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# Photoinhibition and continuous growth of the wild-type and a high-light tolerant strain of *Chlamydomonas reinhardtii*

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## Abstract

The volumetric productivity of the high-light tolerant strain *hit2* of *Chlamydomonas reinhardtii* was found to be higher than that of the parental strain *CC124* during continuous growth at PPFD from 200 to 1,500 μmol $m^{-2}$ $s^{-1}$. At PPFD of 1,250 μmol $m^{-2}$ $s^{-1}$, *hit2* produced 2.53 ± 0.18 and *CC124* produced 2.05 ± 0.12 g(biomass) $dm^{-3}$ $d^{-1}$. The rate constant of photoinhibition of *hit2* was less than half of that of *CC124*, suggesting that *hit2* produces more biomass than *CC124* because *hit2* does not need to allocate as much resources for PSII repair as *CC124*. Growth in high light triggered similar loss of chlorophyll, increase in the carotenoid-to-chlorophyll ratio, and decrease in PSI fluorescence in both strains. Thermoluminescence B band was shifted toward the Q band in *hit2*, suggesting that low redox potential of the $Q_B/Q_B^-$ pair contributes to the photoinhibition tolerance of *hit2*.

*Additional key words*: algae; biomass production; carotenoid; growth rate; nonphotochemical quenching; turbidostat.

## Introduction

Photosynthetic organisms differ in light intensity optima and abilities to acclimate to light intensities. The characteristics of light and shade plants and the mechanisms of plant acclimation to light or shade have long been studied. Algae are also able to acclimate to light intensity, just like plants. Nonphotochemical quenching of excitation energy (NPQ) that dissipates excess absorbed light energy is a major factor in algal high-light acclimation (for review on NPQ mechanisms, *see* Wobbe *et al.* 2016). While NPQ dissipates energy absorbed by PSII, de- and recoupling of LHCIIs between photosystems adjusts the balance of light absorbance, providing a two-stepped photoprotective mechanism (Roach and Na 2017). Parts of the LHCIIs are rapidly decoupled from PSII in high light, *via* interaction with light-harvesting-complex-stress-related-3 protein (LHCSR3) (Roach and Na 2017). This decoupling enables rapid quenching of excitation energy, due to shorter lifetime of excited LHCIIs when detached (Ünlü *et al.* 2014), prior to Stt7-dependent LHCII phosphorylation and the associated state transition. In plants and green algae, NPQ is regulated by an acidification of the thylakoid lumen. In addition, xanthophylls contribute to NPQ but also quench the harmful singlet oxygen, and a large increase in zeaxanthin, anteraxanthin, and lutein occurs in *C. reinhardtii* cells during acclimation to high light (Bonente *et al.* 2012). In *C. reinhardtii*, the amount of active PSI is reduced in high light while the number of PSII centers is maintained (Bonente *et al*. 2012); the advantage of this acclimation is not known. For most green algae, moderate PPFD values from 50 to 400 μmol $m^{-2}$ $s^{-1}$ are found to be good for growth in various algal species (Sforza *et al.* 2012, Singh and Singh 2015).

The growth rate of a photosynthetic organism increases with light intensity to a certain range, and therefore the ability to utilize high light for photosynthesis is a key property of algae considered for high-value compound production or bioenergy applications. The photosynthetic rate can be limited by the damaging effects of light. Photoinhibition of PSII is the best studied mechanism by which light damages the photosynthetic machinery (for review *see* Tyystjärvi 2013), but the importance of photoinhibition for the rate of growth in high light is poorly understood.

The green alga *C. reinhardtii* is usually cultured in laboratories at a PPFD of 100 μmol $m^{-2}$ $s^{-1}$ or less (Roach *et al.* 2017, Xing *et al.* 2017, Andrade *et al.* 2018, Lin *et al.* 2018, Yang *et al.* 2018), and earlier growth tests suggest that wild-type *C. reinhardtii* does not grow rapidly in strong, oversaturating light (Schierenbeck *et al.* 2015, Lin *et al.* 2018). However, spontaneous mutations improving the ability to withstand high light occur frequently (Förster *et al.* 2005). A study revealed that high-light tolerant mutants, induced with ultraviolet radiation followed by selection in high light, can take over a wild-type *C. reinhardtii* culture under a strong selection pressure in high light. Whole

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*Abbreviations*: DCBQ – 2,6-dichloro-1,4-benzoquinone; DCMU – 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DM – dry mass; NPQ – nonphotochemical quenching; PBR – photobioreactor.
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genome sequencing of two such mutants, *hit1* and *hit2*, capable of growing at light intensities not tolerated by the parental control strain *CC124*, revealed single nucleotide substitutions in the gene *Cr-COP1* (Cre02.g085050) for both strains, which were confirmed to be causative for the high-light tolerance *via* segregation analyses (Schierenbeck *et al.* 2015). *Cr-COP1* is a homolog of the *Arabidopsis thaliana* gene *COP1* coding for a protein that functions in sensing and signaling ultraviolet radiation and is required for induction of several ultraviolet B (UVB)-induced genes in *Chlamydomonas* (Tilbrook *et al*. 2016). UVB treatment of the *hit1* mutant of *Cr-COP1* induces decrease of the variable to maximum fluorescence ratio similarly, whether or not the cells have been pretreated with an UVB dose that causes acclimation in the wild type (Tilbrook *et al*. 2016).

In the present study, we report a comparison of the growth rates of the *hit2* strain and the parental wild-type strain *CC124* in a wide range of light intensities. Cultures were grown in a turbidostat, continuously diluting the culture with fresh growth medium to maintain a constant biomass concentration of the algal suspension. A relatively low biomass concentration ensures that the algae stay in the exponential growth phase throughout the experiment. Furthermore, we compared photoinhibition of PSII in the two strains to see whether also photoinhibitory damage to PSII is slow in a high-light tolerant strain. In all experiments, the algae were grown in a mineral medium to ensure similar photosynthetic properties between experiments.

## Materials and methods

**Algal strains and inoculum**: The *hit2* strain of *C. reinhardtii* has been described earlier (Schierenbeck *et al.* 2015). The parental strain *CC124* was used as a control. Preculturing of the cells was done in Tris-acetate-phosphate medium (TAP) (Gorman and Levine 1965), at PPFD of 80 μmol $m^{-2}$ $s^{-1}$ and 27°C for 5 d, after which they were transferred to Sueoka's high salt (HS) medium (Sueoka 1960) for the inoculum. Inoculum was prepared in an autoclaved flask, from which the culture was transferred to the photobioreactor (PBR) through sterile tubing in standard HS medium in RT (24°C) and PPFD of 200 μmol $m^{-2}$ $s^{-1}$, while bubbling the medium in PBR with 3% $CO_2$.

