The Americas Journal of Plant Science and Biotechnology ©2011 Global Science Books

Living in the O-zone: Ozone Formation, Ozone-Plant Interactions and the Impact of Ozone Pollution on Plant Homeostasis

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ABSTRACT

Ozone (O_3) is a key constituent of the terrestrial atmosphere. Unlike in the stratosphere, where O_3 provides an essential barrier to incoming UV radiation, within the troposphere, it is a major secondary air pollutant that is estimated to cause more damage to plant life than all other air pollutants combined. In the troposphere, O_3 is produced by photochemical oxidation of primary precursor emissions of volatile organic compounds (VOCs), carbon monoxide (CO), and sulfur dioxide (SO_2) in association with elevated levels of oxides of nitrogen ($NO_x \equiv NO + NO_2$). Because of its strong oxidizing potential, ozone is damaging to plant life through oxidative damage to proteins, nucleic acids and lipids either directly or as a result of reactive oxygen species (ROS) derived from O_3 decomposition. In plants, ROS, directly or indirectly derived from O_3 exposure, are routinely scavenged by an array of enzymatic and non-enzymatic antioxidant defense mechanisms. The various ROS generated by O_3 have strong influence on the plant's biochemical and signalling network eliciting a wide range of responses including cell death.

Keywords: hydrogen peroxide, mitogen-activated protein kinase, reactive oxygen species, signalling, transgenic trees
Abbreviations: **APX**, ascorbate peroxidase; **CAT**, catalase; **Fe^{2+}**, ferrous ion; **Fe^{3+}**, ferric ion; **GSH**, reduced glutathione; **H_2O_2**, hydrogen peroxide; **HHP**, hydroxyhydroperoxide; **$HO^{\bullet}$**, hydroxyl radical; **HR**, hypersensitive response; **MDHA**, monodehydroascorbate; **NADPH**, nicotinamide adenine dinucleotide phosphate; **NO_x**, nitrogen oxides; **$O(^3P)$**, ground state oxygen; **$O(^1D)$**, electronically excited state of oxygen; **SA**, salicylic acid; **SOD**, superoxide dismutase; **VOC**, volatile organic compounds

CONTENTS

OZONE FORMATION AND DISTRIBUTION

In the atmosphere of early Earth, before photosynthetic archaea and bacteria evolved, free oxygen was practically non-existent. Around 2.7 billion years ago, free oxygen started to gas out from the oceans into the atmosphere where it gradually accumulated, ultimately converting the early reducing atmosphere to an oxidizing one. It was not until the Paleoproterozoic era, between 2.5 and 1.6 billon years ago, that significant levels of free oxygen started to accumulate in Earth's atmosphere (Holland 1994). In early days, the majority of this free oxygen combined with dissolved iron in the oceans forming banded iron formations, which are still present today (Eigenbrode *et al*. 2006, and references within). The presence of large amounts of free and dissolved oxygen in the oceans and atmosphere of early Earth, led to a massive ecological change that disadvantaged the already existing anaerobic organisms. The evolution of photosynthesis and respiration of oxygen made the evolution of eukaryotic cells and eventually multicellular organisms such as plants and animals possible.

In the upper layers of the stratosphere, O_2 molecules absorbed UV radiation (UVR) from the sun in a photochemical reaction that eventually led to the formation of ozone (O_3). The resulting stratospheric O_3 layer that surrounds the Earth acted as a "filter" against a significant amount of the incoming UVR that once had passed through the atmosphere. This allowed for the colonization of the Earth's oceans and land masses by early life forms.

O_3 forms readily in the stratosphere, above about 30 km,

Received: 4 December, 2009. Accepted: 10 December, 2010.

Invited Review

primarily as a result of photo-dissociation of diatomic oxygen by high energy, UVR of wavelengths less than 240 nm (λ<240 nm). A theory for the photochemical mechanism of O_3 production in the stratosphere was first proposed by Chapman (1930) and thus often is referred to as the "Chapman mechanism." The reaction sequence starts with the ultraviolet photolysis of diatomic oxygen (R1), which creates two oxygen radicals followed by each of these oxygen radicals reacting with another molecule of molecular oxygen, forming O_3 (R2). These oxygen atoms usually exist in the triplet or ground state i.e. $O(^3P)$, however, at wavelengths less than 175 nm, both a triplet state and an excited state i.e. $O(^1D)$ oxygen atoms are formed. This oxygen atom, in an excited state, on collision with some diatomic molecule (M) will result in an oxygen atom in the ground state. In these reactions, ($h\nu$) denotes a quantum of radiation of appropriate energy, and the symbol (M) refers to a third molecule, such as N_2 or O_2, which stabilizes (conservation of energy and momentum) the reaction by absorbing a portion of the energy released in O_3 formation. In order for a balance to be maintained, without the O_3 concentration increasing indefinitely, there must be additional reactions that intervene to destroy O_3. Chapman proposed that O_3 in the stratosphere is destroyed through photolysis of O_3, reactions (R3 and R4). This dissociation produces both atomic oxygen and molecular oxygen, with the atomic oxygen in an excited state. This newly formed atomic oxygen atom can then combine with molecular oxygen to produce an O_3 molecule as in reaction (R2), or with O_3 to produce two molecules of oxygen, reaction (R4). This continuing process of O_3 production and destruction, called the O_3-oxygen cycle, described by Chapman (1930), thus maintains an O_3 layer in the stratosphere, where about 90% of the Earth's O_3 is located (**Schemes 1** and **2**).

In the 1960s, it was discovered that models based solely on the Chapman cycle over predicted the concentration of stratospheric O_3 (reviewed in Solomon 1999). It is now recognized that several additional mechanisms (cycles) intervene in the destruction of O_3 (Crutzen and Oppenheimer 2008; McConnell and Jin 2008; Velasco *et al*. 2008). Two of these mechanisms are shown in reactions R5-R6 and reactions R7-R9, respectively. The first of these mechanisms is the HO_x (hydroxyl ($^{\bullet}OH$) and hydroperoxyl ($HO^{\bullet}_2$) radicals) or (odd hydrogen catalytic cycles) mechanism and the second being a cycle involving nitrogen monoxide ($NO^{\bullet}$) and nitrogen dioxide (NO_2) (odd nitrogen catalytic cycle). In the reaction where N_2O combines with atomic oxygen to produce two molecules of $NO^{\bullet}$, oxygen is in an excited state $O(^1D)$ and thus must first lose energy by contacting a third body (M) to produce a ground state oxygen. In addition to these mechanisms, there are other mechanisms involving halogens like bromine and chlorine (Cl-Br catalytic cycle) (Crutzen and Oppenheimer 2008; McConnell and Jin 2008).

O_3 produced in the stratosphere can enter the troposphere via what is known as the stratosphere-troposphere exchange phenomenon (Shapiro 1980). This process appears to involve large-scale eddies in the jet stream region, and results in net movements of O_3 from the stratosphere to the troposphere. This process, though it exists, accounts for only a small part of the tropospheric (ground-level) O_3, whereas; the majority of ground-level O_3 is formed *in situ* by photochemical reactions involving VOCs and nitrogen oxides (NOx), which are produced in large quantities in and around heavily industrialized areas (Chameides *et al*. 1988, and references within; Poisson *et al*. 2000).

