UNDERSTANDING AND MANAGING OXIDATIVE STRESS IN TURFGRASS

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Traditionally, reactive oxygen intermediates (ROIs) were considered to be toxic by-products of aerobic metabolism, which were disposed of using antioxidants. However, in recent years, it has become apparent that turf plants actively produce ROIs as signaling molecules to control processes such as programmed cell death, abiotic stress responses, pathogen defense and systemic signaling. Recent advances including microarray studies and the development of mutants with altered ROI-scavenging mechanisms provide new insights into how the steady-state level of ROIs are controlled in cells. In addition, key steps of the signal transduction pathway that senses ROIs in plants have been identified. These raise several intriguing questions about the relationships between ROI signaling, ROI stress and production and scavenging of ROIs in the different cellular compartments.

Oxidative Stress and the Role of Reactive Oxygen Species

The field of redox biology has recently witnessed a dramatic reappraisal of the importance of ROS. Due to the reactivity of ROS and because they are unavoidable by-products of oxygenic

photosynthesis, only the more negative aspects of ROS generation are often considered in relation to observations. Thus, light-driven ROS production is generally described as harmful because it has the potential to cause irreversible damage to photosynthetic components. It is generally often suggested that ROS production should be minimized at all costs. However, despite their potential for causing harmful oxidations, it is now well established that ROS are also powerful signaling molecules that are involved in the control of turf plant growth and development as well as priming responses to stress stimuli. In many studies involving photosynthetic ROS generation, the signaling function of these powerful metabolites is largely ignored, and interpretations are all too frequently based solely on the notion that ROS exert their principal effects through chemical toxicity and their abilities to cause damage. Within this context, the term "oxidative stress" has become largely synonymous with "oxidative damage" to cellular components, particularly in situations where oxidative inactivation exceeds that of repair or replacement. Furthermore, it is often suggested that the accumulation of damaged cellular components and associated loss of function leads to cell death, but the mechanisms that cause cell death in this case are generally vague or undefined. Relatively few studies to date have considered photosynthetic ROS generation in the context of the light-driven production of powerful signaling molecules, whose abundance provides essential information to the cell concerning imbalances between energy-generating and energy-utilizing processes in the PET system. Within this context, increased ROS production leading to enhanced oxidation in high light may be considered to be a powerful signal that not only decreases PSII(Photosystem II) activity but also stimulates gene expression, particularly with regard to acclimation and defense gene. While concepts of ROS function in signaling or damage in photosynthesis are largely irrelevant to the description of the basic biochemical mechanisms by which ROS oxidize cellular components, the philosophical choice between "damage" and "signaling" in data analysis remains crucial to the evaluation of the physiological significance of these mechanisms.

Oxygen Production and the Regulation of Photosynthesis

Oxygenic photosynthesis is a dynamic and flexible process that powers life on earth, in which water oxidation on the lumen side of PSII is an indispensable step.

The light-driven PET system drives electrons from water through to NADP, generating the proton gradient that facilitates ATP synthesis. Intriguingly, a key feature of PSII is its vulnerability to light-induced damage, which is considered to be a consequence of the production of singlet oxygen in the PSII reaction center, leading to irreversible oxidation of the D1 protein. This sensitivity means that the PSII reaction center has to be rebuilt about once every 30 min even under relatively low irradiances. The damage and repair process thus occurs under all light intensities. A limitation of the PET system only occurs when the rate of damage exceeds that of repair, and this makes a major contribution to the processes called photoinhibition. In many circumstances where the rate of damage is fast, such as at high light intensities, the rate of repair is also rapid, so a high level of PSII activity can be maintained.

It is widely accepted that efficient regulation of PET serves to minimize the production of singlet oxygen at PSII as well as the generation of superoxide and hydrogen peroxide (H_2O_2), which occurs predominantly on the reducing side of PSI .Within this context, reversible decreases in the efficiencies of both photosystems are intrinsic to the regulation of light use by photosynthesis .Inherent limitations on the capacity for electron transport through the cytochrome b_6/f complex favor over-reduction of PSII, which exacerbates PSII turnover. Of the

strategies that can be employed to protect PSII under conditions of limiting PET capacity, the most important is non-photochemical quenching (NPQ), which dissipates the excitation energy of chlorophyll *a* molecules safely as heat.

The majority of electron flow follows a linear, noncyclic route from water through PSII, the cytochrome b_6/f complex and PSI to NADP, leading to the generation of both NADPH and ATP. However, the operation of a cyclic electron flow pathway around PSI provides a mechanism whereby ATP production can be increased relative to NADPH. The operation of cyclic electron flow is also considered to prevent over-reduction of the acceptor side of PSI. This is important because superoxide and H_2O_2 are generated by PET components on the acceptor side of PSI. Cyclic electron flow may also help to limit singlet oxygen production at PSII because it enhances protonation in the lumen, which triggers protective NPQ mechanisms. The exact contribution of the linear and cyclic pathways to overall electron flow depends on cell type and environmental conditions. However, in C_3 leaves under optimal growth conditions, the major function of cyclic electron flow around PSI is considered to be the augmentation of ATP production relative to NADPH in order to balance the energy budget of the chloroplast.

While many fundamental questions remain concerning the components involved in cyclic electron flow and the extent to which this pathway operates in C_3 turf plants, such as Kentucky Bluegrass, perennial ryegrass, fescue, creeping bentgrass, there is a general consensus of opinion that cyclic electron flow is an essential component of the repertoire of chloroplast mechanisms that serve to coordinate energy metabolism and balance redox status. A further mechanism of regulation for photosynthesis that prevents over-reduction of the PSI acceptor side and the chloroplast stroma is the "malate valve" system, which transfers reducing equivalents to the cytosol. This pathway, which is activated by the thioredoxin (TRX)-regulated activation of chloroplastic NADP-dependent malate dehydrogenase, is an essential component for the stromal redox homeostasis network because it allows the export of excess reducing power and thus relieves electron pressure in the chloroplast. In this way, the regulation of metabolite distribution can be used to balance cellular redox status in order to achieve metabolic and photosynthetic control. However, to date, little attention has been paid to how the regulated distribution of other metabolites, particularly antioxidants, may alter the stromal redox homeostasis network and affect the regulation of photosynthesis.

