Acclimation to Singlet Oxygen Stress in *Chlamydomonas reinhardtii*†

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In an aerobic environment, responding to oxidative cues is critical for physiological adaptation (acclimation) to changing environmental conditions. The unicellular alga *Chlamydomonas reinhardtii* was tested for the ability to acclimate to specific forms of oxidative stress. Acclimation was defined as the ability of a sublethal pretreatment with a reactive oxygen species to activate defense responses that subsequently enhance survival of that stress. *C. reinhardtii* exhibited a strong acclimation response to rose bengal, a photosensitizing dye that produces singlet oxygen. This acclimation was dependent upon photosensitization and occurred only when pretreatment was administered in the light. Shifting cells from low light to high light also enhanced resistance to singlet oxygen, suggesting an overlap in high-light and singlet oxygen response pathways. Microarray analysis of RNA levels indicated that a relatively small number of genes respond to sublethal levels of singlet oxygen. Constitutive overexpression of either of two such genes, a glutathione peroxidase gene and a glutathione S-transferase gene, was sufficient to enhance singlet oxygen resistance. *Escherichia coli* and *Saccharomyces cerevisiae* exhibit well-defined responses to reactive oxygen but did not acclimate to singlet oxygen, possibly reflecting the relative importance of singlet oxygen stress for photosynthetic organisms.

Reactive oxygen species (ROS) production is an unavoidable consequence of life in an aerobic environment, and reliance upon oxygentic photosynthesis presents plants and algae with sources of ROS not generally shared by their nonphotosynthetic counterparts. Nearly any form of biotic or abiotic stress affects the chloroplast, where the photosynthetic electron transport chain brings together photosensitizing pigments, redox-active electron carriers, and oxygen generation in a polysaturated lipid environment. Disruptions in the balance between incoming excitation energy and terminal electron acceptors can result in ROS production and eventual cell death. High-light (HL) stress, for example, leads to increased production of singlet oxygen (1O₂·), hydrogen peroxide, and superoxide in the chloroplast (31, 44), while hypersensitive responses to tobacco mosaic virus in tobacco result in down-regulation of the proteins necessary to repair ROS-mediated damage to photosystem II (71). Understanding how plants and algae respond to ROS and limit ROS-induced damage is therefore necessary to piece together responses to biotic and abiotic stress.

As a result of the capacity of ROS for damaging cellular constituents, including proteins, nucleic acids, and membranes (41), ROS are often cast in a purely destructive role. Evidence is emerging, however, that sublethal levels of ROS can be important signaling intermediates (4, 30), activating pathways that bolster defense responses and enhance survival of subsequent stress (11, 13, 47, 78). For example, in the yeast *Saccharomyces cerevisiae*, sublethal levels of hydrogen peroxide activate the YAP1 (yeast activator protein 1) transcription factor (15, 85), which then promotes expression of a number of antioxidant-related genes (35, 54), including thioredoxin (51), glutathione peroxidase (48), and gamma-glutamylcysteine synthetase (GSH1), the rate-limiting enzyme in glutathione biosynthesis (86). *S. cerevisiae* also acclimates to superoxide (25, 49) and lipid hydroperoxides (17), and these responses are often characterized by specificity to the chemical identity of the ROS. Therefore, to understand the mechanisms by which cells sense and respond to oxidative stress, it is necessary to investigate responses to individual ROS.

Although ROS sensors in *E. coli* and *S. cerevisiae* have been characterized, the absence of obvious homologs of these sensors in algae and plants suggests that mechanisms for responding to ROS may differ in photosynthetic organisms (reviewed in reference 5). Furthermore, the abundance of photosensitizing pigments required for photosynthesis means that plants and algae may be subject to oxidative stresses, such as 1O₂·, that are not as important for nonphotosynthetic organisms.

Despite the possible importance of 1O₂· stress responses in photosynthetic organisms, little is known about what systems may exist to counteract 1O₂· damage. 1O₂· is a highly reactive, excited state of oxygen that can be formed when excited triplet chlorophyll (3Chl*) in photosystem II interacts with ground-state oxygen. Environmental stress that upsets the balance between light harvesting and energy utilization lengthens the lifetime of chlorophyll (1Chl*) (reaction 1), increasing the likelihood that 1Chl* will undergo intersystem crossing to form 3Chl* (reaction 2). 3Chl* is longer-lived than 1Chl* and reacts more readily with ground-state 1O₂ (reaction 3). The physical
interaction between \(^2\text{Chl}\) and oxygen produces \(^1\text{O}_2\) (reaction 3), liberating oxygen from the spin restriction that normally limits its reactivity with singlet-state biological molecules (39).

The three reactions are as follows: reaction 1, \(\text{Chl} + \text{light} \rightarrow \text{Chl}^\ast\); reaction 2, \(\text{Chl}^\ast \rightarrow \text{Chl}\); reaction 3, \(\text{Chl} + \text{O}_2 \rightarrow \text{Chl} + \text{O}_2^\ast\).

While pigments, such as chlorophyll and protoporphyrin, can generate \(^1\text{O}_2\) endogenously, exogenous photosensitizers, such as rose bengal (RB), generate \(^1\text{O}_2^\ast\) as well (77). \(^1\text{O}_2\) is highly reactive and can modify lipids (36), nucleic acids (58), and proteins (14). Experiments using lipophilic photosensitizers in \(E.\ coli\) established that a \(^1\text{O}_2^\ast\) molecule could not travel more than 0.07 \(\mu\)m within a cell before either being quenched or reacting with another molecule (60), but recent work using a microscope capable of detecting near-infrared phosphorescence from \(^1\text{O}_2\) has indicated that \(^1\text{O}_2^\ast\) generated in the cytoplasm is capable of moving across cell membranes (75).

Despite the transience of \(^1\text{O}_2^\ast\), several lines of evidence indicate that \(^1\text{O}_2\) can impact gene expression in photosynthetic organisms. Previous work in the single-celled alga 

\textit{Chlamydomonas reinhardtii} established \(^1\text{O}_2\)-mediated regulation of a putative glutathione peroxidase gene (\textit{GPXH}) (21, 23, 55). The photosynthetic proteobacterium 

\textit{Rhodobacter sphaeroides} also induces a glutathione peroxidase in response to singlet oxygen (37), and multiple \textit{R. sphaeroides} operons have been identified that appear to be under \(^1\text{O}_2^\ast\) control (3). Recently, work with protoporphyrillide-accumulating \(flu\) mutants in \textit{Arabidopsis thaliana} has shown that \(^1\text{O}_2^\ast\) generated by protoporphyrillide accumulation in the chloroplast can trigger gene expression changes in the nucleus, many of which are specific to singlet oxygen and are not mimicked by treatment with hydrogen peroxide or superoxide (34, 63). \(^1\text{O}_2^\ast\) responses in \(flu\) mutants include growth arrest and programmed cell death, both of which are controlled by the nucleus-encoded, chloroplast-localized protein EX1 (\textit{EXECUTER 1}) (83). Despite this array of physiological responses to singlet oxygen, acclimation to singlet oxygen has not yet been demonstrated in any of these organisms.

