 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, radiative decay (fluorescence) and conversion to the triplet chlorophyll state. The latter interacts with oxygen to produce $^1O_2$.

There are two strategies for defence against $^1O_2$ in the thylakoid membranes. The first is the regulation of the light-harvesting apparatus to minimize triplet chlorophyll production, and the second is the rapid quenching of both the triplet chlorophyll state and $^1O_2$ by membrane-bound quenchers. Two major processes decrease the lifetime of excited singlet-state chlorophyll; the first is photochemistry and electron transport in the reaction centres and the second process involves thermal dissipation of excess excitation energy that quenches singlet-excited chlorophyll to the ground state. Thermal energy dissipation plays a pivotal role in photoprotection since it limits the rate of reduction of the first stable electron acceptor of PSII (Q_A).

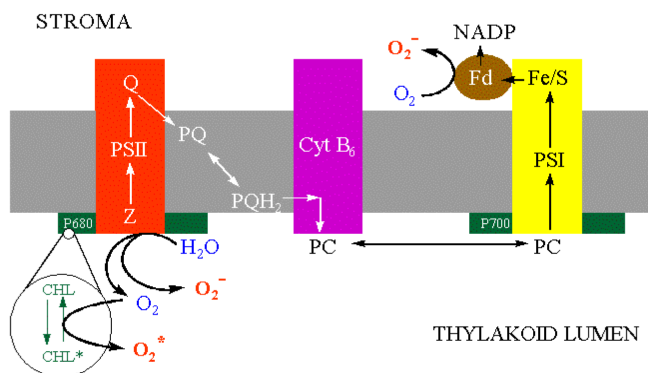


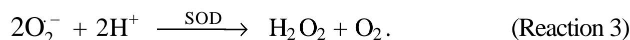
Figure 1. Production of superoxide radical and singlet oxygen in chloroplast at the site of PSI and PSII.

Superoxide production

Photo-reduction of dioxygen in 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[17]. Subsequently, in the 1970s the primary reduced product was identified as the superoxide anion radical (O_2^-). Under most circumstances, the control of electron flow between PSII and PSI regulates the reduction state of the acceptor side of PSI. This means that the redox state of PSI acceptors does not significantly limit electron transport[18]. The regulated activation of Benson–Calvin cycle and control of the rate of electron flow are important factors determining the redox state of the ferredoxin pool[19,20]. 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[5] resulting in the formation of superoxide radical O_2^- (Figure 1). There are two sites of O_2^- production on the reducing side of PSI[17,21]. 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).

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

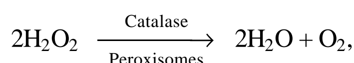
$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2, \quad \text{(Reaction 2)}$$



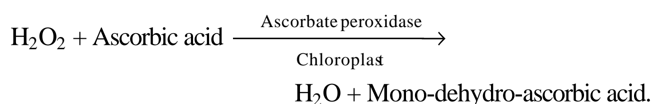
$$2O_2^- + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2. \quad \text{(Reaction 3)}$$

Production and scavenging of hydrogen peroxide in chloroplasts

Hydrogen peroxide is produced by the dismutation of superoxide radicals in a reaction mostly catalysed by superoxide dismutase (Reaction 3)[22]. 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[23,24]. 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[25].



$$2H_2O_2 \xrightarrow[\text{Peroxisomes}]{\text{Catalase}} 2H_2O + O_2,$$



$$H_2O_2 + \text{Ascorbic acid} \xrightarrow[\text{Chloroplast}]{\text{Ascorbate peroxidase}} H_2O + \text{Mono-dehydro-ascorbic acid.}$$

Superoxide generation in plant mitochondria

Several pathways of oxygen consumption are potentially available to isolated plant mitochondria or sub-mitochondrial particles[26]. These can be identified as follows:

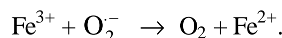
1. Oxygen consumption via cytochrome oxidase to produce water, a process which accounts for more than 95% oxygen consumption in normal, cyanide-sensitive mitochondria (Figure 2).
2. Direct reduction of oxygen to superoxide anions in the flavoprotein region of NADH dehydrogenase segment of the respiratory chain. The component responsible is likely to be the flavoprotein (of either internal or external dehydrogenase) or perhaps an iron–sulphur centre (Figure 2). The process may be identified by its insensitivity to KCN, antimycin A and salicylhydroxamic acid and by the sensitivity of the assayed epinephrine oxidation rate to superoxide dismutase.
3. Oxygen reduction to superoxide anions in the ubiquinone–cytochrome region of the respiratory chain. The process may be identified by its insensitivity to salicylhydroxamic acid and antimycin A, its sensitivity to KCN and the sensitivity of the assayed rate to superoxide dismutase. In these schemes, fully-reduced ubiquinone donates an electron to cytochrome C_1 and leaves an unstable, highly-reducing semiquinone species, which would normally reduce cytochrome b_{566}. It is presumably this unstable semiquinone or a closely interacting species which reduces the oxygen to superoxide anion, since only a species at this site would have enough reducing potential for the reaction. (The oxygen–superoxide couple has an Em at pH 7 of around – 330 mV (ref. 27).)