THE ANTIOXIDANT NETWORK OF CHLOROPLASTS

Photosynthesis is an important source of cellular oxidants in the turf grass system. Even under optimal conditions, the calculated rate of H_2O_2 formation by the PET chain in the chloroplasts during photosynthesis in C_3 turf leaves is nearly as high (4 μmol m^{-2} s^{-1}) as the amount produced in the peroxisomes as a result of the glycolate oxidation in the photorespiratory pathway. While it is often stated that the amount of H_2O_2 in chloroplasts can increase by several orders of magnitude during stress, such notions must be viewed with caution because of the inherent complexities of obtaining accurate measurements of H_2O_2 contents in intact tissues and isolated organelles. Current methods do not allow accurate estimations of the H_2O_2 concentration of the stroma under either optimal or stress conditions. Regardless of any uncertainties about absolute values for H_2O_2 levels in photosynthetic tissues, it has long been recognized that H_2O_2 is a potent inhibitor of photosynthesis, because even at low concentrations it can inhibit

CO_2 fixation. The reduction of ground-state molecular oxygen to superoxide on the acceptor side of PSI in the Mehler reaction is the first step of a series of reactions that together has been called "the water-water cycle". This incorporates superoxide dismutase (SOD), ascorbate peroxidase (APX), and the AsA-GSH cycle in a mechanism that builds up the trans- thylakoid proton gradient and facilitates ATP formation at the expense of NADPH and reductant APX utilizes AsA as a specific electron donor to reduce H_2O_2 to water with the concomitant generation of monodehydroascorbate (MDA), a univalent oxidant of AsA. While MDA is spontaneously converted to AsA and dehydroascorbate (DHA), it is also rapidly reduced to AsA by the action of a NADPH-dependent MDA reductase. DHA reductase (DHAR) utilizes GSH to reduce DHA and thereby regenerate AsA. GSH is then regenerated from oxidized glutathione, also called glutathione disulfide (GSSG), by the action of glutathione reductase (GR) using NADPH. While different APX and SOD isoforms are located in the stroma and thylakoid membrane, the chloroplastic GR and DHAR enzymes are localized in the stroma. A characteristic property of APX, particularly the chloroplastic APX forms, is their susceptibility to oxidative inactivation in the absence of AsA. Under low AsA concentrations, the activity of chloroplastic APXs is rapidly lost in the presence of H_2O_2, with a half-inactivation time of less than 30 s. In contrast, the cytosolic and peroxisomal APXs only lose activity after more than 1 h. The chloroplastic APXs are the primary targets for inactivation if chloroplast AsA accumulation is impaired. Depletion of chloroplast AsA and inactivation of chloroplast APXs, therefore, have been considered as limitations of photosynthetic efficiency in stress conditions and thus potential targets for improvement.

In addition to the AsA-GSH cycle, other proteins that are important in chloroplast ROS detoxification are peroxiredoxin (PRX; particularly 2-CysPrx and PrxQ), glutathione peroxidase (GPX), sulfiredoxin, and cyclophilin, which function together with TRX and TRX-like proteins in the chloroplasts. The thiol-based catalytic mechanism used by PRX to reduce H_2O_2consists of a peroxidative reduction, followed by regeneration that can involve a variety of electron donors such as TRX, glutaredoxin, cyclophilins, GSH, and AsA. GPXs can use both GSH and TRX as reducing substrates, and they can detoxify lipid peroxides as well as H_2O_2. Because TRX is a more efficient substrate than GSH and their high rates of TRX-dependent peroxidase activity, plant GPXs have been assigned to the PRX protein family. In the GPX system, the regeneration of reduced TRX is linked to the PET chain through either ferredoxin-TRX reductases or NADPH-dependent TRX reductases. While the catalytic rates of plant GPXs and their affinities for H_2O_2 are rather low compared with APX, the PRX and GPX pathways provide an alternative pathway to the water-water cycle in the light particularly if the AsA-GSH cycle is impaired. However, lipid peroxides are also efficient substrates for the chloroplast GPXs. The detoxification of lipid peroxides, which exacerbate the lipid peroxidation cascade reactions, may be as equally important as H_2O_2 detoxification in terms of maintaining optimal photosynthetic functions in the light. AsA-GSH cycle and the PRX-dependent detoxification pathways may be equally important in vivo and serve interfacing functions. Our view is that the two detoxification pathways are tailored to suit specific niches in defense metabolism and that their relative importance probably varies according to the prevailing environmental conditions.

The observation that chloroplastic MDA reductases and APXs are enhanced in antisense Arabidopsis (*Arabidopsis thaliana*) plants with suppressed chloroplast-located PRX suggests that regulatory compensation mechanisms exist between the pathways, so that one pathway may be enhanced to compensate for losses in the other pathway. Such observations of interactions

between the AsA-GSH pathway and the PRXs demonstrate cross talk between the individual ROS-metabolizing pathways of the chloroplasts. The AsA-GSH pathway has a higher specificity for H_2O_2 and the chloroplast APX has higher activities than PRXs, but the PRXs have a broad specificity toward lipid peroxides and/or reactive nitrogen species, such as nitric oxide (NO). For example, values for the catalytic efficiencies (K_{cat}/K_m; L mol^{-1} s^{-1}) for plant 2-CysPrx with H_2O_2range between 2.5×10^4 and 1.8×10^3, with a value of 7.3×10^3 with *tert*-butylhydroperoxide. In comparison, the value for AsA-dependent H_2O_2 reduction is 0.9×10^6 L mol^{-1} s^{-1}.

