RNAi Knock-Down of LHCBM1, 2 and 3 Increases Photosynthetic H₂ Production Efficiency of the Green Alga Chlamydomonas reinhardtii

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Abstract

Single cell green algae (microalgae) are rapidly emerging as a platform for the production of sustainable fuels. Solar-driven H₂ production from H₂O theoretically provides the highest-efficiency route to fuel production in microalgae. This is because the H₂-producing hydrogenase (HYDA) is directly coupled to the photosynthetic electron transport chain, thereby eliminating downstream energetic losses associated with the synthesis of carbohydrate and oils (feedstocks for methane, ethanol and oil-based fuels). Here we report the simultaneous knock-down of three light-harvesting complex proteins (LHCBM1, 2 and 3) in the high H₂-producing Chlamydomonas reinhardtii mutant Stm6Glc4L01 using an RNAi triple knock-down strategy. The resultant mutant exhibited a light green phenotype, reduced expression of LHCBM1 (20.6% ± 2.7%), LHCBM2 (81.2% ± 0.037%) and LHCBM3 (41.4% ± 0.05%) compared to 100% control levels, and improved light to H₂ (180%) and biomass (165%) conversion efficiencies. The improved H₂ production efficiency was achieved at increased solar flux densities (450 instead of ~100 μE m⁻² s⁻¹) and high cell densities which are best suited for microalgae production as light is ideally the limiting factor. Our data suggests that the overall improved photon-to-H₂ conversion efficiency is due to: 1) reduced loss of absorbed energy by non-photochemical quenching (fluorescence and heat losses) near the photobioreactor surface; 2) improved light distribution in the reactor; 3) reduced photoinhibition; 4) early onset of HYDA expression and 5) reduction of O₂-induced inhibition of HYDA. The Stm6Glc4L01 phenotype therefore provides important insights for the development of high-efficiency photobiological H₂ production systems.

Introduction

The development of clean fuels for the future is one of the most urgent challenges facing our society for three reasons; to reduce CO₂ emissions, increase energy security and enable sustainable economic development. The importance of fuels is emphasized by the fact that, they directly supply ~80% of global energy demand while electricity provides only ~17% [1]. In terms of fuel security it is notable that in 2010 global energy use rose to ~0.5 ZJ (1 ZJ = 10²¹ J) [2] and that the total global fuel reserves (oil, coal, gas and uranium) were reported at only ~82 ZJ [3].

The capability to produce sufficient renewable fuel to supply global demand is dependent on the availability of a renewable energy source that is large enough to drive this process. Solar energy is by far the largest and most equitably distributed renewable energy source available, supplying ~3020 ZJ yr⁻¹ to the Earth’s surface [4]. Photosynthetically active radiation (PAR – conventionally 400≤λ≤700 nm [5]) makes up 43% of the irradiance delivered by the standard AM1.5 reference solar spectrum [6]. Therefore ~1300 ZJ yr⁻¹ is available globally to drive photosynthesis. Photosynthesis is a solar-to-chemical-energy conversion process that can be used to produce biomass and biofuels. Economic feasibility of photosynthetic fuel production systems is not limited by global solar irradiance, but rather by the areal productivities currently achievable cost-competitively. These depend strongly on the energy conversion efficiency of photosynthesis, which can be improved through engineering.

Microalgae systems are rapidly emerging as one of the most promising platforms for the production of renewable fuels and have a number of advantages compared to higher plants. In particular, they can theoretically address the key areas of concern related to the food vs. fuel conflicts of 1st generation biofuel systems.
(e.g. corn ethanol) [7,8] and assist with the development of a more sustainable food and fuel future. This is because well-designed microalgae systems can be located on non-arable land, cultivated at least in part using saline and waste water, enable increased nutrient recycling and achieve higher yields than crop plants, due to the ability to optimize light distribution, CO₂ supply and production conditions [9]. Importantly, algae, besides being able to produce biomass and oil which can be converted into bio-fuels such as bio-diesel, methane and ethanol, also have the ability to produce hydrogen from water [10]. Solar powered H₂ production is theoretically the most efficient method of biofuel production as it is closely coupled to the photosynthetic electron transport chain and additionally offers the potential to produce a CO₂ - negative bio-fuel, reducing CO₂ emission and assisting in carbon sequestration [11].

The first step of photosynthesis and all biofuel production is light capture. As a result, its optimization is essential for the development of all high-efficiency microalgal processes. In algal cells, light capture is performed by the chlorophyll-binding proteins of the Light Harvesting Complexes I (LHCII) and II (LHCII), which transfer the derived excitation energy to Photosystems I (PSI) and II (PSII), respectively. A secondary but very important role of the LHCII (LHCBM) proteins is that under high light conditions, they are involved in the photoprotective processes of non-photochemical quenching (NPQ) [12–15].

Given the need for microalgae to balance light capture and protection against photodamage, wild type algae [16] have generally evolved large LHC antenna systems to capture incident light efficiently under low light conditions, and the ability to dissipate the excess (~80%) under high light conditions to avoid photodamage [16]. WT cells appear to have a competitive advantage, as they capture the bulk of the incident light at an illuminated surface and dissipate the excess, with the result that cells deeper in culture suffer from light limitation. Even though microalgae down-regulate LHCII levels naturally to a degree, under high light conditions, at 25% of full sunlight (full sunlight cells deeper in culture suffer from light limitation. Even though illuminated surface and dissipate the excess, with the result that advantage, as they capture the bulk of the incident light at an photodamage [16]).

Light that it requires but allow the excess light that would otherwise 'selfish organism strategy'; i.e. each cell uses the light it needs and wastes the rest as heat, which cannot be used to drive photosynthesis in other cells.

