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1 Oxidative Stress: An Introduction

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MOLECULAR OXYGEN AND ITS REACTIVE DERIVATIVES

Atomic oxygen is the most abundant element in the earth's crust; molecular oxygen in the atmosphere and water is required to support all forms of aerobic life. The present oxygen reservoir (37 Emol, 1 Emol = 10^{18} moles) has built up as a result of photosynthesis, a process that liberates dioxygen from water. It is kept approximately constant by respiration, in which O_2 is used as the ultimate electron acceptor. In addition, oxygen atoms are ''fixed'' into various organic molecules by a variety of enzymes (e.g. oxygenases) and non-enzymatic processes (Gilbert, 1981; Elstner, 1982, 1987). Aerobic organisms must, however, cope with the adverse effects of oxygen. At higher-than-atmospheric concentrations, dioxygen may inhibit or inactivate certain enzymes and it also competes with photosynthetic CO_2 fixation by ribulose-1,5-bisphosphate carboxylase/oxygenase, increasing the energetic cost of photosynthesis. Still, the toxic effect of oxygen is mainly exerted by its reactive derivatives, whereas ground-state dioxygen is rather unreactive and can peacefully co-exist with organic matter. This characteristic is explained by the parallel spins of two unpaired electrons of dioxygen, imposing an energetic barrier on its reaction with non-radical compounds (the ''spin restriction''). In order to become chemically reactive, dioxygen must be physically or chemically activated (Table 1).

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Physical activation occurs mainly by transfer of excitation energy from a photo-activated pigment such as an excited chlorophyll molecule to dioxygen. The latter absorbs sufficient energy and, as a result, the spin of one electron is inverted. The first *singlet state of oxygen* (designated $^1O_2$ or $^1\Delta gO_2$) is a prevalent reactive species. It is highly diffusible and capable of reacting with organic molecules (whose electrons are usually paired), and damaging photosynthetic membranes.

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Chemical activation is the other mechanism to circumvent spin restriction. It occurs by univalent reduction of dioxygen, i.e. addition of electrons one by one. Four electrons (and four protons) are required for the full reduction of dioxygen to water; all three intermediates of univalent reduction, namely superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($OH^{\bullet}$), are chemically reactive and biologically toxic (Elstner, 1987; Hamilton, 1991; McKersie and Leshem, 1994; Yu, 1994). This toxicity is reflected by their short half-lives before reacting with cellular components, as compared to that of dioxygen (>100 sec; Table 1). Reactive oxygen species colliding with an organic molecule may extract an electron from it, rendering it a radical capable of propagating a chain reaction, e.g. the peroxyl ($ROO^{\bullet}$) and alkoxyl ($RO^{\bullet}$) radicals.

Superoxide is the first reduction product of ground state-oxygen, capable of both oxidation and reduction. It may react to produce several other reactive species, and may undergo spontaneous or enzymatic dismutation to H_2O_2.

Hydrogen peroxide is not a free radical, but participates as oxidant or reductant in many cellular reactions. Unlike superoxide, H_2O_2 is highly diffusible through membranes and aqueous compartments and it may directly inactivate sensitive enzymes at a low

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Table 1. Formation and characteristics (compared to molecular oxygen) of major reactive oxygen species

Reaction		*ΔG kcal/mole*	*Reactive species*	*Half-life (37°), sec*
Ground state oxygen			dioxygen biradical (•O-O•)	>100
Physical activation				
Chlorophyll* + O_2	→ chlorophyll + $^1O_2$	+ 22.0	singlet oxygen (O-O:)	1×10^{-6}
Chemical activation				
$O_2 + e^-$	→ $O_2^{\bullet -}$	+7.6	superoxide radical (•O-O:)	1×10^{-6}
$O_2^{\bullet -} + e^- + 2H^+$	→ H_2O_2	–21.7	hydrogen peroxide (H:O-:H)	
$H_2O_2 + e^- + H^+$	→ HO + H_2O	–8.8	hydroxyl radical (H:O)	1×10^{-9}

concentration. Much like superoxide, H_2O_2 is rather stable and therefore less toxic than other reactive oxygen species; the main threat imposed by both superoxide and H_2O_2 lies in their ability to generate highly reactive hydroxyl radicals.

The *hydroxyl radical* is the most powerful oxidizing species in biological systems. It will react non-specifically with any biological molecule, and this will limit its diffusion within the cell to a distance of two molecular diameters from its site of production. No specific scavengers of $OH^{\bullet}$ are known, although several metabolites, such as urea or glucose, were proposed as hydroxyl scavengers in animal systems. Recently, a role for $OH^{\bullet}$ in cell wall polysaccharide metabolism has been proposed (Fry, 1998).

The different reactive species described above will cause, to varying extents, (i) inhibition of sensitive enzymes (some specific examples are discussed below), (ii) chlorophyll degradation or ''bleaching'', (iii) lipid peroxidation; free radicals, H_2O_2, and singlet oxygen readily attack unsaturated fatty acids, yielding lipid hydroperoxides, and, in the presence of metal catalysts, alkoxyl and peroxyl radicals that propagate chain reactions in the membranes, changing and disrupting lipid structure and membrane organization and integrity (Yu, 1994). In addition, some aldehydes and hydrocarbons produced by lipid peroxidation exert cytotoxic effects in animal systems (Esterbauer *et al.,* 1990). (iv) Indiscriminate attack by hydroxyl radicals of organic molecules, including DNA. A variety of oxidatively altered DNA species can be identified following $OH^{\bullet}$ attack, including base alterations and strand breaks that may be difficult to repair or tolerate (Kasai *et al.,* 1986). Proteins exposed to $OH^{\bullet}$ undergo typical modifications, including specific amino acid alterations, polypeptide fragmentation, aggregation, denaturation, and susceptibility to proteolysis (Wolff *et al.,* 1986).

BIOLOGICAL SOURCES OF REACTIVE OXYGEN

It is well-established that the formation of reactive oxygen species accompanies normal metabolic processes in all aerobic organisms. We will describe in some detail the source of different reactive species in plant cells. Figure 1 illustrates many of the physiological pathways that are discussed along the chapter. However, many pioneer discoveries on oxygen radicals were made with a facultative aerobe, *Escherichia coli* (Fridovich, 1991). In aerobically grown *E. coli*, $O_2^{\bullet -}$ is produced by reduction of dioxygen during membrane-associated electron transport. Only 0.04% of the electrons ''leak'', but a mechanism to prevent superoxide accumulation is required. Superoxide levels were measured using

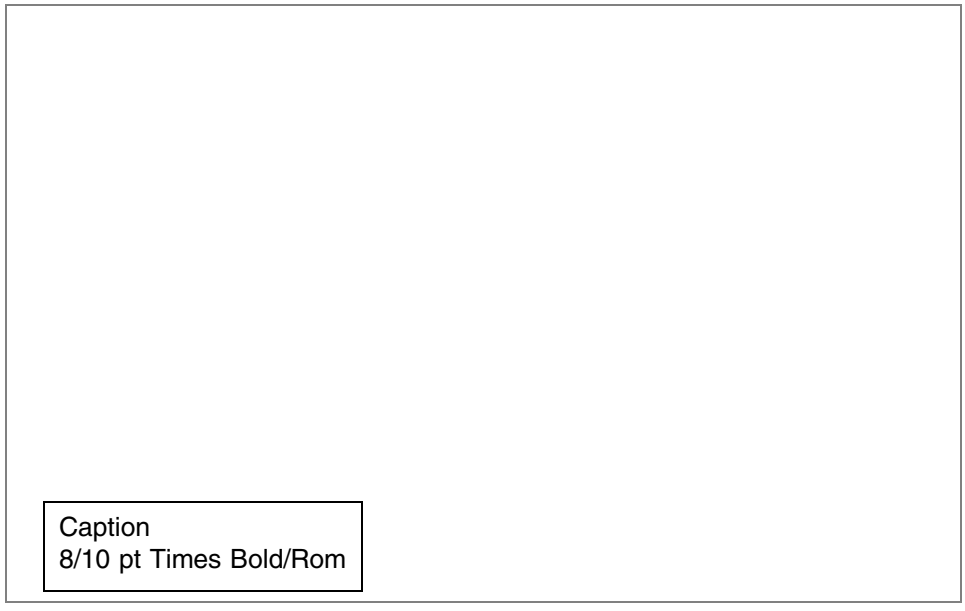

Figure 1. Biochemical chart of the main physiological processes involving reactive oxygen species in the chloroplast.