Like ROS, NO is an important plant signaling molecule. NO reacts rapidly with the superoxide anion to produce peroxynitrite. It is likely that peroxynitrite is produced in chloroplasts of turf grass leaves, where it may fulfill signaling functions. Moreover, in marked contrast to the situation in animals, peroxynitrite does not appear to be toxic to plants, which appear to function without problem even in the presence of high levels of this metabolite. A key feature of the regulation of NO metabolism is its reaction with GSH to form nitrosoglutathione, which can then react with other thiols to form nitrosothiols. Nitrosoglutathione serves as a storage pool of NO and probably also fulfills as yet unknown signaling functions. Like glutathionylation, protein *S*-nitrosylation can modify both protein function and activity.

While the AsA-GSH cycle operates largely within the stroma, it is also linked to the functions of a hydrophobic antioxidant, α-tocopherol (Toc), which is maintained in its reduced form by AsA. Carotenoids and tocopherols are the most abundant groups of lipid-soluble antioxidants in chloroplasts. Toc is accumulated to high concentrations in chloroplasts, where it serves to prevent lipid peroxidation by removal of singlet oxygen and lipid peroxyl radicals. The resultant tocopheroxyl radicals are reduced back to Toc by AsA and the action of the AsA-GSH cycle. The Arabidopsis *vitamin E deficient1*(*vte1*) mutants that are deficient in Toc show a significant accumulation of AsA and GSH. In contrast, VTE1-overexpressing plants, which accumulate Toc, have lower AsA and GSH levels. Such observations provide further evidence of the close relationships that exist between chloroplast antioxidants, which compensate for each other. Moreover, these data not only demonstrate the high degree of interaction between the chloroplast antioxidant pathways but also suggest that there are multiple sites of reciprocal control. However, while the biosynthetic pathways for AsA GSH and Toc are now well established, much remains to be understood regarding how the synthesis and accumulation of these essential antioxidants is controlled and regulated in a coordinated manner. The factors that control the intracellular partitioning of metabolites between the different compartments of the cell and the antioxidant transport systems are of particular importance to the overall regulation of photosynthesis and its effective operation over a wide range of environmental conditions. While recent years have witnessed an increase in our understanding of the roles of ROS in the signaling systems that coordinate antioxidant gene expression, very little information is available to date on how the low-M_r antioxidants participate in this control. Therefore, the balance between ROS production and ROS scavenging in chloroplasts is delicate and must be strictly controlled. AsA and GSH are the most abundant and best characterized water-soluble antioxidants in plants, and they accumulate to millimolar concentrations in chloroplasts. Although these metabolites scavenge ROS separately, they have long been considered to function together in the AsA-GSH cycle and the water-water cycle to metabolize H_2O_2 and to dissipate excess excitation energy in chloroplasts.

A common feature among the different ROS types is their capacity to cause oxidative damage to proteins, DNA, and lipids. These cytotoxic properties of ROS explain the evolution of complex arrays of non-enzymatic and enzymatic detoxification mechanisms in plants. Increasing evidence indicates that ROS also function as signaling molecules in plants involved in regulating development and pathogen defense response. Reactive oxygen species (ROS) are produced as a normal product of plant cellular metabolism. Various environmental stresses lead to excessive production of ROS causing progressive oxidative damage and ultimately cell death. Despite their destructive activity, they are well-described second messengers in a variety of cellular processes, including conferment of tolerance to various environmental stresses. Whether ROS would serve as signaling molecules or could cause oxidative damage to the tissues depends on the delicate equilibrium between ROS production, and their scavenging. Efficient scavenging of ROS produced during various environmental stresses requires the action of several non-enzymatic as well as enzymatic antioxidants present in the tissues. In this paper, we describe the generation, sites of production and role of ROS as messenger molecules as well as inducers of oxidative damage. Further, the anti-oxidative defense mechanisms operating in the cells for scavenging of ROS overproduced under various stressful conditions of the environment have been discussed in detail.

Production of Oxidative Stress in Cells

There are many potential sources of ROIs in plants. Some are reactions involved in normal metabolism, such as photosynthesis and respiration. These are in line with the traditional concept, considering ROIs as unavoidable byproducts of aerobic metabolism. Other sources of ROIs belong to pathways enhanced during abiotic stresses, such as glycolate oxidase in peroxisomes during photorespiration. However, in recent years, new sources of ROIs have been identified in plants, including NADPH oxidases, amine oxidases and cell-wall-bound peroxidases. These are tightly regulated and participate in the production of ROIs during processes such as programmed cell death (PCD) and pathogen defense.

An unavoidable consequence of aerobic metabolism is production of reactive oxygen species (ROS). ROS include free radicals such as superoxide anion (O2•−), hydroxyl radical (•OH), as well as non-radical molecules like hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), and so forth. Stepwise reduction of molecular oxygen (O_2) by high-energy exposure or electron-transfer reactions leads to production of the highly reactive ROS. In plants, ROS are always formed by the inevitable leakage of electrons onto O_2 from the electron transport activities of chloroplasts, mitochondria, and plasma membranes or as a byproduct of various metabolic pathways localized in different cellular compartments. Environmental stresses such as drought, salinity, chilling, metal toxicity, and UV-B radiation as well as pathogens attack lead to enhanced generation of ROS in plants due to disruption of cellular homeostasis. All ROS are extremely harmful to organisms at high concentrations. When the level of ROS exceeds the defense mechanisms, a cell is said to be in a state of "oxidative stress." The enhanced production of ROS during environmental stresses can pose a threat to cells by causing peroxidation of lipids, oxidation of

proteins, damage to nucleic acids, enzyme inhibition, activation of programmed cell death (PCD) pathway and ultimately leading to destruction.