To learn more about how \textit{C. reinhardtii} responds to phototoxic stress, we assayed for the ability to acclimate to specific forms of ROS. We found that sublethal levels of \(^1\text{O}_2^\ast\) triggered a clear enhancement of defenses against \(^1\text{O}_2\). Characterization of this response revealed that the abundance of transcripts of a small subset of genes was enhanced in response to \(^1\text{O}_2^\ast\) pretreatment. Constitutive overexpression of either of two of these genes—a glutathione peroxidase gene and a glutathione \(S\)-transferase gene—was sufficient to promote \(^1\text{O}_2^\ast\) resistance. The inability of \textit{S. cerevisiae} and \(E.\ coli\) to acclimate to \(^1\text{O}_2^\ast\) suggests the importance of \(^1\text{O}_2^\ast\) responses for photosynthetic organisms.

\section*{Materials and Methods}

\textbf{Strains and growth conditions.} The wild-type \textit{C. reinhardtii} strain used in this work was \(4A^+\) (16). The \(pc1\) \(y7\) double mutant strain was produced by crossing \(CC-2471\) (\(pc1\) \(m+t\)) to \(CC-1174\) (\(y7\) \(m-t\)) and selecting for strains that failed to grow in both the light and dark (28). The \(pc1\) and \(y7\) parental strains were obtained from the \textit{C. reinhardtii} Stock Center.

Cells were grown photoautotrophically in minimal (HS) medium or phototrophic in acetate-containing (TAP) medium under low-light (LL) conditions (50 \(\mu\)mol photons \(m^{-2}\) \(s^{-1}\)) as described previously (7). Cultures were grown to mid-exponential phase (1 \(\times\) \(10^6\) to 2 \(\times\) \(10^6\) cells/ml). For LL to HL transitions, cells were shifted to 500 \(\mu\)mol photons \(m^{-2}\) \(s^{-1}\), RB (Sigma), hydrogen peroxide (EM Science), methyl viologen (Sigma), neutral red (Sigma), tert-butyl hydroperoxide (Sigma), and metronidazole (Sigma) were each dissolved in water and added directly to the growth medium immediately prior to use. Deuterium oxide, neutral red, and tert-butyl hydroperoxide assays were carried out in 100-\(\mu\)l volumes in 96-well trays at 50 to 60 \(\mu\)mol photons \(m^{-2}\) \(s^{-1}\).

Deuterium oxide experiments were performed in TAP medium containing 85% (vol/vol) deuterium oxide (Sigma). Cells were incubated in this mix for 2 h prior to RB treatment.

Experiments with \(E.\ coli\) were performed using strain DH5\(a\) in Luria broth at 37°C. Experiments with \textit{S. cerevisiae} were performed at 30°C in yeast extract-peptone-dextrose (YPD) medium using strain YPH500 (73). Maximum pretreatment concentrations used were the highest concentrations of RB that did not result in cell death.

\textbf{Tocopherol and pigment analysis.} Wild-type cells were grown photoautotrophically in 100-ml cultures under LL. RB treatments were administered as described above, and 2-ml samples were taken for acetone extraction and high-performance liquid chromatography determination of tocopherol and pigment content as described previously (6).

\textbf{RNA isolation.} Samples were harvested by centrifugation (3,200 \(\times\) \(g\), 4°C, 3 min) and then resuspended in 0.1 volume of \(H_2O\) at 4°C. An equal volume of 2× hybridization lysis buffer (0.6 M NaCl, 10 mM EDTA, 0.1% [wt/vol] deoxytocyl sulfate [SDS]) was added to the cell suspension, which was subsequently incubated at 65°C for 5 min. Then, 0.132 volume of 2 M KCI was added, and the samples were incubated on ice for 15 min. Samples were then centrifuged at 12,000 \(\times\) \(g\) for 10 min at 4°C. Supernatants were extracted twice with phenol-chloroform and once with chloroform before precipitating RNA overnight using 0.33 volume of 8 M LiCl at 4°C. This was followed by a final ethanol precipitation. RNA quality was assessed using the ratio of absorbance at 260 nm and 280 nm and by ethidium bromide staining following gel electrophoresis. RNA for both microarrays and RNA gel blot analysis was isolated in this manner.

\textbf{Microarray experimental design.} Cultures (100 ml) of \textit{C. reinhardtii 4A}+ were grown photoautotrophically at 50 \(\mu\)mol photons \(m^{-2}\) \(s^{-1}\) until they reached a density of 1.5 \(\times\) \(10^6\) cells/ml. “Pretreated” samples were treated with 2 \(\mu\)M RB, whereas “unpretreated” cultures received a mock inoculation with an equal volume of water. Cells were incubated for 2 h before RNA was harvested.

Microarray slides were printed as part of the \textit{C. reinhardtii} Genome Project (46, 72). Fragments corresponding to the last 400 base pairs at the 3’ ends of 2,761 \textit{C. reinhardtii} cDNAs were amplified and spotted onto polyamine-coated slides (Corning, Acton, MA). Each slide contained four replicate spots arrays in four distinct grids. A total of four slides were used, encompassing two biological replicates, each with a dye-switch control.

\textbf{Microarray probe labeling.} Reverse transcription of RNA samples was carried out at 42°C for 2 h in the presence of deoxynucleoside triphosphates containing a 1:1 ratio of amino-allyl dUTP to dITP. Samples were treated with EDTA to stop the reaction and with NaOH to destroy RNA. After neutralizing the sample with HCl, cDNA was purified using a Microcon 30 column, dried, and resuspended in 0.1 M NaHCO\(_3\) buffer (pH 9.0). RNA samples were labeled using Post-Labeling Reactive Dye Packs from Amersham Biosciences (Amersham, Little Chalfont, Buckinghamshire, United Kingdom). Probes were purified individually using QiAquick columns (QIAGEN, Valencia, CA) and then combined.

\textbf{Microarray hybridization conditions.} Slides were blocked in a sucrose anhydride-sodium borate solution for 20 min and then rehydrated in a boiling water bath for 1 min. Slides were then rinsed in ethanol and dried by brief centrifugation. Prehybridization was carried out for 20 min at 50°C in a solution containing 3.5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% (wt/vol) SDS, and 10 mg/ml bovine serum albumin. After prehybridization, slides were rinsed with water and then with isopropanol and dried. For hybridization, a 2× hybridization buffer containing 6× SSC, 0.2% (wt/vol) SDS, 0.1 mg/ml poly(dA), and 0.1 mg/ml yeast RNA was prepared and mixed with an equal volume of labeled cDNA. Slides were hybridized overnight in a 50°C water bath. The next day, slides were washed once in 2× SSC, 0.03% (wt/vol) SDS, once in 1× SSC, and once in 0.05× SSC, each for 5 min. Slides were then dried and stored until scanning.

