β-Cyclocitral Does Not Contribute to Singlet Oxygen-Signalling in Algae, but May Down-Regulate Chlorophyll Synthesis

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Abstract: Light stress signalling in algae and plants is partially orchestrated by singlet oxygen (1O2), a reactive oxygen species (ROS) that causes significant damage within the chloroplast, such as lipid peroxidation. In the vicinity of the photosystem II reaction centre, a major source of 1O2, are two β-carotene molecules that quench 1O2 to ground-state oxygen. 1O2 can oxidise β-carotene to release β-cyclocitral, which has emerged as a 1O2-mediated stress signal in the plant Arabidopsis thaliana. We investigated if β-cyclocitral can have similar retrograde signalling properties in the unicellular alga Chlamydomonas reinhardtii. Using RNA-Seq, we show that genes up-regulated in response to exogenous β-cyclocitral included CAROTENOID CLEAVAGE DIOXYGENASE 8 (CCD8), while down-regulated genes included those associated with porphyrin and chlorophyll anabolism, such as tetrapyrrole-binding protein (GUN4), magnesium chelatases (CHL1, CHL2, CHL4, CHLH1), light-dependent protochlorophyllide reductase (POR1), copper target 1 protein (CTH1), and coproporphyrinogen III oxidase (CPX1). Down-regulation of this pathway has also been shown in β-cyclocitral-treated A. thaliana, indicating conservation of this signalling mechanism in plants. However, in contrast to A. thaliana, a very limited overlap in differential gene expression was found in β-cyclocitral-treated and 1O2-treated C. reinhardtii. Furthermore, exogenous treatment with β-cyclocitral did not induce tolerance to 1O2. We conclude that while β-cyclocitral may down-regulate chlorophyll synthesis, it does not seem to contribute to 1O2-mediated high light stress signalling in algae.

Keywords: high light stress; singlet oxygen; signalling; GPX5; beta cyclocitral; acrolein; glutathione peroxidase; carbonyl; transcription

1. Introduction

Photosynthetic organisms often encounter suboptimal conditions, leading to the absorption of excess light energy and ‘high light’ (HL) stress. Therefore, light harvesting must be regulated, requiring acclimation to the current environment [1]. As part of acclimation, signals from the chloroplast can alter transcription in the nucleus in so-called retrograde signalling [2,3]. For example, levels of tetrapyrrole intermediates (chlorophyll precursors) in the chloroplast provide feedback cues to the nucleus during chlorophyll synthesis [4,5]. Stress signalling is also partially orchestrated by reactive oxygen species (ROS), including singlet oxygen (1O2), which is produced by energy transfer from excited chlorophyll to molecular oxygen in photosystem II (PSII) [6–8]. Singlet oxygen oxides almost anything in its path and the 1O2 signal leaves the chloroplast in the form of down-stream reaction products. β-cyclocitral, a 1O2-derived breakdown product of β-carotene, has emerged as an aldehyde electrophile involved in 1O2 retrograde signalling of Arabidopsis thaliana [9]. However, the contribution of β-cyclocitral to 1O2 signalling in algae is unknown.

Other 1O2-derived molecules with potent signalling activity include α,β-unsaturated carbonyl derivatives, known as reactive carbonyl/electrophile species (RES). These are produced as a consequence of lipid peroxidation [10,11]. Thylakoid membranes are particularly
enriched in 1-linolenoyl/2-linolenoyl (di-Cl8:3) found in monogalactosyldiacylglycerol, a polar lipid that improves fluidity for membrane functionality, but is highly prone to peroxidation by 1O2. One of the most abundant RES produced by lipid peroxidation in chloroplasts due to HL stress is acrolein [10,11], which can activate a significant proportion of transcriptional changes that occur in response to 1O2 [12,13]. In Chlamydomonas reinhardtii, an electrophile response element (ERE)-containing bZIP transcription factor called SOR1 participates in 1O2 signalling [12,13]. SOR1 up-regulates transcription of a large suite of genes, including glutathione transferases (e.g., GSTS1) and an isoflavone reductase-like protein (IRL1), which contribute to RES-associated antioxidant defences [12,14,15].

