Fun in the Sun: Singlet Oxygen Harnessing the Power of Light in Response to Biotic Stresses

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Abstract

Singlet Oxygen (SO) is among the most potent reactive oxygen species, and readily oxidizes proteins, lipids, and DNA. It can be generated at the plant surface by phototoxins in the epidermis, acting as a direct defense against pathogens and herbivores (including humans). SO can also accumulate within mitochondria, peroxisomes, cytosol, and the nucleus through multiple enzymatic and non-enzymatic processes. However, the primary location of SO in plants is in the chloroplast, where it results from transfer of light energy from PhotosystemII to triplet oxygen. SO accumulates in response to diverse stresses that perturb chloroplast metabolism, and while its short half-life precludes exiting the chloroplast, it participates in retrograde signaling through the EXECUTER1 sensor, generation of carotenoid metabolites, and possibly other unknown pathways. SO thereby reprograms nuclear gene expression and modulates hormone signaling and programmed cell death. While SO signaling has long been known to regulate plant responses to high-light stress, recent literature also suggests a role in plant interactions with insects, bacteria, and fungi. The goals of this review are to provide a brief overview of SO, summarize evidence for its involvement in biotic stress responses, and discuss future directions for the study of SO in signaling and defense.

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ABSTRACT (199 words)

Singlet Oxygen (SO) is among the most potent reactive oxygen species, and readily oxidizes proteins, lipids, and DNA. It can be generated at the plant surface by phototoxins in the epidermis, acting as a direct defense against pathogens and herbivores (including humans). SO can also accumulate within mitochondria, peroxisomes, cytosol, and the nucleus through multiple enzymatic and non-enzymatic processes. However, the primary location of SO in plants is in the chloroplast, where it results from transfer of light energy

from PhotosystemII to triplet oxygen. SO accumulates in response to diverse stresses that perturb chloroplast metabolism, and while its short half-life precludes exiting the chloroplast, it participates in retrograde signaling through the EXECUTER1 sensor, generation of carotenoid metabolites, and possibly other unknown pathways. SO thereby reprograms nuclear gene expression and modulates hormone signaling and programmed cell death. While SO signaling has long been known to regulate plant responses to high-light stress, recent literature also suggests a role in plant interactions with insects, bacteria, and fungi. The goals of this review are to provide a brief overview of SO, summarize evidence for its involvement in biotic stress responses, and discuss future directions for the study of SO in signaling and defense.

KEY WORDS: singlet oxygen, reactive oxygen species, retrograde signaling, chloroplast, insect resistance, pathogen resistance, biotic stress, herbivory, aphids, *Pseudomonas syringae* , *Alternaria alternata,* phytoalexins, phytoanticipins

SUMMARY STATEMENT: This review summarizes the evidence for a role of singlet oxygen (SO) in mediating plant responses to herbivores and pathogens, and makes a case for the importance of further studies on this potent molecule for signaling and defense. Evidence reviewed here is primarily from *Arabidopsis thaliana* , although are species are covered where information is available.

Introduction

Singlet oxygen ($^1O_2$, or SO) is a reactive oxygen species (ROS) predominantly produced in the chloroplast during photosynthesis at PSII by energy transfer from excited chlorophyll or charged reactions centers to molecular oxygen (ie.$^3O_2$ or triplet oxygen) (Dmitrieva et al., 2020). Although it has been studied most extensively in the context of high-light stress, SO accumulates in response to many other abiotic stresses such as heat, heavy metals, mechanical injury, and osmotic stress (Pospíšil & Prasad, 2014; Chen & Fluhr, 2018). As one of the most short-lived ROS, SO is highly unstable and quickly reacts with nearby biological molecules such as lipids, proteins, and carotenoids that can trigger chloroplast-to-nucleus retrograde signaling to influence nuclear gene expression (Triantaphylidès & Havaux, 2009; Galvez-Valdivieso & Mullineaux, 2010). At sublethal doses of SO, retrograde signaling can contribute to adaptation to abiotic stresses by activating hormone signaling and expression of genes involved in detoxification and management of oxidative stress (Ramel et al., 2012a, 2013). Furthermore, many of the responses triggered by SO overlap with disease resistance pathways, including induction of numerous transcription factors in common (Ochsenbein et al., 2006; Mor et al. 2014; Zhang et al., 2014). Thus, SO may have multiple roles in stress-responsive signaling and could potentially contribute to biotic as well as abiotic stress responses. The goals of this review are 1) to provide a brief overview of SO's chemical properties, synthesis, and signaling in plants; 2) to summarize the current state of knowledge of the role(s) of SO in biotic stress; and 3) to propose a path forward to elucidate these roles.

Chemical Properties of Singlet Oxygen

Unlike many other molecules, molecular oxygen is most stable in a triplet state rather than a singlet state, and this property makes life in an oxygenated environment possible. Triplet oxygen ($^3O_2$), the ground state of molecular oxygen, has two unpaired, spin-parallel electrons (Figure 1A), whereas SO, the lowest excited state of molecular oxygen, has two valence electrons spin-paired in a single orbital and a second orbital left empty (Figure 1B). The terms "triplet" and "singlet" oxygen refer to the possible number of electron spins that each form can take; the triplet form has three possible arrangements of electron spins, whereas SO has only one possible arrangement. The triplet configuration of molecular oxygen limits its ability to react directly with most stable organic molecules, which typically have singlet ground states. This limitation prevents runaway oxidation at moderate temperatures and makes life as we know it possible. Singlet oxygen reacts far more readily with organic compounds than triplet oxygen, and can participate in ene reactions and Diels-Alder cycloadditions that triplet oxygen cannot (Figure 1C-D). Consequently, the lifetime of SO in vitro in water and most organic solvents is in the order of microseconds, despite being relatively stable in gaseous form (Koh & Fluhr, 2016; Thorning et al., 2022). Due to this high reactivity, SO is among the most potent reactive oxygen species (ROS), and readily oxidizes molecules with carbon-carbon double bonds. It damages proteins

by reacting with cysteine, histidine, methionine, tryptophan, and tyrosine residues, disrupts membranes by oxidizing polyunsaturated fatty acids to form lipid hydroperoxides, and mutates DNA, causing G to T point mutations (Di Mascio et al., 2019; Agnez-Lima et al., 2012). Hydroperoxides generated by SO can also cause free radical chain reactions, amplifying the oxidative response (Dogra & Kim, 2020). While its high chemical reactivity can make SO toxic, this same trait also enables it to mediate plant interactions with biotic stressors.

