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Optimization of photosynthetic light energy utilization by microalgae

Zoee Perrine [a], Sangeeta Negi [b,c], Richard T. Sayre [b,c,*]

[a] Phycal Incorporated, St Louis, MO 63132, United States
[b] New Mexico Consortium, Los Alamos, NM 87544, United States
[c] Los Alamos National Laboratory, Los Alamos, NM 87544, United States

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ABSTRACT

Over 50% of the energy losses associated with the conversion of solar energy into chemical energy during photosynthesis are attributed to kinetic constraints between the fast rate of photon capture by the light harvesting apparatus and the slower downstream rate of photosynthetic electron transfer. At full sunlight intensities, energy flux from the light harvesting antennae to the reaction centers may be 100-folds greater than the overall linear electron flow resulting in the dissipation of up to 75% of the captured energy as heat or fluorescence. One possible means to couple energy capture and photosynthetic electron transfer more efficiently is to reduce the optical cross-section of the light harvesting antennae. We show that by partially reducing chlorophyll *b* levels in the green alga, *Chlamydomonas reinhardtii*, we can tune the peripheral light harvesting antennae size for increased photosynthetic efficiency resulting in more than a two-fold increase in photosynthetic rate at high light intensities and a 30% increase in growth rate at saturating light intensities. Unlike chlorophyll *b*-less mutants which lack the peripheral light harvesting antennae; transgenics with intermediate sized peripheral antennae have the advantage that they can carry out state transitions facilitating enhanced cyclic ATP synthesis and have robust zeaxanthin–violaxanthin cycles providing protection from high light levels. It is hypothesized that the large antennae size of wild-type algae and land plants offers a competitive advantage in mixed cultures due to the ability of photosynthetic organisms with large light harvesting antennae to shade competing species and to harvest light at low flux densities.

1. Introduction

Single celled microalgae are among the most productive autotrophic organisms in nature due to their high photosynthetic efficiencies and the lack of heterotrophic tissues [1–4]. Yet, photosynthetic effi-

to the poor kinetic coupling between light capture by the light harvesting apparatus and down-stream photochemical and electron transfer processes. During photosynthesis, light captured by the peripheral light-harvesting antenna complexes (LHC) is transferred at nearly 100% efficiency (via quantum coherence processes) to the proximal antenna complexes of the photosystem II (PSII) and photosystem I (PSI) reaction center (RC) complexes where the primary charge separation occurs [7]. Wild-type (WT) algae typically possess large PSII peripheral antennae complexes (LHCII), which maximize light capture at both high and limiting light intensities [2]. However, light harvesting antenna size is not optimized for achieving maximal apparent quantum efficiency in monocultures where competition for light between different species is absent. In nearly all photosynthetic organisms, photosynthesis light saturates at ~25% of the full sunlight intensity [8]. This is due to the fact that at saturating light intensities, the rate of photon capture substantially (>100×) exceeds the rate of ... ge fraction of ... orescence by non-photochemical quenching (NPQ) processes [9]. These dissipative energy losses account for the greatest inefficiencies (~50%) in the conversion of light into chemical energy during photosynthesis [5,6]. Since light is a resource for photosynthetic organisms, it is expected that competition for this resource drives the evolution of antennae size. Ironically, having large, inefficient antennae may increase evolutionary fitness since organisms that compete better for light effectively shade those that are less efficient at capturing light. In mixed species communities, being best at capturing light may be a selective advantage but in monocultures being more efficient at light utilization (energy conversion) may be the better fitness or growth strategy.

To date, the most effective strategy to increase photosynthetic light utilization efficiency is to reduce the size of the light-harvesting antenna per RC complex [5,8,10]. By reducing the effective optical cross section of the antennae complexes the probability of saturating electron transfer at full sunlight intensities is reduced. Significantly, a reduction

Abbreviations: Chl, chlorophyll; LHC, light harvesting complex; P_{MAX}, maximum light-saturated photosynthesis rate; PSI, photosystem I; PSII, photosystem II; WT, wild type.

* Corresponding author. Tel.: +1 505 412 6532.
E-mail address: rsayre@newmexicoconsortium.org (R.T. Sayre).

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in antennae size/RC is also predicted to reduce cell shading and increase the penetration of photosynthetically active radiation to greater depths in the culture water column (Fig. 1A). In *Chlamydomonas reinhardtii*, it has been demonstrated that mutants with reduced antenna size can be generated by eliminating chlorophyll (Chl) *b* synthesis as well as by reducing expression of LHC genes [10,11]. Previous studies have shown that microalgae lacking the peripheral LHCII have increased photosynthetic rates; however, few studies have demonstrated an increase in growth rate with reduced peripheral antennae size under fully autotrophic growth conditions [8,11–15]. To date, nearly all growth studies with algae having altered antennae sizes have been done under mixotrophic (plus acetate) growth conditions.

In addition to harvesting light members of the LHCII gene/protein family also play important roles in; 1) balancing energy distribution between the photosystems (state transitions), 2) regulating cyclic photophosphorylation or ATP synthesis, and 3) mediating the dissipation of excess captured energy as heat through NPQ [9,16,17]. Thus, while the complete elimination of LHCII reduces kinetic constraints between light capture and energy conversion, elimination of all of the peripheral LHCII would be expected to impair the distribution of energy between the two photosystems, reduce the ability to modulate ATP/NADPH ratios, and increase susceptibility to photodamage. Hence, the complete lack of a peripheral antenna may not be the optimal solution for enhancing energy conversion efficiency and growth of algal monocultures.

To determine if there are more optimal antenna sizes for more efficient net photosynthesis, we generated transgenic *C. reinhardtii* strains having a range of LHCII antenna sizes that were intermediate between WT and a Chl *b* less strain which entirely lacks LHCII. We hypothesized that reducing but not eliminating the Chl *b* content would result in algal transgenics with intermediate LHCII levels. We demonstrate that transgenic algae having intermediate LHCII content are capable of state transitions as well as non-photochemical quenching of excess energy via the violaxanthin–zeaxanthin cycle. Algae with intermediate antennae sizes also have substantially higher growth rates than WT or Chl *b* lacking algal strains when grown autotrophically at saturating (in WT) light intensities while having growth rates similar to WT at low light intensities. We propose that these observations also have implications for improving the light harvesting efficiency of photosynthesis in the canopies of terrestrial plants. Leaves having smaller antennae in the upper canopy and larger antennae in the lower canopy may also have increased the apparent photosynthesis efficiency and improved productivity when grown in monocultures.

