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## Free Radical Biology and Medicine

journal homepage: [www.elsevier.com/locate/freeradbiomed](http://www.elsevier.com/locate/freeradbiomed)

## Distress and eustress of reactive electrophiles and relevance to light stress acclimation via stimulation of thiol/disulphide-based redox defences

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## ARTICLE INFO

## Keywords:

Photosynthesis  
Light stress signalling  
Reactive electrophile species  
Acrolein  
Glutathione  
Glutathione transferase

## ABSTRACT

Photosynthetic organisms suffering from light stress have to cope with an increased formation of reactive short-chain aldehydes. Singlet oxygen generated from highly-charged reaction centres can peroxidise the poly-unsaturated fatty acid (PUFA)-rich thylakoid membranes they are embedded in. Lipid peroxides decay to release  $\alpha,\beta$ -unsaturated aldehydes that are reactive electrophile species (RES). Acrolein is one of the most abundant and reactive RES produced in chloroplasts. Here, in the model chlorophyte alga *Chlamydomonas reinhardtii*, a clear concentration-dependent "distress" induced by acrolein intoxication was observed in conjunction with depletion of the glutathione pool. The glutathione redox state ( $E_{\text{GSSG}/2\text{GSH}}$ ) strongly correlated ( $R^2 = 0.95$ ) with decreasing  $F_v/F_m$  values of chlorophyll fluorescence. However, treatment of *C. reinhardtii* with sub-toxic acrolein concentrations increased glutathione concentrations and raised the protein levels of a glutathione-S-transferase (GSTS1), mimicking the response to excess light, indicating that at lower concentrations, acrolein may contribute to high light acclimation, which could be interpreted as "eustress". Furthermore, similar patterns of chloroplastic protein carbonylation occurred under light stress and in response to exogenous acrolein. Priming cells by low doses of acrolein increased the alga's resistance to singlet oxygen. A RNA seq. analysis showed a large overlap in gene regulation under singlet oxygen and acrolein stresses. Particularly enriched were transcripts of enzymes involved in thiol/disulphide exchanges. Some of the genes are regulated by the SOR1 transcription factor, but acrolein treatment still induced an increase in glutathione contents and enhanced singlet oxygen tolerance of the *sor1* mutant. The results support a role for RES in chloroplast-to-nucleus retrograde signalling during high light acclimation, with involvement of SOR1 and other pathways.

### 1. Introduction

In 1936, the "General Adaptation Syndrome" of stress was published [1], which described three phases of the stress response; an initial 'alarm' to the increased stress load that results in acclimation of the organism, enabling 'resistance' whereby the acclimated organism has enhanced protection and repair mechanisms. However, there are limits to stress tolerance that eventually 'exhaust', leading to disorders and irreparable damage. This model was repeatedly supported in both medicine and plant science [2,3] and is sometimes typified by the expression "what doesn't kill you makes you stronger". Since elevated stress is associated with enhanced production of reactive oxygen species (ROS), improved stress tolerance often involves elevated antioxidant

defences. Sensing increased ROS production is part of the 'alarm' phase, and organisms can be 'challenged' or 'primed' to increase their stress tolerance, leading to 'resistance'. For example, weak hydrogen peroxide pre-treatments of tobacco plants [4] or low singlet oxygen pre-treatments of green algae [5] significantly increased the tolerance of these organisms to higher doses of the respective ROS.

A unique feature of thylakoid membranes, which house the photosynthetic and electron-transporting apparatus, is their high galactolipid composition. Thylakoid membranes also separate the stroma from the lumen, over which a proton-motive force is created to drive ATP synthesis. Monogalactosyl-diacylglycerol is rich in linolenic acid, a C18:3 poly-unsaturated fatty acid (PUFA) [6], whose unsaturated carbons are vulnerable to oxidation, either enzymatically, i.e. via

**Abbreviations:** 4-HHE, 4-hydroxy-hexenal; 4-HNE, 4-hydroxy-nonenal; AOX, alternative oxidases of electron transport chains; bZIP, basic leucine zipper (transcription factor);  $E_{\text{GSSG}/2\text{GSH}}$ , glutathione half-cell reduction potential (in mV);  $F_v/F_m$ , maximum quantum yield of PSII;  $\gamma$ -GCL,  $\gamma$ -glutamyl cysteine ligase/synthase (coded for by *GSH1*); GPX, glutathione peroxidases; GRX, glutaredoxins; GSH, the thiol form of glutathione; GSSG, the disulphide form of glutathione; GSTS1,  $\sigma$ -class glutathione-S-transferase 1; MSR, methionine sulfoxide reductases; NRX, nucleoredoxins; NTR, NADPH-dependent thioredoxin reductases; PUFA, Poly-unsaturated fatty acid; PRX, peroxiredoxins; PSII, photosystem II; RES, reactive electrophile species; RB, Rose Bengal; SOD, superoxide dismutases; SOR1, singlet oxygen resistant 1 (transcription factor); SRX, sulfiredoxins; TRX, thioredoxins