Hydroxyl radical: The most reactive oxidant in cells

Hydrogen peroxide and superoxide radical ($O_2^{\cdot -}$) by themselves are relatively less damaging, but they can form species damaging the essential cellular components such as hydroxyl radicals ($OH^{\cdot}$) that can initiate lipid peroxidation and also attack DNA, proteins and many small molecules. Fenton[28,29] in the late nineteenth century described the oxidizing potential of hydrogen peroxide with ferrous salts. Forty years later, Haber and Weiss[30] identified hydroxyl radical ($OH^{\cdot}$) as the oxidizing species in these reactions.

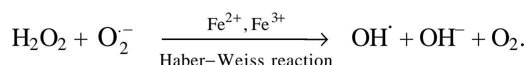
$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\cdot} + OH^{-}.$$

In biological systems availability of reduced ferrous ion may limit the reaction, but ferric ion can be recycled to reduced ferrous state by reducing agents such as $O_2^{\cdot -}$.



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

Therefore, this reaction can be summarized as:



$$H_2O_2 + O_2^{\cdot -} \xrightarrow[\text{Haber–Weiss reaction}]{Fe^{2+}, Fe^{3+}} OH^{\cdot} + OH^{-} + O_2.$$

Thus in the presence of trace amounts of iron ion, superoxide and hydrogen peroxide will form the destructive hydroxyl radical, and initiate the oxidation of organic substrates. Metal ions such as Cu^{+}, Cu^{2+} can replace Fe^{2+}, Fe^{3+} in these reactions.

Oxidation of organic substrates by hydroxyl radical

Oxidation of organic substrates may proceed by two possible reactions: (1) addition of $OH^{\cdot}$ to an organic molecule, or (2) abstraction of a hydrogen atom from it. In the addition reaction the $OH^{\cdot}$ add to organic substrate

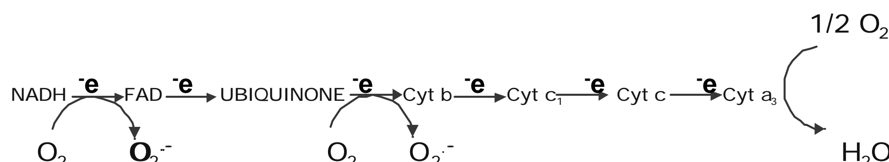
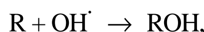
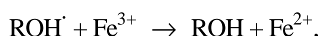


Figure 2. Sites of superoxide radical formation in mitochondrial electron transfer system.

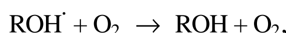
forming a hydroxylated product, which is further oxidized by Fe^{3+} ion, O_2 or other agents to a stable oxidized product. The hydroxylated product can also dismutate to form cross-linked products.



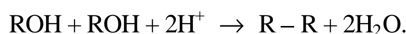
$$R + OH^{\cdot} \rightarrow ROH,$$



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

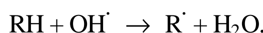


$$ROH^{\cdot} + O_2 \rightarrow ROH + O_2,$$

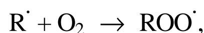


$$ROH + ROH + 2H^{+} \rightarrow R - R + 2H_2O.$$

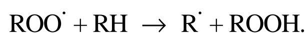
In the abstraction reaction, the $OH^{\cdot}$ radical oxidizes the organic substrate by forming water and an organic radical. The latter product has single unpaired electron and thus can react with oxygen in triplet ground state. The addition of triplet oxygen to the organic radical can lead to the formation of a peroxy-radical, which can readily abstract hydrogen from another organic molecule leading to the formation of a second organic radical. This chain reaction is far more damaging than any other reaction catalysed by reactive oxygen species.



$$RH + OH^{\cdot} \rightarrow R^{\cdot} + H_2O,$$



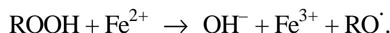
$$R^{\cdot} + O_2 \rightarrow ROO^{\cdot},$$



$$ROO^{\cdot} + RH \rightarrow R^{\cdot} + ROOH.$$

This hydrogen abstraction reaction of hydroxyl radical is best demonstrated by lipid peroxidation of linolenic acid in cell membranes[31] (Figure 3).

The lipid peroxides (ROOH) are unstable in the presence of Fe^{2+} or other reduced metal ions (such as Cu^{+}), as they participate in a Fenton reaction leading to the formation of reactive alkoxy radical.



$$ROOH + Fe^{2+} \rightarrow OH^{-} + Fe^{3+} + RO^{\cdot}.$$

This alkoxy radical is as damaging as the hydroxyl radical, thus starting a cascade of oxidative reactions.