\textbf{Microarray image analysis.} Slides were scanned on an ArrayWoRx Biochip Reader and quantitated using the SoftWoRx Tracker Microarray program, both from Applied Precision, LLC (Issaquah, WA). Each represented gene had to have a valid data point from each biological replicate to be considered further. Abnormal spots were flagged manually and excluded from further analysis, as was any spot in which the mean spot intensity did not exceed two times the
median background intensity, or in which the signal-to-noise ratio value was less than 1. Spots that were >20% saturated were also excluded.

**Data analysis.** Each slide contained four replicates of each spot arrayed in separate grids. For data normalization, each grid (containing a single set of 2,761 C. reinhardtii cDNA PCR products) was normalized individually. Preliminary analysis indicated that considerably fewer than 5% of the data points showed a greater than twofold change, suggesting that scaling could be an appropriate method for normalization of this data set. For comparison, data were also normalized manually in Excel (Microsoft). The median background intensity was subtracted from the mean spot intensity in each channel. A regression line was fit to a C3 versus Cy5 plot, and Cy3 values were divided by the slope of the line. Tailing was often observed at high and low intensity values. To account for this tailing, linear scaling and intensity-dependent normalization were performed using the SNOMAD program with a span of 0.7 and a trim of 0.1 (10). Data generated by both normalization methods were nearly identical. The data presented in Table 1 are ratios calculated from SNOMAD-normalized data.

To account for the many levels of replication in our experimental design, statistical analysis (analysis of variance) was performed using mixed-model analysis of variance performed by the 1/maanova program (9), with treatment (pretreated or not pretreated) and dye as fixed effects and array, biological sample, and spot (referring to the four replicate spots present on each array) as random effects. Data deleted using the criteria described in the preceding paragraph were imputed using the nearest-neighbor algorithm provided in the Statistical Analysis of Microarrays program (82) before performing analysis of variance. The data presented in Table 1 represent those genes for which the difference between pretreated and unpretreated expression levels exceeded 1.5-fold and the false discovery rate-adjusted tabulated P value supporting differential expression was less than 1 × 10−4. P values were calculated using the F₂ test statistic described by Cui et al. (12).

**RNA gel blot analysis.** Primers were designed based upon the expressed sequence tag (EST) contig assembly sequences (72). The primers used to amplify GSTS1 were 5′-TACGACTTCCCTCGCAGTGTTTCTTG-3′ and 5′-CGGAGACACGAGTCGGTTCTTG-3′. The primers used to amplify PHC8 were 5′-CAGCCTGCTACCAAAATTACAC-3′ and 5′-TGGCCCTCATCCTTCTACCTCC-3′. Primers for amplification of a portion of contig number 20021010.5327 (referred to as GSTS2) and 3′-CTGTAAACCAAACTGCTGCCAAGCTGTTGTAAGGGTTG-3′. The primers used to amplify GSTS2 were designed based on the contig 20021010.3547. GSTS1 primer sequences were 5′-AAGGCCCTACTACCAAGCAGAC-3′ and 5′-CTGTAAACCAAACTGCTGCCAAGCTGTTGTAAGGGTTG-3′. PCR products were cloned into the pGEM-T Easy vector (Promega, Madison, WI). GPXH probes were generated from vectors described by Leisinger et al. (55, 56). The APX2 probe was described by Ledford et al. (53). To generate RNA probes, vector inserts were transcribed in the antisense direction in the presence of digoxigenin-labeled dUTP (Roche Molecular Biochemicals, Germany).

RNA from C. reinhardtii cells was prepared as described above, and 5 μg of RNA per sample were fractionated using denaturing gel electrophoresis (67). Blots were hybridized in DIG-EasyHyb solution (Roche Molecular Biochemicals, Germany) at 67°C, and high-stringency washes were carried out at 68°C using 0.2× SSC, 0.1% (wt/vol) SDS.

**Overexpression of GPXH and GSTS1.** PSAD flanking sequences were used to drive constitutive overexpression of GPXH and GSTS1 (24). GPXH cDNA was amplified from the vector described by Leisinger et al. (56), using primers designed to engineer an Ndel site at the beginning and an EcoRI site at the end. The GPXH_Ndel forward primer sequence was 5′-TACCAACAAAGCTCATATA TGGGAAACCCCGAGTTTACG-3′, and the GPXH_EcoRI reverse primer sequence was 5′-CAGCTGTGCTGCAAGATTCTTATGCTGCTTGC-3′ (restriction sites underlined). Similarly, GSTS1 was amplified from genomic DNA using the following primers: GSTS1_Ndel 5′-TCAACAACAGCCCATATA TGGCCTCCAAGGCTGTAA-3′ and GSTS1_EcoRI 5′-CAAGCTGCGCTGCAAGA AAGTCTACCGCGCTGC-3′. PCR products were digested with EcoRI and Ndel and cloned into the pSL18 vector containing PSAD 5′ and 3′ untranslated regions as well as a paromomycin-selectable marker (24, 26). The GPXH and ProAPX2 GSTS1 were transformed into C. reinhardtii 4A-4 by the method of Dent et al. (16). Transformants were selected on TAP plates containing 10 μg/ml paromomycin (Sigma).

**Microarray data accession numbers.** Raw microarray data have been deposited into the National Center for Biotechnology Information Gene Expression Omnibus database at http://www.ncbi.nlm.nih.gov/geo under series accession number GSE4681.

**RESULTS**

*C. reinhardtii* exhibits a robust acclimation response to 1O₂•. To learn more about how photosynthetic organisms respond to photodynamic stress, the model alga, *C. reinhardtii*, was tested for the ability to acclimate to specific ROS. Acclimation was scored as the ability of pretreated cells to survive what would otherwise be a lethal challenge level. (B) Assay for acclimation to RB. Wild-type cells were grown photoautotrophically and pretreated in HS liquid medium for 2 h with 2 μM RB and then challenged for 1 h using the indicated concentrations. Pretreatment and challenge were carried out in 100-ml cultures at 50 μmol photons m−2 s−1. Aliquots were serially diluted and plated on TAP medium, and colonies were counted to assay the viability of the original cultures.