Despite their destructive activity, ROS are well-described second messengers in a variety of cellular processes including tolerance to environmental stresses. Whether ROS will act as damaging or signaling molecule depends on the delicate equilibrium between ROS production and scavenging. Because of the multifunctional roles of ROS, it is necessary for the cells to control the level of ROS tightly to avoid any oxidative injury and not to eliminate them completely. Scavenging or detoxification of excess ROS is achieved by an efficient anti-oxidative system comprising of the nonenzymic as well as enzymic antioxidants. The enzymic antioxidants include superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (GPX), enzymes of ascorbate-glutahione (AsA-GSH) cycle such as ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and glutathione reductase (GR). Ascorbate (AsA), glutathione (GSH), carotenoids, tocopherols, and phenolics serve as potent nonenzymic antioxidants within the cell. Various workers have reported increased activities of many enzymes of the antioxidant defense system in plants to combat oxidative stress induced by various environmental stresses. Maintenance of a high antioxidant capacity to scavenge the toxic ROS has been linked to increased tolerance of turf grass plants to these environmental stresses. Considerable progress has been made in improving stress-induced oxidative stress tolerance in crop plants by developing transgenic lines with altered levels of antioxidants. Simultaneous expression of multiple antioxidant enzymes has been shown to be more effective than single or double expression for developing transgenic plants with enhanced tolerance to multiple environmental stresses. The present review focuses on types of ROS, their site of production, and their role as messenger and inducer of oxidative stress. Further, role of antioxidative defense system in combating danger posed by overproduced ROS under stresses has been discussed in detail.

Reactive Oxygen Species, Sites of Production, and Their Effects

ROS are a group of free radicals, reactive molecules, and ions that are derived from O_2. It has been estimated that about 1% of O_2 consumed by plants is diverted to produce ROS in various subcellular loci such as chloroplasts, mitochondria, peroxisomes. ROS are well recognized for playing a dual role as both deleterious and beneficial species depending on their concentration in plants. At high concentration ROS cause damage to biomolecules, whereas at low/moderate concentration it acts as second messenger in intracellular signaling cascades that mediate several responses in plant cells.

Types of ROS

The most common ROS include $^1O_2$, $O2^{\bullet-}$, H_2O_2, $^{\bullet}OH$. O_2 itself is a totally harmless molecule as in its ground state it has two unpaired electrons with parallel spin which makes it paramagnetic and, hence, unlikely to participate in reactions with organic molecules unless it is activated. Activation of O_2 may occur by two different mechanisms: (i) absorption of sufficient energy to reverse the spin on one of the unpaired electrons and (ii) stepwise monovalent

reduction (Figure 1). In the former, $^1O_2$ is formed, whereas in latter, O_2 is sequentially reduced to $O2^{\bullet-}$, H_2O_2, and $^{\bullet}OH$.

Electrons in the biradical form of oxygen have parallel spin. Absorption of sufficient energy reverses the spin of one of its unpaired electrons leading to formation of singlet state in which the two electrons have opposite spin. This activation overcomes the spin restriction and $^1O_2$ can consequently participate in reactions involving the simultaneous transfer of two electrons (divalent reduction). In the light, highly reactive $^1O_2$ can be produced via triplet chlorophyll (Chl) formation in the antenna system and in the reaction centre of photosystem II. In the antenna, insufficient energy dissipation during photosynthesis can lead to formation of chlorophyll (Chl) triplet state, whereas in the reaction centre it is formed via charge recombination of the light-induced charge pair. The Chl triplet state can react with $^3O_2$ to give up the very highly destructive ROS $^1O_2$:Chllight⟶3Chl,(1)3Chl+3O2⟶Chl+1O2,(2)

Further, limited CO_2 availability due to closure of stomata during various environmental stresses such as salinity, drought favors the formation of $^1O_2$. The life time of $^1O_2$ within the cell is probably 3 μs or less. A fraction of $^1O_2$ has been shown to be able to diffuse over considerable distances of several hundred nanometers (nm). $^1O_2$ can last for 4 μs in water and 100 μs in a nonpolar environment. $^1O_2$ reacts with most of the biological molecules at near diffusion-controlled rates. It directly oxidizes protein, unsaturated fatty acids, and DNA. It causes nucleic acid modification through selective reaction with deoxyguanosine. It is thought to be the most important species responsible for light-induced loss of photosystem II (PSII) activity which may trigger cell death. $^1O_2$ can be quenched by β-carotene, α-tocopherol or can react with the D1 protein of photosystem II as target.

Due to spine restriction, molecular O_2 cannot accept four electrons at a time to produce H_2O. It accepts one electron at a time and hence during reduction of O_2 stable intermediates are formed in the step-wise fashion. $O2^{\bullet-}$ is the primary ROS formed in the cell which initiates a cascade of reactions to generate "secondary" ROS, either directly or prevalently through enzyme- or metal-catalysed processes depending on the cell type or cellular compartment. $O2^{\bullet-}$ is a moderately reactive, short-lived ROS with a half-life of approx. 1 μs. $O2^{\bullet-}$ is a nucleophilic reactant with both oxidizing and reducing properties. Anionic charge of $O2^{\bullet-}$inhibits its electrophilic activity toward electron-rich molecules. $O2^{\bullet-}$ has been shown to oxidize enzymes containing the [4Fe-4S] clusters (aconitase or dehydratase as examples) and reduce cytochrome C. $O2^{\bullet-}$ can accept one electron and two protons to form H_2O_2. It is easily dismutated to H_2O_2 either nonenzymatically or by SOD catalyzed reaction.

Hydrogen peroxide:2O2•−+2H+⟶H2O2+O2,(3)2O2•−+2H+SOD⟶H2O2+O2,(4)

H_2O_2 is generated in the cells under normal as well as wide range of stressful conditions such as drought, chilling, UV irradiation, exposure to intense light, wounding and intrusion by pathogens. Electron transport chain (ETC) of chloroplast, mitochondria, endoplasmic reticulum and plasma membrane, β-oxidation of fatty acid and photorespiration are major sources of H_2O_2 generation in plant cells. Photooxidation reactions, NADPH oxidase as well as xanthine oxidase (XOD) also contribute to H_2O_2 production in plants. It is also generated in tissues requiring it as a substrate for biosynthesis such as for lignification and suberization. $H_2O_2$is moderately reactive and is relatively long-lived molecule with a half-life of 1 ms H_2O_2 has no

unpaired electrons, unlike other oxygen radicals, it can readily cross biological membranes and consequently can cause oxidative damage far from the site of its formation. Because H_2O_2 is the only ROS that can diffuse through aquaporins in the membranes and over larger distances within the cell and is relatively stable compared to other ROS, it has received particular attention as a signal molecule involved in the regulation of specific biological processes and triggering tolerance against various environmental stresses such as plant-pathogen interactions at low concentration. At high concentration, H_2O_2 can oxidize the cysteine (–SH) or methionine residues ($–SCH_3$), and inactivate enzymes by oxidizing their thiol groups, such as enzymes of Calvin cycle, Cu/Zn-SOD, and Fe-SOD. When hydrogen peroxide accumulates at levels of 10 μM, the enzymes in the Calvin cycle, such as fructose-1,6-bisphosphatase, sedoheptulose-1,7-bisphosphatase, and phosphoribulokinase, lose 50% of their activity. It also oxidizes protein kinases, phosphatases, and transcription factors containing thiolate residues. At high concentrations, it orchestrates programmed cell death.