**Experimental set up and biomass production**: The volumetric productivities of both *CC124* and *hit2* were measured at PPFD of 200 to 1,500 μmol $m^{-2}$ $s^{-1}$ (total of six steps: 200, 500, 750; 1,000; 1,250; and 1,500 μmol $m^{-2}$ $s^{-1}$), 27°C, by growing the algae in a 19.5 × 10.0 × 2.5 cm flat panel PBR (*Photobioreactor FMT-150*, *PS Instruments*, Brno, Czech Republic) in final culture volume of 400 $cm^3$, operated as a turbidostat. The bioreactor was set to keep the optical density at 735 nm ($OD_{735}$) of the culture at 0.6–0.65, by dilution with HS medium on demand. At this $OD_{735}$, the average dry biomass content was 0.58 ± 0.03 g $dm^{-3}$ for *CC124* and 0.63 ± 0.06 g $dm^{-3}$ for *hit2*. $CO_2$ was provided by continuous bubbling of 3% $CO_2$. In the turbidostatic phase, the pH of the medium was 6.13–6.67, which was maintained *via* the phosphate buffer of the HS medium. Variation in the pH was due to the combination of bubbling and pumping of the medium. Temperature was controlled and kept constant with an external water bath. Each experiment was started at PPFD of 200 μmol $m^{-2}$ $s^{-1}$ and the PPFD was then increased to 1,500 μmol $m^{-2}$ $s^{-1}$ in six stages, as indicated. The time it took for the whole volume of the reactor to be changed decreased from the maximum of 6.41 × $10^4$ s (17.8 h) at PPFD of 200 μmol $m^{-2}$ $s^{-1}$ to the minimum of 1.99 × $10^4$ s (5.52 h) at PPFD of 1,250 μmol $m^{-2}$ $s^{-1}$. Each PPFD was maintained for 5–6 d to ensure continuous growth, during which 3.2–8.2 liters, or 8.02–20.54 times the volume of the PBR of algal suspension was pumped out of the PBR. The first day in each PPFD was not taken into account to let the cells acclimate to each PPFD prior to analyzing the volumetric productivity. The volumetric productivity was then calculated from the mass of the culture pumped out by using the known relationship between $OD_{735}$ and dry biomass.

**Dry biomass measurements**: The relationship between $OD_{735}$ and dry biomass was obtained by measuring dry mass from triplicate 5-$cm^3$ samples directly from the PBR and weighing them to obtain fresh mass of the sample. Samples were then diluted with 5 $cm^3$ of 0.5 M ammonium formate and filtered through a weighed sterile glass microfiber filter with retention size of 1.6 μm (*VWR*, USA). The filter was washed with 40 $cm^3$ of 0.5 M ammonium formate and dried for 24 h at 96°C. The dried filter was weighed again and the biomass amount was calculated according to the equation: DM [g $dm^{-3}$] = $(m_2 - m_1)/V$(sample), where $m_1$ is the mass of the filter and $m_2$ is the mass of the filter and the dried biomass. The biomass was measured in different culture densities to obtain the proportionality between $OD_{735}$ for calculating the exact biomass in the culture pumped out from the PBR.

**Photoinhibition measurements *in vivo* and *in vitro***: Cells were grown in 40 $cm^3$ of HS medium under continuous light and constant temperature (PPFD of 80 μmol $m^{-2}$ $s^{-1}$, 27°C) for five days until the cell density, determined spectrophotometrically (*Lambda Bio 40*, *Perkin Elmer*, Waltham, MA, USA) reached 8.2 × $10^6$ cells $cm^{-3}$, which corresponded to 25 μg(chlorophyll) $cm^{-3}$ in both strains. Ten $cm^3$ of culture was illuminated at PPFD of 950 μmol $m^{-2}$ $s^{-1}$ and 27°C in a 100-ml beaker with constant stirring with a magnetic bar. The light path in the sample was 7 mm. In experiments done in the presence of lincomycin that blocks PSII repair, lincomycin was added before illumination in a final concentration of 0.5 mg $cm^{-3}$. All values were averaged from 3–5 biological replications.

Aliquots (1 $cm^3$) for oxygen evolution measurements were withdrawn at 10-min intervals. For photoinhibition of intact cells, oxygen evolution was measured *in vivo* in saturating light, PPFD of 1,500 μmol $m^{-2}$ $s^{-1}$, using a Clark-type oxygen electrode (*Hansatech Instruments Ltd*., Norfolk, United Kingdom). 2,6-dichloro-1,4-benzoquinone (DCBQ) was used as an electron acceptor in final concentration of 0.5 mM and 0.5 mM ferricyanide was included to maintain

DCBQ in oxidized state. The percentage of remaining PSII activity was obtained by comparing the rate of oxygen evolution measured from a treated sample to an untreated control of the same biological replicate.

Isolated thylakoids were used for *in vitro* photoinhibition. Cells were cultivated in HS medium under continuous light and constant temperature (PPFD of 80 µmol $m^{-2}$ $s^{-1}$, 27°C). Thylakoids were isolated from concentrated cultures with cell density of 16.0 × $10^6$ cells $cm^{-3}$. Cells were harvested by centrifuging at 6,000 × *g* at room temperature for 5 min, resuspended to a thylakoid isolation buffer (40 mM HEPES; pH = 7.4, 0.3 M sorbitol, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM betaine monohydrate, 1% BSA)and broken with a French Press at 1.0 kbar. The suspension was filtered through *Miracloth* (pore size of 22–25 µm), centrifuged at 2,000 × g and 5°C for 5 min and suspended in an osmotic shock buffer (10 mM HEPES; pH = 7.4, 5 mM sorbitol, 10 mM $MgCl_2$). The suspension was again centrifuged at 4,000 × *g* and 5°C for 5 min and resuspended in storage buffer (10 mM HEPES; pH = 7.4, 0.5 M sorbitol, 10 mM $MgCl_2$, 5 mM NaCl). The thylakoid suspension containing 35 µg(chlorophyll) $cm^{-3}$ was illuminated in the same system as whole cells (PPFD of 950 µmol $m^{-2}$ $s^{-1}$, 27°C), and aliquots for oxygen-evolution measurements ($H_2O$ to DCBQ) were taken every 10 min.