E. coli membrane preparations of mutant strains devoid of superoxide dismutase (*sodA, sodB*) (Carlioz and Touati, 1986; Imlay and Fridovich, 1991). Such mutants exhibit slow growth and several auxotrophies, demonstrating the vital role of SOD in decreasing steady state levels of $O_2^{\bullet -}$ down to 2×10^{-10} M. It is interesting to note that one of the most superoxide-sensitive enzymes in this system is *E. coli* aconitase of the citric acid cycle (Gardner and Fridovich, 1991). The reversible oxidative inactivation of aconitase by $O_2^{\bullet -}$ has been suggested to fulfill a defensive role of ''circuit breaking'' to cut off NADPH production, thus avoiding further build-up of superoxide by respiration. Examples of metabolic inactivation/deviation pathways used to avoid reducing conditions in the photosynthetic apparatus are discussed below.

Reactive Oxygen Formation in Plant Chloroplasts

Chloroplasts are the major source of reactive oxygen forms in plants: they harvest light energy at high efficiency, produce reducing equivalents, such as NADPH, and generate fluxes of dioxygen: indeed the most ''radical-prone'' conditions one could imagine. Several independent pathways, or sites of oxygen activation, have been described in chloroplasts, leading to the production of all of the above reactive species (Elstner, 1982; 1991; Asada, 1994; Foyer and Harbinson, 1994). The most important is the reducing side of photosystem I (PSI), where an electron may be passed from a membrane-bound carrier to O_2 (the ''Mehler reaction''), instead of flowing to downstream carriers that finally reduce $NADP^+$ (Figure 1). Under conditions that limit the availability of electron acceptors from PSI, i.e., when the Calvin cycle does not consume NADPH rapidly enough,

superoxide will form within the membrane. At low pH, superoxide may spontaneously dismutate to the more diffusible H_2O_2; otherwise it may interact with plastocyanine or cytochrome f and reduce them, resulting in a superoxide-mediated cyclic electron flow around PSI (Hormann *et al.,* 1993). This mechanism actually suggests a regulatory role for superoxide production, namely to divert, or cycle, excessive flow of electrons and, at the same time, prevent the diffusion of radicals away from the membrane.

Hydrogen peroxide is mostly produced in chloroplasts by disproportionation of superoxide by SOD, which is much faster than spontaneous dismutation. Another source of H_2O_2 is photorespiration that is initiated by the oxygenase activity of ribulose-1,5-bisphosphate carboxylase in the chloroplast stroma, followed by the production of H_2O_2 in the peroxisomes (Figure 1). Photorespiration may be interpreted as a protective mechanism that recycles electron acceptors and allows photosynthetic electron flow to continue under conditions of low carbon fixation. Compared to electron-cycling around PSI (discussed above as a possible protective pathway), the photorespiratory cycle would dissipate both ATP and NADPH (Wu *et al.,* 1991). An important mechanism of regulation that couples carbon fixation in the stroma to photosynthetic electron flow, is the reversible inactivation of Calvin cycle enzymes when electron carriers of the light reaction are oxidized, and their re-activation when the carriers are reduced. Such regulation is mediated by thioredoxin, stromal pH, and other factors (Figure 1; Foyer *et al.,* 1992). Hydrogen peroxide will disrupt this delicate mechanism by oxidizing thiol groups and inactivating the Calvin cycle enzymes irreversibly; it must therefore be kept below micromolar concentrations in chloroplasts (Foyer and Harbinson, 1994). It will also inactivate copper/zinc (Cu/Zn) SOD.

Hydroxyl radicals may be formed in all living cells in a reaction catalyzed by the transition metal ions, iron and copper, when both superoxide and H_2O_2 are present (Halliwell and Gutteridge, 1992):

(1) the Fenton reaction, resulting in the production of $OH^\bullet$ from H_2O_2:

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

(2) recycling of ferrous ion by superoxide, which acts as a reductant, allowing reaction (1) to continue:

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

(3) the net sum of reactions (1) and (2) is the so-called Haber-Weiss reaction:

$$H_2O_2 + O_2^{\bullet -} \rightarrow O_2 + OH^\bullet + OH^-$$

Because a common source of $H_2O_2$is dismutation of $O_2^{\bullet -}$, SOD is both a scavenger and a source of reactive species. The main danger of superoxide and hydrogen peroxide is indirect, and occurs when they are allowed to accumulate in the same cellular site, and that iron and copper metabolism are intimately connected with oxidative stress (see Chapter 7, this volume). According to a somewhat challenging report by Yim *et al.* (1990), hydroxyl radicals are liberated to the solution by the Cu/ZnSOD enzyme itself that reacts with its hydrogen peroxide product; the $OH^\bullet$ radical may then be the direct cause underlying the well known phenomenon of Cu/ZnSOD inactivation by H_2O_2.

Singlet oxygen is formed in chloroplasts when photo-excited chlorophyll in the triplet state reacts with dioxygen. Again, rates are higher when ATP and NADPH utilization by the Calvin cycle reactions is low. Many stress factors that limit CO_2 assimilation (e.g. those that cause stomatal closure) may enhance the process. Surplus excitation energy must therefore be dissipated, for instance via fluorescence or quenching by carotenoid pigments. In addition, singlet oxygen may be produced by certain plant peroxidases.

Reactive Oxygen Produced in Other Cellular Compartments

Mitochondria

Mitochondria consume oxygen during respiratory electron transport. Different sites of electron leakage and release of superoxide and hydrogen peroxide in respiration have been proposed. One of the sites is specific to plant mitochondria, namely the cyanide-insensitive alternative oxidase (Rich and Bonner, 1978; Elstner, 1991; McKersie and Leshem, 1994).

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Endoplasmic reticulum, peroxisomes, and glyoxysomes

The smooth endoplasmic reticulum and the microsomes derived from it harbour various oxidative processes. Mixed-function oxygenases, such as cytochrome P450, perform important hydroxylation reactions in the mevalonic acid pathway, adding oxygen atoms to substrate molecules. NAD(P)H is the electron donor and superoxide may be released by such reactions. Peroxisomes and glyoxysomes are single membrane organelles that compartmentalize enzymes involved in the β-oxidation of fatty acids, and the C2 photorespiratory cycle, where glycolate oxidase transfers electrons from glycolate to oxygen and produces H_2O_2 (Lindqvist *et al.,* 1991). Xanthine oxidase, urate oxidase, and NADH oxidase generate superoxide (Elster, 1991; McKersie and Leshem, 1994).

Plasma membrane and the appoplast compartment

NAD(P)H oxidases are ubiquitous components of plasma membranes and may produce superoxide and H_2O_2 (Vianello and Macri, 1991). Oxygen activation occurs also in the apoplast: being the first site of pathogen invasion, it contains a first line of plant defense reactions and these involve reactive oxygen (see below). The most common biosynthetic pathway in the apoplast is lignin biosynthesis, where phenypropanoid precursors of lignin are cross-linked by H_2O_2 in reactions initiated by peroxidases (Gross, 1980). The required NADH is generated by a cell wall malate dehydrogenase, and then used to form H_2O_2, possibly by an NADH oxidase. Amine oxidases produce activated oxygen in the cell wall by using diamines or polyamines to reduce a quinone with concomitant formation of peroxides (Vianello and Macri, 1991; Elstner, 1991; McKersie and Leshem, 1994).

DEFENSE AGAINST OXIDATIVE STRESS

Before discussing specific components that protect plants from reactive oxygen, we should note that the exquisitely sophisticated mechanisms that regulate electron transport and

photosynthesis in general, are, in fact, the primary defense against oxidative stress. These mechanisms are responsible for the fine coupling of the light and dark reactions of photosynthesis and for the adaptation of the light-harvesting apparatus to changing conditions. Overexcitation and overreduction of the photosynthetic apparatus are thus avoided, and the formation of reactive oxygen is reduced to a minimum.

Nevertheless, we know that a trickle of active oxygen species is constantly produced in all cellular compartments as a byproduct of normal cellular metabolism and that cell survival will depend upon adequate protection. All aerobic forms of life have evolved multiple defense lines, that include both scavenging enzymes and non-enzymatic antioxidants. Such multiplicity is required because reactive oxygen species (ROS) are produced in different cellular and extracellular compartments, and because reactive species differ in properties such as diffusibility, solubility, and propensity to react with various biological molecules. We thus need a correspondingly diverse set of defense molecules to act in both aqueous and membranal phases, in all cellular compartments, to promptly inactivate radicals as soon as they are formed.

A sequence of detoxification steps is often required to avoid the conversion of one reactive species into a second, more harmful one. The most notable example is the conversion of $O_2^{\bullet -}$ to H_2O_2 by SOD; an insufficience in the next step, the H_2O_2 detoxification, would lead to H_2O_2 accumulation, inactivation of SOD, and formation of $OH^{\bullet}$ radicals. This also implies that multistep defense systems, if tilted out of balance, may collapse and get out of control, e.g. under extreme stress situations, as well as in aging, cancer, and degenerative syndromes. The coordination of the multiple defense components into one integrated and efficient network is not well understood, and constitutes a challenge to both plant and animal oxidative stress researchers. An outline of the defense systems found in plants (with some reference to non-plant systems) is given below.