The overall efficiency of the culture can theoretically be improved, by artificially reducing the light-harvesting antenna size. This strategy is designed to enable each cell to capture the light that it requires but allow the excess light that would otherwise be wasted, to penetrate further into the culture. This illuminates the cells that would otherwise be shaded. This strategy can be therefore broadly likened to ‘social engineering’ in that the light is more evenly distributed between all cells in the culture. Thus, while the maximum light harvesting efficiency of individual cells should theoretically remain the same as long as the incident light exceeds saturating levels at the surface, the overall PBR efficiency can be increased. Due to the decreased energy wastage through NPQ, the concentration of algae cells can be increased, thereby improving the yield per unit volume of culture and reducing PBR costs. This offers a theoretical route to increase the efficiency and cost competitiveness of photo-biological fuel production.

In the green microalga C. reinhardtii, at least 25 LHC genes have been identified [18]. These exhibit a high degree of gene and protein sequence similarity across plant and algal families [14]. The LHCII proteins can be divided into the major and minor LHCII proteins. The major LHCII proteins are reportedly transcribed from 9 different genes LHCBM 1–9 which are numbered according to the relative expression levels initially observed [19,20]. These LHCBM proteins are reported to trimmerize and their primary role is to capture solar energy and funnel it via the minor LHCII proteins, CP29 (LHCb⁴) and CP26 (LHCb⁵), to the PSII core (CP47, CP43, D1, D2, cyt b555, PSBO and additional small and extrinsic subunits). In PSII solar energy is captured by nine LHCA proteins (LHCA1-9) and the derived excitation energy channeled to the PSI reaction center (psaA and psaB) [14,21–23]. In higher plants the LHCI subunits reportedly form a crescent shaped belt on one side of the PSI monomer. This is termed the “LHCl-belt”, and reportedly consists of two dimeric subcomplexes. These subcomplexes are referred as LHClI-730 and LHClI-680 in higher plants and LHClI-705 and LHClI-680 in Chlamydomonas, where the numbers refer to their characteristic fluorescence peaks [24,25].

Melis and coworkers and later others [16,26–28] tested the hypothesis that artificial antenna reduction could improve light-to-biomass conversion efficiency and showed improvement under lab conditions. However, none of these strains were optimized for improved levels of H₂ production in liquid culture.

Hydrogen production is typically induced by sulfur deprivation [10,29]. Under these conditions the repair of the photo-inhibited D1 protein of PSII which contains methionine (and so S) is inhibited, reducing O₂ production. This in turn lifts the inhibition of HYDA expression, inducing H₂ production. Down regulation of PSII however also reduces the yield of H⁺ and e' from H₂O to H₂.

Here we report the specific triple knock-down of the three most abundant LHClII proteins (LHCBM1, LHCBM2 and LHCBM3) in the high H₂ producing background of Stm6Glc4 [30], with the aim of further increasing the efficiency of photobiological H₂ production. Stm6Glc4 is based on the Stm6 mutant, which under H₂-producing conditions was shown to have upregulated Alternative Oxidase (AOX) activity [31] and to be locked into linear electron transport [32], resulting in increased O₂ consumption and linear electron pathway from water to HYDA. The results show that reducing the LHC antenna system allows the rate of O₂ production by photosynthesis to be brought into balance with the rate of O₂ consumption by respiration. This in turn facilitates the induction of HYDA and provides a mechanism for continuous photosynthetic H₂ production from water.

Materials and Methods

Strains and Culture Conditions

The high H₂ producing C. reinhardtii strain Stm6Glc4 described in [30] and Stm6Glc4LO1 were cultivated under mixotrophic (TAP [pH 7.2] [33] and photoautotrophic conditions (PCM (Photoautotrophic Chlamydomonas Medium) (pH 7), NH₄Cl [30 mM], CaCl₂*2H₂O [850 μM], MgSO₄*7H₂O [1.5 mM], KH₂PO₄ [10 mM], FeSO₄*7H₂O [1 μM], CuSO₄*5H₂O [4.6 μM], MnCl₂*4H₂O [7.25 μM], ZnSO₄*7H₂O [77 μM], H₃BO₃ [184 μM], Na₂MoO₄*2H₂O [0.09 mM], CoCl₂*6H₂O [6.7 mM], Na₂SeO₃ [0.1 mM], VO₃*H₂O [0.009 mM], Na₂SO₄*5H₂O [273 μM], Na₂EDTA [537.3 μM], Tris [100 mM], Vitamin B1 [52 μM], Vitamin B12 [0.1 μM]) under continuous white light (50 μM m⁻² s⁻¹), unless stated otherwise. Positive transformants were selected and cultivated under low light conditions (10 μM m⁻² s⁻¹) on TAP agar plates supplemented with 1.5 mM tryptophan, 60 μM 5-fluoroindole (5-FI) and hygromycin [30].