The photoinhibitory decrease of PSII activity, measured *in vitro* or in the presence of lincomycin *in vivo*, was analyzed by obtaining the best fit to the first-order kinetics $dA(t)/dt = -k_{PI} \times A(t)$, where $k_{PI}$ is the rate constant of photoinhibition, t is time, and A(t) is the measured PSII activity at time t. The rate constant of repair of PSII was estimated from the change in PSII activity during illumination *in vivo* in the absence of lincomycin, by obtaining the best fit to the differential equation $dA(t)/dt = k_{PI} \times A(t) + k_{REC} \times (A(0) - A(t))$, with $k_{PI}$ now fixed to the value obtained from the corresponding experiment in the presence of lincomycin. $k_{REC}$ is the rate constant of recovery. The *Copasi* software (Hoops *et al.* 2006) was used for fitting. *See* Campbell and Tyystjärvi (2012) for the equations.

**Chlorophylls (Chl) and carotenoids (Car)**: The concentrations of Chl *a* and *b*, and the total amount of Car were quantified spectrophotometrically (*Lambda Bio 40*, *Perkin Elmer*, Waltham, MA, USA) (Wellburn 1994). A 1-$cm^3$ sample of the culture was centrifuged at 20,000 × *g* for 10 min and the pellet was resuspended in 1 $cm^3$ of methanol. After thorough mixing, Chl was extracted in darkness and cold (+4°C) for 24 h. Absorbance was measured at 470, 652.4, and 665.2 nm after centrifugation at 20,000 × *g* for 10 min.

**Emission spectra** were measured from samples taken directly from the PBR during turbidostatic growth. The samples were immediately frozen in liquid nitrogen and stored at –70°C. For the measurements, each sample was diluted to final Chl concentration of 1.5 µg $cm^{-3}$ and 50 $mm^3$ samples were used for measurements. A dilution series was done to ensure that self-absorption is negligible in these conditions. The samples were then illuminated with blue light of 442 nm in liquid nitrogen temperature and the fluorescence emitted by the sample was measured spectrophotometrically (*Lambda Bio 40*, *Perkin Elmer*, Waltham, MA, USA). The raw spectra were normalized to the value at 685 nm.

***In vivo* thermoluminescence** was measured with a home-built apparatus (Tyystjärvi *et al.* 2009). Cells were grown in HS medium under continuous light and temperature conditions (PPFD of 80 µmol $m^{-2}$ $s^{-1}$ and 27°C), and steady $CO_2$ concentration (1%). The conditions were chosen to be similar as in continuous-growth experiment except that a moderate PPFD was chosen since we wanted to probe the intrinsic features of the strains without eventual effects of high-light treatment. Cultures were used once they had reached $OD_{730}$ of 1.5. Samples of 200 $mm^3$ containing 4–8 µg(Chl) in HS medium were placed on white filter paper. The sample was dark incubated for 5 min on the filter paper at 20°C, after which it was cooled to either –10°C (B band measurements) or –20°C (Q band measurements), charged with a single-turnover flash from a xenon flash lamp (E = 0.976 J). Photon emission was then recorded during warming to 60°C at a heating rate of 0.66°C $s^{-1}$. The Q band was measured in the presence of 20 µM DCMU, added before applying the sample on the filter.

## Results

**Biomass production**: Volumetric productivity was measured during turbidostatic growth in a flat panel PBR from three biological replicates. The productivity increased significantly in both strains (from 0.90 ± 0.07 to 2.06 ± 0.11 g $dm^{-3}$ $d^{-1}$ for *CC124* and from 0.93 ± 0.12 to 2.23 ± 0.09 g $dm^{-3}$ $d^{-1}$ for *hit2*) when PPFD was elevated from 200 to 750 µmol $m^{-2}$ $s^{-1}$ (Fig. 1). Further increase in PPFD caused first saturation and later a decrease in the productivity of *CC124*, which had a shortest doubling time of 1.74 × $10^4$ ± 540 s (4.82 h) at PPFD of 1,000 µmol $m^{-2}$ $s^{-1}$, whereas the productivity of *hit2* still significantly increased from PPFD of 750 to 1,000 µmol $m^{-2}$ $s^{-1}$. *Hit2* had the biggest volumetric productivity at PPFD of 1,250 µmol $m^{-2}$ $s^{-1}$, where it produced 2.53 ± 0.18 g $dm^{-3}$ $d^{-1}$ and had a doubling time of 1.51 × $10^4$ ± 1.26 × $10^3$ s (4.20 h). The difference between strains at PPFD of 1,250 µmol $m^{-2}$ $s^{-1}$ was tested to be statistically significant with *Student*'s *t*-test (*P*=0.042). Even *hit2* showed a decrease in volumetric productivity when light intensity was increased from PPFD of 1,250 to 1,500 µmol $m^{-2}$ $s^{-1}$ (Fig. 1).

**Photoinhibition and D1-repair cycle**: For photoinhibition experiments, the cells were illuminated at PPFD of 950 µmol $m^{-2}$ $s^{-1}$ in the presence of lincomycin that blocks concurrent recovery. This measurement showed much slower photoinhibition in the *hit2* strain than that in *CC124* (Fig. 2*A*). After 2,400 s (40 min) of illumination, the oxygen evolution rate of *hit2* was still 29% of the control value, whereas *CC124* produced no oxygen. The rate constant of photoinhibition ($k_{PI}$) was 6.30 × $10^{-4}$ $s^{-1}$ for *hit2* and 1.37 × $10^{-3}$ $s^{-1}$ for *CC124*.