Superoxide Dismutase

Superoxide dismutases (SOD) are metalloenzymes first discovered by McCord and Fridovich (1969) that convert $O_2^{\bullet -}$ to H_2O_2 in all aerobic organisms as well as some anaerobes (Hassan, 1989) in the following reaction:

$$2\ O_2^{\bullet} + 2H^+ \xrightarrow{\text{SOD}} O_2 + H_2O_2$$

Because superoxide is the first product of univalent reduction of oxygen and also the first species to form in many biological systems, SOD is considered as the ''primary defense'' against oxygen radicals (Bannister *et al.,* 1987). SOD is the fastest enzyme known and its three-dimensional structure has been intensively studied (Kitagawa *et al.,* 1991; Getzoff *et al.,* 1992). The dismutation is catalyzed by the metal ion (Cu, manganese, or iron) at the active site. Superoxide is attracted to such a site by appropriately positioned, positively charged amino acid residues. SODs are classified as MnSODs, FeSODs — both types are phylogenetically related —, and a third unrelated class of Cu/ZnSODs. Plants contain all three types, and distinct SOD isozymes have been identified in the cytosol, mitochondria, and chloroplasts (e.g. Kwiatowski *et al.,* 1985; Palma *et al.,* 1986; Kanematsu and Asada, 1990; for a review, see Bowler *et al.,* 1992). SOD also exists in peroxisomes (Sandalio and del Rio, 1988), glyoxisomes (Bueno and del Rio, 1992), and in the extracellular space

(Castillo *et al.,* 1987; Schinkel *et al.,* 1998). Genes and cDNA-encoding SODs have been cloned from many plant species (Perl-Treves *et al.,* 1988; Bowler *et al.,* 1992; Kliebenstein *et al.,* 1998). All plant SODs are encoded by the nuclear genome (Perl-Treves *et al.,* 1990) and organellar isozymes are transported post-translationally to the appropriate compartment.

SODs are differentially regulated and respond to a variety of stress conditions, such as paraquat application, drought (Perl-Treves and Galun, 1991; Mittler and Zilinskas, 1994), and chilling (Karpinski *et al.,* 1994; see also Chapters 3 and 8, this volume). Several experiments on SOD overproduction in transgenic plants (but also in animal systems) have been reported, implicating SOD in stress tolerance (e.g., Perl *et al.,* 1992; Allen *et al.,* 1997; Van Camp *et al.,* 1997).

Ascorbate Peroxidase, Glutathione Reductase, and Mono-Dehydroxyascorbate Reductase

The product of SOD, hydrogen peroxide, requires further detoxification. This is achieved by other enzymes and non-enzymatic antioxidants that may differ among the various cellular compartments. In the chloroplasts, the so-called ''Halliwell-Asada pathway'' of detoxification has been studied in detail (Figure 1) (Foyer and Halliwell, 1976; Nakano and Asada, 1981; for a review, see Creissen *et al.,* 1994). H_2O_2 is reduced to water by ascorbate peroxidase (APX). This heme-containing enzyme uses a large pool of 10 mM ascorbate present in the chloroplast and oxidizes it to mono-dehydroxyascorbate (MDA; Figure 2) in the following reaction:

$$2\ \text{ascorbate} + H_2O_2 \xrightarrow{\text{APX}} 2\ \text{MDA} + 2H_2O$$

In the chloroplasts, both stromal and thylakoid-bound forms of APX were found (Miyake and Asada, 1992), and cytosolic isozymes were described and cloned (Mittler and Zilinskas, 1992; for a review, see Creissen *et al.,* 1994). Although glutathione (GSH) is present in a similarly large pool in plastids, its utilization for direct reduction of H_2O_2 is not an important process in plants. Animals differ in this respect: their glutathione peroxidases (GSH-PRX) are very important both in mitochondria and cytosol and include selenium-containing and selenium-independent enzymes (Yu, 1994). In the plastids, MDA may give rise to dehydroxyascorbate (DHA). Both must be reduced to regenerate the ascorbate pool, which can be achieved by several reactions:

(1) non-enzymatic reduction by ferredoxin:

$$\text{MDA} + \text{FD}_{\text{red}} \rightarrow \text{ascorbate} + \text{FD}_{\text{ox}}$$

(2) reduction of MDA by MDA reductase (MDAR) in the stroma, using NADPH:

$$2\ \text{MDA} + \text{NADPH} \xrightarrow{\text{MDAR}} 2\ \text{ascorbate} + \text{NADP}^+$$

(3) reduction of DHA to ascorbate by DHA reductase (DHAR) with GSH as the reducing substrate:

$$\text{DHA} + 2\ \text{GSH} \xrightarrow{\text{DHAR}} \text{ascorbate} + \text{GSSH}$$

Scavenging is thought to occur at the thylakoid surface, near PSI, minimizing the risk of escape and reaction of ROS with each other. A second line of defense operates in the stroma, to protect the sensitive enzymes of the Calvin cycle. An APX-based cycle for removal may also operate in the plant cytoplasm (Dalton *et al.,* 1987).

Glutathione Reductase

Glutathione reductase (GR) completes the ''Asada-Halliwell pathway'' by regenerating the glutathione pool with NADPH as electron donor (Foyer and Halliwell, 1976). It should be noted how the repair or prevention of oxidative damage finally consumes reducing equivalents from the light reaction.

$$GSSG + NADPH \xrightarrow{GR} 2\,GSH + NADP^{+}$$

GR is a flavoprotein, of which homologous enzymes were studied in humans, animals, and microorganisms (Karplus *et al.,* 1989). In plants, most GR activity is found in chloroplasts. Mitochondrial and cytosolic isozymes were described as well, and pea GR was cloned (Creissen *et al.,* 1994). GR and APX activities increase in response to ethylene, ozone, SO_2, and NO_2 (Creissen *et al.,* 1994). Pea GR and *E. coli* GR were overproduced in transgenic tobacco. Results were rather variable: some of the lines with elevated GR activity exhibited increased paraquat or ozone tolerance, whereas others did not (Aono *et al.,* 1993; Broadbent *et al.,* 1995; Creissen *et al.,* 1996).

Catalase

Catalases (CATs) efficiently scavenge H_2O_2 and do not require a reducing substrate to perform the task:

$$2\,H_2O_2 \xrightarrow{CAT} 2\,H_2O + O_2$$

In animal cells, peroxisomal and cytosolic catalases are the primary scavengers of H_2O_2. In leaf tissue, catalase is localized in peroxisomes, to scavenge the H_2O_2 produced by glycolate oxidase in the C2 photorespiratory cycle. Other oxidases are also present in the peroxisomes and produce H_2O_2 as part of ureide, and fatty acid metabolism (Willekens *et al.,* 1995a). In chloroplasts, little or no catalase was found (Asada, 1994), but a mitochondrial isozyme was identified in maize (Scandalios *et al.,* 1980). The peroxisome is linked to the photosynthetic metabolism via the photorespiratory process, and, according to recent findings, may take part in oxidative stress tolerance. Catalase cDNAs were cloned from several plants (Scandalios, 1994; Willekens *et al.,* 1995a). Catalase isozymes differ in biochemical properties, as well as in developmental specificity: some seem related to germination, their principal role involving probably fatty acids conversion, whereas others are related to lignification, photorespiration, or aging processes. A *CAT-2*-deficient maize mutant had no phenotypic lesion (possibly because in C_4 plants photorespiration is less important), but a low-catalase mutant of barley was injured under photorespiratory conditions (Kendall *et al.,* 1983). Catalase undergoes photoinactivation and requires continuous *de novo* synthesis. Stress factors that affect protein synthesis (e.g. heat,

chilling) may lead to catalase inactivation. This effect may be compensated for by decreased H_2O_2 production through PSII photoinhibition (see below), as well as compensatory increases in ascorbate, glutathione, and APX (Volk and Feierabend, 1989). Acclimation to chilling by pre-exposure treatment probably includes induction of catalase (Prasad *et al.,* 1994). The *CAT-2* gene of *Nicotiana* is induced by UV-B, ozone, and SO_2 (Willekens *et al.,* 1995b). Recently, a role for H_2O_2 and catalase in the hypersensitive reaction (HR) and systemic-acquired resistance (SAR) responses to plant-pathogen infection has been proposed. The model proposed by Durner and Klessig (1995) (see below) suggests that salicylic acid inhibits catalase and that the resulting H_2O_2 burst is part of the pathogenesis signal transduction chain. Interestingly, the APX enzyme is also inhibited by salicylic acid (Durner and Klessig, 1995).