Growth Rate μ_max

To identify conditions yielding maximum microalgae growth rates in TAP and PCM, a number of factors were tested including...
light intensity (low light: 35 μE m⁻² s⁻¹ and high light: 450 μE m⁻² s⁻¹), culture volume (100, 112.5, 125, 137.5 and 150 μL), trials corresponding to a culture depth of 2.7, 3, 3.5, 4 and 5 mm, respectively) and inoculation density (OD_{750} = 0.1 and 0.3). These experiments were conducted in a specially designed robotic Tecan Freedom Evo system which was fitted with three microwell shakers each capable of holding six 96 well microwell plates, a bank of fluorescent lights positioned 1.2 m from the microwell surface and a CO₂ controller which maintained CO₂ concentrations at 12±0.2% CO₂ during photoautotrophic conditions. The lights consist of 12 cool white fluorescent lights (Philips PL-L53W/840 Cool White, Philips International B.V. Netherland) and 11 warm white fluorescent lights (Philips PL-L55W/830 Warm White, Philips International B.V. Netherland) which were installed in an alternating arrangement. The chamber temperature was 33 ± 1°C. Algal cultures were adjusted to the desired OD_{750} (OD_{750} = 0.1 and 0.3, respectively) in TAP or PCM (supplemented with 1.5 mM tryptophan and 60 μM 5-FI for Stm6Glc5L01) and aliquoted into microwell plates. OD_{750} measurements were recorded at 3 h intervals using a dedicated spectrophotometer (Tecan Infinite D) and fragment B combination was cloned as a  cohesive sequence to fragment A (bp 1033–1160) with sub-
sequent EcoRI and SacI restriction sites. A linker region taken from LHCBM2 (bp 11–103) with a preceding SacI site and fragment B were amplified and joined via multiple template PCR. The linker and fragment B combination was cloned as a SacI fragment into SacI site downstream of fragment A to complete the inverted repeat sequence. The LHCBM3 target sequence was synthesized from bp 628–728 of the LHCBM3 5’UTR followed by a BamHI site, a linker of LHCBM3 5’UTR (bp 255–404) and the reverse oriented 5’UTR sequence (bp 255–404) was synthesized. Additional cloning details are provided in the supplementary materials (Figure S1). The inverted repeat sequences were flank- ed by EcoRI restriction sites to facilitate subsequent cloning into the transformation vector pBDH-R, and yielded vectors pMO75, pMO53 and pMO75, respectively. To create pBDH-R the Ribulose-1,5-bisphosphate-carboxylase/-oxyge
nase small subunit (RBCS) promoter, the intron fragment (with the start codon deleted) of RBCS was produced via fusion PCR and cloned into the XhoI and MluI site of pALKK1 [27] to replace its RBCS promoter/ ATG/intron/BLE region. This deletes an undesired BLE gene fragment present in pALKK1. To produce the triple knock-down strains, equal amounts of each vector pMO52, pMO53 and pMO75 were mixed and transformed into the strain Stm6Glc4 [30] using biolistic bombardment [35].

**RNA Isolation, cDNA Synthesis and Quantitative Real-time PCR (qRT-PCR)**

Isolation of total RNA was performed using a PureLink™ RNA Mini kit (Invitrogen) following the protocol optimized for animal and plant cells according to manufacturer’s directions. As Stm6Glc4 is a cell wall deficient cell line the homogenization step was not carried out. DNase treatment was performed according to the PureLink™ RNA Mini kit manual using RQ1 RNase-free DNase (Promega). First strand cDNA synthesis was performed using iScript™ cDNA Synthesis kit (Bio-Rad Laboratories) and 1 μg total RNA according to manufacturer’s instructions. Quantitative real-time PCR analysis was carried out in triplicate using an Applied Biosystem 7500 Real Time PCR System with software SDS version 1.2.3 and the standard method ‘absolute quantifica-
tion’ two step RT-PCR for thermal profile and dissociation stage (stage 1:1 replication at 50°C, 1 min; stage 2:1 replication at 95°C, 10 min; stage 3:40 replications at 95°C, 15 sec; stage 4: dissociation; 1 replication at 95°C, 0.13 sec; 60°C, 1 min; 95°C, 0.13 sec). The experiment was carried out on a 96 well plate and each reaction contained 7.5 μL SYBR® Green Master Mix (Applied Bioscience), 5 μL of cDNA [1.6 ng μL⁻¹], 2 μL of each primer [2 μM] and 3.5 μL of water. Relative expression levels were normalized using the C. reinhardtii reference gene CBLP [36]. Primer sequences for LHCBM1, LHCBM2, LHCBM3 and CBLP were designed as reported previously [27,36].

**FACS Analysis and Chlorophyll Measurements**

Cells were grown to late log phase in TAP liquid culture (OD_{750} = 1.0), 1 mL of the culture was used for analysis in a FACSCanto II Flow Cytometer (Becton Dickson). Comparative chlorophyll fluorescence data was recorded using a 488 nm blue excitation laser and the 670 nm long pass detector to measure emission.
Total chlorophyll concentration was measured according to previously reported methods [37]. 1 mL of algae culture was sampled at late log phase (OD_{750} = 1.0). The cells were then pelleted (500xg, 5 min, 20°C) and the clear supernatant discarded before being resuspended in 1 mL of ice cold 80% acetone. After vortexing the sample, the precipitate was pelleted (14100xg, 4 min) before measuring the absorption (A) of the supernatant. The A_{470} was set to zero and A_{647} and A_{664} measured subsequently. Chlorophyll content was calculated as previously described [37].

**Demonstration of Algal Fluorescence at Different Depths**

*Stm6Glc4L01* and *Stm6Glc4* were adjusted to same cell density (7.5×10^5 cells mL\(^{-1}\)) and transferred to a 100 mL glass cylinder positioned on a Safe-Imager™ blue-light transilluminator (Invitrogen) fitted with light emitting diodes producing a narrow emission peak centered at ~470 nm. Chlorophyll fluorescence was visualized by an ‘amber’ (530 nm long path) filter (Invitrogen).

**H₂ Volume and Gas Composition Measurements**

For H₂ production *Stm6Glc4L01* and *Stm6Glc4* were grown to late log phase in TAP medium. Cells were harvested, washed twice with sulfur-free TAP medium and resuspended in sulfur-free TAP medium containing glucose [1 mM] [30] and adjusted to the same chlorophyll concentration (14.5 µg mL\(^{-1}\)). H₂ measurements were performed under continuous white light (450 µE m\(^{-2}\) s\(^{-1}\)) using a custom-built PBR system with a dedicated gas collection tube mount [32]. The evolved gas was sampled using a gas-tight Hamilton syringe and injected at regular intervals into a gas chromatograph (Agilent Micro GC3000) fitted with a PlotU pre-column (3 m x 0.32 mm) and MolSieve 5APlot column (10 m x 0.32 mm). Argon (32.5 psi, pound per square inch) was used as the carrier gas and H₂, O₂ and N₂ concentrations were monitored.