In a living algal cell, the photoinhibitory damage is

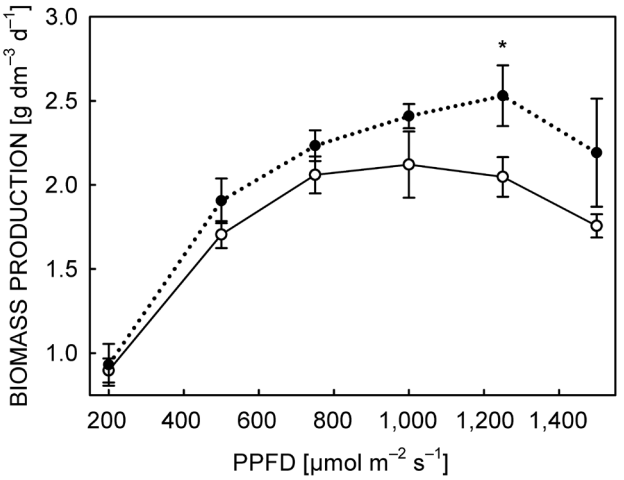


Fig. 1. Volumetric productivities of *CC124* (*open circles*, *solid line*) and *hit2* (*solid circles*, *dotted line*). The cells were grown in continuous light in HS medium in a 400-cm$^3$ flat panel photobioreactor at 20°C at $OD_{730}$ = 0.65 and at the biomass density of 0.58 ± 0.03 g dm$^{-3}$ (*CC124*) or 0.63 ± 0.06 g dm$^{-3}$ (*hit2*). The culture was kept for 5 d at each PPFD. $CO_2$ was provided by continuous bubbling of 3% $CO_2$. Biomass was measured by drying and weighing the product. Each experimental point represents average volumetric biomass production during the last 4–5 d at each PPFD from three biological replications and the error bars show SD. *The star* indicates a statistically significant difference between *CC124* and *hit2* obtained with *Student*'s *t*-test ($p$<0.05).

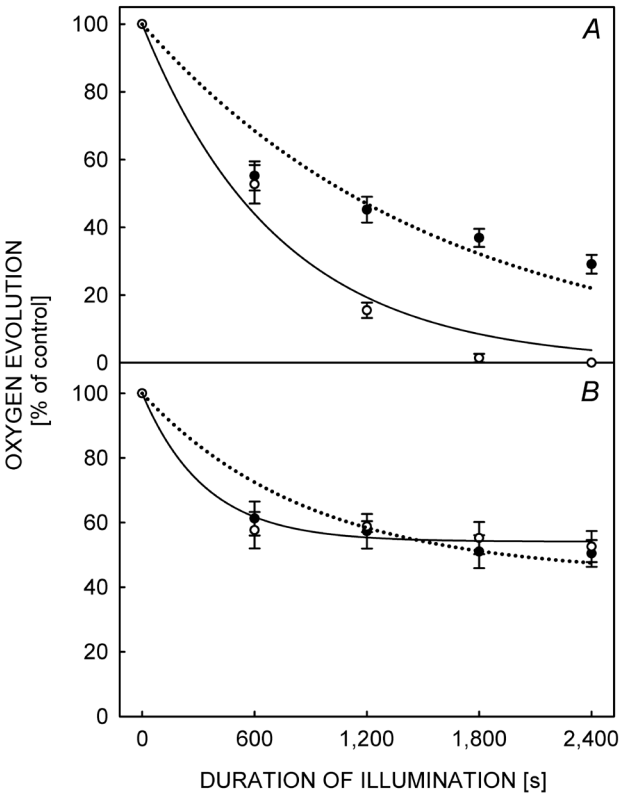


Fig. 2. Photoinhibition of *CC124* (*open circles*, *solid line*) and *hit2* (*solid circles*, *dotted line*) cells of *Chlamydomonas reinhardtii* in the presence (*A*) and absence (*B*) of lincomycin. Live cells (25 µg chlorophyll cm$^{-3}$, cell density of 8.2 × 10$^6$ cells cm$^{-3}$), collected during exponential growth in HS medium, were illuminated in HS medium at PPFD of 950 µmol m$^{-2}$ s$^{-1}$. The number of active PSII units was quantified with the light-saturated rate of oxygen evolution ($H_2O$ to DCBQ) measured from aliquots removed from the illuminated suspension. *The lines* in (*A*) represent the best fit to a first-order reaction equation and the lines in (*B*) represent the best fit to an equation describing photoinhibition and repair as opposing first-order reactions. Each data point represents an average of 3–5 biological replicates and the error bars, drawn if larger than the symbol, show standard error of mean.

continuously repaired. To observe the combined effects of the damaging reaction and the concurrent recovery, we also measured photoinhibition in the absence of lincomycin. The strains behaved very similarly in the absence of lincomycin, but *hit2* decreased slightly below *CC124* after 2,400 s (40 min) of treatment (Fig. 2*B*). After 600 s (10 min) of high-light treatment, the oxygen-evolution rate of *CC124* was 57% of the control value whereas the *hit2* strain had 61% of the control activity left; after 2,400 s (40 min) of treatment, oxygen evolution had equilibrated to 52% of the control value in *CC124* and to 50% in *hit2*. Modeling the concurrent recovery from photoinhibition by assuming that photoinhibition and recovery are opposing first-order reactions (Campbell and Tyystjärvi 2012) revealed that the rate constant of recovery ($k_{REC}$) was much higher for *CC124* (1.62 × 10$^{-3}$ s$^{-1}$) than that for *hit2* (4.88 × 10$^{-4}$ s$^{-1}$), suggesting that *CC124* compensates for rapid photoinhibition by a more active repair cycle.

Some photoprotective mechanisms, like NPQ, function only in intact cells whereas other mechanisms may also protect isolated thylakoid membranes. To distinguish between these alternatives, we did *in vitro* photoinhibition experiments by illuminating isolated thylakoids at PPFD of 950 µmol m$^{-2}$ s$^{-1}$. Thylakoids isolated from the *hit2* strain lost PSII activity more slowly than thylakoids of *CC124* (Fig. 3) but the difference was not as large as *in vivo*. The $k_{PI}$ value was 1.45 × 10$^{-3}$ s$^{-1}$ for *CC124* and 1.06 × 10$^{-3}$ s$^{-1}$ for *hit2* thylakoids.