Chamnongpol *et al.,* (1996) described transgenic tobacco plants deficient in specific CAT isozymes. Under dim illumination, the plants looked like wild type, but upon exposure of CAT-1- deficient lines to 500 μE m^{-2} sec^{-1} light, necrotic lesions appeared. Under high light, CAT seemed necessary for good protection against photooxidation. The relationship between H_2O_2 overproduction and defense activation of pathogenesis related proteins via salicylic acid was investigated with such plants (Chamnongpol *et al.,* 1998).

Additional Proteins and Enzymes

Thioredoxin is a small ubiquitous protein that plays a redox-regulatory role in plants (see above). It has also been suggested to protect organisms by scavenging reactive oxygen, as well as regenerating oxidized proteins (Fernando *et al.,* 1992; Takemoto *et al.,* 1998).

Extracellular scavenging systems (for instance in the plasma) are of great importance in animals. Considering the role of transition metal ions in oxidative stress, proteins involved in the homeostasis of Cu and Fe are sometimes regarded as part of the defense against, or regulatory aspects of, oxidative stress. Transferrin, a plasma Fe carrier protein, ferritin, an intracellular Fe-storing complex, as well as ceruloplasm, a Cu-binding glycoprotein, have all been suggested to have antioxidant functions *in vivo*. Nevertheless, when fully loaded with metal, these proteins may actually enhance oxidative stress, and their physiological role in this regard remains unclear (Gutteridge and Halliwell, 1992). Investigations of the role played by the metabolism of metal ions in plant stress (see Chapter 7, this volume) may unravel additional proteins that control the availability of Fe and Cu to the Haber-Weiss reaction.

In the conceptual framework of defense mechanisms, some authors have included additional cellular processes that are activated after severe oxidative damage, such as phospholipases and proteases that degrade and repair biological macromolecules, whose activities are typically induced in animal and bacterial cells by oxidative stress (Davies, 1988; Yu, 1994). According to these authors, such mechanisms allow the cell to regain its homeostasis and should be regarded as secondary defenses. The large and variable class of plant glutathione-*S*-transferases (GST) may qualify as ''secondary defense enzymes'' (Marrs, 1996). These enzymes catalyze the conjugation of a GSH molecule to a variety of chemical compounds, for example, in the detoxification of herbicides. The conjugate is marked for secretion to the apoplast or vacuole through glutathione pumps. GST conjugations are also important in the synthesis of secondary metabolites. In our context, GSTs detoxify toxic breakdown products of lipid peroxidation or oxidative DNA

degradation; they may also function as peroxidases and scavenge radicals. Interestingly, GSTs are induced, among others, by ROS, ozone, wounding, ethylene, heavy metals, and pathogen attack.

Attempts to isolate additional, yet unknown components of oxidative stress defenses have been reported. For example, Kushnir *et al.* (1995) used an *Arabidopsis* cDNA library to transform yeast and identified clones that imparted oxidative stress tolerance to the yeast host. Three clones that encoded previously unknown plant proteins have been selected, one of which is involved in glutathione metabolism. Lin and Culotta (1995) looked for yeast genes that would complement the growth deficiency of SOD-depleted yeast strains and isolated a novel gene (*Atx1*) encoding a small protein similar to bacterial metal transporters. *Atx1* protects against H_2O_2, $O_2^{\bullet -}$, and $OH^{\bullet}$, and is induced by oxygen. Homologous sequences apparently exist in higher plants.

Non-Enzymatic Antioxidants

Ascorbate

Ascorbate (Figure 2), also known as vitamin C, is an important antioxidant in animal systems, where it was shown to react not only with hydrogen peroxide, but also with $O_2^{\bullet -}$, $OH^{\bullet}$, and lipid hydroperoxides (Yu, 1994). Its role as the APX substrate that scavenges H_2O_2 in the chloroplast stroma has been discussed above. Ascorbate is water soluble, but has an additional role on the thylakoid surface in protecting or regenerating oxidized carotenes and tocopherols. The ascorbate pool is also important in the cytosol (Foyer and Harbinson, 1994, and refs. therein). Tappel (1977) proposed that the antioxidant synergism between vitamins C and E in animal tissues (the former present in 100-fold higher concentrations than the latter) is due to the reduction of tocopherol radicals by ascorbate. On the other hand, a too high (>1 mM in animals) concentration of ascorbate may reduce Fe^{+3} to Fe^{+2} and enhance the Haber-Weiss reaction (Girotti, 1985). Once again, we realize how in oxidative defense supra-optimal levels of one scavenger may worsen the situation.

Tocopherol (vitamin E)

The major isomer of vitamin E is α-tocopherol (Figure 2), a phenolic antioxidant present in both plants and animals. Being a lipid-soluble molecule, it is very important as a chain terminator of free-radical reactions that cause lipid peroxidation (Burton *et al.,* 1982). The high degree of lipid unsaturation in chloroplast membranes requires large amounts of α-tocopherol. Plants synthesize tocopherol by enzymes localized in the inner chloroplast membrane (Soll *et al.,* 1984). Animals, though, must acquire it through their diet (a particularly rich source being vegetative oils). The numerous health benefits of vitamin E have been documented (Nesarentam *et al.,* 1992).

Carotenoids

Carotenoids are lipid-soluble molecules that protect both plants and animals against oxidative damage; β-carotene (Figure 2) is the main precursor of vitamin A. Plant

Figure 2. Chemical structure of important cellular antioxidants.

carotenoids are formed from isopentenyl diphosphate in the chloroplasts and chromoplasts (Beyer, 1989). In the photosynthetic apparatus, β-carotene quenches both excited triplet-state chlorophyll and singlet oxygen, preventing them from initiating lipid peroxidation (Parker and Joyce, 1967). An excited carotene molecule can return to the ground state

either by energy transfer to other pigments in the antenna (acting as a secondary photosynthetic pigment), but also by heat dissipation (acting as a quencher of superfluous excitation energy).

A particular class of carotenoids, the xantophylls, constitute a pool that undergoes prominent changes in response to strong light. The size as well as the composition of the pool change to allow better photoprotection. Zeaxanthin, the de-epoxidized form (Figure 2), better dissipates excitation energy and is derived from the epoxidized pigment violaxanthin. The rapid changes between the two forms, brought about by special enzymes, constitute the ''xantophyll cycle'' (Demming-Adams and Adams, 1996).

Glutathione

Glutathione (GSH), the ubiquitous γ-tripeptide (Glu-Cys-Ala) (Figure 2) effectively reduces and detoxifies many oxidant species. Enzymatic reactions involving GSH have been discussed above. GSH is important for recycling all the above vitamins, while its own regeneration depends on NAD(P)H consumption. A decrease in reduced glutathione pools accompanies many stress and disease conditions, but it is not always clear whether such a decrease is the cause of the disease or stress situation, or rather reflects a greater demand for GSH (Yu, 1994).

Miscellaneous compounds

Flavonoids are a group of phenolic compounds that may have antioxidant activity (Yuting *et al.,* 1990). Their synthesis in plants increases under strong or UV-enriched light, and they may protect the cell against lipid peroxidation (Torel *et al.,* 1986). Uric acid may play an antioxidant role in animals, both in intra- and extracellular compartments (Davies *et al.,* 1986). Sugar alcohols, such as mannitol, are produced by many plants undergoing osmotic stress. They may serve as compatible solutes and osmoprotectants, but may also function as antioxidants (Smirnoff and Cumbes, 1989; Stoop *et al.,* 1996). Mannitol is a more efficient carbon sink for light reaction products (such as NADPH), and may therefore alleviate photooxidative stress under some circumstances. The potential of plant antioxidant compounds, such as resveratrol from grapes and polyphenols from tea, in human therapeutics has recently drawn much attention (Waffo *et al.,* 1998; Katiyar and Mukhtar, 1997).

OXIDATIVE STRESS AS RELATED TO OTHER PLANT STRESSES

Plants are exposed to abrupt daily and seasonal changes in the environment and they display a wide spectrum of developmental responses and biochemical adaptations to stress conditions. The idea that tolerance mechanisms to several kinds of stress are interconnected and partially overlapping is certainly interesting from a basic, as well as an applied, perspective and seems, today, more plausible than ever. The new and exciting data on the intricacies of signal transduction pathways in plants, and the multiple roles that oxygen radicals play in plant metabolism may indeed point to shared, rather than separate, protective pathways in the plant (Leshem and Kuiper, 1996; Smirnoff, 1998).