**FIG. 1.** *C. reinhardtii* acclimates to singlet oxygen. (A) Schematic of acclimation assays. *C. reinhardtii* was assayed for an ability to acclimate to oxidative stress following pretreatment with sublethal levels of that stress. Cells were pretreated and challenged with reactive oxygen species in liquid medium and then plated on agar medium to assay survival. Acclimation was defined as the ability of pretreated cells to survive what would otherwise be a lethal challenge level. (B) Assay for acclimation to RB. Wild-type cells were grown photoautotrophically and pretreated in HS liquid medium for 2 h with 2 μM RB and then challenged for 1 h using the indicated concentrations. Pretreatment and challenge were carried out in 100-ml cultures at 50 μmol photons m−2 s−1. Aliquots were serially diluted and plated on TAP medium, and colonies were counted to assay the viability of the original cultures.
Acclimation to $^{1}\text{O}_2$ stress is likely the product of pretreatment-induced, transient changes in cell physiology that enhance $^{1}\text{O}_2$ resistance. 

**Acclimation is specific to $^{1}\text{O}_2$.** To explore possible overlap between responses to different ROS, we then tested the ability of $^{1}\text{O}_2$ pretreatment to induce acclimation to other ROS-generating compounds, including hydrogen peroxide, methyl viologen, metronidazole, tert-butyl hydroperoxide, and neutral red (another photosensitizing dye). The only evidence of cross-tolerance was the result of $^{1}\text{O}_2$ pretreatment observed in the case of neutral red (Fig. 4A), which has been shown to produce $^{1}\text{O}_2$ in treated C. reinhardtii thylakoids (23). Interestingly, pretreatment with RB increased methyl viologen sensitivity but had no impact on sensitivity to metronidazole. This could be related to differences in chemical structure or sites of action between these two compounds (70). The experiment was also reversed, this time pretreating with neutral red, hydrogen peroxide, methyl viologen, metronidazole, and tert-butyl hydroperoxide, and challenging with RB. Only the neutral red pretreatment was able to enhance resistance to RB (data not shown). These results suggest that the acclimation occurs specifically in response to $^{1}\text{O}_2$ and activates defenses that are specific to protection against $^{1}\text{O}_2$-mediated damage.

**HL treatment induces acclimation to $^{1}\text{O}_2$ stress.** $^{1}\text{O}_2$ is produced endogenously during exposure to HL when triplet chlorophyll interacts with ground-state oxygen. To find out whether there is an overlap between the response to HL and acclimation to $^{1}\text{O}_2$ stress, unpretreated (LL-grown) cells were shifted to HL and then assayed for resistance to $^{1}\text{O}_2$. Cells that had been pretreated with HL for 1 h were more resistant to $^{1}\text{O}_2$ (Fig. 4B).

**Acclimation to $^{1}\text{O}_2$ stress does not alter the composition or content of carotenoids or vitamin E.** Several small-molecule antioxidants are capable of quenching $^{1}\text{O}_2$ or scavenging...
1O₂*-produced damage products (69). Included in this group of antioxidants are carotenoids and tocopherols (vitamin E). In C. reinhardtii, it has previously been shown that alterations in carotenoid composition can affect RB sensitivity (6, 7), but there were no changes in β-carotene, lutein, or the xanthophyll cycle pigments zeaxanthin, antheraxanthin, and violaxanthin during the 2-hour pretreatment or the 1-hour challenge (Fig. 5). Similarly, no changes in vitamin E content or composition were observed (Fig. 5). Based on these data, changes in the abundance or composition of carotenoids and vitamin E do not account for the increased resistance to 1O₂*-seen following pretreatment.

Microarray analysis of gene expression during acclimation to 1O₂*-stress. In addition to small-molecule antioxidants, expression of nucleus-encoded antioxidant enzymes could enhance survival of 1O₂*-stress. To identify the genes that changed expression in response to the 1O₂*-pretreatment, we used the cDNA microarrays generated as part of the C. reinhardtii Genome Project (46, 72). These partial-genome arrays (v1.0) had 2,761 spots, representing approximately 20% of the genome. RNA isolated from pretreated cells was compared

![FIG. 4. Cross-acclimation between 1O₂* and other sources of oxidative stress. (A) Cross-acclimation between 1O₂* and other ROS. Cells were grown photoautotrophically, pretreated (+) with 2 µM RB for 2 h at 50 µmol photons m⁻² s⁻¹, and then challenged for 1 h at the same light intensity with either RB (0, 6, 8, 10, and 12 µM), neutral red (NR; 0, 4, 6, 8, 10 µM), tert-butyl hydroperoxide (tBOOH; 0, 0.25, 0.5, 0.75, and 1 mM), metronidazole (MZ; 0, 2, 3, 4, 6 mM), and methyl viologen (MeV; 0, 0.5, 1, 1.5, and 2 µM). (B) Cross-acclimation between 1O₂* and HL. Cultures were grown photoautotrophically at 50 µmol photons m⁻² s⁻¹ (LL) and then pretreated by shifting to 500 µmol photons m⁻² s⁻¹ (HL) for 1 h prior to challenging with the indicated concentrations of RB for 5 h at LL.](image)

![FIG. 5. Acclimation does not involve changes in cellular carotenoid or α-tocopherol content or composition. Photoautotrophically grown cells were pretreated with 2 µM RB for 2 h at 50 µmol photons m⁻² s⁻¹ and then challenged for 1 h with 12 µM RB. Error bars represent standard errors from four biological replicates. Gray shaded bars, cells not pretreated; white bars, cells not pretreated but challenged; speckled bars, pretreated cells; striped bars, pretreated and challenged cells. The xanthophyll cycle pool is the sum of violaxanthin, antheraxanthin, and zeaxanthin. β-car, β-carotene; α-toc, α-tocopherol; Chl a, chlorophyll a.](image)

![FIG. 6. Pretreatment with 1O₂*-causes changes in gene expression. (A) Volcano plot of microarray results comparing pretreated cells with unpretreated cells. Log₂-transformed gene expression ratios are plotted on the x axis against log₁₀-transformed P values (log₁₀pval). Genes with statistically different expression levels (P value of ≤1 × 10⁻⁴) and changes greater than 1.5-fold are considered to be induced, and genes with statistically different expression levels and changes less than −1.5-fold are considered to be repressed. These genes fall within the boxes in the upper right and upper left corners of the graph, respectively. (B) RNA gel blot confirmation of microarray results. Cells were grown in TAP medium at either 50 µmol photons m⁻² s⁻¹ (“light”) or in the dark. Pretreatment was 2 µM RB for 2 h, and challenge was 10 µM RB for 1 h. Five micrograms of RNA was loaded in each lane, and a methylene blue stain of rRNA was used as a loading control. Blots shown here are representative of two biological replicates. The levels of GPXH (glutathione peroxidase 1), GSTS1 (glutathione S-transferase 1), GSTS2 (glutathione S-transferase 2), PHC8 (pherophorin C8), and APX1 (ascorbate peroxidase) genes are shown. 5327 refers to the EST contig 20021010.5327.](image)
directly to RNA from mock-pretreated cells. Four slides were used to assay two biological replicates, each with a dye-switch control. In addition, each slide contained four replicate spots.