Both $O2•−$ and H_2O_2 are only moderately reactive. The cellular damage by ROS appears to be due to their conversion into more reactive species. The formation of ˙OH is dependent on both H_2O_2 and $O2•−$ and, thus, its formation is subject to inhibition by both SOD and CAT.
The Haber-Weiss reaction generates ˙OH from H_2O_2 and $O2•−$. It consists of the following two reactions:$Fe3++O2•−⟶Fe2++O2$,(5)
First, Fe(III) is reduced by $O2•−$, followed by oxidation by dihydrogen peroxide (Fenton reaction)$Fe2++H2O2⟶Fe3++OH−+•OH$,(6)
reaction:$O2•−+H2O2⟶•OH+OH−+O2$.

Metal catalysis is necessary for this reaction since the rate of uncatalyzed reaction is negligible. ˙OH is the most reactive among all ROS. It has a single unpaired electron, thus, it can react with oxygen in triplet ground state. ˙OH interacts with all biological molecules and causes subsequent cellular damages such as lipid peroxidation, protein damage, and membrane destruction. Because cells have no enzymatic mechanism to eliminate ˙OH, its excess production can eventually lead to cell death. Under illumination, formation of ˙OH by the Fenton reaction at the active site of the enzyme RbcL leads to its fragmentation in chloroplast lysates. The oxidation of organic substrates by ˙OH may proceed by two possible reactions, either by addition of ˙OH to organic molecules or due to abstraction of a hydrogen atom from it. Because of short lifetime and the strongly positive redox potential (close to +2 V) of “free” ˙OH, its sites of reaction are close to its point of formation. In this context, organic oxygen radicals such as alkoxy, peroxy, semiquinones, reduced hydrogen peroxide, and hydrogen peroxide-electron donor complexes (crypto-OH), as well as metallo-oxygen complexes, have been proposed as the ultimately active species besides destructive free ˙OH.

Sites of Production of ROS

ROS are produced in both unstressed and stressed cells at several locations in chloroplasts, mitochondria, plasma membranes, peroxisomes, apoplast, endoplasmic reticulum, and cell walls (Figure 2). ROS are always formed by the inevitable leakage of electrons onto O_2 from the

electron transport activities of chloroplasts, mitochondria, and plasma membranes or as a byproduct of various metabolic pathways localized in different cellular compartments.

Chloroplasts

In chloroplasts, various forms of ROS are generated from several locations. ETCs in PSI and PSII are the main sources of ROS in chloroplasts. Production of ROS by these sources is enhanced in plants by conditions limiting CO_2 fixation, such as drought, salt, and temperature stresses, as well as by the combination of these conditions with high-light stress. Under normal conditions, the electron flow from the excited PS centers to NADP which is reduced to NADPH which, then, enters the Calvin cycle and reduces the final electron acceptor, CO_2. In case of overloading of the ETC, due to decreased NADP supply resulting from stress conditions, there is leakage of electron from ferredoxin to O_2, reducing it to $O2^{\bullet-}$. This process is called Mehler reaction:$2O2+2Fdred \longrightarrow 2O2^{\bullet-}+2Fdox$(8)

Leakage of electrons to O_2 may also occur from 2Fe-2S and 4Fe-4S clusters in the ETC of PSI. In PSII, acceptor side of ETC contains QA and QB. Leakage of electron from this site to O_2 contributes to the production of $O2^{\bullet-}$,

The formation of $O2^{\bullet-}$ by O_2 reduction is a rate-limiting step. Once formed $O2^{\bullet-}$ generates more aggressive ROS. It may be protonated to $HO2^{\bullet}$ on the internal, "lumen" membrane surface or dismutated enzymatically (by SOD) or spontaneously to H_2O_2 on the external "stromal" membrane surface. At Fe-S centers where Fe^{2+} is available, H_2O_2 may be transformed through the Fenton reaction into the much more dangerous $OH^{\bullet}$.

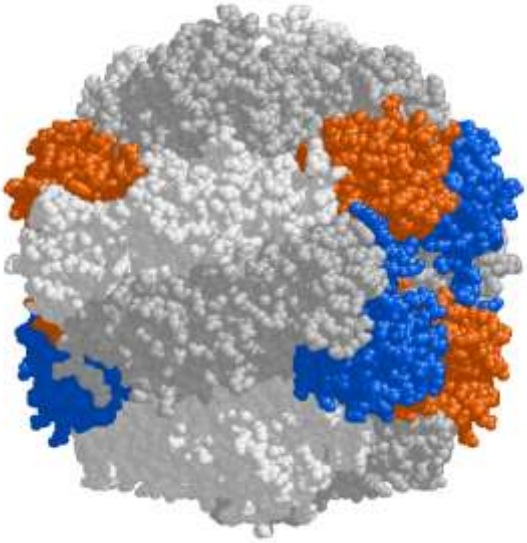
Mitochondria

Mitochondria can produce ROS in several sites of ETC. In mitochondria direct reduction of oxygen to $O2^{\bullet-}$occurs in the flavoprotein region of NADH dehydrogenase segment (complex I) of the respiratory chain. When NAD^+-linked substrates for complex I are limited, electron transport can occur from complex II to complex I (reverse electron flow). This process has been shown to increase ROS production at complex I and is regulated by ATP hydrolysis. Ubiquinone-cytochrome region (complex III) of the ETC also produces $O2^{\bullet-}$from oxygen. It is believed that fully reduced ubiquinone donates an electron to cytochrome C_1 and leaves an unstable highly reducing ubisemiquinone radical which is favorable for the electron leakage to O_2 and, hence, to $O2^{\bullet-}$ formation. In turf grass plants, under normal aerobic conditions, ETC and ATP syntheses are tightly coupled; however, various stress factors lead to inhibition and modification of its component, leading to over reduction of electron carriers and, hence, formation of ROS.