Although they are potent signalling molecules, RES are also toxic to cells by forming Michael addition adducts with nucleophilic thiolate anions [16], such as redox-active cysteine residues of proteins. As a thiol, the antioxidant glutathione (GSH), can detoxify RES. Chlamydomonas reinhardtii rapidly responds to low concentrations of the RES acrolein (≤600 ppm) by increasing GSH contents, but is critically GSH-depleted at higher acrolein concentrations [13]. Another component of RES detoxification putatively includes glutathione peroxidases (GPX), whereby GPX5 (also known as GPXh) transcription is strongly up-regulated by sub-lethal levels of 1O2 [17,18] and acrolein [13]. Moreover, overexpression of GPX5 in C. reinhardtii can increase tolerance to 1O2 [18].

In summary, low levels of ROS/RES can activate signalling pathways implicated in acclimation, whereas an excess ROS/RES load leads to intolerable stress. Thus, stress responses can be distinguished into eustress and distress: Eustress leads to increased stress tolerance via acclimation in which 1O2 signalling can be involved, and distress leads to loss of viability due to excess stress (e.g., high RES load) beyond a level that can be compensated for by acclimation [18–20].

Here, we explored the potential role for β-cyclocitral in 1O2-mediated signalling and inducing 1O2 tolerance in the unicellular model green alga, C. reinhardtii. First, we confirmed that β-cyclocitral could enter cells by measuring increased concentrations of the molecule in treated cells, and observing the concentration-dependent effect on chlorophyll fluorescence. Then, using RNA-Seq analysis, we analysed the transcriptional response of cells to β-cyclocitral and compared this to previously published data of differential gene expression induced by the photosensitizer rose bengal (RB), to reveal if elements of 1O2 signalling were activated by β-cyclocitral. Further experiments investigated if β-cyclocitral can induce 1O2 tolerance, and comparisons are drawn with responses to acrolein, a 1O2-derived signal resulting from lipid peroxidation of the thylakoid membrane.

2. Results

As an aldehyde, β-cyclocitral could be cytotoxic as well as a signalling molecule. Therefore, to assess toxicity, the impact of various concentrations of β-cyclocitral on photosynthesis was probed via chlorophyll fluorescence. The maximum quantum yield of PSII \( (F_v/F_m) \), which is an often-used health marker of photosynthetic organisms, decreased in both wild types (WT), cell-wall containing WT-4A and cell-wall-less cw15, in response to β-cyclocitral treatment with \( \geq 10 \mu L/Petri \) dish (Figure 1A), corresponding to \( \geq 50,000 \) ppm atmospheric concentration (see methods for calculation). In contrast, NPQ that is also measured via chlorophyll fluorescence was affected at much lower β-cyclocitral concentrations (Figure 1B), including at 0.12 \( \mu L/Petri \) dish (Figure S1), which corresponds to the 600 ppm treatment used for the RNA-Seq analysis. Cellular concentrations of β-cyclocitral before treatment were 0.2 nmol g\(^{-1}\) fresh weight, and increased 400 fold 2 h after exogenous treatment at 600 ppm (Figure S2). There was no difference in the influence of β-cyclocitral on NPQ or \( F_v/F_m \) between WT-4A and cw15 strains (Figure 1). Furthermore, the reduction of NPQ was also found in the npq4, stt7, and npq4stt7 mutants (Figure S1), and thus was independent of LHCSR3- and STT17-mediated NPQ, which are the major NPQ mechanisms in C. reinhardtii [1,21]. Therefore, our results are indicative of a direct physical effect of β-cyclocitral on NPQ that occurs at the level of the thylakoid membrane, similar to an uncoupler. To test this, the activity of the pH-dependent violaxanthin cycle was measured.
in response to HL. Less zeaxanthin accumulated in β-cyclocitral-treated cells (Figure S1), indicating that the proton gradient was dissipated by β-cyclocitral.

Figure 1. Effect of exogenous β-cyclocitral on \( F_v/F_m \) and NPQ in Chlamydomonas reinhardtii. (A) Measurements of \( F_v/F_m \) and (B) NPQ in 4 h HL-acclimated cultures were made after 4 h of treatment under very LL (see methods), of cell-wall-containing wild type (WT-4A; black bars) and cell-wall-less (cw15; grey bars) cultures, \( n = 3 \pm SD \), with distinct letters indicating significant differences (\( p < 0.05 \)).