SO Production by Photosensitizers in Plant Biotic Interactions

The earliest work to suggest a role for SO in plant biotic interactions was focused on Type II phototoxins found in plants and fungi. Phototoxins, also called photosensitizers, are compounds whose toxicity is dependent upon the absorption of light energy. Whereas Type I phototoxins act by generating free radicals, Type II compounds generate SO; energy absorbed from light is transferred from the excited Type II photosensitizer to ground state triplet oxygen, which causes the unpaired electrons to shift to opposite spin states, significantly increasing the reactivity of the oxygen molecule (Baptista et al., 2017). In plants, Type II phototoxins can act as phytoalexins—defensive plant secondary metabolites that are induced by biotic stress—and phytoanticipins, chemical defenses that are produced constitutively (Flors & Nonell, 2006). The most extensively studied examples are furanocoumarins and phenalenones, which can have activities against phytopathogenic fungi, bacteria, nematodes, and herbivorous insects.

Phototoxic furanocoumarins are common in the epidermis of species in the Umbelliferae, and Rutaceae, and in plants such as wild parsley or citrus they are a source of phytophotodermatitis—light-dependent skin irritation in humans (Nguyen et al., 2020). Thus, it is logical to hypothesize that they may function as an anti-herbivore defense. The linear furanocoumarin xanthotoxin has known toxicity to the southern fall armyworm (*Spodoptera aridania* Cramer), and this toxicity was enhanced when artificial diet containing xanthotoxin was treated with ultra-violet (UV) light, which promotes SO production by phytoalexins (Berenbaum,1978). Furthermore, when plants from Umbelliferae and Rutaceae were treated with UV light, they generated a high flux of SO in the stable gas-phase on the leaf surface that was projected to be sufficient to damage herbivores on the plant (Berenbaum & Larson, 1988). Together, these results suggest that gaseous SO produced by phototoxins at the leaf surface contribute to plant defenses against herbivores, possibly through direct toxicity to the pest.

Another group of Type II photosensitizing phytoalexins, the phenalenones, have light-dependent, SO-mediated toxicity against root-knot nematodes (Song et al., 2017) and the fungal pathogen*Fusarium oxysporum* (Lazzaro et al., 2004), and, in banana, are associated with resistance to the burrowing nematode (Holscher et al., 2014). It is unclear whether SO-dependent toxicity mediates the effects of phenalenones on such soil-born pathogens *in planta* given their limited light exposure. However, these compounds are in banana also correlated with resistance to the foliar pathogen *Mycosphaerella fidjiensis* (Otalvaro et al., 2002), and are generally regarded as broad-spectrum light-activated phytoalexins (Flors & Nonell, 2006). Further work is needed to determine if SO is produced *in vivo* by these compounds and influences the infection process.

Besides having directly toxic effects on pests and pathogens, SO could potentially also impact host plant resistance by modulating programmed cell death (PCD) in the host. SO is known to regulate PCD in abiotic stress responses (Laloi & Havaux, 2015), and this capability merits further investigation in the context of phototoxin production and biotic interactions. Furthermore, while plants may utilize SO-generating phototoxins for defense, there is also evidence that certain necrotrophic pathogens produce Type II phototoxins such as cercosporin and DHN-melanin that act as virulence factors (Beltran-Garcia et al., 2014; Koh et al., 2023). The fungal toxin cercosporin, for example, changes leaf conductance by permeabilizing guard cell membranes, inhibits photosynthesis, directly oxidizes host RNA, and triggers SO-associated transcript profiles (Koh et al., 2023). This light-dependent damage causes cell death and foliar lesions in the host plant, facilitating the infection process by necrotrophic fungi in the genus *Cercospora* (Rezende et al., 2020). Thus, studies on both plant- and pathogen-derived phototoxins indicate that Type II photosensitizers are utilized as weapons on both sides of the arms race between plants and their biotic attackers.

Intracellular SO Accumulation in Plants

In addition to production of gaseous SO by photosensitizers at the surface of the epidermis, SO may be generated in multiple intracellular locations, often as a byproduct of primary metabolism or other enzymatic reactions. Due to its higher reactivity in solution, SO is estimated to be ~1,000-fold less persistent in cells than in a gas phase (Flors & Nonell, 2006), and measurement *in vivo* remains challenging (see Dmitrieva et al. 2020; Prasad et al., 2018; and You et al., 2018). However, it has been detected in chloroplasts, mitochondria, peroxisomes, the cytosol and the nucleus (Mor et al., 2014; Koh et al., 2022). Mor and coworkers (2014) reported that SO could be generated in the dark and in non-photosynthetic tissues. In the mitochondria, SO is produced by electron transport-linked phosphorylation, and in the peroxisomes, SO may be generated by Fenton reactions involving iron-containing proteins and ascorbate (Sandalio & Romero-Puertas, 2015). At the plasma membrane or other membranes, SO may result from lipoxygenase activity and decomposition of lipid peroxides. Lipoxygenases for example generate SO in response to osmotic stress and mechanical wounding, and mediate cell death in roots in response to osmotic stress (Prasad et al., 2017; Chen and Fluhr, 2017; Chen et al., 2021). Another light-independent route for intracellular SO production is the Haber-Weiss reaction between superoxide and hydrogen peroxide (Mor et al., 2014). Detection with the fluorescent probe Singlet Oxygen Sensor Green (SOSG) suggested that SO levels in the mitochondria and peroxisomes of dark-adapted root tips increased in response to treatment with the bacterial elicitor flagellin (flg22) (Mor et al., 2014). Therefore, light-independent SO production in these organelles could potentially contribute to plant biotic interactions, and warrants further investigation. However, the majority of SO in plants is produced in a light-dependent fashion the chloroplast, and consequently this organelle is the focus of most research on SO in plant stress responses.