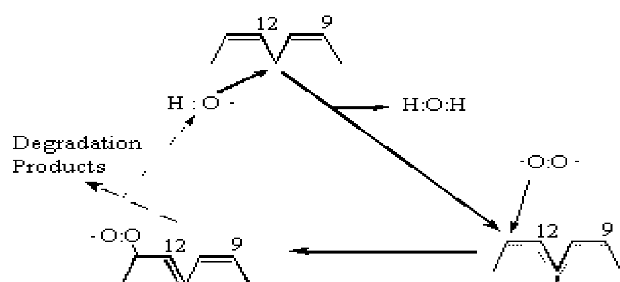


Figure 3. Hydroxyl radical-mediated peroxidation of linolenic acid in lipids.

Conditions enhancing oxidative stress

The generation of toxic O_2 species is increased under stress conditions[32]. Plants which are exposed to severe stress, have been shown to increase susceptibility to photo-inhibition with subsequent development of chlorosis[33]. Photo-oxidative damage is exacerbated by atmospheric pollutants, herbicides, heavy metals and natural compounds like cercosporin (which originates from fungi of the genus *Cercospora*).

Pollutants

Atmospheric pollutants such as ozone (O_3) and sulphur dioxide (SO_2) have been implicated in free-radical formation[34,35] and are considered to be one of the major factors influencing modern forest decline. Ozone, which originates from a natural photochemical degradation of nitrous oxide (NO_x), seems to be a greater threat to plants than SO_2 (ref. 36). Mehlhorn[34] suggested that the phyto-toxicity of O_3 is due to its oxidizing potential and the consequent formation of radicals that induce free-radical chain reactions. The O_3 concentration in the intercellular air spaces of leaves is close to zero[37]. Thus ozone is unlikely to reach the chloroplast, but it nevertheless causes pigment bleaching and lipid peroxidation[38]. Stimulation of synthesis and degradation of the PSII-DI protein occurs in spruce trees following O_3 treatment[39] and a decrease in the activity and quantity of Rubisco has been found in poplar following exposure to O_3 (ref. 40).

Exposure to SO_2 results in tissue damage and release of stress ethylene from both photosynthetic and non-photosynthetic tissues[41]. Fumigation with SO_2 causes a shift in cytoplasmic pH. The proton concentration of the cytoplasm is one of the most important factors regulating cellular activity. When cells are exposed to SO_2 an appreciable acidification of the cytoplasm occurs, because this gas reacts with water to form sulphurous acid which may then be converted into sulphuric acid[42,43]. The oxidation of sulphite to sulphate in the chloroplast also gives rise to the formation of O_2^{-} (ref. 44). The oxidation of sulphite is initiated by light and is mediated by photosynthetic electron transport. This results in loss of photosynthetic function caused by inhibition of the activity of SH-containing, light-activated enzymes of the chlorplast[45,46].

Herbicides

Several herbicides have been found to generate active oxygen species, either by direct involvement in radical production or by inhibition of biosynthetic pathways. The bipyridinium herbicides generate oxygen radicals directly in light. Compounds such as paraquat (also

known as methyl viologen) induce light-dependent oxidative damage in plants. Members of this group are called 'total-kill' herbicides[47]. The di-cationic nature of these compounds facilitates their reduction to radical cation. The PSI-mediated reduction of the paraquat di-cation results in the formation of a mono-cation radical, which then reacts with molecular oxygen to produce $O_2^{\cdot-}$ with the subsequent production of other toxic species, such as H_2O_2 and $OH^{\cdot}$ (ref. 32).

The diphenyl ethers, cyclic imides and lutidine derivatives act by inhibition of biosynthetic pathways with the subsequent accumulation of reactive, radical-forming intermediates. The mode of action of these herbicides is based on their ability to induce the abnormal accumulation of photosensitizing tetrapyrroles, specifically protoporphyrin[48]. It is somewhat anomalous that the reaction product protoporphyrin IX, accumulates in conditions where the enzyme which catalyses its formation is expected to be inhibited.

Other compounds such as diuron, that block photosynthetic electron transport and inhibitors of carotenoids biosynthesis such as norflurazon, initiate photo-oxidative processes most probably via the generation of $^1O_2$ (ref. 15). Herbicides which block photosynthesis cause increased excitation energy transfer from triplet chlorophyll to oxygen, while those which inhibit carotenoids biosynthesis eliminate important quenchers of the triplet chlorophyll and $^1O_2$.

Metals

Accumulation of phytotoxic metals results from industrial and agricultural practices. Zn, Cu and Cd are widespread pollutants resulting in stunted growth, chlorosis and necrosis[49]. Copper (Cu^{2+}) ions cause light-mediated lipid peroxidation, pigment bleaching[50] in rye and decline in endogenous catalase (CAT) level[51]. Cu^{2+} ions are redox active and catalyse Fenton-type reactions producing $OH^{\cdot}$ (ref. 32). Lipid peroxides also originate from the induction of lipoxygenase in the presence of Cu^{2+}. This enzyme is known to initiate lipid peroxidation. Cadmium treatment decreases chlorophyll and heme levels of germinating mungbean seedlings by induction of lipoxygenase with the simultaneous inhibition of the antioxidative enzymes, SOD and CAT[52]. Such inhibition results from binding of the metal to the important sulphydryl group of enzymes, which increase the phytotoxic action of metals[53]. Iron has a pivotal and dual role in free-radical chemistry in all organisms. Free Fe can participate in Fenton reactions and catalyse the generation of hydroxyl radical and other toxic oxygen species. On the other hand, Fe is a constituent of the antioxidant enzymes catalase, ascorbate peroxidase, guaiacol-peroxidase and ferro-superoxide dismutase. When plants are exposed to a variety of adverse conditions, including chilling, high light, drought and paraquat, oxidative stress ensures primarily due to the decrease in antioxidant defences but also due to the increase in free-radical production mediated by catalytic Fe[54].