**Pigment changes**: Car are highly active quenchers of singlet oxygen [for review, *see* Ramel *et al.* (2012)] and their abundance might therefore be of importance for both photoinhibition and biomass production in high light. The amount of Chl in a cell might play a role in the same phenomena. In *Chlamydomonas* cells grown in continuous culture, the Chl-to-biomass ratio decreased with increasing growth irradiance to one third at PPFD of 1,500 µmol m$^{-2}$ s$^{-1}$, in comparison to the value measured from cells grown at PPFD of 200 µmol m$^{-2}$ s$^{-1}$ (Fig. 4*A*). Also the Car-to-biomass ratio decreased with growth irradiance but only to 50% (*CC124*) or to 60% (*hit2*) of the value obtained at 200 µmol m$^{-2}$ s$^{-1}$ (Fig. 4*B*). The steeper PPFD dependence of the Chl-to-biomass ratio than the Car-to-biomass ratio led to 179.2–197.8% increase in the ratio of Car to Chls (Fig. 4*C*) in both strains when going from PPFD of 200 to 1,500 µmol m$^{-2}$ s$^{-1}$. In low light, both pigment-to-biomass ratios were slightly lower in *hit2* than that in *CC124*, but

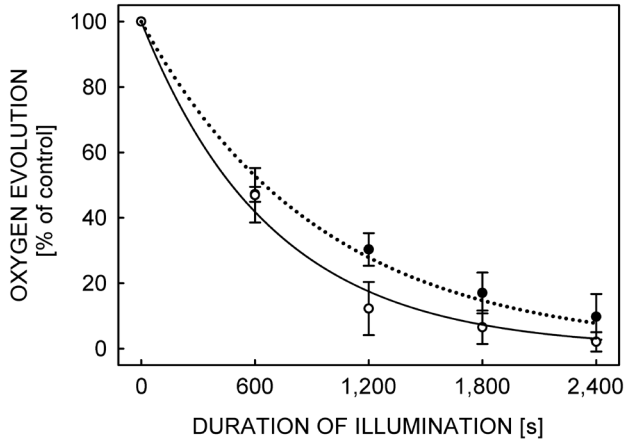


Fig. 3. Photoinhibition of isolated thylakoids of *CC124* (*open circles*, *solid line*) and *hit2* (*solid circles*, *dotted line*). Isolated thylakoids [35 μg(Chl) $cm^{-3}$] were illuminated at PPFD of 950 μmol $m^{-2}$ $s^{-1}$ at 27°C in a buffer solution containing 10 mM HEPES, pH = 7.4, 0.5 M sorbitol, 10 mM $MgCl_2$, and 5 mM NaCl, and the light-saturated rate of oxygen evolution ($H_2O$ to DCBQ) was measured from aliquots removed from the illuminated suspension. *The lines* represent the best fit to a first-order reaction equation. Each data point represents an average of three biological replicates and the error bars show SD.

the differences vanished at the highest light intensities.

The Chl *a*/*b* ratio of the *hit2* strain was significantly smaller (around 2.0) than that of *CC124* (around 3.0) (Fig. 5). The Chl *a*/*b* ratio of *CC124* decreased to 2.46 at 1,500 μmol $m^{-2}$ $s^{-1}$ and that of *hit2* was slightly elevated at the lowest PPFD tested but otherwise the Chl *a*/*b* ratios remained stable throughout the tested growth PPFD range (Fig. 5).

**Stoichiometry of photosystems**: Emission spectra of cells grown in different PPFD values were measured at 77 K from three replicates to learn how acclimation to different light intensities affects photosystem stoichiometry. In *C. reinhardtii*, PSII fluorescence peaks at 686 nm and the PSI peak is at 710–720 nm (Garnier *et al.* 1986). Responses of the parental strain *CC124* and the *hit2* mutant were virtually identical, and in both strains the 715-nm fluorescence peak decreased strongly with increasing growth PPFD (Fig. 6). The response was somewhat weaker in *hit2*, as a ratio of 715 nm to 686 nm fluorescence decreased from 1.72 to 0.75 in *hit2* and from 1.86 to 0.61 in *CC124* when going from PPFD of 200 μmol $m^{-2}$ $s^{-1}$ to PPFD of 1,500 μmol $m^{-2}$ $s^{-1}$. However, the ratio of PSI fluorescence to PSII fluorescence decreased in similar manner in both strains (Fig 6*C*).

**Recombination reactions in PSII**: Thermoluminescence is emitted as a result of recombination of a charge pair, either $S_2/Q_B^-$ (B band, measured without additions) or $S_2/Q_A^-$ (Q band, measured in the presence of DCMU). Thermoluminescence measurements from three biological replicates of *hit2* and *CC124* revealed that Q bands of both strains peak at 14.5°C (Fig. 7). The Q band peak was slightly wider in the case of *hit2*. The B band measurements, in contrast, revealed a difference between *CC124* and *hit2*. The peak temperatures were at 20.6°C for *CC124* and 17.3°C for *hit2*. The difference strongly suggests that the redox potential of the $Q_B/Q_B^-$ pair of the *hit2* strain is shifted toward the negative direction.

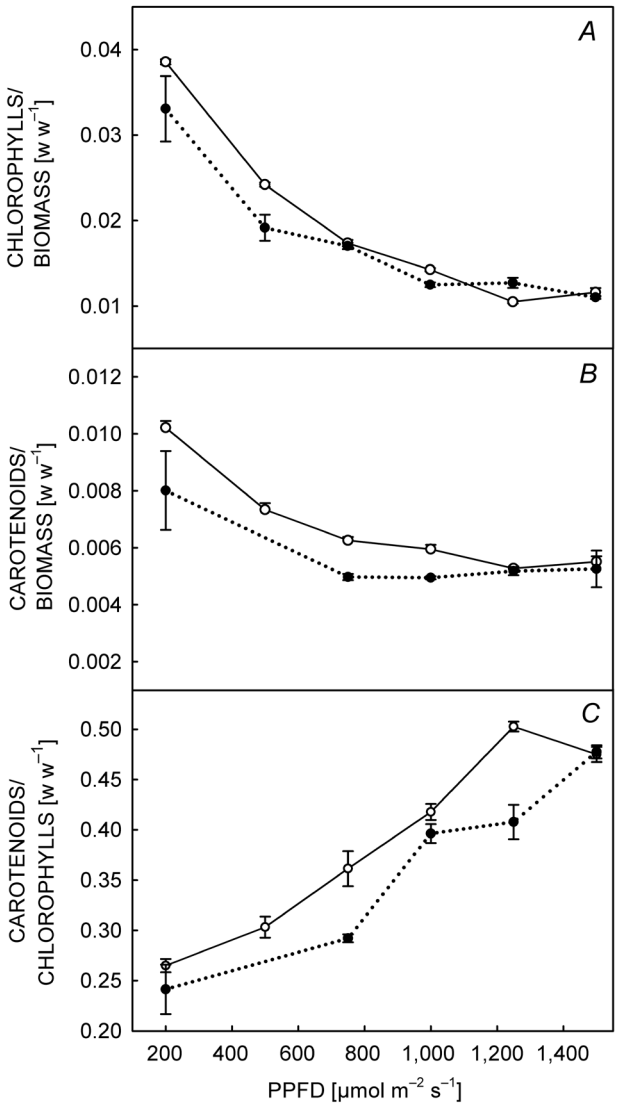


Fig. 4. Ratios between chlorophylls and biomass (*A*), carotenoids and biomass (*B*), and chlorophylls and carotenoids (*C*) in *CC124* (*open circles*, *solid line*) and *hit2* (*solid circles*, *dotted line*) grown at different PPFD values. Chlorophylls and carotenoids were quantified spectrophotometrically according to Wellburn (1994) after 24 h methanol extraction from samples extracted from PBR used in a continuous growth mode. Dry biomass was measured from samples taken from the culture. Each data point represents an average of three biological replicates and the error bars show SD.