SO is generated in the chloroplast as a byproduct of normal metabolism and in response to stress. Photosystem II (PSII), the predominant source of SO, continually produces this ROS during photosynthesis when excess light energy is passed from the photosystem to nearby ground state atmospheric oxygen (ie. triplet oxygen) (Apel & Hirt, 2004). This can occur from either excited chlorophylls or energy charge separation of the PSII reaction center (Dmitrieva et al., 2020). Energy can also be passed to triplet oxygen at PSII when the electron transport chain between PSII and PSI is over-reduced (Asada, 2006). In addition, PSI can contribute to SO generation in the chloroplast through a process known as the Mehler reaction. In this case, reduced ferredoxin transfers an electron to $^3O_2$ instead of to its principle target $NADP^+$, generating SO and decreasing production of NAPDH to fuel the Calvin-Benson cycle (Mehler, 1951). In addition to generation of SO at PSII and PSI, Dogra and Kim (2020) also hypothesize that SO could potentially be generated at the grana margin of the thylakoid membrane, where damaged PSII components are transported for repair (Dogra & Kim, 2020). Dysregulation of chlorophyll biosynthesis can also cause leakage of chlorophyll intermediates from the chloroplast into the cytosol, and these intermediates can cause light-dependent SO accumulation in the cytosol (Koh et al., 2022).

Physiological Responses to SO in Plants

Plant responses to SO have primarily been studied using a) exogenous photosensitizers that induce SO such as Rose Bengal or Acridine Orange; b) high-light treatments that induce SO; and/or c) *Arabidopsis thaliana* mutants that exhibit elevated SO accumulation either constitutively (e.g. *chlorina1* , or *ch1*) or conditionally (i.e. *fluorescent in blue light* , or *flu*) (You et al., 2018; Dmitrieva et al., 2020). The *flu* mutant has a normal phenotype when grown under continuous light; however, if transferred to the dark, it accumulates a potent photosensitizer (protochlorophyllide, or Pchlide) in the chloroplast that generates SO when the plants are reilluminated (Meskauskiene et al., 2001; Op den Camp et al., 2003). The amount of SO that accumulates in *flu* mutants exposed to a Light:Dark:Light (L:D:L) shift can to some extent be modulated by manipulating the duration of the dark period and the light intensity after re-exposure (Lee et al. 2007; Hou et al. 2019). While these approaches cannot perfectly duplicate the timing, localization, and intensity of SO accumulation in wild-type plants experiencing stress, they have dramatically advanced our understanding of plant responses to high SO levels.

Cellular responses to SO vary depending upon the dosage of this ROS. Titers of SO and other ROS in cells

are a product of the balance between generation and scavenging. Under optimal growing conditions, even though SO is continuously produced at PSII, it is quickly scavenged by nearby non-enzymatic antioxidants such as carotenoids or tocopherols, limiting its impact on the cell (Asada, 2006). While SO generation is a consequence photosynthesis even in healthy plants, elevated SO levels are observed in response to many environmental stresses that disrupt the photosynthetic machinery at PSII and/or PSI, such as high light, heat, heavy metals, mechanical injury, and osmotic stress (Pospíšil & Prasad, 2014; Chen & Fluhr, 2018). In extreme cases, SO accumulates to toxic levels that cause membrane rupture and consequent cell necrosis as a result of direct interaction of SO with membrane lipids, termed non-enzymatic lipid peroxidation. Between healthy baseline SO levels on one hand and levels that are high enough to cause necrosis on the other, intermediate doses trigger retrograde signaling for a spectrum of plant stress responses ranging from cellular acclimation to programmed cell death (PCD) (Dmitrieva et al., 2020).

The physiological impacts of SO also depend upon its localization. In the cytosol (and possibly also the nucleus), SO oxidizes mRNA and can thereby decrease expression of transcripts that have high turnover (Koh et al. 2021). In the chloroplast, SO damages the D1 protein in PSII, which can inhibit photosynthesis and retard growth unless rates of D1 repair and replacement are high (Dogra and Kim, 2020). SO in the chloroplast also activates retrograde signaling and transcriptional reprogramming, leading to stress acclimation or PCD (discussed in greater depth in the next section). SO localized at cellular membranes oxidizes membrane lipids, causing decreased integrity of the chloroplast membranes, vacuole leakage, and electrolyte leakage across the plasma membrane (Przybyla et al., 2008; Zhang et al. 2014; Koh et al., 2016); membrane damage can also cause cell death. Although it has yet to be tested in plant cells, artificial SO generation at membranes in mammalian cells strongly induces apoptosis, whereas SO accumulation in the mitochondria or nucleus causes less frequent cell death, via necrosis rather than PCD (Liang et al., 2020). In plants, SO-induced necrosis is considered to be relatively rare, and acclimation and even cell death in response to SO are thought to be genetically programmed and mediated through signaling (Op den Camp et al., 2003; Wagner et al. 2004). Research on SO signaling has focused primarily on the chloroplast as the source of signals because it is the greatest source of SO in the cell.