**Results**

**Identification of LHCBM Target Sequences**

To develop specific LHCBM knock-down strains, the identification of highly specific target sequences is essential. As all *LHCBM* genes display a high degree of sequence similarity [27], genomic BLAST analysis of the three target mRNAs, encoding the major light harvesting complex proteins of PSII (*LHCBM1, 2* and *3*), was performed. This analysis aimed to identify target regions that had minimal similarities to other genes and so prevent their co-suppression. Based on this analysis, discrete regions for each single *LHCBM* target (between 95–100 bp) were chosen for the development of RNAi constructs (sequences see Figure S1). To minimize non-specific RNAi effects further, the *LHCBM1*, *2* and *3*-specific RNAi constructs were engineered so that the linkers positioned between the sense and anti-sense regions of the construct were specific to the respective target genes. The plasmid pALKK1 [27] was used to create the transformation vector pBDH-R (Figure 1A), additionally providing an RNAi target site for the endogenous tryptophan synthase. This allowed direct selection of positive transformants on media plates containing 5-fluorotryptophan (5-FI), as tryptophan synthase converts 5-FI into the toxic tryptophan analogue 5-fluorotryptophan [38].

**Development and Selection of Positive Transformants**

Single *LHCBM* knock-out or knock-down transformants have not to date shown any marked phenotype in terms of chlorophyll concentration. This suggests that the knock-down of one LHCBM may be compensated for by the over expression of the remaining LHCBM proteins [39,40]. Consequently a triple *LHCBM1/2/3*
A knock-down strategy was tested. LHCBM1, 2 and 3 were selected as they are reported to be the most abundant LHCB proteins and so would be expected to achieve the greatest amount of antenna reduction [19,20]. To develop triple knock-down mutants of LHCBM1, LHCBM2 and LHCBM3, equal amounts of the target vectors were mixed and used to transform the high H₂ producing C. reinhardtii strain SimGlc4 [30] using biolistic bombardment [33]. Positive transformants were selected on agar plates containing 5-FI since, as based on the vector design, it was expected that transformants with knocked-down tryptophan synthase, and thus resistance to 5-FI, would co-suppress the desired LHC protein. This strategy enabled the testing of all possible combinations of LHCBM1, LHCBM2 and LHCBM3 knock-downs, with the low-antenna (light green) phenotype as the secondary selection criterion. Consistent with our hypothesis that the simultaneous knock-down of these major LHCs could result in a marked reduction in chlorophyll concentration, a number of light green LCHBM1/2/3 multiple knock-down transformants were identified, of which the mutant with the palest phenotype (designated SimGlc4L01 to signify light harvesting mutant combination 01) was chosen for further characterization.

LHC mRNAs and Phenotypic Characterization

Total mRNA levels of LHCBM1, LHCBM2 and LHCBM3 in SimGlc4L01 were determined using quantitative real-time PCR (qRT-PCR) to establish which of the target LHCBM genes were primarily affected. To confirm that equal amounts of cDNA were used in this experiment, data were compared with those of 18S RNA and the mRNA for endogenous C. reinhardtii gene CBLP (Chlamydomonas B-subunit like polypeptide), which was previously shown to be stably expressed throughout growth and H₂ production [36]. Data normalized to CBLP (Figure 1B) showed that LHCBM1 has been down-regulated to 20.6% ± 0.27% and LHCBM3 to 41.4% ± 0.05% of their original levels, respectively. The down-regulation of LHCBM2 was less dramatic (81.2% ± 0.037%) but importantly, LHCBM2 levels did not increase to compensate for the knock-down of LHCBM1 and LHCBM3. As gene dosage compensation has been suggested to be responsible for the unaffected phenotype of single antenna protein knock-outs [39–41], it is possible that the RNAi construct targeting LHCBM2 functions primarily to limit its expression level, preventing such compensation. Data with absolute expression levels in addition to the fold-differences relative to CBLP (Figure 1B) are provided (Table S1). The similarity of expression levels for 18S and CBLP genes between SimGlc4 and SimGlc4L01 suggests that the reduction in LHCBM1 and -3 levels is both meaningful and significant.

Light micrographs of SimGlc4 and SimGlc4L01 recorded under identical conditions (Figure 1C) showed that SimGlc4L01 cells contained considerably lower chlorophyll levels than the SimGlc4 parental control, consistent with the observed down-regulation of LHCBM1, LHCBM2 and LHCBM3 mRNA (Figure 1B) in a manner similar to that previously reported for other antenna mutants [16,26,27]. Auto-fluorescence images (Figure 1D), support the optical microscopy results and confirm that SimGlc4L01 accumulates less chlorophyll per cell, as fluorescence levels are significantly lower than in SimGlc4. The increased chlorophyll a/b ratio (Mean: SimGlc4:2.29 ± 0.05 and SimGlc4L01:2.62 ± 0.09; P-value <0.0021 [37]) is indicative of a specific reduction in the ratio of ChlB-rich LHC proteins compared with the ChlA-rich PSII core complexes [42] confirming LHC antenna size reduction.