Aldehydes can be detoxified by GSH and associated enzymes, such as GSTS1. Thus, the response of β-cyclocitral on GSH concentrations was also measured up to 1500 ppm (0.3 \( \mu L/Petri \) dish), and after 4 h, no effect was observed (Figure S3).

Overall, the lack of decrease in \( F_v/F_m \) and lack of change in GSH concentrations indicated that β-cyclocitral was not a cytotoxic aldehyde at treatment with up to 1500 ppm, a concentration at which the RES acrolein is lethal [13]. Moreover, the significant impact of β-cyclocitral on NPQ in WT-4A and cw15 showed that this aldehyde could enter the chloroplast of C. reinhardtii and that the cell wall was not a hindrance to influx. Therefore, we felt confident to be able to assess the signalling properties of β-cyclocitral in exogenously-treated cells.
One of the most $^{1}\text{O}_2$-responsive genes in *C. reinhardtii* is GPX5, and GPXs likely have a role in mitigating HL stress by detoxifying aldehydes/RES that are produced as a consequence of $^{1}\text{O}_2$ production [18]. We screened three mutants over-expressing GPX5 in the WT-4A background [18] and found clearly elevated protein levels of GPX5 in GPXHOX-11 and GPX5OX-14, relative to WT-4A, in low light (LL)-treated cells (Figure 2A). This led to the selection of GPXHOX-11 for further experiments to see how β-cyclocitrinal, acrolein, and HL influence GPX5 levels, and how elevated levels of GPX5 affect the response to these treatments and subsequent tolerance to $^{1}\text{O}_2$. Levels of GPX5 increased in WT-4A cells after 4 h of HL, indicating that this treatment induced $^{1}\text{O}_2$ production. However, 4 h treatments with 600 ppm of β-cyclocitrinal or 600 ppm of acrolein did not increase GPX5 levels (Figure 2B). After 4 h of HL, WT cells increased the GSH contents, indicating that cells were under mild oxidative stress, whereas in GPXHOX-11, no change in the GSH contents occurred, indicating that cells were under less stress. In support of this, after 4 h of HL, GPXHOX-11 accumulated less aldehydes (propanal and hexanal) and RES (acrolein and 4-hydroxynonenal) than WT-4A, whereas the fold change of β-cyclocitrinal was equally low in both genotypes (Figure 3). After a 4 h treatment with 600 ppm of acrolein, both WT and GPXHOX-11 accumulated GSH (Figure S3).

![Figure 2](image)

**Figure 2.** Protein levels of GPX5 in WT-4A and GPX5-overexpressor (GPXHOX) mutants under LL and in response to HL, β-cyclocitrinal and acrolein. (A) Three GPXHOX lines and WT-4A were analysed for GPX5 protein levels under LL. Shown above is the Ponceau-stained membrane for loading control. (B) The effect of LL, 4 h with HL, and 4 h with 600 ppm of β-cyclocitrinal (β-cc) or with 600 ppm of acrolein (4 h Acro.) under LL, on GPX5 levels. The D1 reaction centre of photosystem II (PsbA) was used for loading control.
A function of $^{1}$O$_{2}$-related signalling is partly towards increasing tolerance of this ROS \cite{18}. Therefore, the influence of exogenous $\beta$-cyclocitral and acrolein treatments under LL (as potential components of $^{1}$O$_{2}$-mediated signalling) on the tolerance to $^{1}$O$_{2}$ was measured. The influence of HL stress, which increases endogenous $^{1}$O$_{2}$ levels, was also included as pre-treatment before testing $^{1}$O$_{2}$ tolerance, and all comparisons were made to LL-treated ‘control’ cells. Tolerance to $^{1}$O$_{2}$ was tested by incubating cells with the photosensitizer RB. Since the amount of $^{1}$O$_{2}$ that RB produces is dependent on the degree of photoexcitation (i.e., intensity and duration of light treatment) and RB concentration, various treatments were conducted to test cell tolerance. These constituted 4 $\mu$M, 7 $\mu$M, and 10 $\mu$M of RB, either for 10 min at 250 $\mu$mol photons m$^{-2}$ s$^{-1}$ to provide a short $^{1}$O$_{2}$ shock, or 24 h at 50 $\mu$mol photons m$^{-2}$ s$^{-1}$ to test longer term endurance. The 24 h endurance test was more severe and led to less cell survival of control cells, under which GPXHOX-11 was significantly less affected than WT-4A (Figure 4), with $p < 0.05$ when comparing genotype as a factor with MANOVA across all RB concentrations. Pre-treatment with HL increased the tolerance of WT-4A to the $^{1}$O$_{2}$ shock treatment with 7 $\mu$M and 10 $\mu$M of RB (Figure 4A), and the 24 h treatment with 4 $\mu$M (Figure 4B), while affecting GPXHOX-11 less. This resulted in HL-treated WT-4A and GPXHOX-11 having a similar $^{1}$O$_{2}$ tolerance (Figure 4). Relative to control cells, pre-treatment with 600 ppm of $\beta$-cyclocitral had no impact on $^{1}$O$_{2}$ tolerance in either genotype under all treatments (average $p = 0.895$), whereas pre-treatment with 600 ppm of acrolein increased the tolerance of both genotypes (average $p = 0.034$). In summary, acrolein and HL induced tolerance to severe $^{1}$O$_{2}$ stress, whereas $\beta$-cyclocitral did not significantly affect tolerance.