Several enzymes present in mitochondrial matrix can produce ROS. Some of them produce ROS directly, for example aconitase, whereas some others like 1-galactono-γ lactone dehydrogenase (GAL), are able to feed electrons to ETC. $O2^{\bullet-}$ is the primary ROS formed by monovalent reduction in the ETC. It is converted quickly either by the MnSOD (mitochondrial form of SOD) or APX into the relatively stable and membrane-permeable H_2O_2. H_2O_2 can be further converted to extremely active hydroxyl radical ($OH^{\bullet}$) in the Fenton reaction.

What is Rubisco

Ribulose-1,5-bisphosphate carboxylase/oxygenase, or commonly referred to as Rubisco is an enzyme involved in the first major step of carbon fixation, a process by which atmospheric carbon dioxide in converted by turf to energy rich molecules such as glucose. The Rubisco enzyme enables the fixation of inorganic atmospheric carbon dioxide into organic matter for use as a source of energy after incorporation into cellular components.



The rubisco active site is arranged around a magnesium ion. The center magnesium ion connects to a small sugar molecule, three amino acids, lysine, and carbon dioxide molecules. The small sugar molecule that is attached to the magnesium ion is similar to the product that is produced from the Calvin Cycle. The lysine that the magnesium ion attaches to is a modified form of lysine. The lysine has a carbon dioxide molecule attached to its end. In turf, this carbon dioxide molecule is an activator that is attached to rubisco. During the daytime when there is sunlight present, the enzyme is turned on and at night when there is no light present, the enzyme is turned off. When the enzyme is turned on, the magnesium binds to ribulose bisphosphate by attaching to two oxygen atoms and the carbon dioxide molecule that is connected to the sugar.

Warm season grasses have several advantages over cool season grasses. First, by concentrating CO_2, Rubisco carboxylation reactions are increased relative to oxygenation, which results in more CO_2 being fixed per photon absorbed in warm season grasses than in cool season grasses. Secondly, raising the CO_2 partial pressure around Rubisco means it operates at close to

its maximum catalytic rate, thus, to achieve a given CO2 assimilation rate requires a smaller investment of protein into Rubisco.

Both biotic and abiotic stresses can have a negative effect on proper Rubisco function, heat stress seems to have the most destructive effect. Rubisco's heat liable nature affects overall photosynthesis and greatly constrains turf productivity under elevated temperatures. I have seen studies that suggest Rubisco is 80% inefficient when turf is under heat stress. In addition to its role in CO2 assimilation, it also regulates Photosystem II photochemistry. Its deficiency results in decreased levels of different Photosystem II proteins (D1, D2, CP43, CP47, and STN7, number of grana stacks in a chloroplast and discs per grana stack. Rubisco appears to carry a double penalty. Firstly, it catalyzes oxygenation of RuBP leading to photorespiration. Secondly, the maximum catalytic rate of Rubisco is remarkably slow compared with most turf enzymes, such that large amounts of the protein are required to achieve photosynthetic rates necessary to support high productivities in cool season turf. Thirdly, since Rubisco's nature is heat liable, it is prone to oxidative stress.

Endoplasmic Reticulum

In endoplasmic reticulum, NADPH-dependent electron transport involving Cyt P_{450} produces $O2^{\bullet-}$. Organic substrate RH, reacts first with Cyt P_{450} and then is reduced by a flavoprotein to form a radical intermediate (Cyt P_{450} R^{-}). Triplet oxygen can readily react with this radical intermediate as each has one unpaired electron. This oxygenated complex (Cyt P_{450}-ROO^{-}) may be reduced by cytochrome b or occasionally the complexes may decompose releasing $O2^{\bullet-}$.

Peroxisomes

Peroxisomes are probably the major sites of intracellular H_2O_2 production, as a result of their essentially oxidative type of metabolism. The main metabolic processes responsible for the generation of H_2O_2 in different types of peroxisomes are the glycolate oxidase reaction, the fatty acid β-oxidation, the enzymatic reaction of flavin oxidases, and the disproportionation of $O2^{\bullet-}$radicals During photorespiration, the oxidation of glycolate by glycolate oxidase in peroxisomes accounts for the majority of H_2O_2 production. Like mitochondria and chloroplasts, peroxisomes also produce $O2^{\bullet-}$ as a consequence of their normal metabolism. In peroxisomes from pea leaves and watermelon cotyledons, at least, two sites of $O2^{\bullet-}$ generation have been identified using biochemical and electron spin resonance spectroscopy (ESR) methods: one in the organelle matrix, the generating system being XOD, which catalyses the oxidation of xanthine or hypoxanthine to uric acid, and produces $O2^{\bullet-}$ in the process and another site in the peroxisomal membranes where a small ETC composed of a flavoprotein NADH and Cyt b is involved. Three integral peroxisomal membrane polypeptides (PMPs) with molecular masses of 18, 29, and 32 kDa were found to be involved in $O2^{\bullet-}$production. While the 18- and 32-kDa PMPs use NADH as electron donor for $O2^{\bullet-}$ production, the 29-kDa PMP was clearly dependent on NADPH and was able to reduce cytochrome c with NADPH as electron donor. Among the three integral polypeptides, the main producer of $O2^{\bullet-}$ was the 18-kDa PMP which was proposed to be a cytochrome possibly belonging to the b-type group. The PMP32 very probably

corresponds to the MDHAR, and the third O2•−-generating polypeptide, PMP29, could be related to the peroxisomal NADPH:cytochrome P450 reductase. The $O_2^{\bullet-}$ produced is subsequently converted into H_2O_2 by SOD.

