Closeout Report for DE-FG36-05GO15041

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Executive Summary

Measured solar-to-biomass energy conversion efficiencies in mass cultures of green microalgae and cyanobacteria are, at best, about 3% and 1%, respectively. The theoretical maximum solar-to-biomass energy conversion efficiency of photosynthesis was measured to be 8-10%. Accordingly, to enhance the productivity of photosynthetic microorganisms in biomass, hydrogen, biofuels, and chemicals production, there is a need to substantially improve the achieved solar-to-biomass energy conversion efficiency. The project addressed the following technical barrier from the Biological Hydrogen Production section of the Fuel Cell Technologies Program Multi-Year Research, Development and Demonstration Plan: **Low Sunlight Utilization Efficiency in Photobiological Hydrogen Production is due to a Large Photosystem Chlorophyll Antenna Size in Photosynthetic Microorganisms (Barrier AN: Light Utilization Efficiency)**. This was achieved upon development and application of the Truncated Light-harvesting Antenna (TLA)-concept in green microalgae and cyanobacteria, entailing the generation of strains with a smaller or truncated light-harvesting antenna size. The work provided first-time evidence of the applicability of the TLA-concept in these photosynthetic microorganisms, which exhibited substantially improved photosynthetic efficiency and productivity in mass cultures. Outcome of was a new field of science and technology that was created from the execution of this R&D. The *TLA technology* promises to enhance the photosynthetic productivity of microalgae, plants, and cyanobacteria by up to 3-fold over currently achieved yields. It is beginning to find application in the commercial sector. In the course of this work, four novel genes were identified in green microalgae, and shown to be determinants of the light-harvesting antenna size, manipulation of which resulted in the generation of TLA strains. TLA strains of green microalgae were deposited in a national library (The *Chlamydomonas* Center) and are available to the field. Twenty-four highly cited peer-reviewed papers were published. Three different pieces of intellectual property and associated patents resulted from this work.

Introduction

Measured solar-to-biomass energy conversion efficiencies in mass cultures of green microalgae and cyanobacteria is about 3% and 1%, respectively. The theoretical maximum solar-to-biomass energy conversion efficiency of photosynthesis was measured to be 8-10% (Ley and Mauzeral 1982; Bjorkman and Demmig 1987; Melis 2009 Pub#5). It follows that there is room to substantially improve the achieved solar-to-biomass energy conversion efficiency of green microalgal and cyanobacterial cultures.

In all photosynthetic systems, over-absorption of bright sunlight and wasteful dissipation of most of it via non-photochemical quenching is the primary and most important source of the lower-than-theoretical efficiency and productivity. Rectifying this pitfall could improve productivity by up to 3-fold in green microalgae, and by up to 8-10-fold in cyanobacteria. Over-absorption of sunlight is attributed to the assembly of large arrays of light-absorbing pigments in the photosynthetic apparatus, specifically chlorophyll (Chl) in green microalgae and phycobilins in cyanobacteria. Up to 600 Chl *a* and Chl *b* molecules can be found in association with the PSII and PSI reaction centers in green microalgae (Melis 1996; Melis et al. 1999). Up to 850 mostly phycobilins can be found in association with the photosynthetic apparatus of cyanobacteria (Glazer and Melis 1987). At high light intensities, the rate of photon absorption by the large antenna of the top layers of cells in the high-density cultivation or mass culture would far exceed the rate at which photosynthesis can utilize them, resulting in dissipation and loss of excess photons via the process of non-photochemical quenching (Niyogi 1999). Up to 80% of the absorbed photons could thus be wasted (Melis et al. 1999), minimizing solar-to-product energy conversion efficiencies and photosynthetic productivity to unacceptably low levels (**Fig. 1**).