## Discussion

Batch and continuous cultures of microalgae have different characteristics. When the density of a batch culture of a photoautotrophic organism increases, the amount of

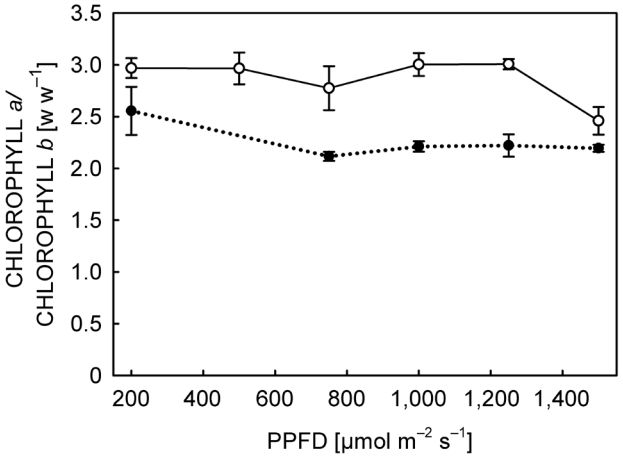


Fig. 5. Chlorophyll *a*/*b* ratio in *CC124* (*open circles*, *solid line*) and *hit2* (*solid circles*, *dotted line*) in increasing PPFD. Chlorophylls *a* and *b* were quantified spectrophotometrically according to Wellburn (1994) after 24 h methanol extraction from samples extracted from a flat-panel PBR operated in continuous mode.

light available per algal cell decreases. The decreasing light causes a shift from exponential to linear growth and may finally lead to cessation of growth much before the nutrients in the medium become depleted. Furthermore, the nutrient content of a batch changes constantly during the growth period, and therefore a rich medium is a necessity. A turbidostat, in turn, maintains the culture at a constant, predefined cell density, thereby stabilizing a major attribute affecting the growth parameters. Furthermore, continuous dilution with fresh medium maintains the medium composition constant for the whole cultivation time. In economical utilization of photosynthetic organisms, a continuous algal culture can maintain a productive process for a long time whereas a batch culture is suitable if the algae are expected to deplete the growth medium of some substance.

The maximum specific growth rates and biomass production of the wild-type strain *CC124* were similar as the maximum growth rates obtained in earlier studies in batch mode with various light conditions (Yang and Gao 2003, Kong *et al.* 2010, Kropat *et al.* 2011). The 4.2-h-doubling time of *C. reinhardtii* calculated here from photoautotrophic medium appears to be mediocre compared to the desert-crust alga *Chlorella ohadii* grown in BG-11 (1.2–1.4 h; Ananyev *et al.* 2017) but well in line with the fast-growing cyanobacterium *Synechococcus* sp. PCC 7002 (3.9 h; Xu *et al.* 2013). In a continuous culture, the high-light tolerant strain *hit2* produced biomass more rapidly than the parental wild-type strain *CC124* at all light intensities, and the superiority of *hit2* was significant at PPFD values exceeding 750 µmol m$^{-2}$ s$^{-1}$ (Fig. 1). The difference in the volumetric productivity between the two strains was only 19.1% at PPFD of 1,250 µmol m$^{-2}$ s$^{-1}$, which is small in comparison to the extreme difference in batch growth rates at PPFD of 2,500 µmol m$^{-2}$ s$^{-1}$, observed by Schierenbeck *et al.* (2015). A large difference in batch growth rates upon switching on a high PPFD after moderate light was repeated in our own experiments (data