Fluorescence Properties and Chlorophyll Measurements

Flow cytometry (Figure 2A) was employed to quantify chlorophyll reduction in SimGlc4L01 compared to the parental strain SimGlc4. For this purpose over 10⁶ cells (2.9×10⁵ cells for SimGlc4; 2.79×10⁵ cells for SimGlc4L01, grown under 50 μE m⁻² s⁻¹ illumination to late log phase) were analyzed and chlorophyll fluorescence measured (670 nm long pass filtered). The relative fluorescence of the SimGlc4 control peaked at 13498 relative fluorescence units (RFU) cell⁻¹ compared to the 6750 RFU cell⁻¹ of SimGlc4L01. Mean fluorescence was measured for SimGlc4 at 15606 RFU cell⁻¹ and for SimGlc4L01 at 8951 RFU cell⁻¹ (where the gate was set as indicated by the red and black lines in Figure 2A and displayed in Figure 2B as percentage of SimGlc4L01 (100%). This suggests that the control cell line SimGlc4 exhibited ~75% higher levels of fluorescence losses than those observed in SimGlc4L01 mutant (100%). Furthermore as the red fluorescence emission observed is due to the transition from the lower energy state of chlorophyll to the ground state, a concomitant heat loss is also expected due to the transition from the higher to the lower energy state transition. Therefore SimGlc4L01 is expected to display less heat losses than parental strain SimGlc4.

Though in practice, fluorescence measured by flow cytometry may not be strictly linear with respect to chlorophyll content [43], it indicates that SimGlc4L01 exhibits a significant reduction compared to the parent strain. As the mean cell diameter of the two strains was similar (Figure 1C) a reduction in cell size cannot explain the reduced mean fluorescence in SimGlc4L01. The reduction in fluorescence in SimGlc4L01 is however consistent with the increased chlorophyll a/b ratio (SimGlc4:2.29 ± 0.05; SimGlc4L01:2.62 ± 0.09, Figure 2C) which is indicative of a reduction in the ratio of ChlB-rich LHC proteins compared with the ChlA-rich PSII core complexes [42] as well as the total reduction in chlorophyll concentration per cell (Figure 2D). The observation that the ChlA/B ratio has only increased from 2.29 to 2.62 is completely consistent with many independent observations including the following. The first is the fact that purified thylakoid membranes containing PSI-LHCII and PSI-LHCl typically have a ChlA/B ratio of about 1.9–2.3. The second is that purified PSI-core complexes from higher plants binding CP29, CP26 and CP24 (all of which bind ChlB), but which are almost completely devoid of LHCII and LHCl, have a ChlA/B ratio of ~7 [44]. The fact that the ChlA/B ratio of SimGlc4L01 cells is closer to 2.3 than 7 is therefore completely consistent with the fact that in addition to the ChlA/B binding proteins CP26 and CP29, the SimGlc4L01 cells also contain the full complement of LHCA ChlA/b proteins of which there are ~9 different types, as well as LHCBM4-9 and residual levels of LHCBM1-3. Collectively this explains why the ChlA/B ratio of SimGlc4L01 cells is significantly higher than that of the control (2.29+/−0.03 vs 2.62+/−0.09) but closer to 2.3 (very thylakoids) than 7 (purified PSI core complexes).

Furthermore a determination of the total chlorophyll content [μg mL⁻¹] showed that at the same cell number, total chlorophyll content was reduced by 50% in SimGlc4L01 compared to SimGlc4 (Figure 2D) (total chlorophyll content: SimGlc4:13.65 μg mL⁻¹, SimGlc4L01:6.77 μg mL⁻¹, P-value <0.0006).

Light Penetration

To measure whether the decrease in LHCBM protein expression level increased light penetration through a liquid culture column, thereby making more light available for cells deeper in the culture, auto-fluorescence of SimGlc4 and SimGlc4L01 at the same cell density was measured (Figure 3A). As expected, SimGlc4 cultures were a darker green color.
compared to those of *Stm6Glc4L01*, due to their larger LHC antenna. The cultures were then placed onto a blue light illuminator and chlorophyll auto-fluorescence imaged using an orange filter to block the blue background light. As illumination was provided from below, the height of the fluorescent column was indicative of the light penetration distance. The fluorescence throughout the *Stm6Glc4L01* culture column illustrates that light penetrated ~4 times further than in *Stm6Glc4* at the same cell density (Figure 3B).

**Growth Rates Under Mixotrophic and Photoautotrophic Conditions**

As light is both necessary and damaging for photosynthesis [45] as it is required to drive charge separation but in excess causes photoinhibition, the maximum rate of photochemistry is achieved at the point at which the photosystems are just saturated, assuming that light is the limiting factor. To minimize the area required for microalgae production and thereby also PBR costs, in theory systems should operate at the maximum flux density (high light) and the system poised at this optimal point by adjusting culture depth and cell density. Theoretically small antenna cell lines could allow more cells to be packed into a given unit volume before light becomes limiting and decrease both cell shading and photoinhibition. To test this hypothesis biomass production (as a proxy for chemical energy/biofuels) in form of maximum growth rates ($\mu_{\text{max}}$) of *Stm6Glc4* and *Stm6Glc4L01* cultures were measured under a range of culture depths and cell densities.

The growth rate of *Stm6Glc4* and *Stm6Glc4L01* was next determined under mixotrophic conditions (TAP medium) which are the best-established conditions for photobiological H$_2$ production. Experiments were conducted in microwell plates using three different volumes (100 mL, 125 mL and 150 mL) to simulate different culture depths (average depths of 2.7, 3.5 and 5 mm, respectively). Since an advantage of *Stm6Glc4L01* was expected primarily in high light conditions, growth was measured under 450 $\mu$E m$^{-2}$ s$^{-1}$ illumination (optimal light intensity for the parental control and *wt* strains is 50–100 $\mu$E m$^{-2}$ s$^{-1}$) with an initial inoculation OD$\text{\textsubscript{750}}$ of 0.1. Under all conditions *Stm6Glc4L01* showed a higher growth rate than *Stm6Glc4* (Figure 4A). In the shortest path length cultures (100 mL = 2.7 mm) *Stm6Glc4L01* showed ~3 times the growth rate of *Stm6Glc4* based on OD$\text{\textsubscript{750}}$ measurements. The increased growth rate could be due to reduced photoinhibition (due to the reduced LHC antenna size), improved light