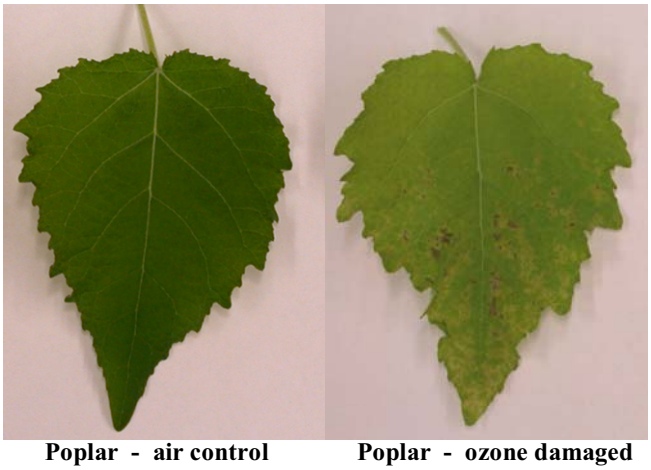
In the troposphere, O_3 is produced by the same reaction responsible for its formation in the stratosphere: the addition of ground state oxygen atoms to molecular oxygen in the presence of a third body, such as N_2 or O_2. In the troposphere, however, only UV radiation with a wavelength greater than (>290 nm), which is not energetic enough for photo-dissociation of diatomic oxygen to occur, exists, due to nearly complete absorption of shorter wavelengths by N_2 or O_2 in the stratosphere and tropopause. In the troposphere, the only significant source known for the ground state oxygen atom, needed for O_3 formation, is through the photo-dissociation of NO_2 (R10). When produced, the ground state oxygen can combine, in the presence of a third body, with molecular oxygen to produce O_3 (R11). The $NO^{\bullet}$ produced in (R10), which is also produced as a result of fuel combustion above 2000°C, spontaneously reacts with this newly formed O_3 (R12) resulting in the destruction of O_3. In an unpolluted troposphere, a steady-state concentration of O_3 can be predicted based on concentrations of reaction partners as well as the intensity of sunlight (Pitts and Finlayson 1975; Barrett *et al*. 1998). This process of O_3 formation and destruction depicted in (R10-R12) is known as the null cycle. This steady-state level of O_3 predicted by the null cycle can be drastically altered as a result of concurrent oxidation of oxidizable species such as carbon monoxide (CO), sulfur dioxide (SO_2), aldehydes, and hydrocarbons in the presence of elevated levels of NOx (Pitts and Finlayson 1975; Barrett *et al*. 1998; Sillman 1999). One such mechanism is shown for the oxidation of CO (R13-R16) which results in the formation of O_3. Reactions (R17 and R18) show the formation of O_3 through the oxidation of various hydrocarbon species.

A number of atmospheric trace gases such as O_3, H_2O_2, formaldehyde (HCHO), nitric acid (HNO_3), NO_2 undergo photolysis when they absorb UVB (280-320 nm) radiation. These photolysis products (O, $NO^{\bullet}$, $^{\bullet}OH$, H, HCO and eventually $HO^{\bullet}_2$ and organic peroxy radicals) exist as highly reactive species in the atmosphere. The increased production of these reactive species adds to the oxidizing capacity of the troposphere, and thus to the oxidant challenge faced by living organisms (Tang *et al*. 1998).

The adverse effect of O_3 on plants was first identified in the 1950s (Hill *et al*. 1961), and since then, has been of growing importance as a major phytotoxin. Studies have shown that O_3 and other atmospheric oxidants are responsible for as much as 90% of agricultural crop loss due to air pollution (Heck *et al*. 1984a, 1984b). In 2000, global crop losses were estimated to be between US $14 and $26 billion, with 40% of this loss in China and India (Van Dingenen *et al*. 2009). Similarly, in forest systems, O_3 is believed to cause more damage to trees than any other gaseous pollutant (Koch *et al*. 1998; Langebartels *et al*. 1998). In the Los Angeles Basin, the Federal Ambient Air Quality Standard of 120 nl L^{-1} (averaged over a 1 hr period) is exceeded 90-100 days a year (Mudd *et al*. 1997) and peak O_3 levels can exceed 400 nl L^{-1} (1 hr daily maxima) (McCurdy 1994). O_3 is not only a problem in cities and industrialized areas, rural locations are also affected by elevated levels of O_3 due to long-distance (local as well as inter-continental) transport of O_3 and its chemical precursors (Prather *et al*. 2003). Because the complex series of reactions are driven by temperature and sunlight, O_3 formation varies hourly, daily and seasonally. On a global scale, mean concentrations of tropospheric O_3 have increased by approximately two-times over the past century due to greatly elevated levels of emissions of fossil fuel and biomass burning (Gauss *et al*. 2006; Denman *et al*. 2007), with the possibility of tropospheric O_3 levels increasing 20-25% by 2050 (Meehl *et al*. 2007). Over the past 20 years in North America, ambient concentrations of O_3 have increased 1 to 2% per year with no indication of leveling off (Stockwell *et al*. 1997) (**Scheme 3**).

EFFECTS OF O_3 EXPOSURE ON PLANTS

It has been known for a long time that O_3 has detrimental effects on vegetation of all types (**Fig. 1**). The effects of this pollutant on plants include diminished photosynthesis (Runeckles and Krupa 1994; Darral 1989; Brendley and Pell 1998), characteristic flecking, chlorosis and formation of necrotic lesions (Manning and Krupa 1992); retarded growth, increased lipid peroxidation (Hewitt *et al*. 1990); membrane damage (Heath 1988); and accelerated foliar senescence (Pauls and Thompson 1980). All of these symptoms can be related to the initial production of reactive oxygen species (ROS) in the affected tissues (Grimes *et al*.



Poplar - air control **Poplar - ozone damaged**

Fig. 1 O_3-induced foliar damage to Poplar showing typical leaf bronzing, patches of dead cells and general yellowing.

1983; Patton and Garraway 1986; for a review, see Runeckles and Chevone 1992).

O_3 and UVR also induce additional stress-related genes such as lipoxygenase (LOX), polyphenol oxidase (PPO), phenylalanine ammonia lyase (Pal), and proteinase inhibitors I and II (Pin) (Lois and Hahlbrock 1992; Maccarrone *et al*. 1992; Bell and Mullet 1993; Sharma and Davis 1994; Sano *et al*. 1994). This indicates that these stressors may share all or part of the subsequent signal transduction cascades.

O_3-INDUCED OXIDATIVE STRESS IN PLANT TISSUES: *IN VIVO* PRODUCTION OF ROS

ROS consist of oxygen-centered redox derivatives of molecular oxygen. The parental molecule for many of these derivatives is the free radical $^{\bullet}O_2^-$. The family of ROS species derived from $^{\bullet}O_2^-$ consists of a number of other free radicals, most notably the $^{\bullet}OH$ and $HO_2^{\bullet}$, which is the conjugate acid to the superoxide anion ($^{\bullet}O_2^-$). In addition to these $^{\bullet}O_2^-$ derived free radicals, various oxygen derivatives that are not free radicals, e.g., H_2O_2, are also formed. Reactive nitrogen species (RNS) such as the parental radical, $NO^{\bullet}$, and an array of redox-active derivatives such as $NO_2^{\bullet}$ and peroxinitrite ($ONOO^{\bullet}$), are also formed, but the impact of RNS is outside of the scope of this review.