Although many genes encoding proteins with possible roles in antioxidant metabolism were included on these partial-genome arrays (53), only a small number of genes changed expression in response to the sublethal pretreatment with RB (Fig. 6A). Each of these genes is listed in Table 1. Among those genes that increased expression was a glutathione peroxidase (GPXH) and a glutathione S-transferase (GSTS1). A cytosolic thioredoxin (TRXH) that has been shown to play a role in resistance to DNA alkylating agents (68) also increased expression, as did a gene encoding a predicted protein with 40% sequence identity to pherophorins from Volvox carteri. In addition, a gene with no sequence similarity to GSTS1 increased expression in response to 1O2\(^{-}\) and 5327 was performed using RNA isolated from heterologous expression within 3 h (Fig. 7B).

**Gene expression changes following RB pretreatment are in response to 1O2\(^{-}\).** To determine whether the gene expression changes observed in the microarray were in response to 1O2\(^{-}\) or simply to the presence of a xenobiotic compound (RB), RNA gel blot analysis of GPXH, GSTS1, PHC8, and 5327 was performed using RNA isolated from heterotrophically grown cells treated with RB in the light and in the dark (Fig. 6B). In all cases, gene expression changed only when cells were treated with RB in the light, indicating that these changes are in response to photosensitized 1O2\(^{-}\) production. GSTS2, another putative sigma class glutathione S-transferase with high sequence similarity to GSTS1 was also assayed, as was expression of an ascorbate peroxidase (APX1) that was not present on the array. Neither of these genes showed altered RNA levels in response to 1O2\(^{-}\) when cells were grown heterotrophically (Fig. 6B). CAH1 transcript was not detected (data not shown), likely because of the presence of acetate in the medium (19).

**Endogenous photosensitizers also induce GPXH and GSTS1 expression.** Although RB appeared to be affecting cell physiology through the production of 1O2\(^{-}\), we wanted to know whether these same changes would occur in response to an endogenous source of 1O2\(^{-}\). To that end, we evaluated gene expression changes in a chlorophyll biosynthesis mutant. In C. reinhardtii, there is both a light-dependent and a light-independent pathway for the conversion of protochlorophyllide to chlorophyllide (28). The pcl1 y7 double mutant is blocked in both pathways, leading to the accumulation of protochlorophyllide and inability to grow under even LL conditions (57). In the absence of light, accumulated protochlorophyllide can act as an endogenous photosensitizer and generate 1O2\(^{-}\) within the chloroplast (63). RNA was isolated from pcl1 y7 and wild-type cells following a shift from the dark to LL, and RNA gel blot analysis was used to evaluate gene expression changes immediately following this shift. Transcript abundance of both GPXH and GSTS1 increased to higher levels in the pcl1 y7 mutant relative to the wild type in response to the transfer from dark to LL (Fig. 7A). GPXH exhibited a more rapid response, with transcript abundance increasing after only 30 min in the light, whereas GSTS1 expression exhibited a slower response, increasing after 3 h.

Given the cross-tolerance observed between HL and 1O2\(^{-}\) produced exogenously by RB (Fig. 4B), it was possible that the genes that increased expression in response to 1O2\(^{-}\) could also be induced by HL treatment of wild-type cells. Previous work has shown that GPXH responds rapidly to changes in light intensity (21, 53). GSTS1 expression was also tested. Expression of both genes was enhanced following exposure to HL, with GPXH again showing a more rapid induction. Induction of GPXH occurred within 30 min, followed by increased GSTS1 expression within 3 h (Fig. 7B).

**Specificity of 1O2\(^{-}\)-induced gene expression changes.** To determine whether other ROS affect expression of the 1O2\(^{-}\)-

### TABLE 1. Differentially expressed genes in microarray analyses of cells pretreated with RB

| EST clone ID | Description | Contig | JGI v3.0 protein ID | Ratio | P value |
|-------------|-------------|--------|---------------------|-------|---------|
| 963046E04   | Glutathione peroxidase (GPXH) | 5826   | 143122              | 6.85  | 1.38 × 10\(^{-10}\) |
| 894082A11   | Putative glutathione S-transferase (GSTS1) | 6612   | 193661              | 1.80  | 1.53 × 10\(^{-6}\) |
| 984036D03   | Putative indigoidine synthase (INDA) | None   | 968088              | 1.70  | 1.23 × 10\(^{-5}\) |
| 963061H06   | Pherophorin C8 (PHC8) | 5156   | 196005              | 1.67  | 5.28 × 10\(^{-5}\) |
| 984077F06   | Thioredoxin h1 (TRXH) | 8550   | 195887              | 1.60  | 5.48 × 10\(^{-6}\) |
| 963017B01   | None | 5327   | 190453              | 1.59  | 8.89 × 10\(^{-8}\) |
| 984022B08   | Carbonic anhydrase (CAH1) | 3976   | 24120               | −2.91 | 4.03 × 10\(^{-6}\) |
| 963041C09   | Thiazole biosynthetic enzyme (THI4a) | 2889   | 185190              | −2.04 | 2.08 × 10\(^{-7}\) |
| 963024B05   | Cysteine synthase (OASSTL5) | None   | 183351              | −1.95 | 1.54 × 10\(^{-7}\) |
| 963041F09   | Argininosuccinate synthase (AGS1) | None   | 58140               | −1.75 | 1.13 × 10\(^{-5}\) |
| 894050G09   | Chloroplast envelope carrier protein (CCP1) | 3478   | 189430              | −1.68 | 5.40 × 10\(^{-5}\) |
| 963035C07   | Carbamoyl-phosphate synthase (CMPS1) | 955    | 128227              | −1.60 | 1.53 × 10\(^{-5}\) |
| 984047A05   | None | 7804   | 189092              | −1.53 | 5.53 × 10\(^{-5}\) |
| 963077B03   | None | 2003   | 158519              | −1.46 | 5.38 × 10\(^{-6}\) |

\(a\) EST identification number.
\(b\) Contig numbers are based on the 20021010 EST assembly (72).
\(c\) v3.0 protein identification (ID) numbers are based on the Joint Genome Institute v3.0 of the C. reinhardtii nuclear genome sequence.
\(d\) Ratios of expression levels calculated from SNOMAD-normalized data. Positive ratios indicate genes that are expressed at higher levels in RB-pretreated cells, and negative ratios correspond to lower levels of expression in pretreated cells.
\(e\) \(P\) values are false discovery rate-adjusted, tabulated \(P\) values generated using the limma program as described in Materials and Methods.
were grown photoautotrophically at 50 μmol photons m⁻² s⁻¹ (LL). The protochlorophyllide-accumulating mutant pc1 y7 and the wild type (WT) were grown in TAP medium in the dark and then shifted to 50 μmol photons m⁻² s⁻¹ (LL). RNA samples were taken at the indicated time points, and 5 μg of total RNA was loaded in each lane. Methylene blue staining of rRNA was used as a loading control, and the mutant and wild-type blots were hybridized together to the same probe. Both blots are representative of two biological replicates.