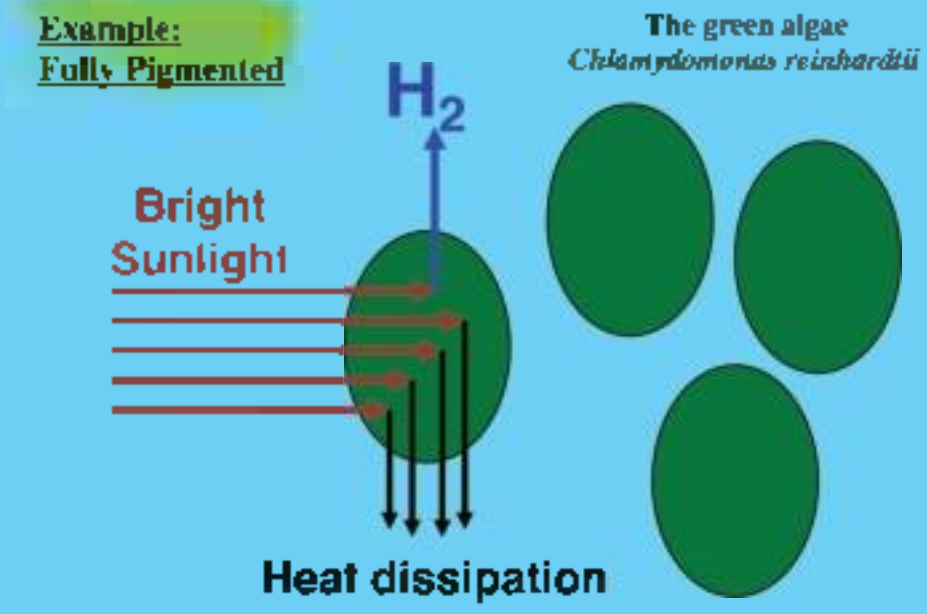

Fig. 1. Schematic presentation of the fate of absorbed sunlight in fully pigmented (dark green) algae. Individual cells at the surface of the culture over-absorb incoming sunlight (i.e., they absorb more than can be utilized by photosynthesis), and 'heat dissipate' most of it. Note that a high probability of absorption by the first layer of cells would cause shading of cells deeper in the culture.

In addition to the wasteful dissipation of excitation, and also due to the high rate of photon absorption by the photosynthetic apparatus, cells at the surface of the mass culture would be subject to photoinhibition of photosynthesis (Powles 1984; Melis 1999), a phenomenon that further compounds losses in productivity (Nakajima et al. 1998). Meanwhile, cells deeper in the liquid culture are deprived of much needed sunlight, as this is strongly attenuated due to the filtering (Naus and Melis 1991) (see also Fig. 1). Alleviating this optical pitfall and validating the notion of improved photosynthetic productivity and solar-to-product energy conversion efficiency of mass cultures was the goal of this EERE-funded R&D (Melis and Mitra 2010 Pub#6&7).

Specific objectives of the DE-FG36-05GO15041 work were the generation of green microalgal and cyanobacterial strains with enhanced photosynthetic productivity and H_2-production under mass culture conditions (Melis 2005 Pub#2). To achieve this, it was necessary to minimize the light absorption capability of individual cells (Mitra and Melis 2008 Pub#4) or chloroplasts (Ort et al. 2011 Pub#8; Blankenship et al. 2011 Pub#9), without adversely affecting their ability to utilize the absorbed sunlight. A cost-effective way to achieve this goal was to genetically minimize the number of chlorophyll (Chl) molecules (green microalgae) or phycobilins (cyanobacteria) that function as light-harvesting pigments in the apparatus of photosynthesis (Polle et al. 2003 Pub#1).

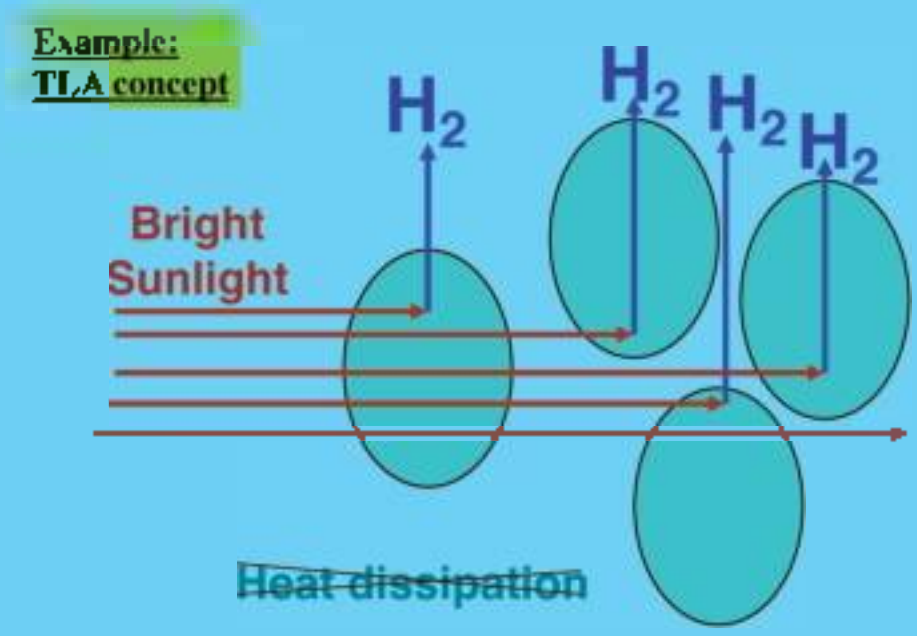

Fig. 2. Schematic of sunlight penetration through cells with a truncated chlorophyll antenna size. Individual cells have a diminished probability of absorbing sunlight, thereby permitting penetration of irradiance and H_2-production by cells deeper in the culture.