O_3 enters plants via the stomata in the leaf surface during normal gas exchange (Kerstiens and Lendzian 1989), diffuses through the inner air spaces and is absorbed into the apoplast of the mesophyll cells (Morgan and Wenzel 1985; Salter and Hewitt 1992; Sharma and Davis 1997; Renaut *et al*. 2009). The O_3 concentration in the apoplastic cavity is close to zero (Laisk *et al*. 1989), indicating that O_3 is rapidly degraded. Initial targets for O_3 include water, susceptible amino acids in plasma membrane proteins, organic metabolites present in the cell wall, apoplastic enzymes, and lipids. When O_3 comes in contact with pure water, $^{\bullet}OH$ and peroxyl radicals and $^{\bullet}O_2^-$ are formed, albeit, these reactions proceed slowly at neutral pH (Heath 1987). However, when phenolic compounds are present, the rate of $^{\bullet}OH$ formation is greatly increased over that of pure water; pointing to the importance of biologically relevant compounds in the production of O_3-derived ROS.

As a result of these above-mentioned interactions, O_3 degrades rapidly to secondary oxidizing products such as $^{\bullet}O_2^-$, $HO_2^{\bullet}$, the conjugate acid of $^{\bullet}O_2^-$, $HO^{\bullet}$, and singlet oxygen ($^1O_2^*$). In addition to these free radical species, various non-radical oxygen-centered species, e.g., H_2O_2 are also formed (Mehlhorn *et al*. 1990; Runeckles and Vaartnou 1997; Pellinen *et al*. 1999). It can also form ozonides and lipid peroxides that can initiate a series of reactions producing further damaging reactive oxygen intermediates (Sharma and Davis 1997). Because of the strong oxidizing potential (+2.07 eV) of O_3, the accepted view is that the primary reactions of O_3 manifest in the apoplastic space.

O_3 has the ability to react with all hydrocarbons, but has a higher affinity for unsaturated hydrocarbons, such as polyunsaturated fatty acids found in lipid bilayers of living cells. The most rapid reaction between O_3 and unsaturated hydrocarbons is through the process of Criegee ozonation (Criegee 1957; Kelly *et al*. 1995). The first step in this reaction mechanism is initiated through the non-radical mediated electrophilic reaction (1, 3-dipolar -cycloaddition) of O_3 with an unsaturated C-C bond resulting in a trioxygen intermediate, 1,2,3-trioxalane (**Fig. 2a**) (Criegee 1957; Squadrito *et al*. 1992). The 1,2,3-trioxolane, also known as a maozonide or primary ozonide, derivative can decompose through *γβ*-scission to give either the carbonyl oxide and aldehyde directly (**Fig. 2b**) or undergo O-O bond homolysis to yield a very unstable biradical species (**Fig. 2e**), which then can partition to give the carbonyl oxide (**Fig. 2f**) or undergoes *β*-scission yielding alkyl and peroxyl radicals (**Fig. 2g**) (Criegee 1957; Squadrito *et al*. 1992; Pryor 1994; Kelly *et al*. 1995). In the absence of water, the aldehyde and carbonyl oxide recombine to form 1,2,4-trioxalane (*cis*- or *trans*-Criegee ozonide or secondary ozonide), which is relatively stable (**Fig. 2c**). In an aqueous environment, however, the carbonyl oxide has a proclivity to react with water, resulting in the production of a hydroxyhydroperoxide (HHP) compound (**Fig. 2d**) with the concomitant formation of H_2O_2 (Squadrito *et al*. 1992, and references within; Kelly *et al*. 1995). This pathway is a consequence of the lower affinity of the carbonyl oxide for the aldehyde species compared to water (Cueto *et al*. 1992; Pryor *et al*. 1992, 1995).

It has been postulated that the H_2O_2 produced here, as well as other sources, may encounter free transition metals, including ferrous (Fe^{2+}) ions, to produce the very reactive $^{\bullet}OH$ through what is known as the Haber-Weiss cycle, via Fe-catalyzed Fenton chemistry, reactions (R19-R24) and discussion below (Fenton 1894; Haber and Willstätter 1931; Haber and Weiss 1932; Haber and Weiss 1934; Grimes *et al*. 1983; Storz *et al*. 1990; Runeckles and Vaartnou 1997; Storz and Imlay 1999). It is also possible for the transition metals to react directly with the HHP compound to produce $^{\bullet}OH$. However, no matter what form the oxidizing species take, it is reasonable to predict that O_3 could be eliciting its downstream effects, at least in part, through the initial rapid oxidation of cell signalling components situated at or near the cell surface.

The Haber-Weiss cycle reactions (R20-R21) were first proposed by Haber and Willstätter (1931) to specifically explain the action of catalase, and should be called the Haber-Willstätter cycle. The Fenton reaction (R19) starts the chain producing $^{\bullet}OH$ ions which is then followed by the chain indicated in reactions (R20 and R21), the Haber-Weiss cycle and ending by chain termination, reaction (R22). Reactions (R23 and R24) were published in Haber and Weiss (1934), but at the time were not considered important. However, it was later shown (Weiss and Humphrey 1949) that reaction (R24), in the presence of ferric (Fe^{3+}) ions, largely replaces reaction (R21), the second reaction (i.e. the reduction of H_2O_2 by $^{\bullet}O_2^-$) of the Haber-Weiss cycle (Koppenol 2001). In an additional paper (George 1947), it was shown that the second reaction (R21), the reaction of H_2O_2 with $^{\bullet}O_2^-$, is far too slow compared to the rapid dismutation of $^{\bullet}O_2^-$ to be of any importance (Koppenol 2001). Other work has shown that oxygen is not produced from reaction (R21) but rather evolves from the reduction of Fe^{3+} ions by $^{\bullet}O_2^-$ in reaction (R24). Thus, (**1**) production of $^{\bullet}OH$ radicals arises from the Fenton reaction (R19) and not from the Haber-Weiss cycle and (**2**) reaction (R21) does not take place and should therefore no longer be discussed as a possible source for $^{\bullet}OH$ radicals in living systems.