Photosensitizing toxins

Natural photosensitizers induce oxidative damage in the light, and make plants sensitive to visible wavelengths of light and cause phytotoxic reactions. Perhaps the best-known fungal photosensitizer is cercosporin[55]. The fungus *Cercospora* causes worldwide destruction of important crop species, including corn, sugar beet, tobacco, coffee, soybean, and banana. Cercosporin is a red, perylenequinone secondary metabolite produced by many species within the genus. When activated by light, it reacts with oxygen to form $^1O_2$ (ref. 56). Ion leakage rapidly results due to changes in membrane composition caused by lipid peroxidation[57]. The membrane damage caused by cercosporin provides nutrients for fungal growth and sporulation in the host.

Drought

A plant's response to drought stress is a complex phenomenon that appears to involve the synthesis of polyamines and a new set of proteins whose function is largely unkown[58]. Abscisic acid is central in the response because it stimulates stomatal guard cells to close, reducing water loss. This process also reduces the availability of CO_2 for photosynthesis, which can lead to the formation of reactive oxygen species from the misdirecting of electrons in the photosystem. Hence, mechanisms that reduce oxidative stress may play an important role in drought tolerance.

In tomato, cytosolic Cu/Zn-SOD was induced strongly by drought, while the chloroplastic Cu/Zn-SOD remained largely unaffected. Glutathione reductase activity increased in drought-stressed wheat and cotton plants[59], and it was proposed that, in addition to removing H_2O_2, this increase might make NADP available that can accept electrons from ferredoxin, thereby minimizing chances of superoxide formation. In drought-tolerant *Hordeum* species, levels of glutathione reductase and ascorbate peroxidase increased, but SOD activity was not examined[60]. Drought-stressed cotton was found to be resistant to a subsequent challenge of paraquat[59], which may indicate the existence of a common protective mechanism against these stresses.

Drought-induced changes in lipid peroxidation and the activities of SOD and catalase were compared in two mosses, the drought-tolerant *Tortula ruralis* and the drought-sensitive *Cratoneuron filicinum*[61]. During stress, the drought-tolerant moss showed lower levels of

lipid peroxidation together with increased levels of both the enzymes: the opposite occurred in the sensitive moss. Glutathione metabolism was subsequently studied in the tolerant moss, and oxidized glutathione (GSSG) was found to be a good indicator of drought stress[62]. Drought-tolerant and intolerant maize inbred were analysed by Malan *et al.*[63] and resistance was found to correlate with Cu/Zn SOD and glutathione reductase activities, although higher levels of one enzyme alone apparently did not confer drought tolerance.

Sairam *et al.*[64,65] showed that H_2O_2 scavenging systems as represented by ascorbate peroxidase, glutathione reductase and catalase are more important in imparting tolerance against drought-induced oxidative stress than superoxide dismutase alone. The relative tolerance of a genotype to water stress as reflected by its comparatively lower lipid peroxidation and higher membrane stability index, chlorophyll and carotenoid contents was closely associated with its antioxidant enzyme system (SOD, APO, GR, CAT).

Free radicals in senescence and ripening

Membrane breakdown and ethylene biosynthesis, which appear to be closely linked, seem to involve free radicals[66]. *In vitro* studies have suggested that the conversion of ACC to ethylene may involve peroxidation reaction[67]. Studies performed with *Dianthus caryophyllus* indicate that senescence can be slowed by retarding peroxidation by neutralizing free radicals. Moreover, inhibition of ethylene bursts slows peroxidation and prolongs the life of cut carnations, suggesting a relationship between free radical generation and ethylene production[68].

Sylvestre *et al.*[69] showed that during petal development in cut carnation, ethylene content increases simultaneously with peroxidation and the activities of SOD and catalase decrease from the initial stage to blooming. McRae *et al.*[70] demonstrated more precisely the role of superoxide anion in this reaction. Baker *et al.*[71] reported that the vase-life of carnations was increased by the use of sodium benzoate (a free-radical scavenger) at a concentration of 10^{-3} M, and that the outburst of ethylene was inhibited. According to Mayak *et al.*[72], the microsomal membranes of carnation petals produce an increasing amount of O_2^- radicals during senescence, and this increase parallels the decrease in membrane fluidity. The addition of a free-radical scavenger, propyl gallate at a concentration of 10^{-2} M, prevents change in the fluidity of the microsomal membranes. The O_2^- anions contribute to breakdown of phospholipids and the fatty acids released may then be peroxidized[72]. This phenomenon leads to the rigidification of the membrane in senescent tissues.