2. Materials and methods

2.1. Vector construction

The plasmid for inducing RNAi-mediated silencing of the chlorophyllide *a* oxygenase (*CAO*) gene in *C. reinhardtii* strain CC-424 (arg2 cw15 sr-u-2-60 mt−, *Chlamydomonas* Genetic Center) was constructed using a genomic-sense/cDNA-antisense strategy. The first two exons and introns of the *CAO* gene were amplified by PCR using GCTTTCGTCATATGCTTCCTGCGTCGCTTC and CTC TGGATCCGTCTGTGTAAATGTGATGAAGC as forward and reverse primers respectively and the resulting product was digested with

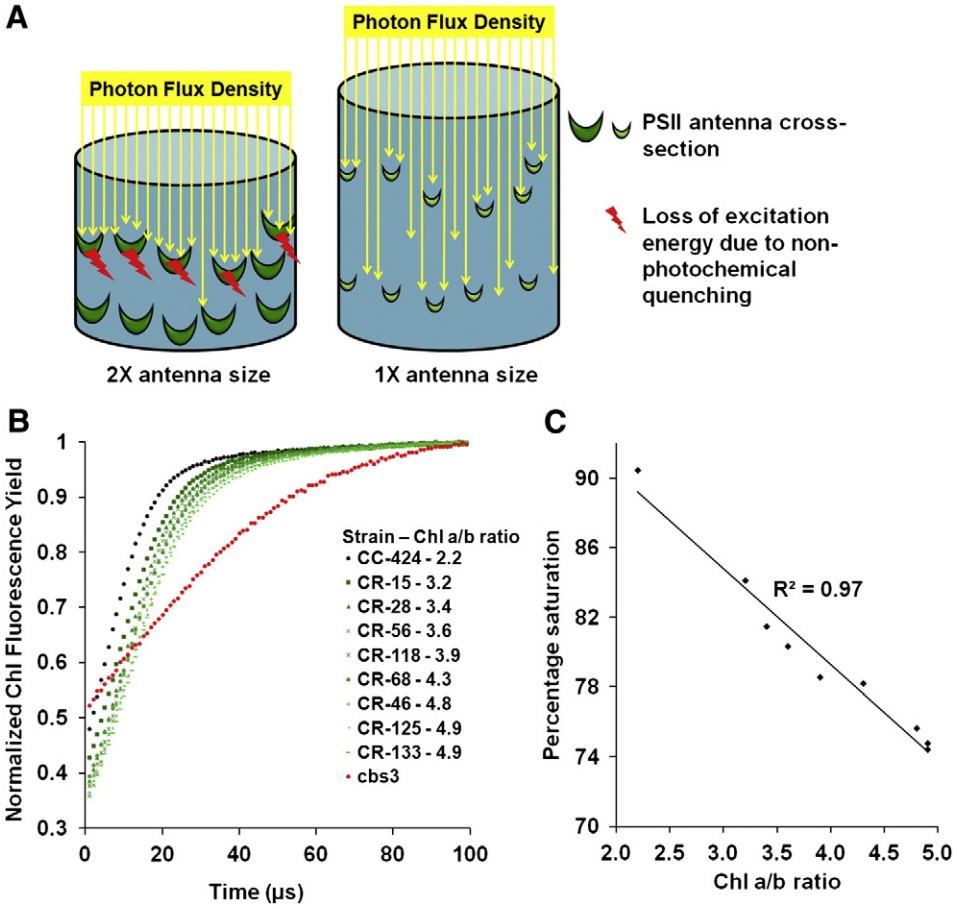

Fig. 1. Algae with truncated LHCII. (A) Model for light absorption and utilization by algae with large and truncated antennae at saturating light intensities. (B) Chl fluorescence rise kinetics in parental (CC-424), Chl *b* reduced transgenics (CR) and Chl *b* less mutant (*cbs3*). Chl fluorescence levels were measured under continuous, non-saturating illumination every 1 µs. (C) Correlation between Chl *a*/*b* ratio and percent closure of PSII RCs.

restriction enzymes NdeI and BamHI. The corresponding cDNA region spanning exons 1 and 2 of the *CAO* gene was amplified using GACGAATTCGTCAGATGCTTCCTGCGTCG and CTCTAGATCTGTCGC CTCCGCCTTCAGCTC as the forward and reverse primers and digested with restriction enzymes EcoRI and BglII. The genomic DNA and cDNA fragments were cloned into the PSL18 vector [18] using the NdeI and EcoRI sites to generate the *CAO*-RNAi vector (Fig. S1A). The *psaD* promoter and terminator cassette of the PSL18 vector was used to drive RNAi. The PSL18 vector contains the paromomycin resistance gene driven by the Hsp70/RbcS2 fusion promoter [19], placed in tandem with the *PsaD* promoter and terminator cassette. *Chlamydomonas* transformants generated using the *CAO*-RNAi vector were selected based on resistance to paromomycin.

2.2. Generation and screening of the CAO-RNAi transformants

For the generation of the *CAO*-RNAi lines (CR), the cell wall-less CC-424 *C. reinhardtii* strain was transformed with 1 μg of ScaI linearized *CAO*-RNAi plasmid by glass bead-mediated nuclear transformation [20]. Transformants were selected on TAP agar plates containing 100 μg/mL of L-arginine and 50 μg/mL of paromomycin. Transformants were further screened by pigment extraction and spectrophotometric analysis of Chl *a*/*b* ratios, which were expected to increase as a consequence of *CAO* gene silencing. For this, cells were grown in culture tubes containing 3 mL of high salt (HS) media [21] + arginine (100 μg/mL) for 5–6 days under continuous illumination of ~50 μmol light m^{-2} s^{-1} and the relative amounts of Chl *a* and *b* were determined as described in Arnon [22]. The presence of the *CAO*-RNAi and paramomycin resistance cassettes in the transgenics was further confirmed by PCR using a forward primer binding within the *PsaD* promoter (GTATCAATATTGTTGCGT TCGGGCAC) and a reverse primer binding within the *CAO*-RNAi cassette (ATCAGTTGCGTGCGCCTTGCCAAACC) to yield an ~780 bp fragment as well as a forward primer binding within the Hsp70/Rbcs2 fusion promoter (GGAGCGCAGCCAAACCAGGATGATG) and a reverse primer (GTCCCCACCACCCTCCACAACACG) binding within the paramomycin resistance gene to yield a 630 bp fragment (Fig. S1B).