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<https://doi.org/10.1016/j.freeradbiomed.2018.03.030>

Received 3 November 2017; Received in revised form 10 March 2018; Accepted 16 March 2018  
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lipoxygenases, or non-enzymatically, i.e. via ROS [7]. In mammals, the C20:4 PUFA arachidonic acid located in phospholipid membranes is a major source of oxidised lipid products. After its release by phospholipases, arachidonic acid is rapidly metabolised by cyclooxygenase, lipoxygenases or p450 to produce eicosanoids that are typically electrophilic and reactive signalling molecules [8]. In plants, very high levels of singlet oxygen production induce lipoxygenase activity, which activates programmed cell death (i.e. in the *Arabidopsis thaliana* *ftu* mutant [9]). At lower physiological levels, singlet oxygen also directly and non-enzymatically induces lipid peroxidation [7]. Elevated singlet oxygen production is also coupled to light stress in the model green alga, *Chlamydomonas reinhardtii* [10–12]. Due to its high reactivity, singlet oxygen could contribute to the initial 'alarm' response to light stress via other plastid components, which generate more stable molecules that can participate in chloroplast-to-nucleus so-called 'retrograde signalling' [7]. These might include light stress-related lipid peroxides, which decay releasing short-chain  $\alpha,\beta$ -unsaturated aldehydes, which are reactive electrophile species (RES) [13].

2-propenal is a RES, which due to its acrid smell is called acrolein. In comparison to other RES, acrolein is particularly abundant in light stressed leaves [14], where it can inhibit thiol/disulphide regulated enzymes of the Calvin-Benson cycle [15] and lower maximum photosystem II (PSII) quantum yields ( $F_v/F_m$ ), while respiration is less affected [16,17]. Treating *A. thaliana* leaves with acrolein stimulated gene expression that mimicked the response to avirulent bacteria, including an upregulation of glutathione-S-transferase 1 (*GST1*) [16]. Certain transcription factors have electrophile-responsive-elements (ERE). In *C. reinhardtii*, a basic leucine zipper (bZIP) transcription factor with an ERE called SOR1 regulates the expression of many nuclear genes in response to singlet oxygen and RES-associated stress [18]. In *Saccharomyces cerevisiae*, singlet oxygen stress is partially regulated by the basic leucine zipper (bZIP) transcription factor YAP1 [19], and *yap1* mutants are sensitive to Rose Bengal, a photosensitiser that produces singlet oxygen [20]. The mammalian bZIP transcription factor Nrf2 activates many defence-related genes in response to RES, including  $\gamma$ -glutamyl-cysteine ligase/synthase ( $\gamma$ -GCL) [21], required for the rate limiting step of glutathione (GSH) synthesis [22]. Treating *A. thaliana* leaves with the RES 12-oxo-phytodienoic acid (OPDA) stimulated synthesis of *GST1* and  $\gamma$ -GCL [23]. As an abundant chloroplast and cellular nucleophile [24], GSH is perhaps the most important scavenger of RES [15]. Nucleophilic protein thiol groups are susceptible to attack from RES, leading to irreversible protein carbonylation that can be used as a marker of oxidative stress [25–27], including photooxidative stress [11]. Overall, RES are clearly important stress-related signalling molecules in various life-forms as distant as yeast, mammals and plants. Considering that RES production could be part of light stress, we here explored the potential role of acrolein in the 'alarm' response of *C. reinhardtii* to high light. An RNA seq. analysis showed that sub-toxic acrolein treatments stimulated a significant amount of thiol/disulphide-based antioxidant defences, including many GSTs, such as *GST1*. The global transcriptional response showed a remarkable positive correlation with how cells responded to exogenously-produced singlet oxygen, highlighting the involvement of this ROS in RES signalling. Glutathione concentrations could also be stimulated by low doses of acrolein, independently of the SOR1 bZIP transcription factor, as shown using the *sor1* mutant. These increased defences enhanced the 'resistance' of cells to singlet oxygen, which occurred well before 'exhaustion' and viability loss that occurred after exposure to an acrolein over-dose. Importantly, the same eustress-associated increase in glutathione and *GST1* contents were found in cells acclimating to a higher light intensity, which contained five to ten-fold increases in acrolein and other RES, including malondialdehyde (MDA) and 4-hydroxy-nonenal (4-HNE).