SO Signaling In Plants

Because of SO's extremely high reactivity in aqueous and organic solutions, and the abundance of ROS scavengers in cells, SO has been estimated to have half-life of 200 ns in biological environments (Gorman & Rodgers, 1992). Therefore, responses to intracellular sources of SO are likely due to interactions with biomolecules close to the site of production that initiate a chloroplast-to-nucleus retrograde signal. These may be β-carotene near PSII reaction centers, lipids of chloroplast membranes, or proteins embedded in the thylakoid membrane (Wagner et al., 2004; Przybyla et al., 2008; Ramel et al., 2012b). In*A. thaliana* , there appears to be more than one distinct pathway for SO retrograde signaling (Figure 2), but detection of SO by the EXECUTOR1 protein in the grana margins or carotenoid signaling in the grana core are of particular importance.

SO Sensing by EXECUTER1. EXECUTER1 (EX1) and its homolog EXECUTER2 (EX2) were originally discovered as a result of work with the conditional *flu* mutant (Meskauskiene et al., 2001), and they modulate most of the phenotypes generated by SO induction in *flu* . The amount of SO that accumulates in L:D:L-exposed *flu* mutants varies depending upon the duration of the dark period and the light intensity of re-exposure (Lee et al. 2007; Hou et al. 2019). As a result, this mutant can be utilized to study both programmed cell death (PCD) in response to high SO dosages, or stress acclimation programs activated by sub-lethal SO doses. Experiments with the *flu* mutant demonstrate that EX1 plays a role in both of these processes, and reduces or blocks the majority of phenotypes caused by *flu* (e.g. Lee et al., 2007; Zhang et al., 2014). Exposing *flu* to 8h of darkness followed by reillumination halts plant growth, induces the formation of lesions on foliage, modulates expression of a large set of SO-responsive genes (SORGs), and activates multiple hormone signaling pathways (Ochsenbein et al., 2006; Lee et al., 2007; Przybyla et al., 2008). Introducing the *ex1* loss-of function mutation into the*flu* background inhibited lesion formation, restored growth, and blocked the induction of ˜80% of SORGs, whereas EX2 was not required for induction

of most SORGs (Lee et al., 2007). EX1 also contributes to the effects of SO on hormone signaling. Przybyla and coworkers (2006) demonstrated that exposing *flu* to L:D:L shift induced enzymatic lipid peroxidation and accumulation of the oxylipin hormones 12-oxo phytodienoic acid (OPDA) and jasmonic acid, whereas these responses were inhibited in the *flu/ex1* double mutant (Przybyla et al., 2008). The L:D:L shift also has been shown to cause a rapid upregulation of *ENHANCED DISEASE SUSCEPTIBILITY1* (*EDS1*) and salicylic acid (SA) accumulation, which consequently activated expression of genes encoding pathogenesis-related (PR) proteins *PR1* and *PR5* (Ochsenbein et al., 2006). In protoplasts, SA contributed to the cell death phenotype observed in*flu* (Danon et al., 2005). Zheng and coworkers (2014) subsequently showed that *PR1* induction was compromised in the*flu /ex1/ex2* triple mutant, suggesting that induction of SA signaling by SO is dependent upon EX1.

The complex molecular processes through which EX1 and EX2 mediate plant responses to SO are not yet fully resolved, but recent studies have made major advances in deciphering them. The EX1 and EX2 proteins are localized to the non-appressed region of the thylakoid membrane called the grana margin (Wang et al., 2016), where, prior to stimulation by SO, they complex with several other proteins, including GENOMES UNCOUPLED4 (GUN4) and GUN5, proteins involved in tetrapyrrole synthesis (Li et al. 2023). In response to SO accumulation, EX1 undergoes oxidative modification, disassociates from the complex, and accumulates in the nucleus, where it interacts directly with WRKY transcription factors and gene promoters to activate expression of SORGs (Li et al. 2023). In parallel, exposure to SO also causes a dose-dependent decline in EX1 abundance that requires a functional copy of the thylakoid membrane-bound metalloprotease FtsH2 (Wang et al., 2016; Dogra et al., 2017). Inactivation of FtsH2 repressed induction of ˜85% of EX1-dependent SORGs in *flu* , implying that proteolysis of EX1 by FtsH2 is important to its function in SO-responsive signaling (Dogra et al., 2017). Like EX1, EX2 can also undergo oxidative modification by SO and proteolysis by FtsH2, and the presence of a functional copy of EX2 slows down proteolysis of EX1 and decreases expression of EX1-dependent SORGs (Dogra et al., 2022). These results suggest that EX2 acts as a negative modulator of EX1 signaling, tapping the brakes on this system by competing with EX1 to interact with SO or FtsH2.

Further work is needed to determine how and why proteolysis of EX1 promotes EX1-dependent regulation. The EX1 proteins found in the nucleus after SO induction are full-length (Li et al. 2023), and so it appears that there are two separate pools of EX1 in the cell—one that moves to the nucleus to act as a transcriptional activator, and one that remains in the chloroplast to be degraded. Somehow these pools act synergistically to promote SORG expression. Another important question that remains to be resolved is the source of ROS that oxidizes EX1 and EX2 after SO induction. The typical site of SO production occurs from active PSII in the appressed thylakoid (grana core) during photosynthesis. However, the reactive nature and short half-life of SO (Gorman and Rodgers, 1992) severely reduce the likelihood of this ROS traveling from the grana core to the grana margin. It is possible that SO in the grana core triggers production of other more stable ROS that move to the grana margins to modify EX1 and EX2. Alternatively, it has been proposed that there is an additional mechanism to generate SO in the grana margin via chlorophyll precursors or damaged PSII subunits sent to the grana margins for repair (Wang et al., 2016; Dogra and Kim, 2020). EX1 and EX2 coprecipitate with multiple proteins including the PSII D1 and D2 proteins and proteins involved in chlorophyll synthesis, including GUN4, GUN5, and Pchlide oxidoreductases (Dogra et al., 2022; Li et al. 2023). GUN4 and GUN5 are upstream of Pchlide synthesis in the chlorophyll synthesis pathway, whereas Pchlide oxidoreductases convert Pchlide to chlorophyllide, and so the balance of activities among these enzymes could regulate Pchlide accumulation. GUN4 has been implicated in SO generation (Tabrizi et al., 2016), and together with GUN5, it may promote Pchlide synthesis and SO generation in the grana margins, causing oxidation of EX1 and activation of EX1 signaling. Additional studies are needed to confirm the source of ROS at the grana margins and the functional significance of EX1's multiple interaction partners.