2.3. Chl fluorescence induction measurements

For Chl fluorescence induction analysis, cell suspensions of the parental WT and transgenic *Chlamydomonas* were adjusted to a Chl concentration of ~2.5 μg Chl/mL. Flash Chl fluorescence induction was measured using the FL-3500 fluorometer (Photon System Instruments) as described in Nedbal et al. [23]. The cells were dark adapted for 10 min prior to the experiment. Chl fluorescence was induced using non-saturating continuous illumination and Chl fluorescence levels were measured every 1 μs using a weak pulse-modulated measuring flash. The values of Chl fluorescence were normalized to the maximum achieved for a given sample. For the state transition experiments, low light grown cultures were dark adapted or pre-illuminated with 715 nm or 650 nm light for 10 min prior to the induction of Chl fluorescence. The actinic flash duration for this experiment was set to 50 μs and Chl fluorescence was measured every 1 μs.

2.4. Non-denaturing gel electrophoresis

The CC-424, CR-118 and CR-133 strains, and the *Chlamydomonas* Chl *b* less mutant, *cbs3* [24], were grown in high salt (HS) under low light intensities (50 μmol light m^{-2} s^{-1}) with continuous shaking at 225 rpm for 6 days. Cells were harvested by centrifugation at 3000×*g* for 5 min at 4 °C. The cell pellet was resuspended in buffer A (0.3 M sucrose, 25 mM HEPES, pH 7.5, 1 mM $MgCl_2$) plus 20 μL/mL of protease inhibitor cocktail (Roche), to yield a final Chl concentration of 1 mg/mL. Cells were then broken by sonication (Biologics, Inc., Model 300 V/T Ultrasonic Homogenizer) two times for 10 s each time (pulse mode, 50% duty cycle, output power 5) on ice. The unbroken cells were pelleted by centrifugation at 3000×*g* for 2 min at 4 °C. The supernatant was centrifuged at 12,000×*g* for 20 min and the resulting pellet was washed with buffer A. The sample was subjected to a second centrifugation step at 11,000×*g* to collect thylakoids. Thylakoid membranes were then solubilized with $LiDodSO_4$ [25]. Briefly, 15 μg Chl equivalent of thylakoids was solubilized in a buffer containing 50 mM Na_2CO_3, 50 mM dithiothreitol, 12% sucrose and 2% lithium dodecyl sulfate to yield a final Chl concentration of 1 mg/mL and a Chl/$LiDodSO_4$ (wt/wt) ratio of 1:20. The sample was gently shaken for 60 s. Equal amounts of the sample buffer (62.5 mM Tris–HCl, pH 6.8 and 25% glycerol) were added to the solubilized thylakoids before loading. The samples were then loaded onto a Ready Tris–HCl Gel (Bio-rad 161-1225) and $LiDodSO_4$ and EDTA were added to the upper reservoir buffer (25 mM Tris, 192 mM glycine) to a final concentration of 0.1% and 1 mM, respectively. Electrophoresis was performed at 4 °C in the dark for 2–2.5 h at 12 mA constant current.

2.5. Quantitative real-time PCR

RNA was isolated from 25 mL of the log phase cultures grown under 50 μmol m^{-2} s^{-1} light using Trizol according to the manufacturer's instructions (TRI Reagent®, Ambion, Catalog # AM9738). DNase (Promega, Catalog # M610A) treated RNA samples (2 μg) were reverse transcribed using the qScript™ cDNA SuperMix kit (Quanta Biosciences). Real-time quantitative RT-PCR was carried out using an ABI-Step one plus using the SYBR Green PCR Master Mix Reagent Kit (Quanta Biosciences). The *Chlamydomonas CBLP* gene was used as internal control and was amplified in parallel for gene expression normalization. The forward and reverse primers used for amplification of the *CBLP* gene were GCAAGTACACCATTGGCGAGC and CCTTTGCACAGCGCACAC respectively and the forward and reverse primers used for the amplification of the *CAO* gene were GACTTCCTGCCCTGGATGC and GGG TTGGACCAGTTGCTGC respectively. The PCR cycling conditions included an initial polymerase activation step at 95 °C for 10 min, followed by 40 PCR cycles at 95 °C for 15 s, 61 °C for 15 s and 72 °C for 30 s and a final melting step of 60–95 °C each for 15 s. The quantification of the relative transcript levels was performed using the comparative CT (threshold cycle) method [26].

2.6. Photosynthetic oxygen evolution

The oxygen evolving activity of the log-phase cultures (0.4–0.7 $OD_{750\ nm}$) of CC-424, CR-118, CR-133, CC-2677 (cw15 nit1-305 mt− 5D, *Chlamydomonas* Genetic Center) and *cbs3* was assayed using a Clark-type oxygen electrode (Hansatech Instruments) using low light (50 μmol photons m^{-2} s^{-1}) grown cultures. Cells were resuspended in 20 mM HEPES buffer (pH 7.4) and the rate of oxygen evolution was measured as a function of increasing light intensity (650 nm wavelength red light). The photon flux density was maintained for 1.5 min at 50, 150, 300, 450, 600, 750 and 850 μE m^{-2} s^{-1} of red light to obtain a light saturation curve of photosynthesis. The same experiment was repeated in the presence of 10 mM $NaHCO_3$. Light saturation curves were normalized on the basis of Chl as well as cell density (OD 750 nm).

2.7. Photoautotrophic growth measurements

Photoautotrophic growth of the CC-424, CR-118, CR-133, CC-2677 and *cbs3 Chlamydomonas* strains was measured in a time dependent manner, in 125 mL flasks in liquid HS media, at either low light (LL, 50 μmol photons m^{-2} s^{-1}) or high light (HL, 500 μmol photons m^{-2} s^{-1}) conditions with constant shaking at 175 rpm. The media was supplemented with 100 μg/mL of L-arginine. The optical density of the cultures was monitored on a daily basis at 750 nm using a Cary 300 Bio UV–vis spectrophotometer.