The following hypothesis concerning the sequence of events (and in particular those believed to affect membrane integrity) has been put forward. Initially, a transformation of the lipids leads to membrane breakdown. Free radicals are then produced by peroxidation, and these free radicals promote the burst of ethylene. The effect of the rise in ethylene is, therefore, to accelerate the senescence. This hypothesis is in agreement with the work of Mayak and Adam[73], who suggested that ethylene synthesis requires membrane deterioration so that ACC, a polar molecule, may approach the ethylene-forming enzyme, the membrane enzyme that transforms ACC into ethylene.

Aerobic respiration, which is strongly inhibited by cyanide (CN^-) and azide (N_3^-) ions, is found to continue even when cytochrome oxidase is blocked by these inhibitors in certain organs and/or tissues, due to the existence of an alternate short branch in the electron transport pathway at the first step involving ubiquinone. This provides a means for continued oxidation of NADH and operation of the TCA cycle. The alternate pathway is highly significant in the respiratory climacteric of ripening fruit and leads to the production of hydrogen peroxide and superoxide, which in turn enhance the oxidation and breakdown of membrane, necessary activities in the ripening process. Solomos[74] pointed out that ethylene may act to implement the alternate pathway in ripening fruits. In tomato it was observed that cyanide-resistant respiration constitutes about 94% of the total respiration in ripe fruits, whereas in unripe fruits it is about 60% of the total respiration[75]. In certain cases, its involvement has been noted in raising the temperature as in ripening banana[76] and ripening mango[77]. Cyanide-resistant respiration increases during ripening of tomato fruits along with the activity of hydrolysing enzymes, suggesting that both the processes are related[78].

Free radicals and antioxidants also play a significant role during the natural senescence process. Dagmar *et al.*[79] have reported in the case of maize that early senescence of cv. X 3342 was due to the enhanced H_2O_2 production and lipid peroxidation, and lower SOD, especially Mn-SOD, AsAPOD, and CAT activity towards maturity compared to the late maturing cv. Deccan 103.

Antioxidative system of plants

Plants possess very efficient scavenging systems for reactive oxygen species that protect them from destructive oxidative reactions. These defences are not restricted to the intracellular compartment, but are also found in the apoplast to a limited extent.

Superoxide dismutase

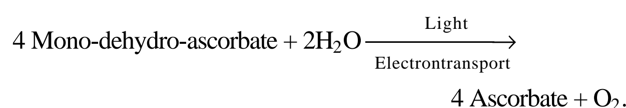
Superoxide dismutase, the family of metalo-enzymes, catalyses the disproportionation of superoxide O_2^- to

molecular oxygen and H_2O_2 (ref. 80). Superoxide dismutase removes superoxide and hence decreases the risk of hydroxyl radical formation from superoxide via the metal-catalysed Haber–Weiss-type reaction. Three isozymes of SOD, namely Mn-SOD, Cu/Zn-SOD and Fe-SOD have been reported in various plant species. Mn-SOD is predominantly found in mitochondria[81,82] and peroxisomes[83–85]. However, there are also reports of its occurrence in the soluble cytosolic fraction[86,87]. Cu/Zn-SOD initially supposed to be located in the cytosolic fraction[82,87,88], has lately also been reported from chloroplastic[82,88,89] and mitochondrial fractions[88,90]. Similarly, Fe-SOD though predominantly detected in chloroplasts[89], has also been reported from cytosolic[86], mitochondrial[91,92] and peroxisomal[92] fractions. It will thus seem that almost all of the isozymes of SOD have been detected in most of the cellular components.

Ascorbic acid and ascorbate peroxidase

Ascorbate is present in chloroplasts, cytosol, vacuole and apoplastic space of leaf cells in high concentrations[93,94]. It is perhaps the most important antioxidant in plants, with a fundamental role in the removal of hydrogen peroxide[95]. Oxidation of ascorbate occurs in two sequential steps, first producing mono-dehydro-ascorbate, and if not rapidly re-reduced to ascorbate, the mono-dehydro-ascorbate disproportionates to ascorbate and dehydro-ascorbate (Figure 4).

Ascorbate peroxidase activity has mainly been reported from chloroplast and cytosol[96]. However some recent studies have also reported its occurrence in mitochondria as well[89,97]. In the chloroplasts, SOD and ascorbate peroxidase enzymes exist in both soluble and thylakoid-bound forms. Superoxide generated at the membrane surface can thus be trapped and converted immediately to H_2O_2 to be scavenged by the membrane-bound ascorbate peroxidase[98–100]. Isolated intact chloroplasts rapidly metabolize exogenously added H_2O_2 (refs 98–100), indicating that *in situ* the chloroplasts may eliminate H_2O_2 generated both internally and externally. Two enzymes are involved in the regeneration of reduced ascorbate, namely mono-dehydro-ascorbate reductase (E.C. 1.6.5.4) which uses NAD(P)H directly to recycle ascorbate (Figure 4) and dehydro-ascorbate reductase. However, the situation is further complicated because mono-dehydro-ascorbate itself is an efficient electron acceptor[101,102]. Mono-dehydro-ascorbate is reduced directly to ascorbate using electrons derived from the photosynthetic electron transport chain as follows:



$$4\ \text{Mono-dehydro-ascorbate} + 2H_2O \xrightarrow[\text{Electrontransport}]{\text{Light}} 4\ \text{Ascorbate} + O_2.$$