Plasma Membranes

Electron transporting oxidoreductases are ubiquitous at plasma membranes and lead to generation of ROS at plasma membrane. Production of ROS was studied using EPR spin-trapping techniques and specific dyes in isolated plasma membranes from the growing and the non-growing zones of hypocotyls and roots of etiolated soybean seedlings as well as coleoptiles and roots of etiolated maize seedlings. NADPH mediated the production of O2•− in all plasma membrane samples. It was suggested that in soybean plasma membranes, O2•−production could be attributed to the action of at least two enzymes, an NADPH oxidase, and, in the presence of menadione, a quinone reductase. NADPH oxidase catalyses transfer of electrons from cytoplasmic NADPH to O_2 to form O2•−. O2•− is dismutated to H_2O_2 either spontaneously or by SOD activity. NADPH oxidase has been proposed to play a key role in the production and accumulation of ROS in plants under stress conditions.

Cell Walls

Cell walls are also regarded as active sites for ROS production. Role of cell-wall-associated peroxidase in H_2O_2generation has been shown. In horseradish, peroxidase associated with isolated cell walls catalyzes the formation of H_2O_2 in the presence of NADH. The reaction is stimulated by various monophenols, especially of coniferyl alcohol. Malate dehydrogenase was found to be the sole candidate for providing NADH. The generation of ROS by cell-wall-located peroxidases has been shown during hypersensitive response (HR) triggered in cotton by the bacterium Xanthomonas campestris pv. malvacearum and potassium (K) deficiency stress in Arabidopsis. Diamine oxidases are also involved in production of activated oxygen in the cell wall using diamine or polyamines (putrescine, spermidine, cadaverine, etc.) to reduce a quinone that autooxidizes to form peroxides.

Apoplast

Cell-wall-located enzymes have been proved to be responsible for apoplastic ROS production. The cell-wall-associated oxalate oxidase, also known as germin, releases H_2O_2 and CO_2 from oxalic acid. This enzyme was reported to be involved in apoplastic hydrogen peroxide accumulation during interactions between different cereals species and fungi. Amine oxidase-like enzymes may contribute to defense responses occurring in the apoplast following biotic stress, mainly through H_2O_2 production. Amine oxidases catalyze the oxidative deamination of polyamines (i.e., putrescine, spermine, and spermidine) using FAD as a cofactor.

ROS as Messengers

At low/moderate concentration, ROS have been implicated as second messengers in intracellular signaling cascades that mediate several plant responses in plant cells, including stomatal closure programmed cell death, gravitropism, and acquisition of tolerance to both biotic and abiotic stresses shows the role of ROS as second messenger.

Turf plants can sense, transduce and translate ROS signal into appropriate cellular responses with the help of some redox-sensitive proteins, calcium mobilization, protein phosphorylation, and gene expression. ROS can be sensed directly also by key signaling proteins such as a tyrosine phosphatase through oxidation of conserved cysteine residues (reviewed in ROS can also modulate the activities of many components in signaling, such as protein phosphatases, protein kinases and transcription factors and communicate with other signal molecules and the pathway forming part of the signaling network that controls response downstream of ROS. The strength, lifetime and size of the ROS signaling pool depends on balance between oxidant production and removal by the antioxidant. Using mutants deficient in key ROS-scavenging enzymes, scientists identified a signaling pathway that is activated in cells in response to ROS accumulation. Interestingly, many of the key players in this pathway, including different zinc finger proteins and WRKY transcription factors, are also central regulators of abiotic stress responses involved in temperature, salinity and osmotic stresses.

ROS are considered second messengers in the abscisic acid (ABA) transduction pathway in guard cells ABA induced H_2O_2 is an essential signal in mediating stomatal closure to reduce water loss through the activation of calcium-permeable channels in the plasma membrane Jannat and coworkers [observed that ABA-inducible cytosolic H_2O_2 elevation functions in ABA-induced stomatal closure, while constitutive increase of H_2O_2 does not cause stomatal closure. Role of ROS as second messenger in root gravitropism has been demonstrated. Based on their work, Joo and coworkers proposed that gravity induces asymmetric movement of auxin within 60 min, and, then, the auxin stimulates ROS generation to mediate gravitropism. Further, scavenging of ROS by antioxidants (N-acetylcysteine, ascorbic acid, and Trolox) inhibited root gravitropism [ROS appear to be involved in dormancy alleviation. In dormant barley grains under control condition, gibberellic acid (GA) signaling and ROS content are low, while ABA signaling is high, resulting in dormancy. Exogenous H_2O_2 does not appear to alter ABA biosynthesis and signaling, but has a more pronounced effect on GA signaling, inducing a change in hormonal balance that results in germination ROS have been shown to play a key role in PCD in barley aleurone cells, initiated by GA. Bethke and Jones observed that GA-treated aleurone protoplasts are less tolerant to internally generated or exogenously applied H_2O than ABA-treated protoplasts and suggested that ROS are components of the hormonally regulated cell death pathway in barley aleurone cells.

Turf plants have evolved a complex regulatory network to mediate biotic and abiotic stress responses based on ROS synthesis, scavenging, and signaling. Transient production of ROS is detected in the early events of plant-pathogen interactions and plays an important signaling role in pathogenesis signal transduction regulators. This production-called oxidative burst could be considered as a specific signal during the interaction process. In HR, SA is thought to potentiate ROS signaling. ROS are shown to act as a second messenger for the induction of defense genes in tomato plants in response to wounding ROS were generated near cell walls of vascular bundle cells of tomato leaves in response to wounding and resulted H_2O_2 from wound-inducible polygalacturonase acted as a second messenger for the activation of defense genes in mesophyll cells, but not for signaling pathway genes in vascular bundle cells.