2.8. Pigment analysis by HPLC

Chlamydomonas cultures were grown in low (50 μmol photons m^{-2} s^{-1}) and high light (500 μmol photons m^{-2} s^{-1}) intensities for 5 days. Cells were centrifuged at 3000 rpm for 3 min and immediately frozen in liquid nitrogen and lyophilized. Carotenoids and Chls were extracted with 100% acetone in the dark for 20 min. After incubation samples were centrifuged at 14,000 rpm for 2 min in a microfuge and the supernatant was transferred to a glass tube and dried under vacuum. The dried samples were resuspended in 1 mL of acetonitrile:water:triethylamine (900:99:1, v/v/v) for HPLC analysis. Pigment separation and chromatographic analysis were performed on a Beckman HPLC equipped with a UV–vis detector, using a C18 reverse phase column at a flow rate of 1.5 mL/min. Mobile phases were (A) acetonitrile/H_2O/triethylamine (900:99:1, v/v/v) and (B) ethyl acetate. Pigment detection was carried out at 445 nm with reference at 550 nm. Pigment standards were bought from DHI, Denmark.

3. Results

3.1. RNAi-mediated silencing of the CAO gene leads to transgenic algae with truncated (intermediately-sized) PSII antenna complexes

To generate transgenic algae with reduced Chl *b* levels and intermediate PSII antenna size, we used an RNAi approach to modulate the expression of *CAO*, the gene responsible for the synthesis of Chl *b* via the oxidation of Chl *a* [27]. A genomic-sense/cDNA-antisense construct spanning the first two exons of the *CAO* gene was used to generate the *CAO*-RNAi transgene (Fig. S1A). After transformation with the *CAO*-RNAi plasmid, transgenics were selected on the basis of paromomycin resistance encoded on the integrating plasmid. Eight independent *CAO*-RNAi (CR) transgenics with Chl *a*/*b* ratios ranging from 3.2 to 4.9 were generated and confirmed by PCR for the presence of the RNAi cassette as well as the paromomycin resistance marker (Fig. S1B). To determine the effects of reduced Chl *b* levels on the PSII antenna absorption cross-section, we measured Chl fluorescence induction kinetics in the CR strains and their parent (CC-424) as well as a Chl *b* less mutant, *cbs3* [24]. The rate at which Chl fluorescence rises is indicative of the rate of closure of PSII RCs and the PSII antenna size under conditions of non-saturating, continuous illumination [23,28]. As shown in Fig. 1B, the CR transgenics had slower Chl fluorescence induction kinetics relative to WT (Chl *a*/*b* = 2.2) reflective of a smaller PSII antenna size and only reached ~75 to 85% PSII RC normalized maximum fluorescence level when the parent strain had reached 90% of saturation. Significantly, the PSII RC closure rate was inversely correlated with the Chl *a*/*b* ratio, implying that the Chl *a*/*b* ratio is a direct indicator of the antenna size over the Chl *a*/*b* ranges tested (Fig. 1C). Reductions in LHCII content in the two CR strains and the *cbs3* mutant were also confirmed using non-denaturing polyacrylamide gel electrophoresis [25]. The two CR transgenics (CR-118 and CR-133), having Chl *a*/*b* ratios representative of an intermediate and the highest CR Chl *a*/*b* ratio, had a ~20–30% reduction in LHCII (CPII band) content relative to WT. The CPII band [29] was absent in the *cbs3* mutant (Fig. 2A). As expected, reductions in CR LHCII content were associated with reductions in *CAO* mRNA levels (Fig. 2B). It is noteworthy that large reductions in *CAO* transcript levels in the CR transgenics relative to their parental WT led to only modest decreases (30–48%) in Chl *b* levels. It has previously been shown that low levels of CAO protein are sufficient to support normal levels of Chl *b* synthesis [30]. Therefore, it is likely that low *CAO* transcript levels in the CR lines are sufficient to support moderate levels of Chl *b* synthesis. Interestingly, chlorophyll pigment analyses of the CR strains grown under low and high light conditions showed some plasticity in Chl *b* levels as a function of growth light intensity. In contrast to the parental WT, Chl *a*/*b* ratios were significantly higher ($p<0.01$) in high-light grown cultures of the CR strains than in low-light grown cultures (Fig. 2C). The CR lines also exhibited substantial decreases in Chl *b* (41–43%) content and antenna size when grown in high relative to low light intensities (Fig. 2D). In addition, we observed a 40–60% decrease in the total Chl content per unit dry weight in high light grown cultures of strains compared to low light grown cultures (Fig. S3).

3.2. CR strains have higher light-saturated photosynthesis rates and higher growth rates than WT cells under high light intensities

To study the effect of reduced LHCII abundance on light-dependent rates of photosynthetic oxygen evolution, we compared rates of photosynthesis in the two CR strains, the *cbs3* mutant, and their parent strains, CC-424 and CC-2677, respectively. The CR lines had 2–2.6 fold higher light-saturated photosynthetic rates (P_{max}) than WT on a Chl basis (Fig. 3A) and up to ~1.5–2 fold greater photosynthetic rates when measured in the presence of saturating inorganic carbon levels (10 mM $NaHCO_3$) (Fig. 3B). The higher photosynthetic rates in the presence of saturating levels of bicarbonate are presumably associated with the active transport of bicarbonate into the cells resulting in the elevation of internal CO_2 concentrations [31]. Similar increases in P_{max} were also observed in the CR transgenics when oxygen evolution rates were expressed on the basis of cell density indicating that the reduction in Chl content per cell did not substantially bias the rates of photosynthesis reported on a Chl basis for the CR transgenics (Fig. S2). In contrast, we observed a ~4 fold increase in P_{max} for the Chl *b* less mutant, compared to its parent measured on a Chl basis, but when expressed on a cell density basis, there was only a 2-fold increase in light-saturated rates of photosynthesis relative to WT indicative of substantial reductions in total Chl/cell (Fig. S2).