Carotenoids and Other SO Signaling Pathways. In addition to the EXECUTOR pathway, which can mediate stress-responsive programmed cell death or at lower SO dosages enable stress acclimation, β-carotene derivatives also play a role in acclimation to high light stress (Ramel et al., 2012a, 2013). The *ch1* mutant, which accumulates excess SO in the grana core, is commonly used to study the role of carotenoids in SO signaling. The reaction of SO with carotenoid scavengers near the reaction center of PSII yields aldehydes and

endoperoxides through oxidative modification (Ramel et al., 2012a). Specifically, oxidation of the carotenoid β-carotene by SO gives rise to β-cyclocitral (β-CC), a volatile, highly reactive electrophilic compound that can then diffuse out of the chloroplast to signal for an acclimation response to high light stress (Ramel et al., 2012a). Importantly, β-CC generation occurs in the grana core of the thylakoid membrane where active PSII reside, whereas EX1 and EX2 are localized in the grana margins, where damaged D1 and D2 proteins of the PSII reaction centers are sent for repair (Dogra and Kim, 2020). Thus, the β-CC and EXECUTER pathways are not initiated in the same area, or by the same SO-generating mechanism, and remain relatively distinct from one another.

Pretreating *A. thaliana* with β-CC upregulated genes associated with oxidative stress, hormone signaling, and detoxification, and rendered plants more tolerant to high light exposure in a dose-dependent manner (Ramel et al., 2012a). It has been proposed that the protein METHYLENE BLUE SENSITIVITY (MBS1) is activated downstream of β-CC to transduce the signal to the nucleus for regulation of plant growth and development under high light stress (Shumbe et al., 2017). In addition, D'Alessandro and colleagues (2018) identified *Scarecrow-Like14* (*SLC14*) as another downstream mediator of the SO signal transduced by β-CC that acts independently of MBS1. SLC14, a GRAS family transcription factor, further regulates the expression of NAC transcription factors, and a transgenic line overexpressing *SLC14* was found to have enhanced resilience to high light stress, indicating *SLC14* is involved in photooxidative adaptation. However, the authors also discovered that only 30% of gene expression changes in *ch1* mutants under high light stress were due to β-CC (Shumbe et al., 2017). Therefore, it is likely that multiple pathways of SO signaling exist for adaptation under high light stress and defense against other abiotic and biotic stressors. Consistent with this hypothesis, Wang and coworkers (2020) report the existence of an EX1-dependent signaling pathway that is negatively regulated by a protein in the chloroplast stroma, SAFEGAURD1, that is degraded in response to SO (SAFE1). SAFE1 protects the grana margins from damage by SO in *flu* plants, and loss of function of SAFE1 in a *flu* / *ex1* background restores the cell death phenotype and many of the transcriptional responses to SO that are seen in *flu* but normally suppressed by *ex1* . Another EX1-independent response factor is OXIDATIVE SIGNAL INDUCIBLE1 (OXI1), a kinase that mediates SO-responsive cell death in the ch1 mutant, probably through a jasmonate-dependent signaling mechanism (Shumbe et al. 2016). It is unclear whether carotenoid signaling promotes OXI1 signaling. Further studies are needed to characterize EX1-independent pathways, examine the potential interconnections among the different SO signaling pathways, and definitively establish their roles in wild-type responses to SO and SO-generating stresses. However, markers associated with known SO signaling pathways give us a good starting point to identify stresses that activate SO signaling in plants.

Evidence of SO Accumulation and Signaling in Biotic Interactions

Several studies indicate that biotic stressors can induce the accumulation of markers associated with elevated SO levels (Figure 3). Oxylipin profiling in *A. thaliana* challenged with virulent and avirulent strains of *Pseudomonas syringae* pv. *tomato* (Pst) revealed that this bacterial pathogen induced 8-fold or higher increases in the hydroxy fatty acids 10-HO-FA and 15-HO-FA (Grun et al., 2007). These two HO-FA species are generated exclusively by non-enzymatic peroxidation of fatty acids by SO, and are considered diagnostic signatures of SO accumulation (Triantaphylidès et al., 2008). Meta-analyses of publicly-available *A. thaliana* transcript profiles also indicated that transcriptional responses to artificial induction of SO by Rose Bengal or by the conditional *flu* mutation overlaped with transcriptional responses to *P. syringae* , a *P. syringae* pv. *pisi* effector protein (AvrRPS4), bacterial molecular patterns (flagellin22, elongation factor thermo unstable EF-Tu), a fungal elicitor (chitin), the fungal pathogen *Peronospora parasitica,* and a molecular pattern associated with pathogen-induced damage to plant cell walls (oligogalacturonides) (Mor et al., 2014). This study identified a suite of over 100 genes that overlap among these plant responses to pathogens and SO. In addition, Zhang and coworkers reported that SO accumulation in Arabidopsis upregulated 22 transcription factors associated with plant resistance to pathogens (Zhang et al., 2014). While this overlap could be due to convergence of different stress responses at some other signaling node, in combination with oxylipin profiles it suggests the possibility that SO could mediate plant responses to *P. syringae* and other pathogens.