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**Figure 2. Chlorophyll fluorescence and chlorophyll yield in *Stm6Glc4* vs. *Stm6Glc4L01*.** (A) Graph derived from flow cytometry analysis showing relative fluorescence units (RFU) per cell. Over 2.7*10^5 cells were analyzed for each cell line. RFU range used to determine the mean is indicated by the red and black bars. (B) Mean chlorophyll fluorescence in percentage normalized to *Stm6Glc4L01* (100%). (C) Chlorophyll a/b ratio of *Stm6Glc4* and *Stm6Glc4L01*. (D) Total chlorophyll content in microgram per milliliter culture. Chlorophyll measurements (C, D) show results of two independent experiments with 7 replicates in total.

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penetration and therefore increased total light-to-biomass conversion efficiency of the culture, or a combination of both factors. As the highest $\mu_{\text{max}}$ h$^{-1}$ values for Stm6Glc4L01 were observed in the shortest path length cultures (2.7 mm) and declined with increasing culture depth (Figure 4A) we concluded that path length and light distribution had a more dominant impact than photoinhibition. In contrast Stm6Glc4 showed no such trend. It was previously reported [31] that Stm6 (and hence Stm6Glc4) is light sensitive, demonstrated here by the poor growth displayed at very low cell densities, where no self-shading occurs at 450 $\mu$E m$^{-2}$ s$^{-1}$ (Figure 4A).

To investigate the growth behavior of Stm6Glc4 and Stm6Glc4L01 under photoautotrophic conditions, we performed a parallel experiment (Figure 4B) to determine the effects of culture depth (100 $\mu$L = 2.7 mm, 112.5 $\mu$L = 3 mm, 125 $\mu$L = 3.5 mm, 137.5 $\mu$L = 4 mm and 150 $\mu$L = 5 mm) at low (35 $\mu$E m$^{-2}$ s$^{-1}$) and high light levels (450 $\mu$E m$^{-2}$ s$^{-1}$).

Previous experiments in TP medium (TAP medium minus acetate) indicated that N and P were limiting and led to an early culture growth plateau. Consequently for this experiment, an improved in-
house medium was developed (Photoautotrophic *Chlamydomonas* Media, PCM) which supplied more nitrogen and phosphate, to ensure that these did not limit growth. All cultures were inoculated at a starting OD$_{750}$ of 0.3 to reduce light stress for *Stm6Glc4* at 450 µE m$^{-2}$ s$^{-1}$.

At a culture depth of 2.7 mm and 450 µE m$^{-2}$ s$^{-1}$ illumination, *Stm6Glc4L01* showed ~120% of the maximum growth rate observed for the parental strain *Stm6Glc4*. Further increases in depth accentuated the advantage of the *Stm6Glc4L01* phenotype, as the growth rate at 5 mm depth was >185% compared to parental strain *Stm6Glc4*. This result shows that a reduction in antenna size improved growth across the conditions tested but particularly in deeper and denser cultures. In contrast, under low light levels (35 µE m$^{-2}$ s$^{-1}$) growth rates of *Stm6Glc4* and *Stm6Glc4L01* were comparable, indicating that the reduced antenna did not disadvantage the cells.

To confirm the OD$_{750}$ measurements, direct biomass yields were also determined both for *Stm6Glc4* and *Stm6Glc4L01* grown in PCM under the best high light conditions tested (450 µE m$^{-2}$ s$^{-1}$, 5 mm culture depth, 1% CO$_2$ atmosphere). OD$_{750}$ was recorded at the starting point (0.113 for *Stm6Glc4*) and 0.169 for *Stm6Glc4L01* and at time intervals (19 h and 43 h) (Figure 4C). To produce sufficient biomass to allow accurately biomass determination, samples were harvested at 43 h (OD$_{750}$: *Stm6Glc4*:0.692 g and *Stm6Glc4L01*:0.803 g) and yielded 2.3 g L$^{-1}$ (*Stm6Glc4*) and 2.5 g L$^{-1}$ (*Stm6Glc4L01*) biomass dry weight, respectively. From this the biomass dry weight/OD$_{750}$ nm ratios of *Stm6Glc4* and *Stm6Glc4L01* were calculated to be 3.3 (2.3/0.692) and 3.1 (2.5/0.803) respectively. Based on these ratios, biomass yields at earlier time points were calculated Figure 4C. Although it appears initially that the differences in biomass yield at the 43 h time point are only approximately 10%, this was because the *Stm6Glc4L01* growth rate had started to plateau presumably due to exhausted nutrient resources. In contrast at the point of maximum growth (19 h) biomass concentrations were by this method calculated to be 0.818 g L$^{-1}$ (*Stm6Glc4*) and 1.35 g L$^{-1}$ (*Stm6Glc4L01*). This represents a 165% increase for *Stm6Glc4L01* over the control *Stm6Glc4* level (100%).