Along these lines, a plethora of physiological studies found correlations between levels of antioxidants and the level of stress tolerance among plant species, varieties, and biotypes. Seasonal and developmental variation in detoxifying agents, as well as their induction by many different stresses has been reported. Some of the data linking oxidative stress to other stresses is discussed below. It appears that, in many cases, radical-induced damage underlies stress situations such as heat, cold, UV, air pollutants, and drought. Another important facet of ROS is their newly discovered involvement in signalling, which may imply that tolerance could be selected (or genetically engineered) against one stress, resulting in cotolerance against other stresses. Alternatively, we could elicit defense responses and render plants physiologically tolerant by proper pretreatments, or by affecting the signal transduction chain. A genetic approach to test the physiological relevance of different protectants and to investigate cotolerance phenomena have seldom been taken (Gressel and Galun, 1994). More recently, however, molecular genetic studies are playing an increasingly important role in addressing such questions.

Photoinhibition

Photoinhibition is defined as a decrease in photosynthetic activity after exposure to strong light. In other words, when the supply of reducing power generated by the light reactions exceeds the demands by the dark reactions, the chloroplast may experience oxidative stress and undergo photooxidation, including pigment bleaching and lipid peroxidation. The term ''photoinhibition'' refers, however, to the faster response of inhibiting photosynthetic electron flow, mainly in PSII, while avoiding further damage (Krause, 1994). Reversible photoinhibition can therefore be regarded as a protective measure to prevent further formation of reactive oxygen and more extreme photooxidative injury. The physiological condition of a given plant will specifically determine whether it will experience photoinhibition at a given light regime. Examples of photoinhibitory conditions include strong light accompanied by low temperatures, or exposure to stronger light without previous gradual adaptation. Other processes that alleviate excess reduction of the photosystems have already been mentioned, e.g. photorespiration and cyclic electron flow. Dioxygen and reactive oxygen species might play a role in photoinhibition, but the mechanism of initial PSII inhibition is still a matter of debate. Does O_2 directly react with reduced plastoquinone (Q_A)? Or does superoxide, generated by the Mehler reaction, inhibit the PSII reaction center? Other possibilities implicate reactions of $O_2^{\bullet -}$, H_2O_2, $OH^{\bullet}$, or singlet oxygen with the D1 reaction center protein, or the P680 chlorophyll (Durrant *et al.,* 1990; Krause, 1994).

Once PSII is inactivated, the D1 reaction center protein becomes altered, undergoes degradation, and must be replaced for the recovery of PSII from photoinhibition. Marking inactivated D1 for degradation depends on oxygen and may be done by singlet oxygen (Sopory *et al.,* 1990).

Recently, attention has been drawn to photoinhibition of PSI as well because of new evidence for its occurrence in higher plants (Sonoike, 1996). In the chilling-sensitive plants, *Cucumis* and *Phaseolus*, PSI was selectively inactivated *in vivo* under chilling conditions; the *psaB* gene product, a PSI reaction center subunit, was degraded following chlorophyll P700 destruction and PSI inactivation. Oxygen was required for this effect, and the addition of active-oxygen scavengers prevented PSI inactivation and PSA-B

degradation. Restoration of PSI activity requires *de novo* PSA-B synthesis and would last a few days; the latter protein has a slower turnover than does the D1 protein of PSII. PSI inhibition is therefore relevant when PSII is still active, namely at chilling temperatures and under dim light.

Herbicides, Toxins, and Triazole Compounds

Several herbicides generate reactive oxygen. Paraquat and diquat are bipyridinium herbicides, known to acquire electrons from PSI and generate superoxide in the light (Foyer *et al.,* 1994). Natural or synthetic photosensitizers induce oxidative damage in the light. The most studied natural photosensitizer is a fungal toxin, cercosporin produced by the pathogen *Cercospora* (Daub and Ehrenshaft, 1993). Cercosporin is activated by light and reacts with oxygen to form $^1O_2$, causing severe lipid peroxidation (Daub and Briggs, 1983). Several herbicides, such as aciflurfen, act as photosensitizing compounds and promote the accumulation of metabolic intermediates of chlorophyll. Upon excitation by light such tetrapyrrole intermediates produce singlet oxygen that kills the plant.

Triazole compounds such as paclobutrazol, uniconazole, and triamimenol are plant growth regulators that inhibit a cytochrome P450-dependent oxidation in the gibberellin biosynthesis pathway. Surprisingly, they have recently been shown to confer tolerance to active oxygen generated by paraquat. Paclobutrazol stimulated an increase in antioxidant enzyme activities in wheat plants, increasing their oxidative stress tolerance (Kauss and Jeblick, 1995). Triazole compounds had been previously shown to enhance tolerance of plants to several environmental stresses (Fletcher and Hofstra, 1988).

Metal Toxicity

Accumulation of phytotoxic metals in the environment results from industrial and agricultural practices. Zn, Cu, Fe, and Cd are widespread pollutants and damage plants in two different modes: by (i) direct inhibition of plant growth and biosynthetic pathways, and (ii) involvement in radical production (Foyer *et al.,* 1994). Plant exposed to elevated levels of copper ions were reported to exhibit lipid peroxidation and pigment bleaching (Sandmann and Gonzales, 1989). Prolonged exposure to $CuSO_4$ resulted also in chlorophyll degradation and in a decline in the endogenous level of catalase. Cu and Fe ions are redox active and catalyze the Fenton reaction (see above). Lipid peroxides also originate from the induction of lipoxygenase in the presence of Cu (Foyer *et al.,* 1994). Treatments with cadmium decreased the chlorophyll and heme levels of germinating mung bean seedlings by inducing lipoxygenase activity with the simultaneous inhibition of the antioxidative enzymes (Somashekaraiah *et al.,* 1992; Van Assche and Clijsters, 1990; Gallego *et al.,* 1996). Recent work demonstrated a link between metal toxicity, oxidative stress, and defense responses in *Arabidopsis* and *Nicotiana* (Xiang and Oliver, 1998; Kampfenkel *et al.,* 1995). In yeast, genes coding for both Fe uptake and oxidative stress response are regulated by the same transcription factor (Dancis *et al.,* 1992).

Air Pollution

Atmospheric pollutants, such as ozone and sulfur dioxide, have been implicated in the formation of free radicals (Cross *et al.,* 1998). Mehlhorn (1990) suggested that the

phytotoxicity of ozone is due to its oxidizing potential and the consequent formation of radicals. Ozone seems to be a greater threat to plants than sulfur dioxide (Heagle, 1989). Plants treated with ozone exhibited lipid peroxidation, pigment bleaching, degradation of the PSII D1 protein, and a decrease in the activity and quantity of ribulose-1,5-bisphosphate carboxylase (Godde and Buchhold, 1992; Landry and Pell, 1993; Foyer *et al.,* 1994). Exposure to sulfur dioxide resulted in tissue damage and release of stress ethylene from both photosynthetic and non-photosynthetic tissues (Peiser and Yang, 1985). When cells are exposed to sulfur dioxide, an appreciable acidification of the cytoplasm occurs because this gas reacts with water to form sulfurous acid that may then be converted to sulfuric acid (Veljovic-Jovanovic *et al.,* 1993).

Ultraviolet Radiation

Increasing fluxes of UV-B (290–320 nm) radiation are reaching the earth's surface as a consequence of stratospheric ozone depletion (Kerr and McElroy, 1993). The deleterious effects of UV-B on plants have been extensively studied (Teramura and Sullivan, 1994; Bornman and Sundby-Emanuelsson, 1995). Damage by UV-B to PSII involves impairment of electron transport (Hideg *et al.,* 1993) and structural damage of the reaction center proteins, primarily D1 (Greenberg *et al.,* 1989). UV-B irradiation induces the accumulation of free radical-scavenging enzymes, such as SOD (Foyer *et al.,* 1994). Illumination of isolated thylakoid membranes by UV-B produced free radicals, mainly hydroxyl and carbon-centered ones, but did not result in singlet oxygen formation. Besides the immediate free-radical production, UV-B irradiation initiated radical-yielding reactions that can be detected in leaves even minutes after the cessation of the treatment (Hideg and Vass, 1996).

Landry *et al.* (1995) utilized *Arabidopsis thaliana* mutants to characterize physiological processes that are critical for protecting plants from UV-B stress. Mutants defective in their ability to synthesize UV-B-absorbing compounds (flavonoids and sinapate esters) were found to be more sensitive to UV-B than the wild type and exhibited the highest levels of lipid and protein oxidation. APX activity increased in response to the UV-B treatment, indicating that the plant responded to UV-B by expressing an oxidative stress response, and that sunscreen compounds reduce oxidative damage caused by UV-irradiation.