Two recent studies also implicate chloroplast retrograde signaling (and possibly SO?) in plant-insect interactions. Mitra and coworkers (2021) reported that applying oral secretions from the Egyptian cotton leafworm (*Spodoptera littoralis*) to mechanically-generated wounds on*A. thaliana* leaves induced accumulation of the SO-responsive metabolite β-cyclocitral, and that induction of β-cyclocitral by this simulated herbivory was higher than in response to wounding alone. β-cyclocitral in turn downregulated the

2-C-methyl-D-erythritol-4-phosphate (MEP) pathway that generates primarily metabolites for use in photosynthesis, and the authors proposed that β-cyclocitral was part of a mechanism to downregulate primary metabolism in favor of defense (Mitra et al., 2021). Artificial treatment of plants with β -cyclocitral reduced *S. littoralis*growth, suggesting that this response helps fend off attack. Further work is needed to determine whether 1) β-cyclocitral is induced by actual as well as simulated caterpillar herbivory; 2) this metabolite is generated by SO, other ROS, or by enzymatic routes (Havaux, 2020); and 3) insect performance is affected by manipulation of endogenous β-cyclocitral and/or SO accumulation. Other evidence for induction of chloroplast signaling by biotic stress comes from a plant interaction with a piercing-sucking insect and an insect-transmitted virus. When*A. thaliana* was challenged with an aphid species (*Macrosiphum euphorbiae*) for which it is a non-host, it accumulated methylerythritol cyclodiphosphate (MEcPP) (Zeng et al., 2022). Zeng and coworkers (2022) also observed MEcPP accumulation in response to Cucumber Mosaic Virus (CMV), a virus transmitted by the green peach aphid *Myzus persicae* , although MEcPP was not measured in response to *Myzus persicae* or other aphids that can infest*A. thaliana* . Like β-cyclocitral, MEcPP is another chloroplastic retrograde signaling molecule that accumulates during high light stress and is probably redox-regulated (Phua et al., 2021). Although the presence of MEcPP does not directly implicate SO, it is indicative of retrograde signaling, and associated with conditions that induce SO. Together, these studies indicate that exposure to insects, salivary elicitors, or insect-transmitted viruses can stimulate retrograde signaling in the chloroplast, and suggest that SO may be involved in at least some of these responses.

Adaptive Significance of Intracellular SO Accumulation and Signaling in Biotic Interactions

If biotic stressors can induce intracellular SO or other components of SO-mediated chloroplast signaling, the next important question is whether these responses contribute to resistance or susceptibility to biotic stress. When oxylipin profiles were compared in *A. thaliana* plants challenged with virulent and avirulent strains of Pst, 12-HO-FAs accumulated more rapidly in the incompatible interaction, suggesting a correlation with resistance (Grun et al., 2007). However, levels of 10- and 15-HO-FAs were not reported in this experiment, and the role of SO in this response is unclear because 12-HO-FAs can result from the action of free radicals as well as SO. Other studies have utilized mutants and/or treatments that induce SO to explore the influence of this ROS on pathogen resistance. In the*flu* mutant, induction of SO by a L:D:L shift also triggered accumulation of salicylic acid and expression of*Pathogensis-Related Protein 1* (*PR1*) (Ochsenbein et al., 2006). Salicylic acid, which is synthesized in the chloroplast, mediates systemic acquired resistance (SAR) to *P. syringae* and many other biotic attackers, and *PR1* is a highly conserved marker of SAR that contributes to multiple forms of disease resistance (Breen et al., 2017). Zhang and coworkers (2014) further reported that subjecting wild-type *A. thaliana* to a pre-treatment (a brief combined exposure to low temperature and light stress) that induced SO-mediated adaptation to subsequent high light exposure also upregulated *PR1*expression and reduced infection by a virulent Pst strain. *PR1*induction was absent in the *ex1/ex2* mutant, and bacterial growth on pre-treated *ex1/ex2* was higher than on pretreated wild-type plants. These results suggest that activation of *EX1* -dependent SO signaling triggers salicylate-mediated resistance to the hemi-biotropic bacterial pathogen *P. syringae* .

Conversely, *EX1* -signaling contributes to the susceptibility of*A. thaliana* to tenuazonic acid, a non-host-specific toxin produced by the necrotrophic fungus *Alternaria alternata* . Although *A. thaliana* is not a host for *A. alternata*(Narusaka et al. 2005), it forms lesions in response to tenuazonic acid, a virulence factor that facilitates the infection of host plants by inducing cell death. The toxin disrupts the electron transport chain at PSII and is expected to promote the generation of SO (Chen et al., 2015). Compared to wild-type, the *ex1ex2* mutant displays less bleaching and transcriptional reprogramming in response to tenuazonic acid treatment (Chen et al., 2015). This suggests that at least some of the effects of this toxin are

mediated through SO signaling, although SO accumulation, cell death, and fungal growth were not directly measured. Jasmonic acid contributes to non-host resistance to *A. alternata*in *A. thaliana* (Narusaka et al., 2005), and many plant pathogens are thought to capitalize on cross-talk between salicylate- and jasmonate signaling to promote virulence (Hou. & Tsuda, 2022). Thus, it is possible that in response to artificially high doses of tenuazonic acid, EX1-mediated induction of salicylate signaling could suppress jasmonate-dependent defenses. However, it is important to note that neither phytohormone was measured in this interaction, and that putative SO accumulation is in some cases accompanied by jasmonic acid induction (Przybyla et al., 2008; Mor et al. 2014). Moreover, because Arabidopsis is a non-host, it is not possible to correlate alternations in host signaling with changes in the extent of fungal infection. Further studies are therefore needed to unravel the roles of SO and EX1 in the interactions between necrotrophic fungi and host- and non-host plants.