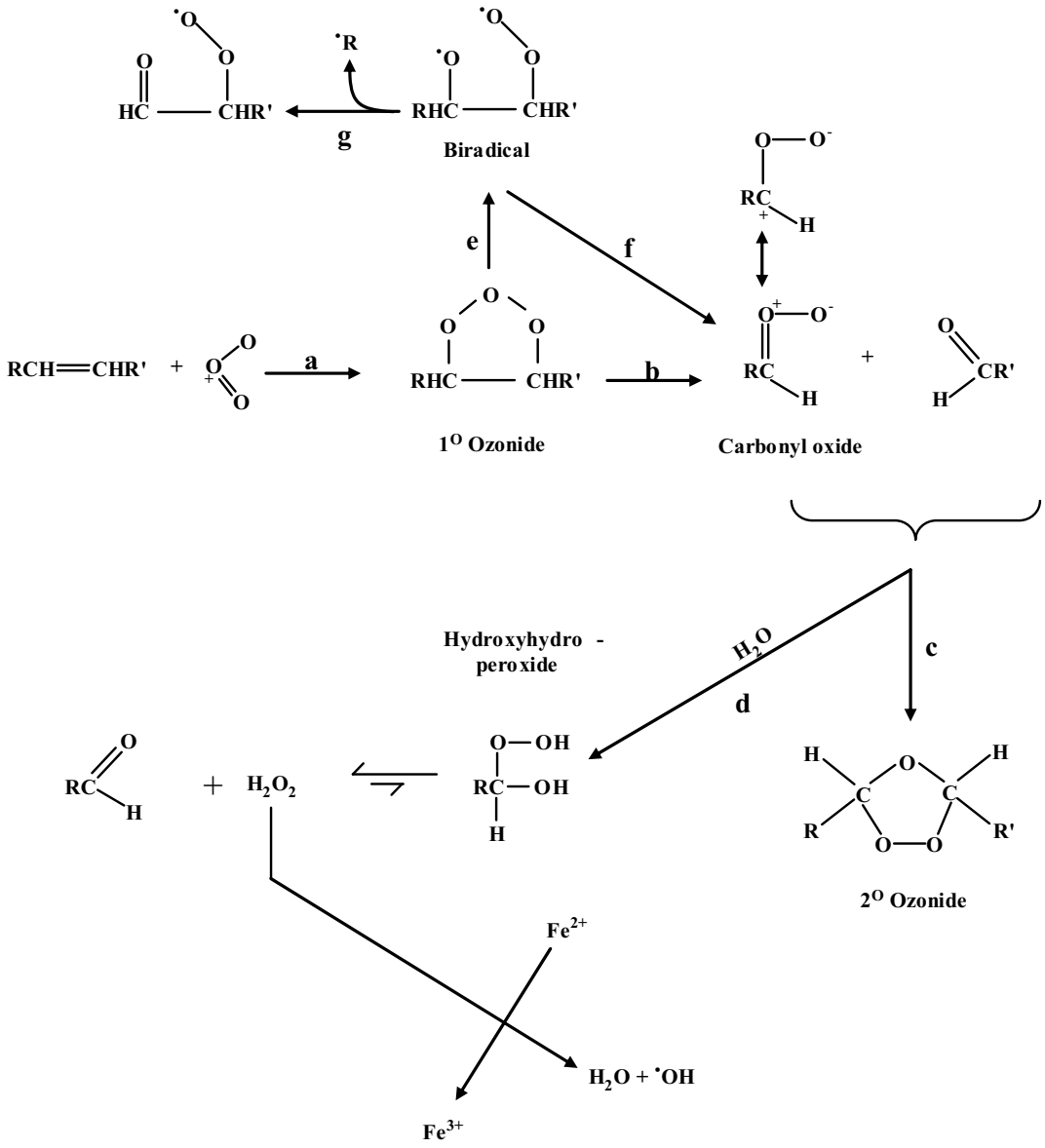


Fig. 2 Reaction scheme for ozonation of alkene bonds. Reactions (a-c) leading to the formation of the secondary ozonide or Criegee ozonide, outline the classical Criegee ozonation pathway. In an aqueous environment, however, reaction pathway (d) predominates, via the hydroxyhydroperoxide species with concomitant H_2O_2 and $^{\bullet}OH$ production, the later if free transition metals such as (Fe^{2+} or Cu^{2+}) are present. The primary ozonide species typically undergoes a $\gamma\beta$-scission producing the carbonyl oxide (b), however, can also yield a biradical species (e) which can partition to give the carbonyl oxide (f), or transition by β-scission to yield peroxyl and alkyl radicals (g).

ORIGINS OF METABOLIC REACTIVE OXYGEN SPECIES

While ROS are produced in cells as a result of environmental stresses such as O_3 and UVR, they are also produced in unstressed cells as a result of normal metabolic events (Foyer *et al*. 1994; Abe *et al*. 1998; Blumwald *et al*. 1998). In green plant parts in the light, the metabolic event with perhaps the greatest propensity for ROS production in plants is photooxidation in chloroplasts and peroxisomes, where the major ROS produced is $^{\bullet}O_2^-$ (Asada 1992; Foyer *et al*. 1994; Foyer and Noctor 2003). In non-green plant parts or in darkness, the mitochondria have been suggested to produce the majority of ROS (Maxwell *et al*. 1999; Møller 2007; Møller *et al*. 2007). This ROS production is generally caused by an over-reduction of the electron transport chain (Møller *et al*. 2007). Within this oxygen-rich microenvironment, $^1O_2^*$ is produced at photosystem I (PSI) while $^{\bullet}O_2^-$ is formed via direct donation of an electron to oxygen from reduced ferredoxin residing in the photosynthetic electron transport chain, photosystem II (PSII) (Asada 2006; Foyer *et al*. 1994; Kangasjarvi *et al*. 1994).

The initial step in the reduction of O_2 by electrons leaking from these high energy systems (**Fig. 3**) produces the short-lived $HO_2^{\bullet}$ and $^{\bullet}O_2^-$ radicals. $HO_2^{\bullet}$ and $^{\bullet}O_2^-$ radicals from hydroperoxides with unsaturated carbon skeletons such as membrane fatty acids, and also oxidize specific amino acids, such as histidine, methionine and tryptophan. $HO_2^{\bullet}$, the conjugate acid of $^{\bullet}O_2^-$ has the ability to move, to some degree, across lipid barriers due to its low electronegative character.

Further reduction of $^{\bullet}O_2^-$ yields H_2O_2, a relatively long-lived molecule with the ability to diffuse greater distances and to readily cross cellular membranes (Brendley and Pell 1998; Branco *et al*. 2004; Bienert *et al*. 2007). H_2O_2 can oxidize -SH groups, a process that is greatly enhanced by the presence of transition metals (catalysts) such as Cu^{2+} (cupric) and Fe^{2+}. With further reduction of H_2O_2, the production of the $^{\bullet}OH$ radical (discussed above); an extremely reactive species with a propensity to oxidize biological targets very near to its site of production, is possible.

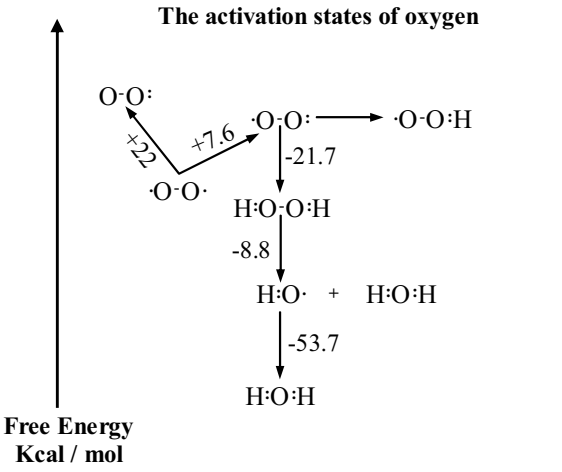


Fig. 3 Representation of various activation states of oxygen found in a physiologically normal plant cell.

Plant mitochondria

Mitochondria are also important producers of cellular ROS (Poyton and McEwen 1996; reviewed in Kowaltowski and Vercesi 1999). In fact, it is estimated that 1-5% of the oxygen reduced by the plant mitochondria, results in ROS production (Boveris and Chance 1973; Møller 2001; Kowaltowski and Vercesi 1999). This 1-5% conversion rate of molecular oxygen to ROS is increased under biotic and abiotic stresses (Tenhaken *et al*. 1995). The main sites for ROS production in plant mitochondria (Rich and Bonner 1978) are similar in mammalian mitochondria (Boveris *et al*. 1976; Turrens and Boveris 1980; Chakraborti *et al*. 1999). The sites of mitochondrial ROS production are complexes I (NADPH-Q-reductase) and the ubiquinone reductase site (complex III) of the respiratory chain. Braidot *et al*. (1999) demonstrated that in pea stem mitochondria, H_2O_2 is produced at complex II (succinate dehydrogenase).

Plant peroxisomes

Plant peroxisomes play important roles in myriad metabolic processes including photorespiration, fatty-acid β-oxidation, the glyoxylate cycle and the generation/degradation of H_2O_2 (reviewed in Corpas *et al*. 2001), subsequently producing the following oxygen and nitrogen species: H_2O_2, $^{\bullet}O_2^-$, and NO. Plant peroxisomes, in addition to chloroplasts and mitochondria, should be considered as cellular compartments able to produce and deliver important oxygen-based signalling molecules into the cytosol.