### Hydrogen Production

As photobiological H$_2$ production is the most efficient light-to-biofuel conversion strategy [46], the effect of specific LHC gene down-regulation in *Stm6Glc4L01* on H$_2$ generation was investigated. For this purpose a time course measuring H$_2$ production rates (mL H$_2$ L$^{-1}$ h$^{-1}$) in *Stm6Glc4* and *Stm6Glc4L01* cultures, adjusted to the same chlorophyll concentration (14.5 µg mL$^{-1}$), was carried out in sulfur deprived media to establish anaerobiosis (Figure 5). At constant chlorophyll concentration and identical volumes, the cell number of *Stm6Glc4L01* was approximately double the cell number of *Stm6Glc4* (*Stm6Glc4*:1×10$^6$ cells mL$^{-1}$; *Stm6Glc4L01*:2×10$^7$ cells mL$^{-1}$). H$_2$ production in the *Stm6Glc4L01* trials started almost immediately after transfer into sulfur deprived media in marked contrast to the ~22 h lag time of the *Stm6Glc4* control (Figure 5A). This suggests that intracellular O$_2$ concentrations were lower in *Stm6Glc4L01* than in *Stm6Glc4* and close to or below the induction threshold for HYDA expression. The reduced lag phase has marked operational benefits, and significantly increases the yield. The H$_2$ production rates of both strains peaked at 47 h after sulfur deprivation, with *Stm6Glc4L01* producing 6.25 mL H$_2$ L$^{-1}$ h$^{-1}$ compared to 3.31 mL H$_2$ L$^{-1}$ h$^{-1}$ for *Stm6Glc4* under the conditions tested. Both strains produced H$_2$ until 188 h after sulfur deprivation with a similar decline in both strains after peak production. Under the conditions tested *Stm6Glc4L01* produced a total volume of 361 mL ±27.6 mL H$_2$ per liter culture compared to 198 mL ±21.2 mL H$_2$ per liter culture produced by *Stm6Glc4*. *Stm6Glc4L01* therefore produced >180% more H$_2$ per unit chlorophyll than the *Stm6Glc4* control (Figure 5B), which via gas chromatography was determined to be >95% pure. These results clearly show that *Stm6Glc4L01* exhibited improved characteristics in terms of early onset and higher rates of H$_2$ production.

### Discussion

#### Photosynthetic Efficiency

Light capture is the first step of all microalgae-based fuel production and its optimization is therefore of fundamental importance for the development of high-efficiency processes. Wild type microalgae antennae have evolved intricate mechanisms to adapt to natural fluctuations in light levels and quality to maximize light capture under low light conditions and dissipate excess light under high light conditions. While algae possess the ability to down-regulate their antenna, even 25% of full sunlight is sufficient to saturate the photosynthetic capacity of most algal species [17]. Under such over-saturated light conditions for a well-mixed culture in an open raceway or closed PBR, energy losses of up to 90% can occur [47]. This is because cells near the illuminated

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**Figure 5.** H$_2$ production of *Stm6Glc4L01* and *Stm6Glc4*. H$_2$ production rate in mL h$^{-1}$ L$^{-1}$ algae culture (A) and total H$_2$ production in mL L$^{-1}$ culture were determined (B). Experiments were performed under sulfur deprivation and with cultures adjusted to same chlorophyll content. Data were compiled using 3 replicates. doi:10.1371/journal.pone.0061375.g005
surface dissipate excess solar energy via NPQ which is then no longer available to cells deeper in the culture and which are therefore light-limited. Consequently, while these mechanisms are finely tuned for adaptation to their natural environment, they are far from optimized for industrial microalgae production, which to minimize area requirement and photobioreactor (PBR) construction costs should ideally be established for high flux density conditions. Photobioreactors can be designed to achieve light dilution (e.g. to 10% of 2000 μE m⁻² s⁻¹), but due to increased complexity and material costs this increases photobioreactor expense. Antenna engineering has the advantage that it can theoretically improve production efficiency and reduce capital costs both of which are major economic drivers for this technology [48].

Given the large number of LHC proteins in eukaryotic photosynthetic organisms, a diversity of function among family members is implied. The generation of *Stm3LR3* [27] involved knocking-down all LHC classes and resulted in a low chlorophyll phenotype. It was however recognized during this initial antenna engineering step that some vital LHC functions might also be affected. This assumption was borne out by subsequent work [49] and the observation that *Stm3LR3* is moderately light-sensitive at low cell densities. For example, *CP29 (LHC4)* is essential for state transitions and for docking the LHCBM1, −2 and −3 trimers to PSII [49], while a role for *CP26 (LHC5)* in preventing photo-inhibition has also been described [13,15,49–51]. It is therefore likely that the pattern of LHC down-regulation displayed in *Stm3LR3* does not preserve all of the functions required to support growth at high light levels [40].

While the high degree of homology in LHcs reflects a common ancestry, it also suggests that specific functions likely reside in the small non-conserved regions. Consequently, precise engineering of specific LHC proteins is important to avoid undesired effects due to co-suppression of other genes. As PSII is the main site of light-induced damage, there is a strong correlation between PSII antenna size and photosynthetic productivity [47,52–54]. This supports the idea that the most abundant LHCII antenna proteins, LHCBM1, −2 and −3, are the most important targets for down-regulation. In this context it is of note that while the exact molecular mechanisms for photodamage of PSII are still under investigation (for review see [54]), it has been shown that reduced chlorophyll content plays a crucial role in preventing photodamage, and thus allows the cells to grow under higher light conditions [55], supporting our findings. The above experiments show that LHCBM1 (20.6% ±0.27%), LHCBM2 (81.2% ±0.037%) and LHCBM3 (41.4% ±0.05%) expression levels were effectively knocked-down (Figure 1B), resulting in the light green mutant *Stm6Glc4L01* (Figure 1C), which had a reduced total chlorophyll content (50% of parental strain (Figure 2D)) and an increased ChlA/B ratio (*Stm6Glc4*;2.29±0.05 vs. *Stm6Glc4L01*:2.62±0.09) (Figure 2C), confirming LHClII depletions. Down-regulation of the LHCII antenna system was further supported by chlorophyll auto-fluorescence measurements obtained by flow cytometry (Figure 2A and B). Due to its reduced antenna size, cultures of *Stm6Glc4L01* exhibited considerably improved light penetration (Figure 3B) over those of the control (*Stm6Glc4*) at the same cell density. Our data also indicates that the improved light penetration is in part due to reduced fluorescence and heat losses at the illuminated culture surface (Figure 2B) and in part due to the reduced chlorophyll content of each cell (Figure 2D) which improves light transmission through the culture (Figure 3B). Together these properties resulted in an increased fraction of cells in the culture being poised close to the optimum point of light saturation (achieving maximal rates of photochem-
H₂O from mitochondria). This allows photosynthetic electron transport to continue to operate, providing an alternative pathway for the production of ATP and NADPH to enable cell survival (11).