FIG. 7. Impact of endogenous photosensitizers on GPXH and GSTS1 expression. (A) RNA gel blot analysis of pc1 y7 and the wild type (WT) were grown photoautotrophically in LL and then shifted to 500 μmol photons m⁻² s⁻¹ (HL). RNA was extracted from heterotrophically grown paromomycin-resistant transformants containing the ProPSAD:GPXH overexpression construct. Five micrograms of RNA was loaded in each lane, and methylene blue staining of rRNA was used as a loading control, and the mutant and wild-type blots were hybridized together to the same probe. Both blots are representative of two biological replicates. (B) RNA gel blot analysis of wild type transferred from LL to HL. Wild-type cells were grown heterotrophically in LL and then shifted to 500 μmol photons m⁻² s⁻¹. Total RNA was extracted at the indicated times, and 5 μg of total RNA was run in each lane. Methylene blue staining of rRNA was used as a loading control, and both blots are representative of two biological replicates.

FIG. 8. Oxidative stress-induced changes in gene expression. Cells were grown photoautotrophically at 50 μmol photons m⁻² s⁻¹ and treated with the indicated compounds (BOOH, tert-butyl hydroperoxide; MZ, metronidazole). Concentrations used did not result in cell death during 12 h of treatment. Five micrograms of total RNA was loaded in each lane, and a methylene blue stain of the blot was used as an rRNA loading control. Blots shown here are representative of two biological replicates.

FIG. 9. Constitutive overexpression of GPXH and GSTS1 confers resistance to \( {O}_2^•\). (A) RNA gel blot analysis of ProPSAD:GPXH transformants. RNA was extracted from heterotrophically grown paromomycin-resistant transformants containing the ProPSAD:GPXH overexpression construct. Five micrograms of RNA was loaded in each lane, and methylene blue staining of the blot was used as a loading control. “pSL18” refers to transformants that contain an empty vector control. (B) RNA gel blot analysis of ProPSAD:GSTS1 transformants. RNA was extracted from heterotrophically grown paromomycin-resistant transformants containing the ProPSAD:GSTS1 overexpression construct. Five micrograms of RNA was loaded in each lane, and methylene blue staining of the blot was used as a loading control. “pSL18” refers to transformants that contain an empty vector control. (C) Phenotypes of ProPSAD:GPXH and ProPSAD:GSTS1 transformants were grown in TAP liquid cultures, then plated onto TAP plates containing either 1.5 μM RB or 4 μM neutral red (NR), and grown at 100 μmol photons m⁻² s⁻¹. pSL18A1 is a control transformant containing the empty vector.

responsive genes, gene expression changes in response to hydrogen peroxide, metronidazole, and tert-butyl hydroperoxide were monitored. As previously reported (55, 56), GPXH expression did not respond as strongly or as quickly to hydrogen peroxide or the superoxide generators metronidazole and methyl viologen but did respond slowly to tert-butyl hydroperoxide, an organic peroxide (Fig. 8). All other \( {O}_2^•\)-induced genes were also induced by these additional ROS. Interestingly, GSTS2 and APX1, which did not increase expression in response to \( {O}_2^•\) in heterotrophically grown cells (Fig. 6), did respond to \( {O}_2^•\) in photoautotrophically grown cells (Fig. 8). CAH1, which decreased expression in response to \( {O}_2^•\) in the microarray analysis (Table 1), also decreased expression in response to all other ROS tested (Fig. 8).

Constitutive overexpression of either GPXH or GSTS1 is sufficient to enhance resistance to \( {O}_2^•\). To investigate the possible connection between enhanced \( {O}_2^•\) resistance and increased expression of GPXH or GSTS1, each gene was constitutively overexpressed using the PSAD promoter (24). Overexpression constructs were transformed into wild-type cells, and transformants were screened based on GPXH or GSTS1 expression (Fig. 9A and B). Because the PSAD 3′ and 5′
untranslated regions are smaller than the untranslated regions flanking the endogenous GPXH gene (362 bp versus 721 bp). ProPSAD:GPXH transformants have two GPXH transcripts, the smaller band produced from the transgene and the larger band corresponding to transcript from the endogenous gene. Of the 16 transformants screened for each construct, 12 overexpressed GPXH, and 8 overexpressed GSTS1. A subset of these is shown in Fig. 9.

ProPSAD:GPXH and ProPSAD:GSTS1 transformants were then assayed for sensitivity to \( \cdot \text{O}_2^\cdot \) by plating cells onto agar medium containing either RB or neutral red (Fig. 9C). Constitutive overexpression of either GPXH or GSTS1 was sufficient to confer enhanced resistance to \( \cdot \text{O}_2^\cdot \), as evidenced by the ability of ProPSAD:GPXH and ProPSAD:GSTS1 strains to withstand higher concentrations of RB and neutral red (Fig. 9C). ProPSAD:GPXH and ProPSAD:GSTS1 strains were also more resistant than the wild type to RB in liquid culture, but the level of resistance was not as high as that induced by pretreatment with RB (data not shown).

**E. coli and S. cerevisiae do not acclimate to \( \cdot \text{O}_2^\cdot \) stress.** There are large mutant collections for *E. coli* and *S. cerevisiae*, two model organisms in which oxidative stress responses have been extensively investigated. In hopes of taking advantage of the tools available in these systems, *E. coli* and *S. cerevisiae* were tested for the ability to acclimate to RB-induced \( \cdot \text{O}_2^\cdot \) stress. Concentrations of RB were established for both *E. coli* and *S. cerevisiae* that would be sublethal (for pretreatments) over different periods of exposure ranging from 15 min to 5 h. No condition was found that enhanced survival after challenge, and instead the pretreatment often had an additive, deleterious effect when followed by a challenge (Fig. 10).