ANTIOXIDANT METABOLITES AND ENZYMES

Plants constitutively produce a basal level of antioxidant metabolites and enzymes which help to protect them from the potential damaging effects of exogenous and endogenous ROS. O_3- and UVR-exposure are also known to increase, in a number of plants, the expression of numerous antioxidant enzymes (**Fig. 4, Table 1**) including: superoxide dismutase (SOD), peroxidases (POXs), catalase (CAT), and ascorbate peroxidase (APX) (Tanaka *et al*. 1988; Conklin and Last 1995; Rao *et al*. 1996; Boldt and Scandalio 1997). SOD catalyzes the dismutation of $^{\bullet}O_2^-$ (and its conjugate acid $HO_2^{\bullet}$) to H_2O_2 and O_2 (Foyer *et al*. 1994; Kangasjarvi *et al*. 1994). There are different types of SOD located in the chloroplasts, mitochondria and the cytosol (Bowler *et al*. 1992). Some peroxidases catalyze reduction of alkyl-peroxides to H_2O and an alcohol in a reaction coupled to oxidation of a reductant (AH_2). Catalase, a hydroperoxidase, catalyzes the decomposition of H_2O_2 into O_2 and H_2O, without the production of free radicals (Foyer *et al*. 1994).

APX is an important part of the ascorbate-glutathione cycle (**Fig. 4**) (Kangasjarvi *et al*. 1994). There are different types of APX located in the cytosol (soluble), cytosol (membrane bound), chloroplast (thylakoid membrane bound), and chloroplast (stromal) (Kubo *et al*. 1992; Jespersen *et al*. 1997; Santos *et al*. 1998). APX catalyses the first step in the ascorbate-glutathione cycle by reducing H_2O_2 to H_2O, where ascorbate is the electron donor (Mittler and Zilinskas 1991; Noctor and Foyer 1998). The ascorbate-glutathione (Halliwell-Asada) pathway starts, as stated above, by the reduction of H_2O_2 to form monodehydroascorbate (MDHA). The regeneration of ascorbate from MDHA can come about by two different mechanisms. The first is catalytic reduction of MDHA by NADH, mediated by monodehydroascorbate reductase, while the second is spontaneous diproportionation of MDHA into ascorbate and dehydroascorbate (DHA). In a connected set of reactions, ascorbic acid can be reclaimed from DHA via the dehydroascorbate reductase reaction, in which reduced glutathione (GSH) is oxidized to form GSSG. Subsequently, GSH is reformed from GSSG by reduction with NADPH, a reaction catalyzed by glutathione reductase.

OXIDATIVE STRESS AND CALCIUM IONS

It has previously been demonstrated that oxidants like O_3, UVR and H_2O_2 can induce a transient increase in intracellular calcium (Ca^{2+}) in many types of plant cells. Clayton *et al*. (1999) demonstrated in aequorin-expressing Arabidopsis plants that O_3 exposure elicited a rapid but transient biphasic increase in cytosolic free Ca^{2+}. Cytosolic calcium homeostasis may be sensitive to the oxidation status of the glutathione pool, which it responds to via the plasma membrane Ca^{2+}-ATPase (reviewed in, Price *et al*. 1996). In this model, an increase in the ratio of oxidized (GSSG) to reduced (GSH) glutathione causes a pronounced reduction in the activity of calcium transport proteins responsible for removing Ca^{2+} from the cytosol, leading to an increase in intracellular Ca^{2+}.

This oxidant-induced elevation in intracellular Ca^{2+} ions may further induce the production of $^{\bullet}O_2^-$ (and eventually H_2O_2) via a Ca^{2+}-dependent NADPH oxidase-mediated mechanism, thus creating a positive feedback loop. Consistent with this mechanism, Larkindale and Knight (2002) showed that heat-induced oxidative damage in Arabidopsis cells was abrogated by pretreatment with various Ca^{2+}-channel blockers, including lanthanum (La^{3+}). However, it has been demonstrated in Arabidopsis that the influx of

Table 1 Major antioxidant enzymes and their reactions.

Superoxide dismutase (SOD)	$O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$
Catalase (Cat)	$2H_2O_2 \rightarrow 2H_2O + O_2$
Ascorbate peroxidase (APX)	$2Asc + H_2O_2 \rightarrow 2MDA + 2H_2O$
Monodehydroascorbate reductase (MDAR)	$MDA + NAD(P)H + H^+ \rightarrow Asc + NAD(P)^-$
Dehydroascorbate reductase (DHAR)	$DHA + 2GSH \rightarrow Asc + GSSG$
Glutathione reductase (GR)	$GSSG + NAD(P)H \rightarrow 2GSH + NAD(P)^-$
Glutathione peroxidase (GPX)	$H_2O_2 + 2GSH \rightarrow 2H_2O + GSSG$
NADPH oxidase-like, alternative oxidase (AOX)	$2e^- + 2H^+ + O_2 \rightarrow H_2O$
Peroxiredoxin (PrxR)	$2P\text{-}SH + H_2O_2 \rightarrow P\text{-}S\text{-}S\text{-}P + 2H_2O$
Glutaredoxin (GLR)	$DHA + 2GSH \rightarrow Asc + GSSG$