In the \textit{wt}, only small amounts of H₂ are typically produced as the large antenna phenotype increases oxygen production in illuminated cells, which is not removed quickly enough by \textit{wt} respiration levels and so leads to a reduction in HYDA transcription and function. Typically, anaerobiosis can be established in \textit{wt} algae cultures through sulfur depletion. This is because the Methionine containing D1 protein of PSII, which is rapidly photo-damaged under high light conditions, can only be repaired in the presence of sulfur. Consequently, by limiting sulfur, PSII-mediated O₂ evolution is gradually reduced over time. When it drops below the rate of O₂ uptake by mitochondrial respiration, the system becomes micro-oxic or anaerobic and induces HYDA expression [58].

The parental strains of \textit{Stm6Glc4L01} are \textit{moc1} mutants (i.e. \textit{Stm6} and \textit{Stm6Glc4} [30–32]). Both have an increased respiration rate due to upregulated AOX activity (high O₂ consumption), but also have a large \textit{wt} like PSII antenna system (Figure 6A). As a result of their increased rate of respiration \textit{Stm6} and \textit{Stm6Glc4} exhibit a higher rate of oxygen consumption than the \textit{wt} and so enter anaerobiosis faster under sulfur deprivation. This in turn results in earlier activation of HYDA expression [59] compared to the \textit{wt} leading to the rapid onset of H₂ production. However the

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure6.png}
\caption{Mechanistic model of improved H₂ production in \textit{Stm6Glc4L01}. (A) \textit{Stm6Glc4} has a large PSII antenna system consisting of LHCBM1-9. LHCBM1-3 are reported to be most abundant. Large antenna size results in increased PSII mediated O₂ production and NPQ losses. NPQ losses reduce system efficiency; intracellular O₂ levels inhibit expression of HYDA until the system is sulfur deprived (sulfur required for the repair of the PSII-D1 subunit. B: \textit{Stm6Glc4L01} has a reduced antenna size which is figuratively shown, and leads to reduced O₂ production and early onset of H₂ production. The light green phenotype allows higher cell densities to be used leading to increased rates of H₂ production.}
\end{figure}
relatively large antenna systems of Stm6 and Stm6Glc4 still supports rates of O2 production which exceed the respiration rate and so sulfur depletion is required to induce H2 production.

In contrast in Stm6GlcAL01 the LHCBM1, LHCBM2 and LHCBM3 have been down-regulated within an upregulated AOX background (Figure 6B). The importance of this is that the simultaneous reduction of O2 production and increased O2 consumption is thought to reduce intracellular O2 levels below the induction threshold for HYDA. This hypothesis is supported by three observations. First, Stm6GlcAL01 cultures showed an almost immediate onset of H2 production upon sulfur deprivation (Figure 5A) indicating that the intracellular O2 levels in sulfur replete medium are already poised closed to HYDA threshold level. Second, during the early stages of H2 production the contaminating O2 concentrations in the gas produced by Stm6GlcAL01 cultures were considerably lower than those produced by Stm6Glc4. Third, the rate of H2 production by Stm6GlcAL01 was almost twice as high as that observed for Stm6Glc4 (Figure 5B) for a given culture volume adjusted to the same chlorophyll concentration, for reasons described above. The finding that the Stm6GlcAL01 is so closely poised to HYDA expression opens up the exciting possibility of using this mutant for continuous H2 production in sulfur replete medium. To date, to our knowledge continuous H2 production in sulfur replete medium has not been reported, presumably because under these conditions O2 production usually exceeds O2 consumption inhibition HYDA expression. For example the strains Stm3Rb [27], Stm6Glc-FT7 [26] and TLA1 [16,60] have not been reported to increase H2 yield in liquid culture which would be desirable for the development of continuous H2 production in scale PBR systems. However, important steps along this development path include the use of sulfur microdosing approaches [61] and the use of alginate solid phase systems which reduced O2 inhibition [60].

In summary, Stm6GlcAL01 exhibited an improved H2 and biomass production efficiency (165–180% improvement over parental strain). Importantly, this was achieved at increased solar flux densities (450 μE m−2 s−1) and high cell densities which are best suited for microalgae production as light should ideally be the limiting factor. Our data suggests that the overall improved photon to H2 conversion efficiency is due to: 1) reduced loss of absorbed energy by non-photochemical quenching (fluorescence and heat losses) near the photobioreactor surface; 2) improved light distribution in the reactor; 3) reduced photo-inhibition; 4) early onset of HYDA expression, 5) reduction of O2 induced inhibition of HYDA. The Stm6GlcAL01 phenotype therefore provides important insights for the development of high efficiency photobiological H2 production systems.

**Supporting Information**

**Figure S1 Initial RNAi oligo-nucleotides used during construction of RNAi vectors.**

(DOC)

**Table S1 Absolute expression data derived during qRT-PCR experiments.**

(DOC)

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**Author Contributions**

Conceived and designed the experiments: MO ILR ES BDH. Performed the experiments: MO JS JW KAR JK. Analyzed the data: MO ILR JW KAR JK AKR. Contributed reagents/materials/analysis tools: OK. Wrote the paper: MO ILR OK BDH.

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