Figure 3. Influence of GPX5 overexpression on aldehyde/RES accumulation. Change in RES concentrations in response to HL (600 $\mu$mol photons m$^{-2}$ s$^{-1}$) for 4 h, relative to levels before treatment at LL (50 $\mu$mol photons m$^{-2}$ s$^{-1}$), in wild-type (black) and GPXHOX-11 (white). MDA: malondialdehyde, $\beta$cc: $\beta$-cyclocitral, 4HNE: 4-hydroxynonenal. $n = 4 \pm$ SD with * denoting significant differences between genotypes ($p < 0.05$).
To investigate if β-cyclocitral could contribute to O$_2$-mediated signalling in C. reinhardtii, an RNA-Seq analysis of cells treated for 2 h with 600 ppm of β-cyclocitral under LL (Table S1) was conducted and compared to the transcriptional response of cells treated with the O$_2$-producing photosensitizer RB at 1 μM under LL (data from [13]). In response to the β-cyclocitral treatment, only six genes were significantly up-regulated, and 57 genes were down-regulated, when considering a fold change of >2 and modified t-test p values of <0.01 (Table S1). Of the genes down-regulated by β-cyclocitral, 18 and 6 were significantly down-regulated and up-regulated, respectively, by RB (Table S1; Figure S4). Differentially expressed genes associated with carotenoid metabolism include carotenoid cleavage dioxygenase 8 (Cre08.g365851), up-regulated five-fold, and β-carotene ketolase (BKT1), up-regulated two-fold, but not significantly (Table S1). Of all significantly down-regulated genes, the only ontological group with >1 hit was ‘porphyrin and chlorophyll metabolism’ with 10 hits: Cre01.g015350 (POR1), Cre01.g050950, Cre02.g085450 (CHLD), Cre05.g246800 (GUN4), Cre06.g306300 (CHLI1), Cre07.g325500 (CHLI1), Cre09.g396300 (PPX1), Cre12.g510050 (CTH1), Cre12.g510800 (CHLI2), and Cre16.g663900. Collectively,
these genes covered several steps of chlorophyll anabolism (Figure S5), but the overall overlap of differential gene expression with RB-treated cells was low, as shown by a R² linear correlation of <0.01 when including all genes, which increases to 0.15, considering only the 63 genes with significantly altered expression (Figure 5). In comparison, this contrasts with the much tighter relationship between differential gene expression shared between acrolein-treated and RB-treated cells [13], which has an R² linear correlation of 0.34 and 0.70 when considering expression of total genes and only significantly affected genes, respectively (Figure 5).

Figure 5. Correlation of differential gene expression induced by treatment with ¹O₂ and acrolein (blue) or ¹O₂ and β-cyclocitral (red). Levels of mRNA are expressed as log₂ fold changes (log₂FC), relative to mock-treated cells, calculated from RNA-Seq analyses; n = 3 for treatments and controls. Treatments with acrolein and β-cyclocitral were at 600 ppm and ¹O₂ was induced by rose bengal (RB; 1 µM under growth light at 50 µmol quanta m⁻² s⁻¹). Continuous and dashed lines of best fit consider all and only significantly affected genes, respectively. See Table S1 for expression levels of each gene. RNA-Seq data for acrolein and RB treatments adapted with permission from [13] 2018, Copyright Elsevier.