**DISCUSSION**

*C. reinhardtii* exhibits an acclimation response to \( \cdot \text{O}_2^\cdot \). Responses to \( \cdot \text{O}_2^\cdot \) have recently been characterized in several photosynthetic organisms, including *C. reinhardtii* (21, 55), *A. thaliana* (63, 83), and *R. sphaeroides* (3, 37), but how these organisms sense \( \cdot \text{O}_2^\cdot \) remains a mystery. We have found that *C. reinhardtii* exhibits a physiological acclimation in response to \( \cdot \text{O}_2^\cdot \) which is summarized in Fig. 11. This response is charac-

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**FIG. 10.** Screens for acclimation to \( \cdot \text{O}_2^\cdot \) in *E. coli* and *S. cerevisiae*. *E. coli* and *S. cerevisiae* cultures were pretreated with the indicated RB concentrations for 2 h, then challenged for 1 h, and plated.

**FIG. 11.** Model of acclimation to \( \cdot \text{O}_2^\cdot \) in *C. reinhardtii*. Pchlide, protochlorophyllide; LOOH, lipid hydroperoxide.

terized by the ability of a sublethal \( \cdot \text{O}_2^\cdot \) pretreatment to transiently enhance \( \cdot \text{O}_2^\cdot \) resistance (Fig. 1 to 3) and can be triggered by both exogenous photosensitizing dyes, including RB, or endogenous pigments, such as chlorophyll (Fig. 4). While acclimation to hydrogen peroxide, superoxide, and lipid peroxides has been demonstrated in *E. coli* and *S. cerevisiae*, *C. reinhardtii* is, to our knowledge, the first example of an organism that exhibits an acclimation response to \( \cdot \text{O}_2^\cdot \). This has been independently verified in a very recent study by Fischer et al. (20). Furthermore, *E. coli* and *S. cerevisiae* did not acclimate to \( \cdot \text{O}_2^\cdot \) under our conditions (Fig. 10), although for *E. coli* we cannot rule out the possibility that an acclimation response might have been obscured by the high DNA damage sensitivity of the recA mutant strain that was used. \( \cdot \text{O}_2^\cdot \) is a particularly important ROS for photosynthetic organisms because of their reliance upon photosensitizing pigments for light harvesting (50), and acclimation to \( \cdot \text{O}_2^\cdot \) in *C. reinhardtii* may reflect the relative importance of \( \cdot \text{O}_2^\cdot \) stress for this photosynthetic alga. It will be interesting to examine acclimation in other photosynthetic organisms, such as *A. thaliana* and Synechocystis sp. strain PCC 6803.

The response to \( \cdot \text{O}_2^\cdot \) was remarkably specific, and no evidence of cross acclimation with other forms of ROS was observed (Fig. 4A). This is particularly significant because approximately 1% of photosensitization reactions involve electron transfer to oxygen, generating superoxide radicals (26, 52). This is the case regardless of whether the source is an exogenous dye, such as RB, or an endogenous pigment, such as chlorophyll or protochlorophyllide (27), but the absence of cross-acclimation between superoxide and \( \cdot \text{O}_2^\cdot \) argues that acclimation is a response to \( \cdot \text{O}_2^\cdot \) and not superoxide. Furthermore, deuterium oxide, which lengthens the half-life of \( \cdot \text{O}_2^\cdot \), enhanced RB toxicity (Fig. 2B), also indicating that \( \cdot \text{O}_2^\cdot \) is responsible for the cell death observed in unacclimated cells. Deuterium oxide enhancement of toxicity does not definitively implicate \( \cdot \text{O}_2^\cdot \); although, because deuterium oxide assays are widely used to test for the involvement of \( \cdot \text{O}_2^\cdot \) (66), it is possible that deuterium oxide slows the decay of other ROS as well (8, 62). Nevertheless, this result taken together with the absence of cross-acclimation between superoxide and \( \cdot \text{O}_2^\cdot \) suggests that acclimation to RB is a specific response to \( \cdot \text{O}_2^\cdot \) production.
The ability of HL to induce acclimation to $^1$O$_2$· stress demonstrates an intriguing overlap between $^1$O$_2$· responses and HL exposure (Fig. 4B) and suggests that endogenous $^1$O$_2$· (produced by excited chlorophyll) can induce acclimation to exogenous $^1$O$_2$· (produced by RB) (Fig. 11). In its natural environment, C. reinhardtii would regularly experience photon flux densities equivalent to our HL treatment, which represents ~25% of full sunlight, and rapid fluctuations in light intensity similar to the 10-fold increase used in our experiments occur frequently in nature. A number of ROS are produced in response to HL, including hydrogen peroxide, superoxide, and $^1$O$_2$· (29, 31, 44). The fact that methyl violoquin, metronidazole, hydrogen peroxide, and tert-butyl hydroperoxide did not induce acclimation to $^1$O$_2$· (Fig. 4A) implies that the HL-generated signal is likely mediated by $^1$O$_2$· rather than hydrogen peroxide, lipid peroxides, or superoxide. However, because tert-butyl hydroperoxide is a shorter molecule than naturally occurring lipids, a role for lipid peroxides cannot be completely ruled out.

Changes in gene expression, but not carotenoid or vitamin E composition, occur during acclimation. Carotenoids are efficient $^1$O$_2$· quenchers, and they increase $^1$O$_2$· resistance when overexpressed in E. coli (79). A. C. reinhardtii double mutant, npq1 lor1 mutant that is unable to synthesize lutein and zeaxanthin is also more sensitive than the wild type to $^1$O$_2$· (7). Furthermore, creating a triple mutant containing npq1, lor1, and npq2, which causes cells to accumulate zeaxanthin, restores RB tolerance to wild-type levels (6). Tocopherols (vitamin E) also play a role in $^1$O$_2$· defense, and the tocopherol-deficient vte1 mutant in A. thaliana accumulates more lipid peroxides in response to $^1$O$_2$· than the wild type (43), but despite the potential for carotenoids and tocopherols to protect against $^1$O$_2$·, changes in carotenoid and tocopherol composition or content did not accompany acclimation to $^1$O$_2$· in C. reinhardtii (Fig. 5). Glutathione can also be an important component of antioxidant defenses against $^1$O$_2$· by providing reducing power for lipid peroxide scavenging enzymes, but previous work has established that neither glutathione content nor glutathione redox state is altered by RB treatment under conditions similar to those used in this work (1 μM RB for 20 to 120 min at 120 μmol photons m$^{-2}$ s$^{-1}$) (22).

Instead, acclimation to $^1$O$_2$· was associated with changes in nuclear gene expression. Microarray experiments using the v1.0 C. reinhardtii cDNA arrays (46, 72) detected only 14 genes that changed expression in response to pretreatment with $^1$O$_2$· (Fig. 6A and Table 1). Six of the 14 genes (GPXH, GSTS1, PHC8, 5327, CAH1, and THI4a) were also tested by RNA gel blot analysis (Fig. 6B and 8; also data not shown), and the changes in gene expression were confirmed in each case. The arrays used in our analysis cover approximately 20% of the genome. Extrapolating from these results to the full genome yields an estimated 70 $^1$O$_2$·-regulated genes in the C. reinhardtii genome.