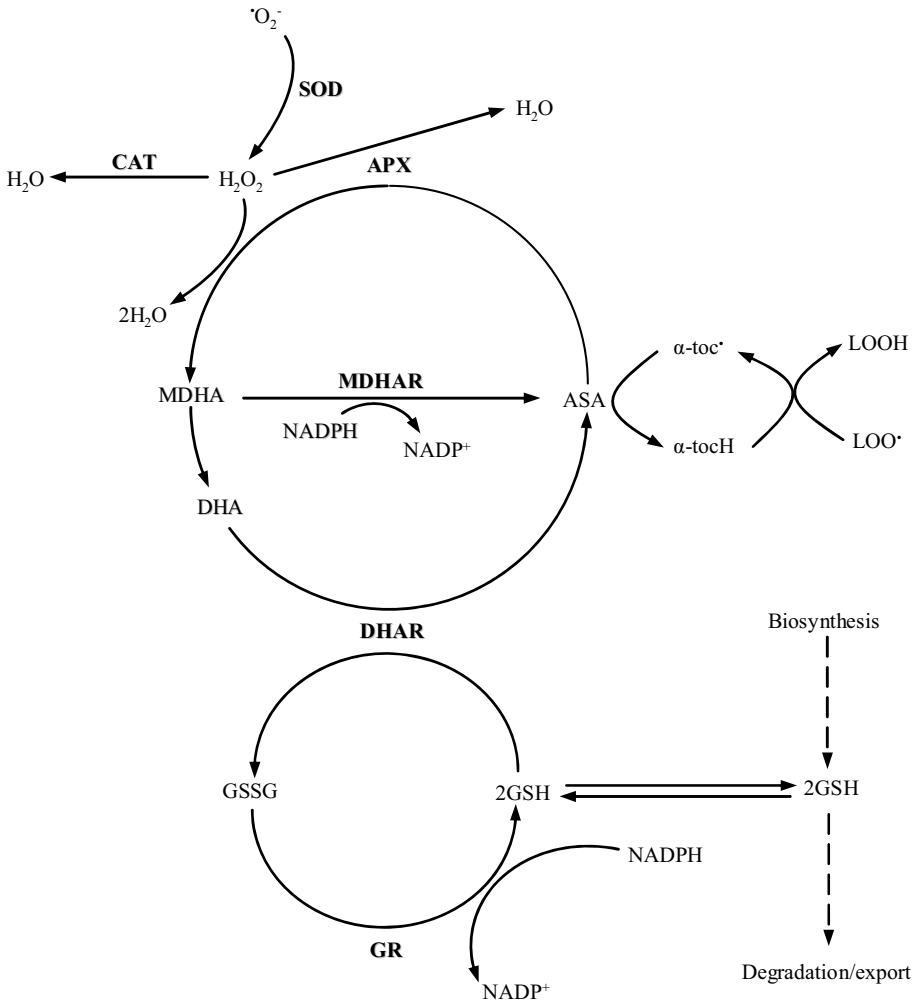


Fig. 4 Schematic representation of the relationships between glutathione biosynthesis and export together with interactions between the reduced and oxidized forms of glutathione and ascorbate in removal of H_2O_2 and regeneration of α-tocopheryl from α -tocopheryl radicals. H_2O_2 is reduced to water by reacting with ascorbate peroxidase (APX) using ascorbate as the electron donor forming the radical monodehydroascorbate (MDHA). The regeneration of ascorbate can proceed from MDHA directly by the action of monodehydroascorbate reductase, or via the spontaneous disproportionation of the MDHA radical into ascorbate and dehydroascorbate (DHA). Then, ascorbic acid is regenerated from DHA in a reaction catalyzed by dehydroascorbate reductase (DHAR), in which reduced glutathione (GSH) is oxidised into GSSG. GSH is regenerated in the company of NADPH by glutathione reductase. H_2O_2 can also be eliminated by catalase (CAT) as well as produced by the action of superoxide dismutase (SOD).

Ca^{2+} also activates a Ca^{2+}/calmodulin protein which binds to, and enhances the activity of, a specific isoform of catalase (AtCat-3). Since the activated Arabidopsis Ca^{2+}/CaM had no effect on bacterial, fungal, bovine or human catalases, it seems that the increase in intracellular Ca^{2+} could be negatively regulating H_2O_2 homeostasis in a plant-specific process (Yang and Poovaiah 2002).

While the role of NADPH oxidase in generating a short-lived "oxidative burst" has been well characterized in some mammalian cells, a role for the same enzyme in plant ROS has been more difficult to demonstrate unequivocally. Several reports have described the impact of the classical NADPH oxidase inhibitor, diphenyleneionium (DPI), on various ROS-linked processes in plants (Levine *et al*. 1994; Samuel *et al*. 2000), which is generally taken as *prima facie* evidence for the involvement of NADPH oxidase. However, it has been pointed out the DPI is also capable of inhibiting other flavoproteins, as well as peroxidases, all of which are both efficient producers of ROS and abundant in plant cells (reviewed in Bolwell 1999). Nevertheless, there is other evidence indicating that NADPH oxidase-like activities may be present in plants. Bioinformatic analysis has shown that both Arabidopsis and tomato possess multigene families of putative mammalian NADPH oxidase subunit homologues (Sagi and Fluhr 2001), while antibodies, raised against human $p22^{phox}$, $p47^{phox}$, and $p67^{phox}$ have been demonstrated to cross-react with appropriate sized protein bands in plant extracts (Tenhaken *et al*. 1995; Desikan *et al*. 1996; Xing *et al*. 1997).

In Arabidopsis, analysis of mutants of the NADPH oxidase, respiratory burst oxidase homologs (rboh), rbohD and rbohF, has demonstrated that these molecules are sources of $^{\bullet}O_2^-$ production in the pathogen-induced oxidative burst (Torres *et al*. 2001). Ca^{2+} ions also interacted functionally with recombinant rboh *in vitro*. Elevated levels of NADPH oxidase activity were induced by Ca^{2+} concentrations between 50 μM and 10 mM, indicating that the plant plasma membrane NADPH oxidase might be directly regulated by Ca^{2+} *in vivo*, unlike the neutrophil $gp91^{phox}$. Another major difference between the plant NADPH oxidase activity and its mammalian counterpart is that the plant enzyme can reduce molecular oxygen to $^{\bullet}O_2^-$ in the absence of any cytosolic subunits. Torres *et al*. (2001) showed that knockouts of *AtrbohF* in Arabidopsis blocked H_2O_2 generation during bacterial and fungal challenge.

CALCIUM IONS IN CELLULAR PROCESSES

Ca^{2+} ions play important roles as a second messenger in metazoans (reviewed in Price *et al.* 1996; Clayton *et al.* 1999). Intracellular Ca^{2+} levels regulate a large number of important cellular processes including gene expression, cell viability, cell proliferation, cell motility, cell shape and volume regulation (Hrabak *et al*. 1996; Sreeganga and Low

1997). Plants, like all other organisms, maintain concentrations of this ion in the cytosol and nucleus three to four orders of magnitude lower than that of other cellular compartments (Felle 1988; Bush *et al.* 1989).

Intracellular Ca^{2+} also rapidly responds to many external activating agents, and the fluxes in this cation play a key role in regulating cell responses to environmental signals. The changes in Ca^{2+} levels within the cell induced by environmental challenges are controlled by ligand-gated and G protein-coupled ion channels in the plasma membrane, and by mobilization of Ca^{2+} from intracellular stores (Sreeganga and Low 1997). The generation of cytosolic Ca^{2+} spikes and oscillations typically involves the coordinated release and uptake of Ca^{2+} from these stores, mediated by intracellular Ca^{2+} channels. These channels are sensitive to several second messengers including cytosolic ADP ribose, inositol triphosphate and Ca^{2+} itself.

Transgenic Arabidopsis plants expressing the calcium reporter protein aequorin have been used to demonstrate a rapid but transient biphasic increase in cytosolic free Ca^{2+} upon O_3 exposure (Clayton *et al*. 1999). Other oxidative stresses such as H_2O_2 can also affect Ca^{2+} fluxes. In tobacco seedlings treated with 10 mM H_2O_2, cytosolic free Ca^{2+} levels showed a transient (1-2 minute) increase, following a lag of 20-40 seconds (Price *et al.* 1994). Salicylic acid (SA) (0.5 mM) treatment of tobacco cell suspension cultures stimulated an immediate and transient burst of $^{\bullet}O_2^-$ production followed by a transient increase in cytosolic free Ca^{2+} (Kawano *et al*. 1998). In parsley cell suspension cultures, a transient Ca^{2+} influx was observed within two to five minutes following elicitation, and this increase was followed by an immediate increase in H_2O_2 (Nürnberger *et al.* 1994). The importance of Ca^{2+} channels for the oxidative burst in plant-pathogen interactions has been demonstrated by Baker *et al.* (1993), who found that the Ca^{2+} channel-blocker La^{3+} inhibited elicitation-induced oxidative burst in tobacco cell suspensions. Another study showed that adding EGTA (a chelator of Ca^{2+}) to suspension-cultured spruce cells significantly reduced the oxidative burst following elicitation, thus confirming the importance of an extracellular source for Ca^{2+} (Schwacke and Hager 1992). Specific inhibitors of Ca^{2+} influx blunted the cell death triggered in soybean cells by either *Pseudomonas syringae* or H_2O_2, while calcium ionophores were able to induce cell death in the absence of elicitation (Levine *et al.* 1996). In tobacco, the O_3-sensitive Bel-W3 cell line was shown to display increased calcium levels compared to the resistant Bel-B cell line and incubation with calcium chelators abrogated the O_3-induced cell death (Kadono *et al.* 2006). Similarly, a number of chemicals including Ca^{2+} chelators, ion channel blockers and ROS scavengers eliminated O_3-induced calcium signature in Bel-W3 line indicating that increase in Ca^{2+} is dependent on multiple upstream responses (Kadono *et al*. 2006). In tobacco 'Xanthi' lines, calcium chelators effectively blocked O_3 and other ROS-induced MAPK activation (Samuel *et al*. 2000), suggesting a role for calcium as a second messenger in O_3 and ROS-induced responses.