To determine the impact of antenna size on photoautotrophic growth, we measured growth rates under limiting and saturating light conditions (50 and 500 μmol light m^{-2} s^{-1}). Growth of the CR transgenics was unimpaired compared to its parental WT under limiting light intensities (Fig. 3C). On the other hand, the *cbs3* mutant had a 25% reduction in stationary phase cell density under low light growth conditions relative to its parent WT strain (CC-2677), presumably due to the smaller optical cross section of the antennae. Under saturating light intensities, however, the CR strains had ~15 to 35% higher stationary phase culture densities than the parental WT, while the *cbs3* strain had a substantially reduced stationary phase cell density (~80% of WT) indicating that photosynthetic and growth rates were not correlated in this mutant presumably reflecting additional impairments in photosynthetic activities (Fig. 3D).

3.3. Reduction of LHCII content in the CR strains does not impair state transitions

In *C. reinhardtii*, the peripheral PSII antenna is able to migrate laterally between PSII and PSI, in a process known as state transitions, to balance the excitation energy distribution between the two photosystems and to regulate the ratio of linear and cyclic electron flows [16]. Linear electron transfer produces ATP and NADPH, while cyclic electron transfer driven by PSI produces only ATP. Increasing the antenna size of the PSI complex facilitates cyclic electron transfer and has been shown to enhance ATP production and support the optimal growth of *Chlamydomonas* [16,32,33]. Thus, LHCII minus strains would presumably have an impaired ability to synthesize ATP by cyclic photophosphorylation. To assess the impact of reduced LHCII content on the ability to carry out state transitions, Chl fluorescence induction kinetics were measured in low-light grown WT, *cbs3* and CR cells that were either dark adapted, pre-illuminated with PSI (715 nm), or pre-illuminated with PSII (650 nm) light. PSI light pre-illumination promotes LHCII migration from PSI to PSII while PSII light does the opposite. An increase in the PSII antenna size would accelerate Chl fluorescence rise kinetics and increase the maximal Chl fluorescence

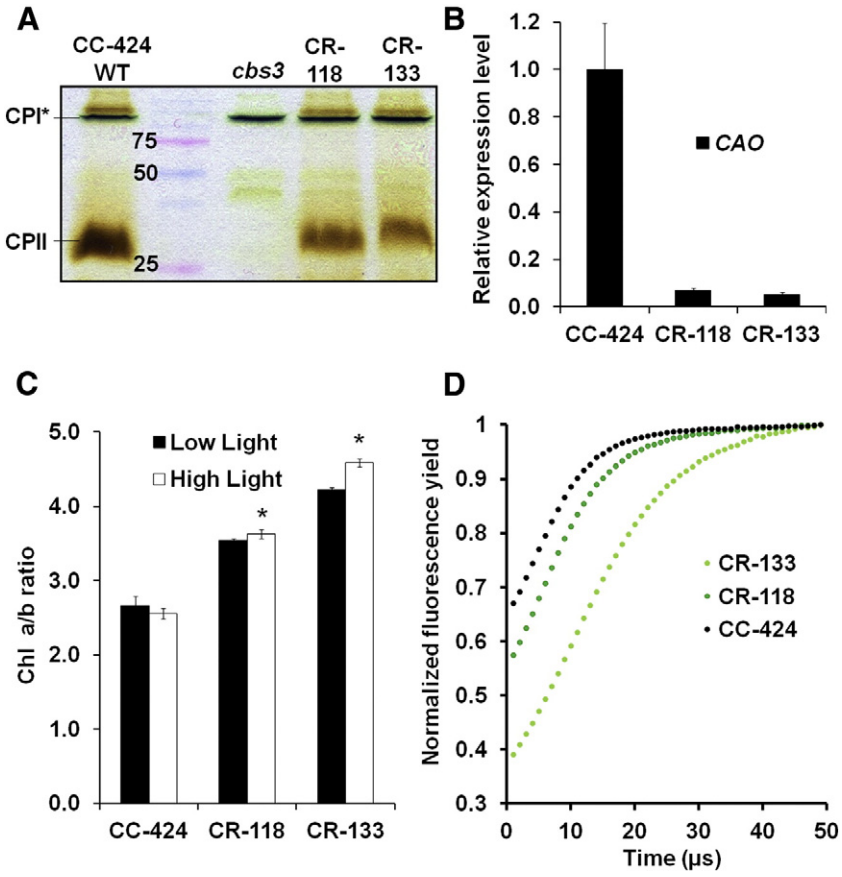

Fig. 2. Analysis of peripheral LHCII content, *CAO* transcript levels, Chl *a*/*b* ratios and Chl fluorescence induction kinetics in parental (CC-424) and Chl *b* reduced transgenics (CR). (A) LHCII abundance on non-denaturing PAGE. (B) Real-time PCR analysis of *CAO* transcript levels in CC-424 and CR strains. (C) Chl *a*/*b* ratios of CR strains grown in low (50 µmol photons m^{-2} s^{-1}) and high (500 µmol photons m^{-2} s^{-1}) light. The asterisk (*) indicates a significant difference in the two light conditions determined by Student's *t*-test, with ($p<0.01$). (D) Chl fluorescence induction kinetics of high-light grown CR transgenics. Chl fluorescence levels were measured under continuous, non-saturating illumination every 1 µs.

level at sub-saturating light intensities. As expected, CR and WT strains had faster Chl fluorescence rise kinetics and achieved greater maximum Chl fluorescence levels following pre-illumination with PSI light (Fig. 4). However, no observable increase in Chl fluorescence yield was observed in the *cbs3* strain following pre-illumination with PSI light, indicating that *cbs3* lacked the ability to carry out state transitions (Fig. 4). The absence of LHCII and state transitions and presumably diminished potential for cyclic photophosphorylation and ATP synthesis, may partially account for the impaired photoautotrophic growth of *cbs3*.