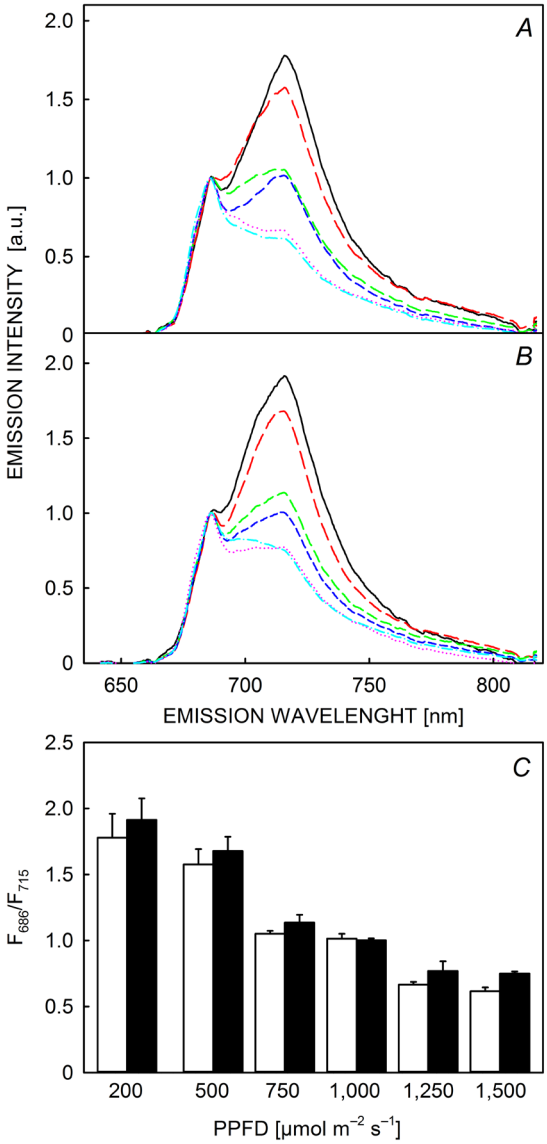


Fig. 6. Emission spectra of *CC124* (*A*) and *hit2* (*B*) grown at PPFD of 200 (*black solid line*), 500 (*red long dashed line*), 750 (*green medium dashed line*), 1,000 (*blue short dashed line*), 1,250 (*purple dotted line*), and 1,500 (*light blue dash-dotted line*) µmol m$^{-2}$ s$^{-1}$ measured at 77 K. Ratio of signal peaks originating from PSI and PSII in increasing light intensities in *CC124* (*white bars*) and *hit2* (*black bars*) (*C*). Emission spectra at 77 K temperature were obtained by illuminating samples with 442 nm blue light and measuring the emitted fluorescence. Panel (*C*) represents the ratio of emission intensity from wavelengths 715 ($F_{715}$) and 686 nm ($F_{686}$). The samples were obtained directly from PBR used in continuous growth in increasing light intensities and frozen immediately in liquid nitrogen. Each line represents an average of three biological replicates that were normalized to value at 686 nm.

not shown), indicating that the properties of the *hit2* strain have not changed. The difference between the strains appears mainly to concern the ability of *CC124* to grow in high light, as the continuous-growth experiment shows that in the conditions applied here (mineral medium, continuous bubbling with $CO_2$) the optimum PPFD for

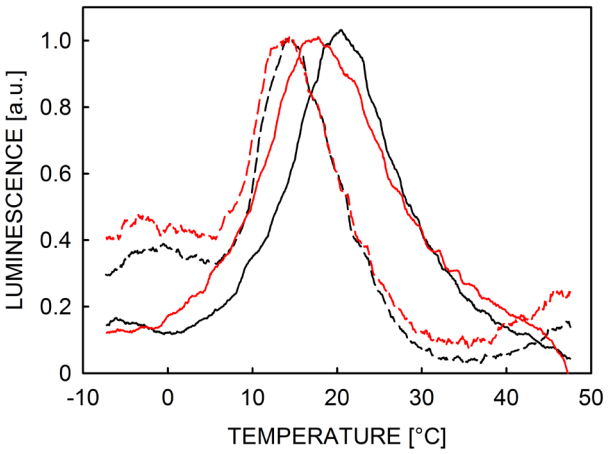


Fig. 7. Thermoluminescence Q band (*dashed lines*) and B band (*solid lines*) measurements of *CC124* (*black*) and *hit2* (*red*). 200 $mm^3$ samples, containing 4–8 µg of chlorophyll, were frozen to –10°C and then excited with a single turnover flash, after which the temperature was gradually increased to 60°C with rate of 0.66°C $s^{-1}$. Q bands were obtained by adding DCMU to the samples at the final concentration of 20 µM. Each peak is an average of three biological replicates.

wild-type *C. reinhardtii* is around 1,000 µmol $m^{-2}$ $s^{-1}$, and even at PPFD of 1,500 µmol $m^{-2}$ $s^{-1}$, wild-type *C. reinhardtii* produces biomass at 82.8% of the maximum rate. Thus, some acclimation responses allowing the *hit2* strain to grow in high light appear to be turned on in low-light-acclimated cells.

Even in *hit2*, many acclimation mechanisms are only turned on during exposure to strong light, and these mechanisms are very similar in *CC124* and *hit2*. Some acclimation mechanisms observed when PPFD is increased in a stepwise manner resemble high-light acclimation in plants and cyanobacteria (Yin and Johnson 2000, Muramatsu and Hihara 2012, Schumann *et al.* 2017). The Chl content of dry biomass decreased when the growth light intensity increased (Fig. 4), obviously reflecting diminishing need for light harvesting. However, the constancy of the Chl *a*/*b* ratio (Fig. 5) indicates that decreasing amount of the Chls reflects a decrease in the total number of photosystems rather than PPFD dependence of the antenna size. Earlier batch growth experiments have produced similar results (Bonente *et al.* 2012). While the Chls decreased, Car decreased much less, which led to an increase in the Car-to-Chl ratio (Fig. 4*C*). An increase in Car might be beneficial in high light because Car quench the harmful, highly reactive singlet oxygen ($^1O_2$) produced by reaction center of PSII (Krieger-Liszkay *et al.* 2008, Mulders *et al.* 2014), and the Car-to-Chl ratio increases in many organisms in response to high light (Solovchenko and Neverov 2017). The decrease in the Chls and increase in the Car-to-Chl ratio were similar in both strains. However, the Car-to-Chl ratio was slightly lower in *hit2* throughout the light intensities studied, which may partially result from the high-light tolerance of *hit2*. Interestingly, *hit2* appears to have less Car than *CC124*. Furthermore, the smaller Chl *a*/*b* ratio of *hit2* (Fig. 5) may suggest that *hit2* has a larger amount of LHCII proteins per reaction centers of PSII and PSI than *CC124*. These data may suggest that the good intrinsic tolerance to photoinhibition in *hit2* (Fig. 2*A*) allows the algae to save resources by synthesizing less Car, and on the other hand, perhaps to maintain a larger PSII antenna as suggested by faster growth and lower Chl *a*/*b* ratio of *hit2* than that of *CC124*.

Fluorescence emission spectra, measured at 77 K, indicated a drastic decrease in PSI emission in both strains when going from moderate to high light (Fig. 6). Simultaneous changes in PSII emission or an increase in PSII emission cannot be ruled out based on our data only. Furthermore, lowering of PSII fluorescence in high light, *e.g.*, due to sustained photoinhibition, cannot explain the data, as the ratio of PSII to PSI fluorescence increases with light intensity. However, decrease in PSI in high light appears to be a general phenomenon, as the same has earlier been seen in *C. reinhardtii* cells grown in batch culture, where the amount of the PsaA protein of PSI was found to be lower in high-light-grown cells, compared to cells grown in lower light intensity (Bonente *et al.* 2012). The present data suggest that the PSI:PSII ratio decreases with PPFD throughout the tested range of light intensities.

Photoinhibition and PSII repair were analyzed with the classical first-order model for the damaging reaction of photoinhibition (Tyystjärvi and Aro 1996). The fit in *Chlamydomonas* is not perfect but the deviations from first order are different for the two strains, suggesting that they result from biological variation and experimental errors rather than indicate that the model is invalid. For repair of PSII, we used an oversimplifying model that contains only active and inactive PSII, and therefore the rate constant of repair obtained from the fit should be viewed as an approximation only.