Salt Stress

Salinity affects important metabolic processes located in chloroplasts and mitochondria (Cheeseman, 1988), but little is known about its effect on activated oxygen metabolism of these organelles. Hernandez *et al.* (1995) hypothesized that the decrease in CO_2 concentration in chloroplasts brought about by stomatal closure results in $NADP^+$ shortage and O_2 reduction. Experiments with leaf mitochondria and peroxisomes from NaCl-treated pea plants, have demonstrated a salinity-induced enhancement in $O_2^{\bullet -}$ production, as well as a strong decrease in mitochondrial MnSOD (Hernandez *et al.,* 1993). The possible involvement of activated oxygen species in the mechanism of damage by NaCl stress was further studied in chloroplasts from two pea cultivars with differential sensitivity to 70 mM NaCl. In the tolerant plants, NaCl stress increased Cu/ZnSOD and ascorbate peroxidase activities, as well as the ascorbate pool. In the sensitive plants, the H_2O_2 content and lipid

peroxidation products increased without change in the enzymatic activities (Hernandez *et al.,* 1995). These results support the idea proposed by Singha and Choudhuri (1990) that H_2O_2 played a role in the mechanism of salt injury. Similarly, in radish plants exposed to 100 mM NaCl, the activity of APX increased two-fold, but the levels of the respective transcripts remained unchanged, suggesting that the response was mediated by post-transcriptional events (Lopez *et al.,* 1996). In a recent study in *Citrus*, Cu/ZnSOD and APX were induced by salt stress (Gueta-Dahan *et al.,* 1997), as did SOD isozymes of the halophyte *Mesembryanthemum* (Miszalski *et al.,* 1998).

Drought and Heat Stress

During drought stress, an abscisic acid (ABA) signal causes stomatal closure and the light-exposed, over-reduced photosynthetic apparatus may experience oxidative stress. According to Price and Hendry (1991) who studied the role of oxygen radicals in different grasses exposed to drought, water deficit stress causes an overall inhibition of protein synthesis, inactivation of several chloroplast enzymes, impairment of electron transport, increased membrane permeability, and increased activity of enzymes of the H_2O_2 scavenger system. SOD mRNA were found to be induced by ABA (Guan and Scandalios, 1998). In the resurrection plant *Sorobolus stapfianus*, the levels of glutathione reductase and dehydroascorbate reductase increased upon drought (Sgherri and Navari-Izzo, 1995). Perl-Treves and Galun (1991) observed a drought-induced increase in cytosolic Cu/ZnSOD transcript, and Burke *et al.* (1985) reported an increase in GR activity in cotton upon drought. Malan *et al.* (1990) selected heat- and drought-tolerant maize inbreds and found that they had improved coping with oxidative stress. Correlations between antioxidant defense enzymes and heat stress in tomato and *Vicia* were described as well (Rainwater *et al.,* 1996; Filek *et al.,* 1997). The recovery from drought appears also to be a delicate stage that may be accompanied by oxidative stress, requiring the induction of defense systems (Mittler and Zilinskas, 1994). This observation is reminiscent of the recovery from anoxia in flooded plants, where tolerant plants exhibit increased SOD levels (Monk *et al.,* 1987; Biemelt *et al.,* 1998), as well as the phenomenon of oxidative injury upon reperfusion that threatens ischemic patients.

Chilling and Freezing

Combinations of low temperatures and strong light impart photooxidative stress on plants: Wise and Naylor (1987) demonstrated that reactive oxygen and lipid peroxidation are involved in chilling injury of cucumbers. Evergreen forests are seasonally exposed to such a stress, which is further exacerbated by air pollutants (Karpinski *et al.,* 1994; see Chapter 4, this volume). Michalski and Kaniuga (1982), who compared the effect of chilling in tomato, a sensitive species, and spinach, that is cold tolerant, reported that during a cold and dark treatment, sensitive leaves become depleted of Cu ions and of Cu/ZnSOD activity, and experience oxidative injury upon illumination. This observation implies that chilling stress also weakens protection against photooxidation.

Transgenic alfalfa with elevated Cu/ZnSOD showed improved tolerance to freezing stress (McKersie *et al.,* 1993), whereas transgenic tobacco with elevated SOD had an increased chilling stress tolerance (Sen Gupta *et al.,* 1993). Acclimation of maize seedlings

to otherwise lethal chilling temperatures by a milder cold pretreatment was accompanied by catalase and peroxidase transcript accumulation (Prasad *et al.,* 1994). Other correlations between cold acclimation and antioxidant defense have recently been reported (O'Kane *et al.,* 1996; Tao *et al.,* 1998). An H_2O_2 treatment could induce defense enzymes and confer chilling tolerance, but, if delivered at cold temperatures, the induction would not work. It remains to be seen whether H_2O_2 induction operates *in vivo* during acclimation to cold stress.

THE ROLE OF REACTIVE OXYGEN IN DISEASE RESISTANCE: REACTIVE SPECIES AS SIGNALS?

The involvement of reactive oxygen in plant-pathogen and plant-pest interactions is one of the most exciting developments in the field of oxidative stress responses. Reactive oxygen species apparently play a multiplicity of roles as cell suicide agents, antimicrobial compounds, lignification substrates, and, most interestingly, as signal molecules (Mehdy, 1994).

The Respiratory Burst

An important resistance response of a plant against an invading pathogen is the hypersensitive response (HR), which induces localized cell death around the invasion site. This response often involves a respiratory burst and the rapid production of oxygen radicals: the accumulation of H_2O_2 and oxygen radicals is one of the earliest events following host-pathogen recognition. In addition to its oxidative potential in killing or inhibiting the growth of pathogens, H_2O_2 participates in a number of responses, such as phytoalexins biosynthesis (Degousee *et al.,* 1994), lignification, cross-linking of cell wall glycoproteins (Brisson *et al.,* 1994), and transcription of defense proteins (Levine *et al.,* 1994). Alvarez *et al.,* (1998) demonstrated that, after the primary burst around the inoculation site, secondary ''micro bursts'' occur that are required for systemic resistance. By using transgenic plants, Harding and Roberts (1998) evidenced the involvement of calmodulin signalling in the plant oxidative burst. Transgenic potato plants overexpressing the glucose oxidase gene from *Aspergillus niger* provided a direct link between H_2O_2 generation and disease resistance (Wu *et al.,* 1995): the increased H_2O_2 levels conferred resistance against *Erwinia carotovora* and *Phytophthora infestans*. The first plant gene encoding a catalytic subunit of a respiratory burst related-oxidase has only recently been cloned (Groom *et al.,* 1996) and it shares homology with mammalian NADPH oxidase, that produces, in neutrophils, an outwards-directed burst of superoxide during inflammation.

Role of Hydrogen Peroxide and Salicylic Acid

In some cases the pathogen elicits a systemic response in the plant, and resistance of distant tissues to subsequent infections (systemic acquired resistance or SAR) is induced by a yet unknown mobile signal. Considerable evidence supports the involvement of salicylic acid (SA) in the induction of SAR (Yalpani *et al.,* 1991). Chen *et al.* (1993)

demonstrated that SA binds to a catalase isoform in tobacco leaves and inhibit its activity, causing increased levels of H_2O_2 and concluded that SA could act via H_2O_2 in inducing SAR. Pretreatments of parsley cell cultures with SA, or methyl jasmonate, greatly enhanced the elicited H_2O_2 burst (Kauss *et al.,* 1994; Kauss and Jeblick, 1995). Vernooij *et al.,* (1994) asked whether SA is the mobile signal in SAR. Grafting experiments were performed, using transgenic plants that express a bacterial SA-degrading enzyme. Transgenic root stocks, although unable to accumulate SA, were capable of delivering a signal that rendered the non-transformed scions resistant to pathogen infection (Vernooij *et al.,* 1994). This result indicated that the translocated signal is not SA, although the latter's presence is required in the distant tissue to induce SAR.

Role of Extracellular Superoxide in HR

Certain plant mutations induce dead cell-lesions in the absence of pathogens. Such a mutant, of which lesions spread beyond the well-localized boundaries of the wild-type response was studied. Appearance of the lesions could be triggered by supplying superoxide to extracellular spaces by xanthine and xanthine oxidase, but not by H_2O_2. SOD, but not CAT or APX, attenuated the response (Jabs *et al.,* 1996). This study significantly demonstrates that extracellular $O_2^{\bullet -}$ is a component of the cascade leading to programmed cell death during HR. The *lsd*1 mutation probably impairs the plant's ability to confine the response, or lowers the threshold for its initiation.

Plant Tumor Formation

Another interesting plant-pathogen interaction is tumor formation by *Agrobacterium*. From a new study by Jia *et al.* (1996), this process appears also to involve an oxidative burst! Nononcogenic strains or inoculation with *E. coli* did not provoke the oxidative burst, whereas SOD-overproducing plants had a smaller burst, and a smaller tumor. The relationship between such a burst and the well-established hormone-induced proliferation in the gall is yet unexplained. Is there a possible parallel with the role of radicals in animal tumors?