3. Discussion

Ten years ago, the discovery that β-cyclocitral in A. thaliana effects transcription of several genes know to be affected by ¹O₂ [9] made a coherent link between HL-induced ¹O₂ production and ROS-associated retrograde signalling. Subsequently, it was found that β-cyclocitral functions up-stream of MBS1 [22], a zinc finger protein that regulates ¹O₂-dependent gene expression, not only in A. thaliana but also in C. reinhardtii [23]. However, the fact of whether β-cyclocitral actually has a role in ¹O₂ signalling in alga remained unknown. Since then, other RES (i.e., acrolein), related to lipid peroxidation rather than carotenoid cleavage, emerged as retrograde signals acting in ¹O₂-mediated stress acclimation [13]. Here, we investigated how β-cyclocitral modulates the physiology and transcription in C. reinhardtii and made comparisons with transcriptional responses of cells to the photosensitizer RB and the RES acrolein.

The very minor effect of β-cyclocitral on Fᵥ/Fₘ showed how tolerant cells were of this molecule. For example, decreases in Fᵥ/Fₘ occurred at >1000 fold concentrations...
While we did not find a similar transcriptional response to a lack of inducing with highest tolerance to 1

whereas in C. reinhardtii, was indeed shown by the lower accumulation of zeaxanthin under HL, which is a process occurring in response to 600 ppm of acrolein, the dose that induced a eustress response 

Previously, we showed that exogenous acrolein treatments induce a ‘eustress’ (i.e., acclimation) response by up-regulating thiol-disulfide-dependent defence mechanisms required for tolerating 1 O2 [13]. Of note, around half of global gene expression (up and down) occurring in response to 600 ppm of acrolein, the dose that induced a eustress response with highest tolerance to 1 O2 was shared with the gene regulation in response to RB [13]. While we did not find a similar transcriptional response to β-cyclocitral (Figure 5), in line with a lack of inducing 1 O2 tolerance (Figure 4), there was evidence that β-cyclocitral may have some signalling properties in C. reinhardtii. Collectively down-regulated genes (Table S1) covered many steps of chlorophyll anabolism (Figure S5), such as porphobilinogen deaminase/HemC (Cre16.g663900.11.2), coproporphyrinogen III oxidase (CPX1), and protoporphyrinogen oxidase (PPX1), which are involved in early steps of porphyrin synthesis, as well as genes coding for proteins that insert Mg2+ into protoporphyrin, including tetrapyrole-binding protein (GUN4) and magnesium chelatase (CHLI1, CHLI2, CHLD, CHLH1) to form Mg-protoporphyrin IX (MgP), the first dedicated intermediate of the
chlorophyll branch. In *C. reinhardtii*, CHLI2 seems to be redundant to CHLI1 [30]. Gene expression associated with later steps of chlorophyll synthesis, such as copper target 1 protein (CTHI) and light-dependent protophylophylide reductase (POR1) were also down-regulated by β-cyclocitral. In *A. thaliana*, β-cyclocitral also down-regulated expression of CHLI2 and a few other genes involved in porphyrin/chlorophyll biosynthesis, such as PORB, CHLM and HEME1 alongside an up-regulation of CLH1 and CLH2 involved in chlorophyll catabolism (Ramel et al., 2102), indicating a conserved signalling role for β-cyclocitral in decreasing chlorophyll contents, which existed before the evolution of vascular plants. Chlorophyll synthesis needs to be tightly regulated because MgP and protophylophylide are, similar to free chlorophyll, highly efficient photosensitizers [6]. MgP provides feedback on chlorophyll synthesis by repressing nuclear transcription in a signalling pathway that requires GUN4 [4]. There are contrasting reports on whether the GUN4-MgP complex produces more or less $^{1}$O$_{2}$ than MgP alone, and if $^{1}$O$_{2}$ is a component of the retrograde signal [31,32].