3.4. High light grown CR strains have an increased level of photoprotective pigments

The peripheral PSII antenna binds an array of carotenoids involved in energy capture or dissipation. Under high light intensities acidification of the chloroplast lumen activates de-epoxidases that convert violaxanthin into zeaxanthin. Violaxanthin transfers energy to Chl facilitating light harvesting at low light intensities while zeaxanthin dissipates excess Chl excited states at high light as heat [34]. We hypothesized that modulating LHCII content in the CR and *cbs3* transgenics would also alter cellular carotenoid abundance and composition at different light intensities. To examine the effects of reduced antenna size on carotenoid levels, we carried out pigment analyses of low and high light grown strains (Fig. 5 and Figs. S3, S4). As expected, we observed a decrease in carotenoid levels in low-light grown CR (76–80% of WT) and *cbs3* (76% of WT) strains (Fig. S4). The high-light grown CR parental WT strains had a 2.8 and 3 fold increase in antheraxanthin and zeaxanthin pools respectively, compared to low-light grown cells (Fig. 5A). However, high-light grown CR lines displayed a 15–30% increase in de-epoxidation status (antheraxanthin + zeaxanthin / violaxanthin + antheraxanthin + zeaxanthin) compared to their WT parental strain. Hence, even greater increases in the levels of antheraxanthin and zeaxanthin were observed in high-light grown CR-118 (5 and 5.6 folds) and CR-133 (5.3 and 6.8 folds) than in its parental (CC-424) WT (3 folds), which is indicative of a more active xanthophyll cycle in the CR transgenics (Fig. 5B and C). Further, a 1.2 fold increase in lutein content was observed in high-light grown CR-133 relative to low-light grown cells (Fig. 5C). In contrast, the *cbs3* parent strain (CC-2677) had no change in its carotenoid de-epoxidation state or xanthophyll cycle carotenoid levels under high-light relative to low-light growth (Fig. 5D). However, the CC-2677 strain had higher beta-carotene (2 folds) levels when grown under high versus low-light growth conditions (Fig. S4), suggesting that this strain differs in its carotenoid regulation from the WT parent (CC-424) of the CR transgenics. Unexpectedly, high-light grown *cbs3* exhibited a 1.8 fold increase in its carotenoid de-epoxidation state compared to its parent (CC-2677) and had a 2-fold increase in zeaxanthin content, however, the total levels of de-epoxidated carotenoids were substantially lower in CC-2677 derived lines than in CC-424 derived lines (Fig. 5E and F). Similar to its parent strain, an elevation (2-fold) in beta-carotene levels was also observed in high-light grown *cbs3* relative to low-light growth (Figs. 5E, S4). Overall, the differences in carotenoid de-epoxidation

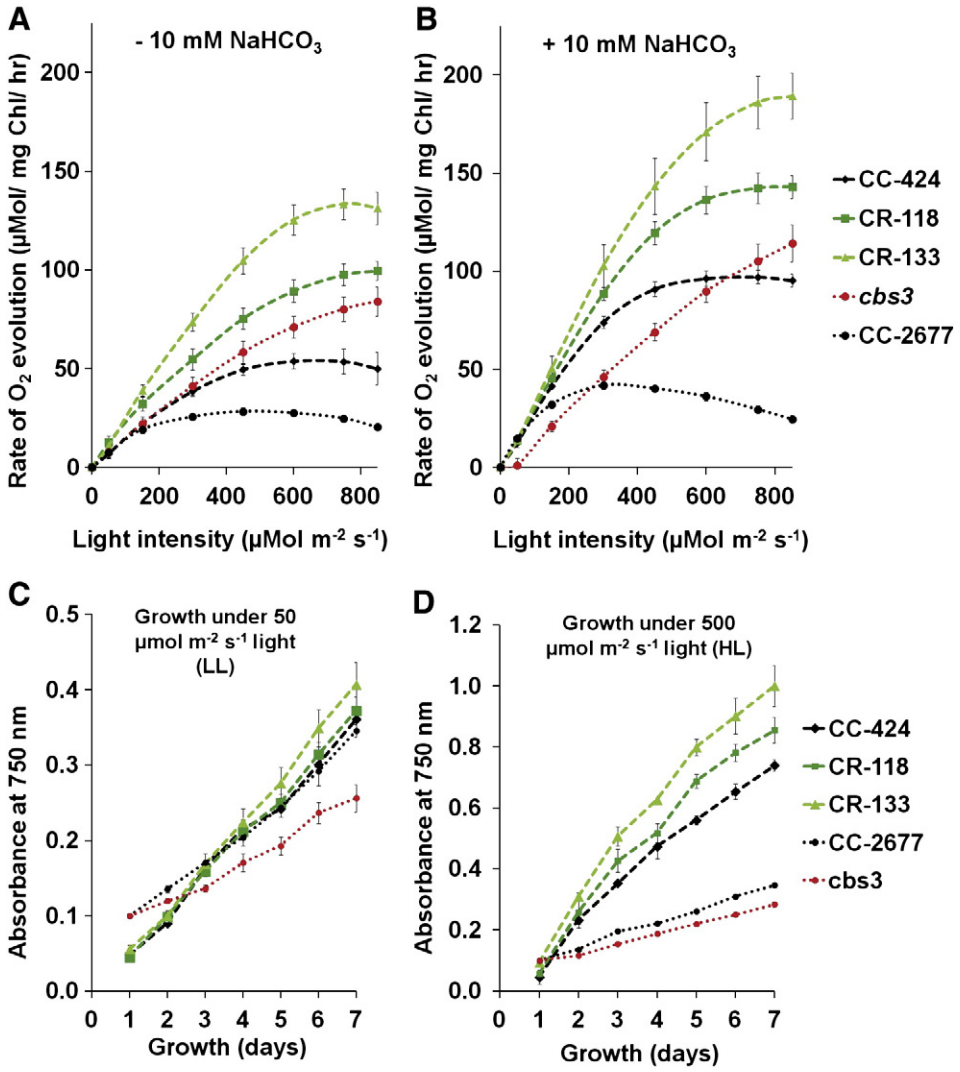

Fig. 3. Photosynthetic oxygen evolution and growth rates in Chl *b* reduced (CR), Chl *b* less (*cbs3)* and parental (CC-424 and CC-2677) strains. Light-dependent rates of photosynthesis for log-phase cultures grown photoautotrophically at 50 µmol photons m^{-2} s^{-1} measured in (A) the absence of $NaHCO_3$ or (B) presence of 10 mM $NaHCO_3$. (C) Photoautotrophic growth under limiting light intensities (50 µmol photons m^{-2} s^{-1}). (D) Photoautotrophic growth under saturating light intensities (500 µmol photons m^{-2} s^{-1}). Results represent the average and SE of three to four independent measurements.

levels observed in the truncated antenna mutants and WT parental strains indicate that xanthophyll cycle activity is not directly correlated with LHCII content in these particular *Chlamydomonas* strains.