Nitric Oxide and Plant Stress?

Recently the endogenous production of the nitric oxide radical by plants has been reported (Leshem and Haramaty, 1996), which raised the possibility that this important gaseous signal molecule, intensively studied in animal systems (Moncada *et al.,* 1991), may operate also as a signal in plants and may be related to stress. First proofs of its involvement in the HR response was published recently (Delledonne *et al.,* 1998).

Plant-Pest Interactions and the Oxidative Burst

Besides its involvement in responses to viral, bacterial, and fungal pathogens, reactive oxygen appears to participate also in plant-pest interactions. Several genes whose expression is up-regulated after nematode infection have been isolated from potatoes

(Niebel *et al.,* 1995). One of them encoded a catalase isoform, *Cat2St*, whose mRNA levels increased throughout the infected root. Bi and Felton (1995) have demonstrated a shift in the oxidative status of soybeans attacked by the insect *Helicoverpa zea*: feeding caused significant increases in lipid peroxidation, and hydroxyl radical formation. The activity of several enzymes, including lipoxygenase, peroxidases, ascorbate oxidase, and NADH oxidase increased. Interestingly, the oxidative changes in the host plant correlated with increased oxidative damage in the midgut of insects feeding on previously wounded plants. Spider mite feeding on soybeans increased plant lipid peroxidation, lipogenase, and peroxidase levels, reduced carotenoid levels, but did not affect catalase and SOD (Hildebrand *et al.,* 1986). Phloem-feeding by aphids increased the glutathione reductase levels in wheat and barley (Argandona, 1994).

REACTIVE OXYGEN, DISEASE AND STRESS: HUMAN AND ANIMAL SYSTEMS

The involvement of reactive oxygen with human disease and aging is a ''hot'' field of study, and the relationships of oxidative stress with a great number of diseases, including diabetes, cataract, and AIDS have been studied. Only a few important examples dealing with cancer and aging will be discussed here.

Oxygen Free Radicals in Inflammation and Cancer

The involvement of free radicals, such as $O_2^{\bullet -}$, $OH^{\bullet}$, and carbon-centered alkyl and peroxyl radicals, in inflammation or cancer, has been extensively studied. An important endogenous cause of chronic oxidative stress in animals and humans is the inflammatory response (Cerutti and Trump, 1991). Activated leukocytes generate $O_2^{\bullet -}$ and hypochlorous acid, which represents an important source of oxygen-free radicals *in situ* (Weiss, 1989). The radicals mediate the killing of target cells, but also induce oxidative stress in adjacent tissues. Activated neutrophils stimulate mutagenesis *in vitro* (Weitzman and Gordon, 1990), and oxidative stress from chronic inflammation promotes cancer development in many organs, which may underlie as much as one third of the world cases of cancer (Ames *et al.,* 1993). Examples of cancer induction by chronic inflammation are ulcerative colitis (Collins *et al.,* 1987), mesothelioma caused by asbestos deposits (Mossman *et al.,* 1990), urinary bladder cancer induced by *Schistosoma haematobium* infections (Rosin *et al.,* 1994), and the induction of hepatocellular carcinoma by viral hepatitis (Shimoda *et al.,* 1994).

Other studies suggest that oxygen free radicals may also directly contribute to cancer development (not via chronic inflammation) (Dreher and Junod, 1996; Comstock *et al.,* 1997; Olinski *et al.,* 1998). Radical-related lesions in proteins and DNA accumulate, and reactive oxygen is believed to stimulate the development of cancer at all three stages of the disease: initiation, promotion, and progression.

Initiation, or the first step of carcinogenesis, requires a permanent modification of the DNA in one cell. The number of oxidative hits to DNA is estimated to be approximately 10,000 per day in humans (Ames *et al.,* 1993). Many modifications result in replicative blocks, whereas others may induce point mutations that escape repair mechanisms, and

accumulates with age (Lindahl, 1993). The frequent 8-OH-Gua modification produces GC to TA transversions that are frequently detected in the RAS oncogene (Bos, 1988), or in modified p53 genes in lung and liver tumors (Hsu *et al.* 1991).

Oxidative stress can also stimulate the proliferation of mutated cell clones, by modulating the expression of specific genes. Oxygen free radicals can induce large changes in cytosolic Ca^{2+}, brought about by the mobilization of intracellular Ca^{2+} stores, or influx of extracellular Ca^{2+}. This mechanism may regulate the transcription of genes involved in cell growth and proliferation (Larson and Cerutti, 1989; Maki *et al.,* 1992; Werlen *et al.,* 1993).

The final stage in cancer development is the acquisition of the malignant properties by the tumor. The accelerated growth of tumors requires additional DNA alterations. It has been hypothesized that an elevated generation of oxygen free radicals in tumor cells increased genomic instability (Toyokuni *et al.,* 1995) and lowered the activity of antioxidant enzymes (Punnonen *et al.,* 1994).

Oxygen Free Radicals and Aging

Oxidative stress has been postulated, years ago, to be a causal factor in the aging process (Harman, 1956). The basic tenet of this hypothesis is that the age-associated decline in the functional capacity of biological systems is primarily due to the accumulation of irreparable oxidative molecular damage (Sohal *et al.,* 1995). The extent of oxidative damage to DNA, proteins, and lipids has been found to increase with age, providing support for this hypothesis (Agarwal and Sohal, 1994). A possible cause for such age-related accumulation of molecular damage could be a corresponding decline in the efficiency of antioxidative defenses. Extensive studies have yielded, however, a large body of confusing, often contradictory, data. A fairly common finding is that the level of some antioxidative defenses increases, while others decline or remains unchanged with age.

Utilizing X-ray irradiation as a source of reactive oxygen species, Agarwal and Sohal (1996) tried to determine whether the susceptibility of tissues to protein oxidative damage increases with age, and whether tissues of longer-lived species are less susceptible. Brain homogenates from 22-month-old rats were, in fact, more susceptible to oxidative stress than those from 3-month-old rats, and a comparison of five different species (mouse, rat, rabbit, pig, and pigeon) indicated that the maximum life span potential of the species was inversely related to their susceptibility to acute oxidative stress. An earlier study of the same group utilized *Drosophila melanogaster* that overproduced Cu/ZuSOD and catalase (Sohal *et al.,* 1995). The transgenic flies were less susceptible to protein and DNA oxidation, and lived 34% longer than control flies. Laboratory-selected long-lived *Drosophila* strains exhibited improved oxidative stress resistance (Arking, 1998) and interestingly, an analogy with late-flowering *Arabidopsis* mutants exhibiting paraquat tolerance was reported (Kurepa *et al.,* 1998).

Age-related loss of different cognitive and motoric abilities in mice was positively correlated with oxidative molecular damage in the cerebral cortex and the cerebellum, respectively (Forster *et al.,* 1996). These results support the view that oxidative stress is a causal factor in brain senescence, and suggest that age-related decline of cognitive and motoric performance progress independently, involving different regions of the brain.

Although longevity is strongly influenced by the environment, its genetic component is also being investigated (Shmookler and Ebert, 1996). Ebert *et al.* (1996) have identified five chromosomal regions that help specify life span in *Caenorhabditis elegans*, by comparing the genotypes of short-lived and long-lived progenies. This mapping study suggested that longevity is positively correlated with the levels of superoxide dismutase and catalase late in life.

REGULATION OF THE OXIDATIVE STRESS RESPONSE

The Regulatory Problem

The different defense proteins and antioxidant molecules must be present in the cell at some baseline level, to provide a constitutive defense against reactive species that form under normal metabolic conditions, or that appear as a result of a sudden stress. The *E. coli* FeSOD is an example of a constitutive defense protein, present even when the cell grows unaerobically, whereas a second isozyme, MnSOD, is induced by oxygen and oxidative stress. Many of the genes that encode defense components in bacteria, animals, and plants, can, in fact, increase their transcription in response to oxidative stress. Other levels of regulation, such as activation of existing proteins, are known as well. Some of the most interesting open questions involve the regulation of the oxidative stress response. How does the cell sense oxidative stress in its different compartments? How is such information transduced to the photosynthetic and respiratory apparati, to quickly elicit all those subtle physiological adaptations that were discussed above? What are the transcriptional regulators of such genes that encode SOD, APX, and GR? No one has yet identified such components in plants; it may be useful therefore, to have a closer look at some non-plant models, because parallel mechanisms may operate in the plant.