In summary, in *C. reinhardtii*, β-cyclocitral does not seem to have a role in $^{1}$O$_{2}$ signalling or inducing $^{1}$O$_{2}$ tolerance. Nonetheless, the influence of carotenoid cleavage products on chlorophyll synthesis and how $^{1}$O$_{2}$ integrates into the retrograde signalling of this pathway warrants further investigation.

4. Materials and Methods
4.1. Strains and Growth Conditions
*Chlamydomonas reinhardtii* WT-4A*+* (CC-4051) and GPXHOX*+* strains GPXHOX-10 (CC-4606), GPXHOX-11 (CC-4607), and GPXHOX-14 (CC-4608) in the WT-4A*+* background, from Ledford et al., [18], were initiated in Tris-Acetate-Phosphate (TAP) liquid media, pH 7.0. For agar-grown cultures, 1 mL of liquid culture was evenly spread across 11 cm Petri dishes half-filled with 1.5% (w/v) TAP agar media. The liquid medium was evaporated for 0.5 h in a sterile air-flow bench before the lid was replaced, but not sealed. Liquid and agar-grown cultures were grown under constant LL (50 $\mu$mol photons m$^{-2}$ s$^{-1}$) at 20 °C in a growth chamber (Percival PGC-6HO, CLF Plant Climatics GmbH, Wertingen, Germany). HL was provided by increasing the light intensity of the growth chamber to 600 $\mu$mol photons m$^{-2}$ s$^{-1}$ for 4 h.

4.2. Chlorophyll Fluorescence Measurements

Pulse-amplitude modulation (PAM) measurements of chlorophyll fluorescence ($F$) parameters were performed with an Imaging PAM (WALZ). After dark treatment, minimum ($F_{o}$) and maximum fluorescence ($F_{m}$) was measured immediately before and during a 200 ms saturating pulse (6000 $\mu$mol photons m$^{-2}$ s$^{-1}$), respectively. The maximum quantum yield of photosystem II ($F_{v}$/$F_{m}$) was calculated via ($F_{m}$−$F_{o}$)/$F_{m}$ after 1.5 h recovery in dark. NPQ was calculated via ($F_{m}$−$F_{m}'$)/$F_{m}$, with $F_{m}'$ measured after 2 min at 750 $\mu$mol photons m$^{-2}$ s$^{-1}$.

4.3. Exogenous β-Cyclocitral Treatments

For treatments, advantage was taken of the volatility of β-cyclocitral (Sigma-Aldrich), which was placed on a paper wick within the middle of a sealed Petri dish to treat agar-grown cells, in an identical approach used for acrolein treatments [13]. B-cyclocitral was diluted in 100% p.a. methanol and 1 $\mu$L containing 0–0.3 $\mu$L of diluted β-cyclocitral (mock = 1 $\mu$L pure methanol) was placed on a paper wick in an Ependorf lid in the centre of a 11 cm Petri dish, which was immediately sealed with Parafilm and left under very LL (2 $\mu$mol photons m$^{-2}$ s$^{-1}$). After 2–4 h, as indicated, cells within 2 cm of the middle of the plate were gently scraped from the agar with a spatula and immediately frozen in liquid nitrogen prior to biochemical analyses. B-cyclocitral concentrations in parts per million (ppm) were calculated on a volume basis, considering a 30 cm$^{3}$ air space in a 11 cm Petri dish, a β-cyclocitral density of 0.943 g mL$^{-1}$, 95% purity, and the particle-related gas
concentration of 0.0241 m³/mol, so that 0.12 µL of β-cyclocitral corresponded to 600 ppm inside the Petri dish.

4.4. HPLC Analysis of Glutathione and Pigments, Western Blotting of Glutathione Peroxidase 5, and LC-MS/MS Measurement of RES

All methods were conducted according to Roach et al., [13], after 4 h treatment. For HPLC analyses (glutathione and pigments), cells were first freeze-dried for 3 days and each replicate was composed of 8–10 mg dry weight. For Western blotting of GPX5 levels and LC-MS/MS analyses of RES, cells were not freeze-dried. Loading of protein extracts and normalisation of pigments was made to total chlorophyll of the extract, according to [13]. For western blotting, the GPXh antibody (AS15 2882, Agrisera, Vännäs, Sweden) at a ratio of 1:10,000 and PsbA antibody (AS05 084, Agrisera, Vännäs, Sweden) at a ratio of 1:25,000 were used.