The regeneration of ascorbate within the chloroplast provides a putative mechanism for the regulation of electron transport[98]. Ascorbate is not only a potent antioxidant, but is implicated in the pH-mediated modulation of PSII activity and its down-regulation associated with zeaxanthin formation[103]. This is a potent mechanism for preventing photo-oxidation. The Mahler-peroxidase reaction sequence helps to generate the low lumen pH values required for the formation of zeaxanthin[104,105]. This xanthophyll pigment has been consistently shown to be involved in the mechanisms of thermal energy dissipation[106].

Glutathione and glutathione reductase

Glutathione, glutamyl cysteinyl glycine (GSH) is the major low molecular weight thiol compound in most

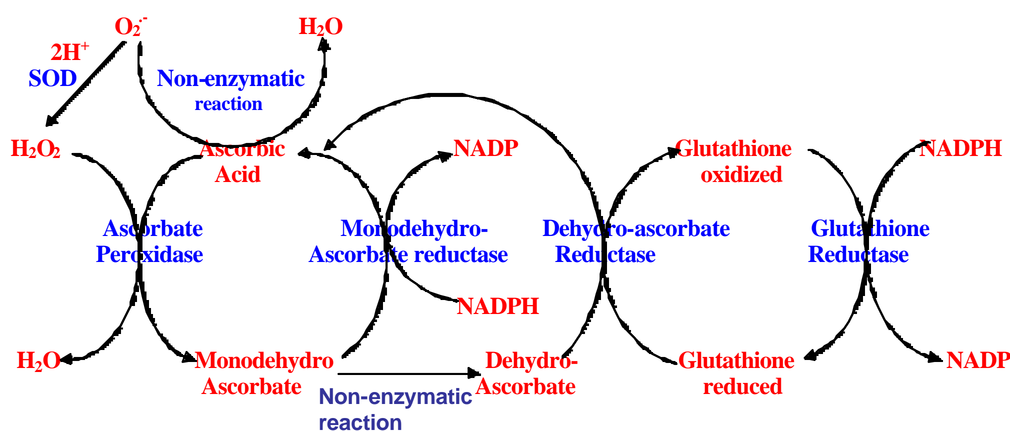


Figure 4. Asada–Halliwell pathway of hydrogen peroxide scavenging and ascorbic acid regeneration involving various antioxidant enzymes.

plants[107,108]. Glutathione acts as disulphide reductant to protect thiol groups on enzymes, regenerate ascorbate and react with singlet oxygen and hydroxyl radicals. In some plants, such as legumes, homoglutathione (glutamyl cysteinyl alanine) may partly or wholly replace glutathione[109]. It acts as a protein disulphide reductant, which detoxifies herbicides by conjugation, either spontaneously or by the activity of one of a number of glutathione-S-transferases, and regulates gene expression in response to environmental stress and pathogen attack[107,110–112]. It also participates in the regeneration of ascorbate from dehydro-ascorbate via the enzyme dehydro-ascorbate reductase (E.C. 7.8.5.1; Figure 4). In such reactions GSH is oxidized to glutathione disulphide (GSSG). GSH is regenerated by glutathione reductase (GR) in a NADPH-dependent reaction (Figure 4). Creissen *et al.*[113] have reported that GR is located in chloroplastic, mitochondrial and cytosolic fractions of maize. Recent studies have further confirmed its existence in chloroplast, mitochondrial and cytosolic fractions[87,89].

$$\text{Dehydro-ascorbate} + 2\ \text{GSH} \xrightarrow{\text{DHAR}} \text{Ascorbate} + \text{GSSG},$$

$$\text{GSSG} + \text{NADPH} \xrightarrow{\text{GR}} 2\ \text{GSH} + \text{NADP}.$$

α-Tocopherol and carotenoids

The membrane-associated antioxidant, α-tocopherol, scavenges singlet oxygen and lipid peroxides. Ascorbate and α-tocopherol are extremely effective antioxidants because they are relatively poor electron donors in physiological conditions and act primarily by transfer of single hydrogen atoms[114]. Ascorbic acid and α-tocopherol can scavenge hydroxyl radicals, singlet oxygen and superoxide radical.

Carotenoids quench singlet oxygen and also protect by absorbing excess excitation energy from chlorophyll by direct transfer[103–106].

Protection against oxygen radicals – The molecular approach

The availability of null mutants in bacteria allows relatively simple complementation with genes of interest. Therefore, our current knowledge about the physiological function of antioxidative enzymes has been significantly influenced by the results obtained in well-characterized bacterial null mutants.