Lignin is important for the plant's response to environmental stress. Researchers characterized a genetic network enabling plants to regulate lignin biosynthesis in response to cell

wall damage through dynamic interactions between Jasmonic acid and ROS. ROS have been shown to play important roles in osmotic stress, low temperature, and heavy metal signal transduction pathway. Genes involved in osmotic stress signaling have been shown to be upregulated by ROS, including the transcription factor DREB2A and a histidine kinase. In Arabidopsis culture cells, it was reported that the MAPK AtMPK6 that can be activated by low temperature and osmotic stress could also be activated by oxidative stress. Studies have suggested that the increased osmotic stress tolerance of transgenic Arabidopsis expressing a salicylate hydroxylase (NahG) gene, might result from decreased SA-mediated ROS generation. Some studies have suggested that ROS play important roles in drought-induced abscisic acid synthesis in plant and suggested that they may be the signals through which the plant can "sense" the drought condition. Using pharmacological inhibitors, it is demonstrated that metals Cd^{2+} and Cu^{2+} induce MAP kinase activation via distinct ROS-generating systems.

ROS and Oxidative Damage to Biomolecules

Production and removal of ROS must be strictly controlled in order to avoid oxidative stress. When the level of ROS exceeds the defense mechanisms, a cell is said to be in a state of "oxidative stress". However, the equilibrium between production and scavenging of ROS is perturbed under a number of stressful conditions such as salinity, drought, high light, toxicity due to metals, pathogens, and so forth. Enhanced level of ROS can cause damage to biomolecules such as lipids, proteins and DNA. These reactions can alter intrinsic membrane properties like fluidity, ion transport, loss of enzyme activity, protein cross-linking, inhibition of protein synthesis, DNA damage, and so forth ultimately resulting in cell death.

Lipids

When ROS level reaches above threshold, enhanced lipid peroxidation takes place in both cellular and organelle membranes, which, in turn, affect normal cellular functioning. Lipid peroxidation aggravates the oxidative stress through production of lipid-derived radicals that themselves can react with and damage proteins and DNA. The level of lipid peroxidation has been widely used as an indicator of ROS mediated damage to cell membranes under stressful conditions. Increased peroxidation (degradation) of lipids has been reported in plants growing under environmental stresses. Increase in lipid peroxidation under these stresses parallels with increased production of ROS. Malondialdehyde (MDA) is one of the final products of peroxidation of unsaturated fatty acids in phospholipids and is responsible for cell membrane damage. Two common sites of ROS attack on the phospholipid molecules are the unsaturated (double) bond between two carbon atoms and the ester linkage between glycerol and the fatty acid. The polyunsaturated fatty acids (PUFAs) present in membrane phospholipids are particularly sensitive to attack by ROS. A single $^{\bullet}OH$ can result in peroxidation of many polyunsaturated fatty acids because the reactions involved in this process are part of a cyclic chain reaction. The overall process of lipid peroxidation involves three distinct stages: initiation, progression, and termination steps. The initial phase of lipid peroxidation includes activation of O_2 which is rate limiting. $O2^{\bullet-}$ and $^{\bullet}OH$ can react with methylene groups of PUFA forming

conjugated dienes, lipid peroxy radicals and hydroperoxides PUFA−H+X•⟶PUFA+X−H.(9)PUFA+O2⟶PUFA−OO•.
The peroxy radical formed is highly reactive and able to propagate the chain reaction:PUFA−OO•+PUFA−OOH⟶PUFA−OOH+PUFA•.

The formation of conjugated diene occurs when free radicals attack the hydrogens of methylene groups separating double bonds and, thereby, rearrangement of the bonds occurs The lipid hydroperoxides produced (PUFA-OOH) can undergo reductive cleavage by reduced metals, such as Fe^{2+}, according to the following reaction:Fe2+complex+PUFA−OOH⟶Fe.
Several reactive species including: lipid alkoxyl radicals, aldehydes (malonyldialdehyde, acrolein and crotonaldehyde), alkanes, lipid epoxides, and alcohols can be easily formed by the decomposition of lipid hydroperoxide. The lipid alkoxy radical produced, (PUFA-O•), can initiate additional chain reactions [PUFA−O•+PUFA−H⟶PUFA−OH+PUFA•. Peroxidation of polyunsaturated fatty acid by ROS attack can lead to chain breakage and, thereby, increase in membrane fluidity and permeability

Proteins

The attack of ROS on proteins may cause modification of proteins in a variety of ways, some are direct and others indirect. Direct modification involves modulation of a protein's activity through nitrosylation, carbonylation, disulphide bond formation, and glutathionylation. Proteins can be modified indirectly by conjugation with breakdown products of fatty acid peroxidation. As a consequence of excessive ROS production, site-specific amino acid modification, fragmentation of the peptide chain, aggregation of cross-linked reaction products, altered electric charge and increased susceptibility of proteins to proteolysis occur. Turf grass tissues injured by oxidative stress generally contain increased concentrations of carbonylated proteins which is widely used marker of protein oxidation Enhanced modification of proteins has been reported in plants under various stresses. The amino acids in a peptide differ in their susceptibility to attack by ROS. Thiol groups and sulphur containing amino acids are very susceptible sites for attack by ROS. Activated oxygen can abstract an H atom from cysteine residues to form a thiyl radical that will cross-link to second thiyl radical to form disulphide-bridge. Several metals, including Cd, Pb, and Hg have been shown to cause the depletion of protein bound thiol groups. Oxygen also can be added to a methionine to form methionine sulphoxide derivative. Tyrosine is readily cross-linked to form bityrosine products in the presence of ROS.
Oxidation of iron-sulphur centers by O2•− is irreversible and leads to enzyme inactivation. In these cases, the metal (Fe) binds to a divalent cation-binding site on the protein. The metal (Fe), then, reacts in a Fenton reaction to form a $^{\bullet}OH$ that rapidly oxidizes an amino acid residue at or near the cation-binding site of the protein. Oxidized proteins serve as better substrates for proteolytic digestion. It has been suggested that protein oxidation could predispose it to ubiquitination, which, in turn, would be a target for proteasomal degradation. The incubation of pea leaf crude extracts with increasing H_2O_2 concentrations, Cd-treated plants and peroxisomes purified from pea leaves showed increase in carbonyl content. Oxidized proteins were more efficiently degraded, and the proteolytic activity increased 20% due to the metal treatment [Several studies have revealed that after a certain degree further damage leads to extensively