O_3, ROS AND HYPERSENSITIVE CELL DEATH

The sign of a successful hypersensitive response (HR) is the formation of restricted lesions at the site of attempted colonization of the challenged plant tissue, clearly delimited from surrounding healthy tissue (Haamond-Kosack and Jones 1996). Associated with lesion formation is the development of immunity to a subsequent attack by a broad range of normally virulent pathogens (Ryals *et al*. 1996). ROS generated through the oxidative burst have been proposed to play a central role in the development of cell death during HR. The use of antioxidant enzymes or ROS scavengers has been shown to interdict the cell death process during a number of incompatible plant-pathogen interactions, while inhibition of endogenous antioxidant mechanisms results in increased ROS levels and subsequently increased cell death (Levine *et al*. 1994; Grant and Loake 2000). H_2O_2 from the oxidative burst has been shown to be both necessary and sufficient to trigger hypersensitive cell death (Tenhaken *et al.* 1995). A several fold higher concentration of exogenously supplied H_2O_2 is however required to induce cell death than to induce defense gene expression (Levine *et al.* 1994), and unlike induction of defense gene transcription following HR, induction of hypersensitive cell death appears to show threshold dependency on H_2O_2 levels (Tenhaken *et al.* 1995). Levine *et al.* (1994) speculated that H_2O_2 can function as a mobile intercellular alarm signal, diffusing from infected cells (with high H_2O_2 levels and undergoing hypersensitive cell death) to adjacent cells. This, in turn, activates cell protection genes (but not hypersensitive cell death) in these neighboring cells, since H_2O_2 has not reached the threshold levels required to trigger hypersensitive cell death. Plant cells rapidly metabolize exogenous H_2O_2, and a sustained oxidative burst is required for induction of hypersensitive cell death (Lamb and Dixon 1997). Treatment of Arabidopsis *lsd1* and *rcd1* mutant plants with a superoxide generating system, but not with H_2O_2, was shown to induced cell death (Jabs *et al*. 1996; Alvarez *et al*. 1998). Despite this evidence, there is still debate whether, and which, ROS are necessary and/or sufficient to orchestrate plant defense responses, including HR cell death (Rao and Davis 2001).

The O_3-derived burst of ROS is thought to mimic the oxidative burst that accompanies recognition of avirulent pathogens, and O_3-induced injury may therefore involve signalling pathways that are shared with those involved in the plant HR (Sharma and Davis 1997; Sandermann *et al*. 1998). In Arabidopsis *rcd1* (radical-induced cell death 1) is an ROS-responsive lesion-mimic mutant, in which O_3 and extracellular superoxide and not H_2O_2 can induce transiently spreading lesions. Upon O_3 exposure, *rcd1* accumulated $^{\bullet}O_2^-$ in the zone ahead of the expanding lesions before appearance of visible symptoms. This response was similar to the HR triggered by an avirulent *Pseudomonas syringae* strain DC3000 in an incompatible interaction in the same mutant (Overmyer *et al*. 2000). O_3-induced cell death in *rcd1* required both SA and cyclic nucleotide-gated ion channels. RCD1 was further deciphered to be a WWE protein-protein interaction domain containing protein involved in abscisic acid, ethylene and methyl jasmonate (MeJA) responses (Ahlfors *et al*. 2005). Further evidence for O_3 mimicking the pathogen-induced cell death process comes from simultaneous analysis of the O_3-sensitive poplar clone NE-245 for a programmed cell death process (PCD) induced by O_3 exposure as well as by avirulent pathogen infection. Both stresses elicited similar patterns of DNA fragmentation, with concomitant PR-1 gene induction (Koch *et al*. 2000). In Arabidopsis, through a reverse genetics approach, a number of O_3-induced transcripts were analyzed for their role in mediating O_3 sensitivity. From this screen, Grim Reaper (GRI) was identified to play a central and positive role in regulating ozone-induced cell death in Arabidopsis. GRI is orthologous to STIG1, a stigma specific gene from tobacco. Despite its poor expression in vegetative tissue in Arabidopsis, *gri* plants displayed enhanced sensitivity to ROS stress that depended on both superoxide and SA for inducing cell death (Wrzaczek *et al*. 2009). The dSPM insertion in the locus At1g53130 resulted in a truncated N-terminal secreted peptide that has been proposed to prime the cell for enhanced sensitivity to ROS-stress (Wrzaczek *et al*. 2009). In tobacco, a pharmacological approach was used to identify that scavenging of $^1O_2^*$, H_2O_2, $^{\bullet}OH$ and redox active metals such as Fe^{2+} resulted in reduction of O_3-induced cell death of the sensitive Bel-W3 line, suggesting a role for the multiple ROS derivatives of O_3 (Kadono *et al.* 2006).

ADDITIONAL SIGNALLING SPECIES

Biosynthesis of SA is triggered by various biotic and abiotic stresses that also generate ROS (Yalpani *et al*. 1994; Sharma *et al*. 1996; Draper *et al*. 1997). SA can induce a

wide array of defense reactions including changes in cellular redox state, cellular defense and cell death (Rao and Davis 2001). O_3 challenge also induces changes in SA metabolism. Exposure of tobacco seedlings to 200 nL L^{-1} O_3-induced accumulation of SA, which increased 66 fold above basal levels within one day after treatment (Yalpani *et al*. 1994). Exogenous SA by itself can induce the production of ROS. Treatment of tobacco suspension cultures with SA induced increased levels of $^{\bullet}O_2^-$ (Kawano *et al*. 1998). One of the proposed roles of SA relates to its inhibitory effect on H_2O_2-metabolizing enzymes such as CAT and APXs. Such inhibition can potentially lead to increased levels of ROS, which would function as second messengers in defense signalling pathways (Klessig *et al*. 2000).

Jasmonic acid (JA) is another signal molecule that appears to play a central role in plant disease resistance (Penninckx *et al*. 1996). JA signalling can, depending on plant species and stimulus, either antagonize or synergize SA signalling and *vice versa* (Dong 1998; Pieterse and van Loon 1999). O_3-exposed Arabidopsis and hybrid poplar plants accumulated increased JA within several hours of treatment (Koch *et al*. 2000; Rao *et al.* 2000). Wounding or MeJA treatment of O_3-sensitive tobacco plants led to reduced O_3-induced cell death in these plants (Orvar *et al*. 1997), and wounding the plants led to reduced accumulation of H_2O_2 levels following O_3 exposure (Schraudner *et al*. 1998). The precise mechanism by which JA regulates cell death is still unclear.