Among the most intensively studied are the null mutants for Mn-SOD and Fe-SOD (*Sod A* and *Sod B* genes), glutathione reductase (*gor* genes) and the two different forms of catalase (*kat G* and *kat E* genes). Cells carrying mutations in *Sod A* or *Sod B* genes lacked detectable Mn-SOD or Fe-SOD activity, respectively[115]. In minimal medium, aerobic growth was affected by methyl viologen, a bipyridly herbicide that exacerbates O_2^- production. The *Sod A* mutant, lacking Mn-SOD activity, was especially sensitive to methyl viologen treatment, although neither single mutation affected aerobic cell growth. *Sod A–Sod B* double mutants were unable to grow on minimal medium under aerobic conditions. These double mutants demonstrate how harmful superoxide anion formation is for bacterial cells. In addition, the double mutant was killed by exposure to H_2O_2 and had a much higher spontaneous mutation frequency in the presence of oxygen than wild type cells with normal SOD activity[116].

In contrast to the harmful effect of the double mutant, reports of the consequences of increased SOD activities by complementation in null mutants and SOD over expression are very inconsistent. Overproduction of plasmid encoding Mn-SOD led to increased sensitivity of *E. coli* cells to methyl viologen[117]. Increased H_2O_2 production might be responsible for this observed increase in sensitivity. Hopkins *et al.*[118] have reported that Mn-SOD and Fe-SOD are not functionally equivalent and that they have different antioxidant roles in *E. coli.* Mn-SOD was more effective than Fe-SOD in preventing DNA damage, while Fe-SOD excelled in protecting a cytoplasmic superoxide-sensitive enzyme.

Only one form of GR has been identified in *E. coli.* Both the GR enzyme proteins and the *gor* gene from *E. coli* have been characterized in detail[119]. Barbado *et al.*[120] demonstrated that a bacterial mutant deficient in GR activity had increased sensitivity to H_2O_2 relative to a catalase-deficient parental strain. The GR-efficient mutant expressing the *gor* gene had a greatly increased GR activity, which was directly related to an increased glutathione content. However, overproduction of GR could not replace SOD in a SOD-deficient mutant, and GR was less effective than Fe-SOD in protecting against methyl viologen toxicity[121].

Catalase prevents oxidative damage to DNA during aerobic growth. *E. coli* produces two distinct forms of catalase, a bifunctional catalase–peroxidase (hydroperoxidase I, HP-I) and a monofunctional catalase (HP-II). However, studies on the physiological role of these catalases in oxidative stress are complicated because the two catalases are genetically controlled by three loci: *kat E*, *kat F* and *kat G* (ref. 122). The genes *kat G* and *Kat E* encode HP I and HP II, respectively and do not appear to be linked. Analysis of the amino acid sequence of the *Kat G* product has shown that it is similar to peroxidase[123], but bears no resemblance to any of the known catalase sequences[124]. The third gene, *kat F*, is required for expression of *kat E*, the *kat F* product appears to be a positive regulator of *kat* E (ref. 125).

Use of native plant gene

The isolation of native plant genes provides an opportunity either to over express the native genes in order to increase enzyme activity or to produce enzyme-deficient plants using antisense technology.

Superoxide dismutase

Several cDNAs that encode either chloroplastic or cytosolic plant Cu/Zn-SOD have been isolated. The cDNAs for chloroplastic Cu/Zn-SOD from tomato[126], pea[127] and petunia[128] and for cytosolic Cu/Zn-SOD of maize[129], pea[130] and *Nicotiana plumbaginifolia*[131] have been isolated. The cDNAs for Mn-SOD and Fe-SOD, which are located in the plant mitochondria and the chloroplast, respectively have also been isolated and cloned from *N. plumbaginifolia*[81] and *Arabidopsis thaliana*[132]. Over expression of native forms of SOD in transgenic plants has been achieved in several laboratories, with apparently conflicting results.

Teppermann and Dunsmuir[133] produced transgenic tobacco plants expressing the Cu/Zn-SOD from petunia in addition to the native forms of the enzyme. The transgenic tobacco leaf discs expressed 30 to 50-fold more Cu/Zn-SOD than controls. However, they were not protected against methyl-viologen-mediated inhibition of $^{14}CO_2$ assimilation nor chlorophyll bleaching during photo-inhibitory conditions[130] nor were they protected against ozone toxicity[134]. These authors suggested that elevating SOD alone could not protect against oxygen toxicity and that it would be necessary to increase simultaneously the enzymes involved in H_2O_2 detoxification. In contrast, high level of overproduction of Mn-SOD leading to protection against methyl viologen has also been reported[135,136].

Glutathione reductase

In comparison to SOD, GR has received little attention. The genetic relationship between pea GR and that from other organisms has been studied[137]. In addition, the native pea GR gene introduced into transgenic tobacco plants[137], and GR-deficient plants, produced by antisense technology, is also being studied.