4.5. Singlet Oxygen Resistance Test

Resistance to singlet oxygen was performed by suspending liquid cultures in fresh TAP media with RB at 0, 4, 7, and 10 µM of RB in a 96-well multiwall plate with a total volume of 200 µL per well. One light treatment consisted of a ¹⁰⁰₂ shock by exposure to 250 µmol photons m⁻² s⁻¹ for 10 min before recovery at 20 µmol photons m⁻² s⁻¹, and another was constant exposure to 50 µmol photons m⁻² s⁻¹. After 24 h, cell density at 650 nm was measured as an indicator of cell number for calculating differences between each RB treatment and cells without RB for each genotype and pre-treatment individually.

4.6. RNA-Seq Analysis

Total RNA was extracted with the Rneasy Plant Mini Kit (Qiagen, Hilden, Germany) and additional on-column Dnase treatment (Rnase-free Dnase set, Qiagen) from ca. 15 mg (fresh weight) per replicate (=1 Petri dish). Cells were harvested after a 2 h treatment with 600 ppm of β-cyclocitral diluted in methanol, or for control with methanol only. Poly A-enriched library preps were sequenced with an Illumina HiSeq2500 using single-end 50 bp read lengths, by the NGS Core Facility of the Vienna Biocentre, Austria, resulting in an average of 24,063,263 reads per replicate, n = 3. Reads were aligned against the C. reinhardtii reference genome (JGI v5.5 release) with STAR version 2.5.1b, created by Dobin et al. [33] (Cold Springs Harbour, New York, NY, USA), using 2-pass alignment mode.

4.7. Data Analysis and Statistics

For all measurements, one Petri dish of cell culture counted as an individual biological replicate. The RNA-Seq analysis data was analysed by the Bioinformatics and Scientific Computing Core of the Vienna Biocenter Core Facilities with the R package limma for selecting genes that were differently expressed more than two-fold between control and treatment (i.e., mock cells v β-cyclocitral-treated cells). The p-values used for filtering differentially expressed genes were cut off at p < 0.01. KEGG pathways and gene ontology annotations (Chlamydomonas-based) were conducted using the Algal Functional Annotation Tool [34].

Significant differences for biochemical measurements and chlorophyll fluorescence at p < 0.05 were calculated in IBM SPSS Statistics, version 24, IBM, (New York, NY, USA) using one-way ANOVA with Tukey’s post hoc test, or for pairwise comparisons using t-test with independent samples. A multivariate general linear model (MANOVA) was additionally calculated to evaluate the influence of genotype on RES tolerance.

5. Conclusions

Overall, we first conclude that β-cyclocitral is not a particularly reactive aldehyde since it does not lead to loss of Fv/Fm or modulate GSH concentrations like RES do. Second, unlike most RES, β-cyclocitral concentrations are not associated with HL stress in C. reinhardtii. Third, GPX5 helps mitigate HL stress by breaking down RES produced by ¹⁰⁰₂.
Fourth, β-cyclocitrail is unable to induce tolerance to $^1$O$_2$, and does not seem to contribute to $^1$O$_2$ signalling in C. reinhardtii, but instead may have a specific role in down-regulating chlorophyll synthesis.

Supplementary Materials: The following supporting information can be downloaded at: [https://www.mdpi.com/article/10.3390/plants11162155][1], Figure S1: Effect of β-cyclocitrail treatments on NPQ, the xanthophyll cycle, and $F_v$/$F_m$ in high light-treated cells. Figure S2: Effect of exogenous treatment with acrolein or β-cyclocitrail on RES levels. Figure S3: Effect of β-cyclocitrail, HL, and acrolein treatments on total glutathione (GHS + GSSG) contents. Figure S4: Overlap in differential gene expression changes between $^1$O$_2$ stress, acrolein, and β-cyclocitrail treatments. Figure S5: Genes with transcription down-regulated by 600 ppm of β-cyclocitrail that are involved in porphyrin and chlorophyll metabolism. Table S1: Transcriptional levels from an RNA-Seq analysis of Chlamydomonas reinhardtii treated with 600 ppm of β-cyclocitrail in comparison to control (mock-treated) identically grown cells.

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