Oxidative-Stress Regulons in Bacteria

Escherichia coli and *Salmonella typhimurium* are enterobacteria that experience oxidative stress when leaving the host's body or when attacked by macrophages. Bacteria have very efficient induction-repression systems; they respond to environmental threats by inducing ''global responses'', i.e. by expressing groups of unlinked genes (named regulons) that are regulated in concert. A few regulons are related to stress. Hypersensitive mutants that are unable to mount the response, as well as mutants that ''respond'' constitutively, have allowed the genetic identification and cloning of important regulatory genes.

Hydrogen peroxide at micromolar amounts induces approximately 30 bacterial polypeptides. This treatment renders the microorganism resistant to otherwise lethal millimolar concentrations of H_2O_2. These polypeptides were found to include CAT and GR, but not SOD. Some of them are also part of the heat-shock and/or superoxide responses (Demple, 1991). The *OxyR* locus was found to encode a positive regulator of this response and to belong to a family of DNA-binding proteins. It promotes the transcription of most of its target genes, but downregulates its own. OXY-R is a redox-sensitive protein that undergoes a reversible, subtle conformational change as a result of oxidation. It will bind to its target gene promoters both at 1, and 100 mM dithiotreitol

(DTT) concentrations, respectively, but its DNAase footprinting patterns are different under such conditions, and only the oxidized OXY-R will promote transcription of defense genes (Demple, 1991; Storz *et al.,* 1990).

Superoxide and paraquat induce an additional set of 40 polypeptides, including *Sod*A, glucose-6-phosphate dehydrogenase (required to generate NAD(P)H), endonucleases involved in DNA repair, and heat shock chaperonins. Such a diverse list of ''regulon members'' supports the above-mentioned inclusive view of cellular defenses that considers the various repair functions as secondary defenses against oxidative stress. A regulatory locus that encodes two proteins, SOX-R and SOX-S, positively regulates nine of the superoxide-inducible genes, and negatively regulates three others. It appears that SOX-S directly activates transcription, whereas SOX-R is a redox-sensor that activates the former. The molecular details of the OXY-R and SOX-R/S functions are still actively pursued (Hidalgo and Demple, 1996; Hidalgo *et al.,* 1998). Two additional regulons, and their respective regulators *katF* and *soxQ*, control responses to carbon starvation, antibiotic resistance, and to oxidative stress.

Individual defense genes are often members of several regulons: *sodA,* for example, is regulated by four different ones! This is a genuinely complex regulatory network, of which the coordination and integration aspects are not yet understood. When turning to eukaryotes, with their multiple organelles, and more diverse array of antioxidant molecules, we should indeed expect very sophisticated regulation.

Yeast and Animal Cells

Understanding the regulation of oxidative response in eukaryotes, and its exploitation for therapeutic purposes has become a major research target (Sen, 1998). In yeast, a transcription factor, YAP-1, was identified, the overproduction of which conferred tolerance to toxic compounds, including H_2O_2 and thiol-oxidants. YAP-1 becomes activated and promotes transcription of target genes under oxidative conditions (Kuge and Jones, 1994). This effect results from an increase in YAP-1 binding to its DNA targets, as shown by gel retardation assays. Genes that are transcriptionally induced by *YAP*-1 and confer oxidative stress tolerance were also identified (Kuge and Jones 1994). One of these encodes thioredoxin, a thiol-protein whose defensive role was discussed above. Other targets of YAP-1 include GSH-1, the rate-limiting enzyme in yeast glutathione synthesis (Wu and Moye-Rowley, 1994). The catalase promoter has been recently dissected and regions responsible for its oxidative stress inducibility have been mapped (Nakagawa *et al.,* 1998).

In mammalian tissues, several examples of induction of defense responses have been documented, for example after exposure of lung tissue to hyperoxic conditions (Harris, 1992). The transcription factor AP-1 (homologous to yeast YAP-1) has been studied in detail. It is a heterodimer made of the JUN and FOS polypeptide products. Transcription of the respective gene is induced by H_2O_2. Its DNA-binding activity responds only poorly to hydrogen peroxide, but is strongly increased by reducing conditions (Meyer *et al.,* 1993). Another transcription factor, NFκB, is transcriptionally induced, and also post-transcriptionally activated to bind DNA, by a variety of oxidants and biological inducers, including viral proteins or inflammation cytokines. All these inductory effects can be abolished by antioxidant treatments. Target genes of NFκB include *TNF*α and β-interferon.

Activation of NFκB is a complicated process: it is present in the cell as an inactive complex, and an inhibitory subunit, IκB, must be released to enable the rest of the complex to enter the nucleus and bind target DNA (Ginn-Pease and Whisler, 1998).

Regulation of Defense Systems in Plants

The existence of genetic mechanisms that regulate and coordinate the oxidative defense can be inferred from physiological and genetic studies in which increased expression of a few defense genes in concert has been reported. A thoroughly analyzed example is a paraquat-tolerant genotype of the weed *Conyza bonariensis*, that exhibits increase in the activities of three enzymes, chloroplast SOD, APX, and GR. The phenotype is inherited as a single locus, probably encoding a regulatory gene (Shaaltiel *et al*., 1988). An alternative explanation would be that the locus regulates only SOD, but the resulting increase in H_2O_2 induces the downstream genes. Indeed, a transgenic plant overproducing Cu/ZnSOD in its chloroplasts had elevated APX as well (Sen Gupta *et al*., 1993). Therefore, a regulatory role for SOD might be claimed. In another study, however, increased MnSOD targeted to the chloroplasts suppressed endogenous SODs and did not elevate downstream enzyme activities (Slooten *et al*., 1995).

We have discussed the multiplicity of defenses and the need to regulate their expression according to changes in plant metabolism and in the environment. By examining the induction of individual defense genes at the molecular level, such genes have been found to be regulated both developmentally and in response to stresses of various origin (Kliebenstein *et al*., 1998). For example, transcript levels of Cu/ZnSODs of tomato respond to light, paraquat application, ethylene, wounding, and drought, and vary according to leaf age (Perl-Treves and Galun, 1991). The cellular mechanism that mediates gene activation in response to all these factors is still unknown; for instance, whether SOD induction during drought occurs as a result of reactive oxygen production or precedes it, and whether its induction by ethylene implies an endogenous role for ethylene in the system. Is ethylene upstream or downstream a putative superoxide signal? Isolation of promoters of oxidative response genes (Hérouart *et al*., 1993; Kardish *et al*., 1994; Van Camp *et al*., 1996) provides important tools to answer such questions, allowing us to ''work our way up'' the signal transduction chain and identify DNA-binding factors, redox sensing-proteins, etc. The first indications for redox sensing in the transcriptional regulation of plant antioxidant genes were provided by Wingsle and Karpinski (1996), who treated spruce needles with either reduced (GSH) or oxidized (GSSG) glutathione and followed the changes in enzymatic defense levels. GSSG increased GR activity by 60%, probably as a result of a posttranslational modification, because the respective protein and transcript levels remained unaffected. Chloroplast Cu/ZnSOD did not respond, while cytosolic Cu/ZnSOD transcript increased by GSSG and decreased by GSH. Quite different results have been reported by Hérouart *et al*. (1993), who followed the expression of a reporter gene fused to the cytosolic Cu/ZnSOD promoter from tobacco. Here, GSH induced transcription in tobacco protoplasts, while GSSG did not affect it. Molecular identification of putative redox-sensitive factors would allow a closer look at these systems.

Post-translational activation of plant enzymes by the cell redox potential is an established phenomenon. The best known example involves activation of Calvin cycle

enzymes by thioredoxin (see above). Another indication for redox modulation involved bacterial cysteine-rich enzymes expressed in transgenic plants. Enzymatic activities of β-glucuronidase and neomycin phosphotransferase II increased 8-fold after dithiothreitol treatment, and both the enzymatic activity and the amount of protein were increased (Garcia-Olmedo *et al*., 1994). A novel mechanism of redox sensing to mediate light regulation was discovered by Danon and Mayfield (1994), who studied chloroplast gene expression in *Chlamydomonas* and found that gene-specific translational activator proteins are imported into the chloroplast, where they bind to mRNAs, and regulate their translation: RNA binding is increased by reductants and decreased by oxidants.

A further level of physiological regulation involves the action of phytohormones that are released in response to various stresses. How do these interact with the oxidative stress response? Ethylene, ABA, jasmonate, and SA have all been implicated in stress responses such as wounding, anaerobiosis, drought, cold, and salinity (Leshem and Kuiper, 1996). Hormones could initiate defense responses before the redox situation of a cell has changed, because they can convey information about an environmental/metabolic stress that is occurring in a distant tissue. Another possibility is that some hormone-metabolic pathways are themselves sensitive to oxidative changes. As a result, the hormone will propagate the defense response to other tissues, or maintain it for extended periods, following a local change in the redox state at its primary site of production.

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