The relationship between photoinhibition and ability to use high light for photosynthesis is not self-evident, as the damaging reaction of photoinhibition occurs at all light intensities (Tyystjärvi and Aro 1996). Comparison of the *CC124* and *hit2* strains reveals a large difference in the rate of the damaging reaction of photoinhibition (Fig. 2*A*) but the difference between the strains disappeared when the cells were illuminated in the absence of lincomycin to observe the equilibration between photoinhibition and repair of PSII (Fig. 2*B*). These data indicate that in the *CC124* strain, rapid repair of photoinhibited PSII compensates for faster photoinhibition. Thus, the repair of PSII flexibly increases its rate when PSII becomes faster damaged, confirming that the repair of PSII is regulated by the demand for active PSII centers (Campbell and Tyystjärvi 2012). The repair of PSII consumes resources (Aro *et al.* 1993, Murata and Nishiyama 2018), and therefore faster biomass production by *hit2* than *CC124* is, at least partly, explained by the fact that the *hit2* uses fewer resources to repair PSII than *CC124*. Rapid growth of the *hit2* strain in high light (Fig. 1) indicates that the repair of PSII in *hit2* cells acclimates to the increase in the rate of the damaging reaction of photoinhibition in high light. The finding that when the cells are suddenly exposed to high light, then PSII repair in *hit2* appears less efficient than in *CC124*, suggests that a strain with a low rate constant of photoinhibition in growth light does not unnecessarily

invest to efficient repair machinery (Fig. 2). However, the good performance of *hit2* in high light indicates that the PSII repair of *hit2* acclimates to the increasing rate of the damaging reaction of photoinhibition when light intensity increases (Tyystjärvi and Aro 1996).

The finding that the difference in the rate of the damaging reaction of photoinhibition between *hit2* and *CC124* was smaller *in vitro* than that *in vivo* (Fig. 3) suggests that the mechanism protecting *hit2* against photoinhibition functions best in the native stromal environment of thylakoids. NPQ is one such mechanism, as NPQ phenotypes cannot be seen in isolated thylakoids with completely unstacked grana (Goss *et al.* 2007), unless artificially induced (Gilmore and Yamamoto 1992, Krieger *et al.* 1992). On the other hand, the $k_{PI}$ value of *hit2* is less than half of that of *CC124*, although NPQ does not lower the $k_{PI}$ value by more than 25% in plants (Sarvikas *et al.* 2006), and the protective efficiency of NPQ is relatively small also in the microalga *Phaeodactylum tricornutum* (Havurinne and Tyystjärvi 2017). However, *hit2* has almost twice as high NPQ than *CC124* (Schierenbeck *et al.* 2015), and it is therefore obvious that the strong NPQ contributes to the slow photoinhibition in *hit2*. NPQ is wasted energy and strong NPQ in *hit2* may partly explain why biomass production of *hit2* is only 10–25% faster than biomass production of *CC124*, although *CC124* can hardly grow at all when suddenly exposed to strong light (Schierenbeck *et al*. 2015).

Altered linear electron transfer may also protect PSII from photoinhibition. *Chlorella ohadii*, a species of green algae impervious to photoinhibition, was found to have its thermoluminescence B band virtually at the same temperature as the Q band (Treves *et al.* 2016). Interestingly, the *PD* mutant of the cyanobacterium *Synechocystis* sp. PCC 6803 shows high tolerance to photodamage (Mulo *et al.* 1998) and has the Q and B bands very close to each other (Keränen *et al.* 1996). The finding that similar features can be seen together also in the *hit2* strain of *C. reinhardtii* may suggest that a negative shift in the redox potential of the $Q_B/Q_B^-$ pair protects against photoinhibition of PSII. Such redox potential shift has two consequences. Firstly, $Q_B^-$ becomes less stable, which favors charge recombination reactions. Secondly, electron transfer from $Q_A^-$ to $Q_B$ becomes less efficient. As recombination reactions hardly can play a protective role, it is possible that the changed electron sharing equilibrium between $Q_A$ and $Q_B$ has a protective effect.

It might be suggested that slow photoinhibition is not the reason for the rapid growth of *hit2* but in fact this strain has unusually rapid metabolism downstream of PSII, and rapid growth results from the rapid metabolism. In this scenario, the sink activity after PSII would be high, and therefore less reactive oxygen species (ROS) would be produced in the thylakoids, which might protect against photoinhibition. High sink activity would also alleviate the 'excitation pressure' of PSII. This scheme has support in the literature, which shows that the PSII repair cycle is highly susceptible to ROS (Nishiyama *et al.* 2001, Hakala-Yatkin *et al.* 2011). The damaging reaction of photoinhibition, however, has often been found to be independent of ROS and independent of electron transfer reactions after PSII (Nishiyama *et al*. 2001, Hakala *et al*. 2005, Takahashi and Murata 2005). In the case of *hit2*, the damaging reaction was affected (Fig. 2), strongly suggesting that if slow photoinhibition and rapid growth have a causal relationship, slow photoinhibition is the cause, not the effect.

The connection between the mutation in the *Cr-COP1* gene and the low-photoinhibition/fast-growth phenotype of *hit2* remains obscure. The same mechanism(s) function in photoinhibition in visible and UV light (*see* Tyystjärvi 2013) but this does not directly explain why a mutation in a gene required for UVB signaling would cause the decrease in $k_{PI}$ and enable rapid growth in visible high light. One possibility is that *hit2*, like *hit1* (Tilbrook *et al*. 2016), is deficient in UV acclimation but *hit2* is locked at an acclimation status in which the alga is more tolerant to visible light than the wild type. We also do not know how the *hit2* mutation causes the change in the redox potential of $Q_B$. The redox potentials of the PSII electron acceptors are known to vary due to the presence or absence of the oxygen-evolving manganese complex (Krieger *et al.* 1995) or bicarbonate on the reducing side of PSII (Brinkert *et al*. 2016), suggesting that factors outside of PSII may have effects on them.

In summary, the results suggest that slow photoinhibition is a factor explaining why the *hit2* strain is high-light tolerant and produces more biomass per time unit even during continuous cultivation. Slower photoinhibition in *hit2* gives this strain an advantage because PSII repair needs fewer resources in *hit2* than in the parental wild-type strain *CC124*. Thus, screening for algae that show slow photoinhibition is a potential method for finding algae that grow rapidly in high light.

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