Gene expression changes in response to the pretreatment were light dependent, indicating that transcript abundance changed in response to $^1$O$_2$·, and not merely in reaction to the presence of a xenobiotic compound, such as RB (Fig. 6B). This was particularly interesting in the case of GSTS1. No longer relegated only to the role of xenobiotic detoxification, glutathione S-transferases are now known to be a diverse group of enzymes responsible for detoxifying endogenous compounds, including lipid peroxides (1, 74). Some play a direct role in signaling (1). In mammalian systems, prosta glandin H synthase-2, also a member of the sigma class of glutathione S-transferases, is induced by ROS (18). Activation of GSTS1 by RB and endogenous photosensitizers only in the light suggests that transcript abundance of this gene is regulated by oxidative stress rather than the mere presence of foreign chemicals (Fig. 6B and 7). The signal that triggers enhanced GSTS1 expression was not specific to $^1$O$_2$·, however, and induction of both GSTS1 and GSTS2 occurred in response to each ROS tested (Fig. 8). $^1$O$_2$· induction of APX1 and GSTS2 was more complicated, occurring only in photoautotrophically grown cultures (Fig. 6B and 8).

Decreased expression of the periplasmic carbonic anhydrase gene, CAH1, was also observed in response to each of the ROS tested (Fig. 8). CAH1 transcript levels respond rapidly to changes in CO$_2$, and mRNA abundance decreases within an hour after the start of CO$_2$ supplementation (32). The signal that triggers these changes in C. reinhardtii is as yet unknown, and the effect of $^1$O$_2$· on CAH1 and CPP1 expression might indicate some cross talk between oxidative stress and regulation of the carbon-concentrating mechanism. In the marine diatom Phaeodactylum tricornutum, increases in cyclic AMP (cAMP) have been suggested to repress transcription of a chloroplastic carbonic anhydrase gene (42). Interestingly, a sequence motif similar to the mammalian cAMP response element has been identified within a region of the GPXH promoter that is responsible for increased transcription of this gene in response to $^1$O$_2$· in C. reinhardtii (55).

Of all the genes that changed expression in response to $^1$O$_2$·, only GPXH showed a stronger, more rapid response to $^1$O$_2$· than to the other ROS tested (Fig. 8). This result confirms previously published work showing that GPXH is induced by photosensitizing dyes and organic hydroperoxides, but not by the superoxide-generating herbicides metronidazole and methyl violoquin (55). Because GPXH and GSTS1 encode proteins with potential antioxidant function, it was possible that changes in expression of these genes could affect $^1$O$_2$· resistance in C. reinhardtii. Overexpression of either GPXH or GSTS1 was sufficient to enhance $^1$O$_2$· resistance (Fig. 9). However, pretreatment with other ROS also enhanced GSTS1 expression (Fig. 8) without increasing $^1$O$_2$· resistance (data not shown), suggesting that transient increases in GSTS1 transcript cannot be sufficient to induce $^1$O$_2$· resistance and implying an additional level of regulation. This added layer of regulation could be at the level of translation, and singlet oxygen has been previously shown to affect translation elongation of the D1 protein in Synechocystis sp. strain PCC 6803 (61).

How do GPXH and GSTS1 enhance resistance to $^1$O$_2$·? Sequence alignments with glutathione peroxidases from other organisms showed that GPXH exhibits features of phospholipid hydroperoxide glutathione peroxidases (data not shown) (5). Given that some glutathione S-transferases also function as lipid peroxidases, the simplest explanation is that overexpressing GPXH and GSTS1 protects cells from $^1$O$_2$· by enhancing lipid peroxidase activity, but other possible functions for these two genes certainly have not been ruled out. For example, both glutathione peroxidases and glutathione S-transferases have been shown to play direct signaling roles as...
Model of acclimation to $1\text{O}_2^*$. Overall, this study has allowed us to derive a model of $1\text{O}_2^*$ acclimation (Fig. 11) in which $1\text{O}_2^*$ from exogenous dyes, such as RB (Fig. 1 and 2), or endogenous pigments, such as chlorophyll or protochlorophyllide (Fig. 7), activates a signal transduction pathway that increases expression of $\text{GPHX}$, $\text{GSTS1}$, as well as other genes (Table 1). How the $1\text{O}_2^*$ signal is perceived and converted to enhanced gene expression remains a mystery. There are three obvious possibilities: $1\text{O}_2^*$ could itself directly modify a protein sensor; a by-product of $1\text{O}_2^*$ damage, such as a lipid peroxide or protein peroxide, could interact with the sensor protein; or $1\text{O}_2^*$ could activate a sensor by perturbing the redox state of the cell (Fig. 11). At present, there is no definitive evidence for or against any of these possibilities. Regulation of $\text{GPHX}$ expression by $1\text{O}_2^*$ has been mapped to two regions of the promoter (55), one of which contains a putative cAMP-response element that is also found in predicted introns of both $\text{GSTS1}$ and $\text{GSTS2}$ (data not shown). Future work evaluating the role of this element in $1\text{O}_2^*$ signaling could be valuable for piecing together how this short-lived signal is sensed.

HL-induced acclimation to $1\text{O}_2^*$ stress demonstrates the presence of a chloroplast-to-nucleus retrograde signaling pathway capable of activating the acclimation response. Studies of $1\text{O}_2^*$ responses in the flu mutant of $A. \text{thaliana}$ have also demonstrated a $1\text{O}_2^*$-activated retrograde signaling pathway (63, 83). In $A. \text{thaliana}$, this pathway is mediated by EX1. flu eti double mutants produce as much $1\text{O}_2^*$ as flu single mutants but do not experience either growth arrest or cell death in response to light/dark cycles (83). This suggests a signaling rather than antioxidant role for the chloroplast-localized EX1 and also indicates that, in the flu mutant, cell death in response to $1\text{O}_2^*$ is genetically programmed rather than the direct product of oxidative damage. Whether this response to $1\text{O}_2^*$ is conserved in $C. \text{reinhardtii}$ is currently unknown, but there is a gene with sequence similarity to EX1 in the current release of the $C. \text{reinhardtii}$ genome. Future work will address the role of this gene in $C. \text{reinhardtii}$.

This work expands our knowledge of biological responses to $1\text{O}_2^*$ and raises questions about the nature of the sensing and signaling pathways involved in acclimation to $1\text{O}_2^*$. The phylogenetic and molecular characterization described here opens the door for genetic approaches to dissect these pathways. The strong acclimation to $1\text{O}_2^*$ exhibited by $C. \text{reinhardtii}$ makes it an ideal model for photosynthetic eukaryotes in which to pursue studies of $1\text{O}_2^*$. 

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ACCLIMATION TO SINGLET OXYGEN

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