Ethylene influences a broad spectrum of physiological processes, both during development and in response to stress (Suzuki *et al*. 1998). Ethylene is a known modulator of organ senescence, a specialized form of PCD. Ethylene production is also induced by various plant pathogens, O_3 and hypoxia (He *et al*. 1996; Pell *et al.* 1997). O_3 exposure leads to ethylene emission in pea seedlings, and this stimulation appears to be linked to the plant's sensitivity towards O_3 (Mehlhorn and Welburn 1987). When O_3-induced ethylene emission was blocked with inhibitors of ethylene biosynthetic enzymes, there was no visible injury induced by O_3. Induction of ethylene biosynthetic enzymes by O_3 was blocked by K252 A, a protein kinase inhibitor, and the same enzymes were induced by calyculin A (a protein phosphatase inhibitor) in the absence of O_3. This pattern suggests that reversible phosphorylation events are an essential element of the regulation of ethylene biosynthesis induced by O_3 (Tuomainen *et al*. 1997).

In the Arabidopsis *rcd1*, an ROS-responsive "lesion mimic" mutant, ethylene production was necessary for propagation of the ROS-induced lesions (Overmyer *et al*. 2000). Both JA and SA signalling pathways are known to interact with ethylene. Coordinated action of both ethylene and JA were required for efficient defense responses (Pieterse and van Loon 1999), while ethylene is believed to increase the plant sensitivity to SA (Lawton *et al*. 1995). Expression of ein2 (ethylene insensitive 2) attenuated the SA-dependent cell death in *acd5* (accelerated cell death 5) mutant (Greenberg *et al*. 2000). O_3-induced lesion propagation was reduced when *rcd1:ein2* double mutant was exposed to O_3. It is generally accepted that the interaction between SA and ethylene signalling pathways fine-tunes the kinetics of lesion formation and propagation (Rao and Davis 2001).

It is interesting that in mammalian systems, ROS can activate various protein kinases including modules of a number of early signal transduction components such as PK-C (Taher *et al*. 1993) and lead to the activation of both ERK1/2 pathway and p38 pathway, which have opposing roles in cancer suppression (Pan *et al*. 2009). In plants, multiple biotic and abiotic elicitors associated with ROS accumulation also induce rapid MAPK activation (Mishra *et al*. 2006). Similar to all these elicitors, exposure of both tobacco and Arabidopsis to O_3 leads to rapid activation of MAPKs, which were identified through in-gel kinase assays and immune-blotting with phosphor-MAPK specific antibodies (Samuel *et al*. 2000; Samuel and Ellis 2002; Miles *et al*. 2002). These MAPKs were further identified to be SIPK and WIPK in tobacco and the orthologous AtMPK6 and AtMPK3 in Arabidopsis (Samuel and Ellis 2002; Miles *et al*. 2005). The O_3-induced MAPK activation was further identified through pharmacological approaches to be mediated through upstream receptor activation, indicating a possible oxidative role for O_3 in altering membrane function and initiating the signal at the cell membrane (Miles *et al*. 2002). Ozone-induced MAPK activation was also observed in other species including poplar, conifers (*Picea*) and moss (*Physcomitrella patens*) (Miles *et al*. 2002).

Through RNA-interference it was also shown that MAPKs, particularly SIPK/AtMPK6, played a central role in mediating O_3-induced cell death. Suppression of SIPK and AtMPK6 in tobacco and Arabidopsis respectively, resulted in increased sensitivity of these transgenics to O_3 exposure (Samuel and Ellis 2002; Miles *et al*. 2005, 2009a). This hypersensitivity to O_3 was also accompanied by misregulation of the co-activated WIPK/AtMPK3 in both systems, suggesting a complex interaction between these kinases. In Arabidopsis, the MAPKK, AtMKK5 was identified as an upstream activator of AtMPK6/AtMPK3 module, as suppression of AtMKK5 resulted in increased O_3 sensitivity and reduced signal transmission to AtMPK6/AtMPK3 (Miles *et al*. 2009b). This is quite consistent with the hypothesis that O_3 exposure leads to rapid ROS accumulation resulting in oxidative phosphorylation and activation of a membrane bound receptor (or multiple receptors) that relays the signal on to the MAPK pathway.

FUTURE PERSPECTIVES

ROS generated from O_3 stress can act as second messengers helping to integrate a plethora of diverse cellular processes including characteristic O_3-induced flecking or HR. These O_3-induced responses are mediated by multiple second messengers and signalling proteins that, along with the various plant phytohormones, orchestrate the transmission and response patterns of various plant and crop species to O_3 stress. While the various signals such as MAPK pathways, Grim reaper and RCD1-regulated ROS signal controls have been identified, the nature of the molecules and signalling mechanisms downstream of these components remain elusive. Both biochemical and genetic approaches in the future should provide insights into these complex regulatory networks. As tropospheric O_3 levels rise, it does become imperative to create O_3-tolerant crop species to sustain productivity. A thorough understanding of the mechanisms behind O_3-induced cellular signalling will allow us to manipulate pathways in crop species, to create ozone-tolerant varieties that will sustain yield under damaging ozone concentrations.

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Scheme 1 Stratospheric ozone formation and destruction.

$O_2 + h\nu \rightarrow 2\ O\ (\lambda < 242\ nm)$	(R1)
$O + O_2 + M \rightarrow O_3 + M$	(R2)
$O_3 + h\nu \rightarrow O + O_2\ (\lambda < 1140\ nm)$	(R3)
$O + O_3 \rightarrow 2\ O_2$	(R4)
$^{\bullet}OH + O_3 \rightarrow HO^{\bullet}_2 + O_2$	(R5)
$HO^{\bullet}_2 + O_3 \rightarrow {}^{\bullet}OH + 2O_2$	(R6)
$N_2O + O \rightarrow 2NO^{\bullet}$	(R7)
$NO^{\bullet} + O_3 \rightarrow NO^{\bullet}_2 + O_2$	(R8)
$NO^{\bullet}_2 + O \rightarrow NO^{\bullet} + O_2$	(R9)

Scheme 2 Tropospheric ozone formation and destruction.

$NO^{\bullet}_2 + h\nu \rightarrow NO^{\bullet} + O\ (\lambda < 420\ nm)$	(R10)
$O + O_2 + M \rightarrow O_3 + M$	(R11)
$O_3 + NO^{\bullet} \rightarrow NO^{\bullet}_2 + O_2$	(R12)
$CO + {}^{\bullet}OH \rightarrow CO_2 + H^{\bullet}$	(R13)
$H^{\bullet} + O_2 + M \rightarrow HO^{\bullet}_2$	(R14)
$NO^{\bullet} + HO^{\bullet}_2 \rightarrow NO^{\bullet}_2 + {}^{\bullet}OH$	(R15)
$NO^{\bullet}_2 + O_2 + h\nu \rightarrow NO^{\bullet} + O_3$	(R16)
$CH_4 + 4O_2 + 2h\nu \rightarrow HCHO + H_2O + 2O_3$	(R17)
$RH + 4O_2 + 2h\nu \rightarrow R'CHO + H_2O + 2O_3$	(R18)

Scheme 3 Fenton and Haber-Weiss reactions.

$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^- + HO^{\bullet}$	(R19)
$H_2O_2 + HO^{\bullet} \rightarrow H_2O + {}^{-}O^{\bullet}_2 + H^+$	(R20)
$H_2O_2 + {}^{\bullet}O_2^- + H^+ \rightarrow O_2 + H_2O + HO^{\bullet}$	(R21)
$Fe^{2+} + HO^{\bullet} + H^+ \rightarrow Fe^{3+} + H_2O$	(R22)
$Fe^{3+} + HO^-_2 \leftrightarrow Fe^{2+} + HO^{\bullet}_2$	(R23)
$Fe^{3+} + {}^{\bullet}O^-_2 \rightarrow Fe^{2+} + O_2$	(R24)