Information about the influence of SO signaling on the outcomes of plant-insect interactions is also limited. Mitra and coworkers (2021) reported that exogenous application of β-cyclocitral to *A. thaliana* decreased growth of the Egyptian cotton leafworm on foliage. This suggests that signaling between the chloroplast and the nucleus can trigger herbivore defenses, and may help balance resource allocation between primary metabolism and defense. However, further work is needed to confirm that this retrograde signaling is induced by real herbivory, and to determine if it involves SO. While the piercing-sucking insect*M. euphorbiae* on a non-host (*A. thaliana*) induces the chloroplast signal MEcPP and the defense signaling molecules pipecolic acid and N-hydroxy-pipecolic acid (Zeng et al., 2022), the adaptive significance of this response is also unclear. Do MEcPP, pipecolic acid, and/or N-hydroxy-pipecolic acid contribute to non-host resistance, and is SO involved? Would similar or different responses be observed in a compatible interaction with other aphid species such as *M. persicae* or *Brevicoryne brassicae* that can successfully colonize*A. thaliana* ? These questions remain unresolved, and even less is known about the potential role of SO in response to other herbivores.

Conclusions and Future Directions

Although SO receives far less attention in the field of plant stress biology than other ROS such as hydrogen peroxide (H_2O_2) and superoxide (O_2^-), a growing body of evidence indicates that it plays a role in plant responses to diverse challenges, including biotic as well as abiotic stresses. Singlet oxygen can be generated in gaseous form by photosensitive phytoalexins at the plant surface, where it is able to act as a direct defense against insect herbivores and other biotic stressors. In addition, SO accumulates within plant cells in the cytosol, peroxisomes, nucleus, mitochondria, and especially in the chloroplasts. Numerous abiotic and biotic stresses perturb the cell's photosynthetic machinery, promoting SO generation, and this positions SO well to sound the alarm and activate adaptive responses (Lu and Yao, 2018). SO can also be generated as the result of the enzymatic activities of lipoxygenases or other stress-responsive peroxidases (Chen et al., 2021; Dmitrieva et al., 2020). This highly reactive ROS is an important player in retrograde signaling and is known to reprogram nuclear gene expression through more than one distinct pathway, including sensing by EX1 at the grana margins and signaling via the β-carotene derivative β-CC in the grana core. SO also modulates phytohormone signaling and, via EX1, can activate programmed cell death. While SO signaling is known in some cases to promote plant adaptation to stress, certain necrotrophic plant pathogens appear to have co-opted this response to facilitate the infection, secreting SO-inducing phytotoxins that form lesions and create infection courts for the pathogen. In short, SO plays diverse and important roles in biotic stress responses, and its adaptive significance for the host plant and for the attacker varies among different interaction pairs. Unfortunately, SO accumulation, signaling, and outcomes for resistance or susceptibility have been characterized in very few biotic interactions so far. To better understand the roles of SO in plant biotic interactions, we propose that the biotic stress community has the following research needs:

1. *Improved methods for direct detection and quantification of SO in vivo* . Several reviews cover the current options for SO detection in destructive assays and *in situ* detection (Dmitrieva et al. 2020; Prasad et al. 2018; You et al., 2018). While fluorescent sensors such as Singlet Oxygen Sensor Green (SOSG) are useful tools, uneven penetration of these exogenous sensors represent a significant

challenge. SO-responsive reporter gene constructs overcome this issue, but have separate concerns about specificity. Therefore, there is a need for improved SO-specific *in vivo*sensors, to characterize accumulation of this molecule in plant interactions with biotic stressors.

2. *Comprehensive identification of SO-responsive signaling pathways, and elucidation of the relationships among them* . The relationships among the known components of SO signaling (e.g. EX1/EX2, β-CC, SAFE1, GUN4, and multiple transcription factors) are not yet well-understood. Moreover, studies suggest the existence of other yet-to-be-identified nodes in SO signaling (e.g. D'Alessandro et al., 2018). As a more comprehensive understanding of the SO response network emerges, it will enable a better understanding of how this network impacts biotic interactions.
3. *Characterization of the effects of different biotic stresses on SO accumulation and the different branches of SO signaling, including comparisons of compatible and incompatible biotic interactions* . As yet, the role of SO has been examined in relatively few biotic interactions, and often in an indirect fashion. As a greater number of biotic interactions are considered from the perspective of SO, we anticipate that important commonalities and differences will emerge among interaction types. Within a particular combination of host plant and attacker species, it can be particularly informative to compare compatible and incompatible interactions, which are governed by known determinants of virulence/avirulence in the attacker and/or resistance or susceptibility factors in the host. These comparisons could help identify correlations between SO responses and the outcomes of the interaction.
4. *More precise tools to manipulate SO accumulation in vivo, including control of the timing, dosage, and localization of its generation* . Because the consequences of SO accumulation likely vary depending upon the timing, dosage, and location of accumulation, tools are needed to manipulate these variables and assess their consequences. Mammalian cell lines have recently been engineered to express a genetically encoded photosensitizer targeted to specific subcellular compartments that can deliver different doses of SO depending upon light exposure (Liang et al. 2020). The development of similar sensors for use in plants could dramatically advance our understanding of SO signaling, and in particular, the role of different organelles in SO-mediated responses.
5. *Evidence on how promotion or attenuation of SO accumulation or SO signaling pathways, singly and in combination, impact the outcome of plant biotic interactions* . For definitive evidence on the adaptive significance of particular signaling events, we ultimately rely on the ability to manipulate these events and test the phenotypic effects of enhancing or abrogating them. For example, null mutations in EX1 and EX2 have been invaluable in identifying the roles of these proteins in SO signaling. Therefore, identifying a more comprehensive set of signaling nodes (#2 above) and developing methods to enhance or inhibit them, in combination with methods to manipulate accumulation of SO itself (#4), are essential steps towards understanding the roles of SO in biotic stress.

In closing, SO signaling is an important emerging area of study in the field of plant stress biology, and advances in this area will likely identify novel mechanisms of plant adaptation to biotic attack in addition to environmental stress. Due to its close relationship with the photosynthetic machinery, SO also is an important linker between primary metabolism and defense. Understanding this linkage is critical to in order to leverage plant defense mechanisms for the protection of crop health and productivity of the face of increasing environmental stresses and changing pest pressures.