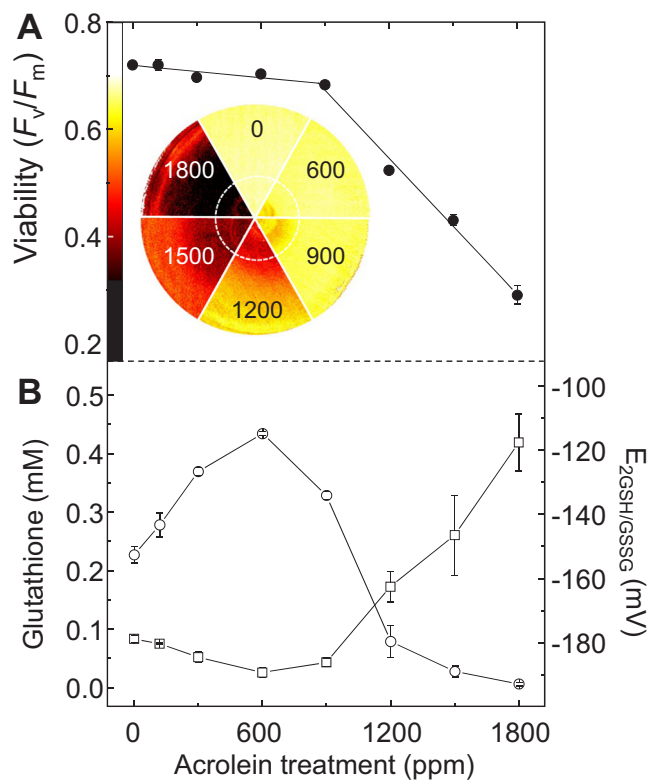


Fig. 1. Effect of acrolein treatments on  $F_v/F_m$ , glutathione contents and redox states as viability indicators of *Chlamydomonas reinhardtii*. (A) Cells were treated with up to 1800 ppm acrolein placed in the centre of a 11 cm Petri dish and the  $F_v/F_m$  was measured 4 h later. The inset shows a false-colour image of  $F_v/F_m$ , demonstrating a diffusion gradient of the influence that acrolein had on  $F_v/F_m$ . The dotted line indicates a 2 cm radius. (B) Cells were treated as in (A) before measurements of glutathione contents (left Y axis; circles) and the glutathione redox state ( $E_{GSSG/2GSH}$ ; right Y axis; squares), as calculated from individually measured GSH and GSSG concentrations,  $n = 3 \pm SD$ .

## 2. Results

### 2.1. Distress and eustress in response to acrolein dose

Acrolein has a boiling point of 53 °C and rapidly evaporates at 25 °C, making it possible to test the effects of volatile acrolein on agar-grown cultures within a sealed Petri dish. Cells tolerated acrolein up to a concentration of 900 ppm, with higher concentrations negatively correlating with the decrease in the viability marker  $F_v/F_m$  (Fig. 1A). Chlorophyll fluorescence imaging, used to measure  $F_v/F_m$ , clearly showed that the effect of acrolein was dependent upon the distance of cells from the source, whereby cells closest to the source were most affected (Fig. 1A, inset). Therefore, for all analyses, cells were only included or taken from within a 2 cm radius of the acrolein source (Fig. 1A, inset). The glutathione contents of cells increased when treated with low acrolein concentrations, doubling 4 h after treatment with 600 ppm acrolein (Fig. 1B), but depleting at 1200 ppm, leading to an oxidative shift (less negative) in the glutathione redox state ( $E_{GSSG/2GSH}$ ) (Fig. 1B). There was a very strong correlation of  $E_{GSSG/2GSH}$  with  $F_v/F_m$  ( $R^2 = 0.95$ ) over all acrolein treatments (Supplemental Fig. 1). The abundance of *GST1* protein, which has previously been associated to singlet oxygen stress [5], dramatically increased in response to acrolein treatment, whereby cells treated with 600 ppm acrolein accumulated the most *GST1* (Fig. 2A). The  $\sigma$ -class of GSTs have high glutathione-conjugating activity with RES [28]. Cells treated with higher acrolein concentrations accumulated lower *GST1* amounts.

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