4. Discussion

We demonstrate that modulating Chl *b* levels, which binds preferentially to peripheral antenna protein complexes, substantially alters the LHCII content (Figs. 1 and 2). We also show that there is an inverse relationship between Chl *a*/*b* ratios and the PSII antenna size (Fig. 1B). In the present work, truncation of LHCII was achieved through RNAi-mediated silencing of the Chl *b* synthesis gene resulting in transgenic algae with intermediate antenna size. Other strategies have also been used to modulate light harvesting antenna size including reduction in LHCII transcript and protein levels [8,10–15]. The overwhelming consensus that emerges from these studies is that mutants with smaller peripheral antenna size have increased light utilization efficiency since they do not saturate rate-limiting, downstream electron transfer processes. However, previous studies had not shown how increased light utilization manifests into photoautotrophic growth under low and high light intensities and what the optimal antennae size was for maximal growth across a range of light intensities. We show that the CR transgenics with intermediate antenna sizes grew at WT rates at low light intensities but had ~15 to 35% higher culture densities than their parental WT strain when grown at saturating light intensities (25% of full sunlight intensity) (Fig. 3). These studies indicate that at low light intensities the size of the peripheral antennae complex is more than sufficient to support the maximal rates of photosynthesis and that the reductions in antennae size within the range tested had no impact on algal growth rates. The large antenna absorption cross-section of wild type algae reduces available light for competing algal species providing a selective advantage even at very low light levels [35]. The trade off for having a large peripheral antennae complex is reduced photosynthetic efficiencies at high light intensities when electron transfer reactions are light saturated.

Previous studies have shown that enhanced cyclic photophosphorylation, associated with increased photosystem I excitation, is required to meet the demands of the inorganic carbon concentrating system and to support sufficient rates of photosynthesis for optimal growth [16,32]. The observation that in *Chlamydomonas*, 80% of the peripheral PSII antenna is involved in state transitions compared to 15–20% in *Arabidopsis* [36], lends further support to the idea that state transitions are critically important for the maintenance of

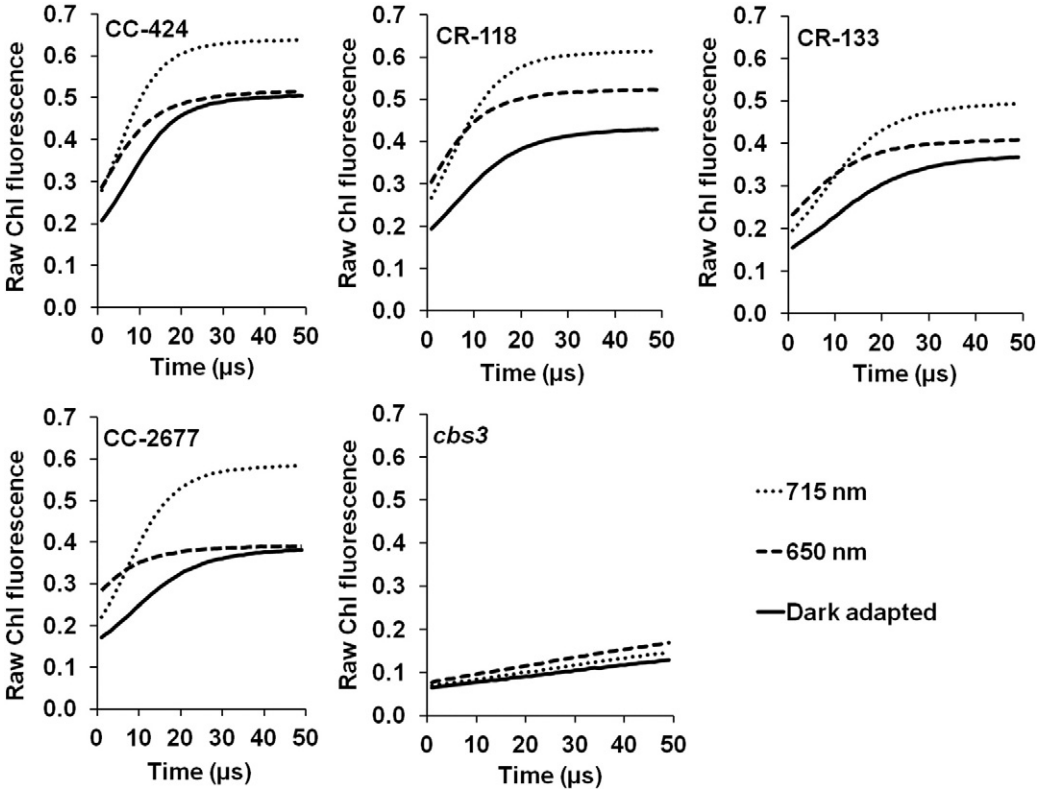

Fig. 4. Chl fluorescence induction kinetics of low-light grown Chl *b* reduced (CR), Chl *b* less (*cbs3*) and parental strains (CC-424 and CC-2677). Cultures were either dark adapted or pre-illuminated with 715 or 650 nm light prior to measurement. For Chl fluorescence induction measurements, Chl fluorescence was measured under continuous, non-saturating illumination every 1 μs.

intracellular ATP levels in *C. reinhardtii* [32] to support the additional ATP demands for active bicarbonate import. Unlike the *cbs3* mutant, the intermediate antennae size CR transgenics retained the ability to carry out state transitions and presumably high rates of cyclic ATP synthesis (Fig. 4). The inability of the *cbs3* strain to carry out state transitions and presumably to support high rates of cyclic photophosphorylation could partially account for its poor photoautotrophic growth relative to the CR transgenics.