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FIGURES

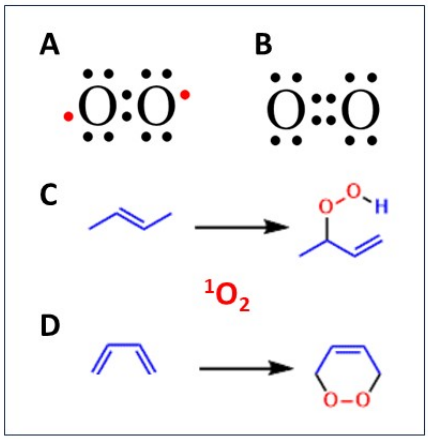
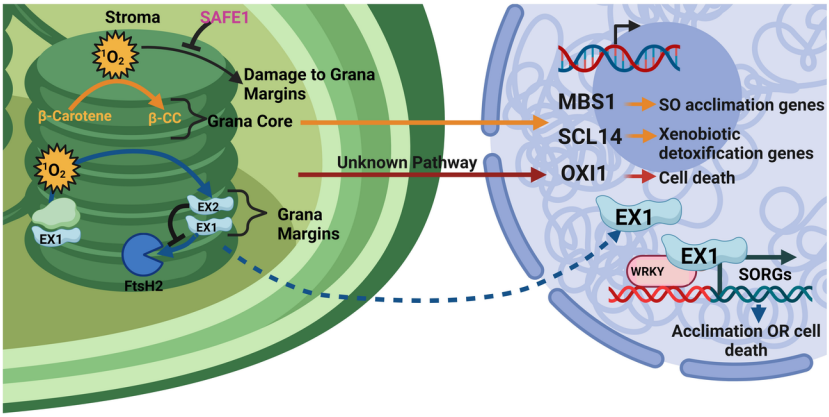


Figure 1. Chemical Properties of Singlet Oxygen. Whereas ground state molecular oxygen is in a triplet state, with two unpaired, spin-parallel electrons (A), singlet oxygen has two valence electrons spin-paired in a single orbital and a second orbital left empty. Consequently, singlet oxygen is more reactive than triplet oxygen, and can react with carbon double bonds in a variety of molecules through ene reactions (C) and Diels-Alder cycloadditions (D) that triplet oxygen cannot perform.



β

Figure 2. SO Signaling. Blue arrows represent EX1 signaling events. Prior to SO stimulation, EX1 is present in the grana margins in complex with several other proteins, but SO accumulation causes EX1 to disassociate as a result of oxidative modification. EX1 can then 1) translocate to the nucleus (dashed arrow) to promote SORG expression in concert with WRKY transcription factors; or 2) undergo proteolysis by FtsH2. Proteolysis also promotes SORG expression through an as-yet-unknown mechanism. Orange arrows represent carotenoid signaling. SO generated in the grana core oxidizes β-carotene, yielding β-cyclocitral (β-CC). This signaling molecule promotes increased expression of MBS1 and SCL14, which in turn upregulate genes for stress adaptation. β-CC or some other unknown signal promotes expression of the OXI1 kinase, which mediates cell death in response to SO from the grana core. SAFEGAURD1 (SAFE1) also acts independently of EX1 to suppress responses to SO caused by damage to the grana margins. This figure was created with Biorender, and was inspired in part by figures from (Woodson, 2019; Wang et al., 2020; Li et al., 2023).

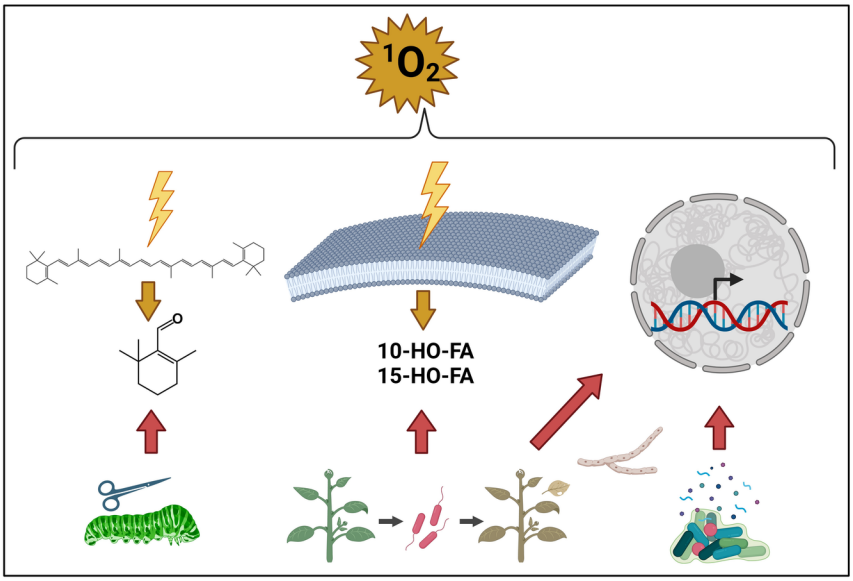


Figure 3. Overlap between Plant Responses to Singlet Oxygen and to Biotic Stressors. SO oxidizes β-carotene to generate the retrograde signaling molecule β-cyclocitral, which is also induced by simulated beet armyworm feeding (mechanical damage paired with caterpillar oral secretions). In addition, SO oxidizes membrane lipids, giving rise to hydroxy fatty acids including 10-HO-FA and 15-HO-FA. These two HO-FAs, which are considered diagnostic markers of SO accumulation, are also induced in Arabidopsis by *Pseudomonas syringae* infection. Gene expression profiles induced in host plants by *P. syringae* , the fungal pathogen *Peronospora parasitica* , or by treatment with effectors associated with other pathogens (e.g. flagellin, chitin, oligogalacturonides) also show considerable overlap with transcriptional reprogramming by SO. This figure was made in Biorender.

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