Ascorbate peroxidase

Ascorbate peroxidase has been purified and characterized from spinach[138], tea[96] and pea leaves[139]. AsAPOD is a heme-protein more similar in primary structure to yeast cytochrome-C POD than to the guaiacol peroxidase, such as horseradish peroxidase[140]. AsAPOD exists in both soluble cytosolic and chloroplastic forms. The chloroplastic form can be distinguished from the cytosolic form by its labiality in the absence of ascorbate, its high specificity for ascorbate and its narrow pH optimum. Four discrete bands of AsAPOD activity can be separated from poplar leaves by activity staining following non-denaturing PAGE. Anion-exchange chromatography has been used to purify AsAPOD, and two isoforms (AsAPOD I and II) have been detected, isoform I is a plastidic protein, while isoform II cytosolic.

Glutathione peroxidase

Glutathione peroxidase activity is absent in leaf extracts. Jablonski and Anderson[141] showed that H_2O_2-dependent oxidation of glutathione could be measured in pea shoot extracts, but this involved more than one protein. Selenium-independent glutathione peroxidase has recently been identified in higher plants, but they are not normally expressed in leaves or roots. A cDNA library from freshly isolated *Nicotiana sylvestris* protoplasts was screened using cDNA from mesophyll cells, stressed leaf strips and cell suspension cultures[142]. A clone with homology to mammalian selenium-dependent glutathione peroxidase (GPOD) was isolated. Selenium-dependent GPOD enzymes are largely absent from higher plants, but selenium-independent GPOD of unknown function are induced under stress conditions[142]. There is no clear evidence from biochemical studies that these GPOD-like enzymes have activity *in situ* and their function is unknown.

Peroxidase

A gene encoding an anionic tobacco peroxidase (POD) has been expressed in both transgenic tobacco[143] and transgenic tomato[144] under the control of the CaMV 35S promoter. Several physiological processes were dramatically affected by POD over-production, and severe wilting was found in transgenic plants.

Catalase

Peroxisomes contain large amounts of catalase, but its properties suggest that the enzyme is inefficient in removing low concentrations of H_2O_2. Catalase-deficient mutants and cDNA for different catalase genes have been isolated and characterized[80]. A barley mutant largely deficient in catalase but with an increased glutathione content, was unable to survive under photorespiratory conditions, but grew well in a high CO_2 atmosphere[145]. In contrast, a maize mutant deficient in two of the catalase isoforms grew well in air and resembled the wild-type in phenotype[80,146]. Photorespiratory CO_2 loss was decreased by 10–20% in a high catalase

mutant of *N. tabacum* var. Havana, with a 40% higher catalase activity than the wild type[147]. This decrease in photorespiration was considered to result from inhibition of the chemical decarboxylation of α-keto acids by peroxisomal H_2O_2. This interpretation implies that introduction of cloned catalase genes into C_3 plants may offer an opportunity to decrease photorespiratory carbon loss, since it appears that endogenous catalase at normal levels is too low to compete effectively with keto acids for peroxisomal H_2O_2.

Conclusions and perspectives

Although oxidative stress is potentially a lethal situation, it is also clear that plant systems exploit the interaction with oxygen. The production and destruction of active oxygen species is intimately involved with processes such as the hypersensitive responses and the regulation of photosynthetic electron flow. The activity of the antioxidative defence system must be equal to the task of destruction of reactive oxygen species in normal metabolism and at times when the plant suffers stress. However, the antioxidative defence system of plants is quite limited in its capacity to respond to stress, the activities of component enzymes or the antioxidant levels usually only double in response to many stress situations. This rather moderate response might be understood if we consider that the system is geared to self-destruction when it comes under threat.

We must consider that antioxidants are not always accessible to some of the sites where they are most needed in times of stress. Examples of this are the limited availability of ascorbate in the apoplastic space during attack by the pollutant ozone[9], the very poor diffusion of ascorbate across the thylakoid membrane that provides ascorbate for the violaxanthin deepoxidase reaction[19,103] and the absence of effective antioxidants at the PSII reaction centre to prevent the oxidative damage associated with photo-inhibition.

Studies carried out by many research groups with prokaryotic and eukaryotic systems have considerably supplemented previous pure physiological or biochemical approaches. Genetic engineering also offer advantages in terms of the study of the physiological roles of enzymes where a classical genetic approach, such as selection of enzyme-deficient mutants, is difficult or almost impossible to carry out. In plant systems, the situation is often considerably complicated by the presence of a large number of isoenzyme forms, for example, the large GR and SOD families of isoenzymes, encoded by different genes. In the future, however, the use of antisense technology combined with selection of specific cDNA clones for isoenzymes may facilitate investigation of such enzyme-deficient mutants.

The ability to generate transgenic plants has provided a powerful tool with which to increase our present understanding of the antioxidative defence network. This work extends and compliments similar research work on prokaryotic systems. From the data accumulated thus far, it is clear that an appropriate physiological balance of all the components of the antioxidative defences is necessary in order to obtain increases in stress tolerance. Current observations suggest that increasing the level of stress tolerance by reinforcing the plant's defence system with new genes is an attainable goal. In future, better appreciation of control of the expression of the native genes, increasing the activities of the enzymes of the antioxidant systems by manipulation of the regulatory processes controlling their expression, may provide an additional means of improvement.

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