Photosynthetic organisms have evolved a variety of strategies to reduce photodamage under saturating light conditions. In excess light, a reduction in the thylakoid lumenal pH activates a rapid and reversible de-epoxidation of violaxanthin to antheraxanthin and zeaxanthin [37,38]. The accumulation of zeaxanthin under high light stress helps reduce photo-damage by, 1) quenching excess Chl excited states through NPQ, 2) scavenging reactive oxygen species, and 3) reducing lipid peroxidation [34]. Further, it is known that the photoprotection by zeaxanthin can occur in the absence of LHCII, although it is enhanced by LHCII [39,40]. Lutein is another photoprotective carotenoid present in LHCII complexes which also plays a vital role in quenching Chl triplets [34,41]. Lutein deficient mutants of *Arabidopsis* have smaller antenna size, reduced LHCII trimer stability, lower levels of NPQ, and impaired state transitions [42,43]. Conversely, *Arabidopsis* lycopene-ε-cyclase mutants that over-accumulate lutein have increased NPQ levels and photoprotection [44]. In the present study, substantial reductions in lutein levels were only observed between the low-light and high-light transitions for the *cbs3* mutant, indicating that the absence of LHCII can impact lutein steady-state levels. Higher levels of xanthophyll cycle carotenoids have also been correlated with higher rates of photosynthetic recovery following photoinhibition and superior levels of biomass production in 'super high yield' cultivars of rice [45,46]. The increased accumulation of the photoprotective pigments zeaxanthin and lutein observed in the CR transgenics relative to their parent strain (CC-424) under high-light growth conditions (Fig. 5) likely contributes to their enhanced growth under high-light conditions (Fig. 3D). Unexpectedly, the presence of intermediate LHCII levels in the CR transgenics apparently facilitates zeaxanthin cycle activity. In green algae, the LHCSR3 protein is known to accumulate in response to high light stress conditions [47]. LHCSR proteins bind Chl *a*, *b* and xanthophylls, particularly lutein and zeaxanthin and become protonated at low pH helping to quench excess Chl excited states [47]. The fact that the CR transgenics retain some level of Chl *b* suggests that the photoprotective function of LHSCR3 is less likely to be impaired than in the LHCII minus strains. The mechanism for the enhanced zeaxanthin levels in the CR transgenics relative to their parental strain, however, remains unknown at the present time.

Collectively, these findings re-affirm the hypothesis that truncation of the peripheral LHCII light harvesting complex in green algae leads to increased photosynthetic energy conversion efficiency by reducing flux constraints between light capture and linear electron flow at high light intensities. However, unlike algae that lack the PSII peripheral antenna, the CR transgenics retain the photoprotective functions of the antenna and to quench excess potentially damaging Chl excited states and combine improved photon capture and energy conversion with the ability to dynamically regulate light distribution between the photosystems to support cyclic photophosphorylation.

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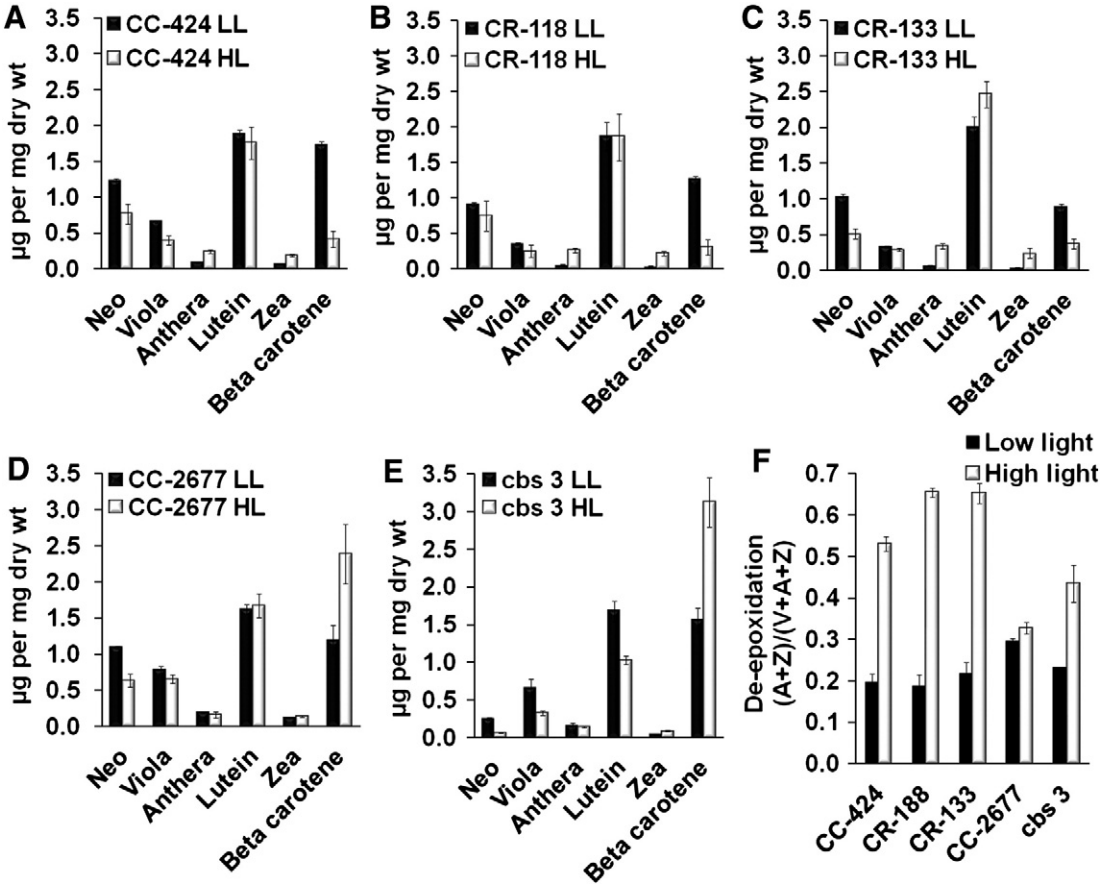

Fig. 5. Carotenoid levels of low (50 µmol photons m^{-2} s^{-1}) and high light (500 µmol photons m^{-2} s^{-1}) grown Chl *b* reduced (CR), Chl *b* less (*cbs3*), and parental (CC-424 and CC-2677) strains. Cells were grown in low and high light intensities for 5 days and pigments (Neo—neoxanthin, Viola—violaxanthin, Anthera—antheraxanthin, Lutein, Zea—zeaxanthin, and beta carotene) were analyzed by HPLC. (A) CC-424 (CR parent), (B) CR-118, (C) CR-133, (D) CC-2677 (*cbs3* parent), (E) *cbs3*. (F) De-epoxidation status in low and high light. Results are the average and SE of three independent experiments.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.algal.2012.07.002.

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