The rationale for this work is that a Truncated Light-harvesting Antenna size (TLA-trait) in green algae or cyanobacteria would prevent individual cells at the surface of a high-density culture from over-absorbing sunlight and wastefully dissipating most of it (e.g. Fig. 1). A TLA-

trait would permit sunlight to penetrate deeper into the culture, thus enabling many more cells to contribute to useful photosynthesis and H_2-production (e.g. **Fig. 2**).

Project Objectives

The overarching question was to generate green microalgal and cyanobacterial strains with enhanced capacity for photosynthetic productivity and hydrogen production under bright sunlight and mass culture conditions. It is understood that such TLA-strains would also find application in fields other than hydrogen production, including biomass, bioenergy, and renewable chemicals generation (Melis 2012 Pub#11). To achieve this important objective, it was necessary to improve the light-utilization efficiency of the cells, and this was achieved by minimizing the number of chlorophyll (Chl) in green microalgae and phycocyanin molecules in cyanobacteria, that function to absorb sunlight in photosynthesis. The effort aimed, therefore, to generate microalgal and cyanobacterial strains with a **truncated light-harvesting antenna (TLA-strains)**. Objectives:

- Minimize, or truncate, the chlorophyll antenna size in green microalgae, and the phycobilisome antenna size in cyanobacteria to maximize culture photobiological solar energy conversion efficiency.
- Demonstrate that a TLA-trait minimizes absorption and wasteful dissipation of bright sunlight by individual cells, resulting in better irradiance distribution, improved utilization efficiency, and greater photosynthetic productivity in high-density mass cultures.

The goal of this research was to generate green algae and cyanobacterial strains that are not subject to this optical pitfall and suboptimal utilization of sunlight in mass culture, and which would operate with improved sunlight utilization efficiency in photosynthetic energy conversion and overall productivity under bright sunlight. To this end, it was necessary to minimize the absorption of light by individual cells so as to prevent the over-absorption and wasteful dissipation of sunlight at the surface of the culture, while permitting greater transmittance of irradiance through the high-density medium. This requirement was recognized long ago (Kok 1953; Myers 1957; Radmer and Kok 1977) but could not be satisfied due to the lack of the necessary technologies by which to approach the problem. The advent of **molecular genetics** in combination with **sensitive absorbance-difference kinetic spectrophotometry** for the precise measurement of the chlorophyll or phycobilisome antenna size in green algae and cyanobacteria, respectively, offered a valid approach by which to pursue a reduction in the size of the light-harvesting antenna molecules.

In laboratory simulations and greenhouse mini scale up experiments, it was shown that a TLA-trait enables a greater solar energy conversion efficiency and photosynthetic productivity than could be achieved with fully pigmented cells.

Technical Barriers Addressed

The project addressed the following technical barriers from the Biological Hydrogen Production section of the Fuel Cell Technologies Program Multi-Year Research, Development and Demonstration Plan: **Low Light Utilization Efficiency in Photobiological Hydrogen Production due to a Large Photosystem Chlorophyll Antenna Size (Barrier AN: Light Utilization Efficiency).**

Technical Targets

The Fuel Cell Technologies Program Multi-Year Plan technical target for this project was to apply the TLA concept in green microalgae and cyanobacteria and to test for and validate the premise of improved sunlight utilization efficiency and culture productivity.

Milestones and Deliverables Summary

The approach to achieving the Project Objectives entailed application of deoxyribonucleic acid (DNA) insertional mutagenesis, screening, biochemical and molecular genetic analyses for the isolation of TLA-strains in the green microalgae *Chlamydomonas reinhardtii* and the cyanobacterium *Synechocystis* sp. Cloning of genes that conferred the TLA property, followed by molecular and biochemical characterization of the gene and its encoded protein were part of this work. Application of these genes and of the respective TLA-strains in mass culture productivity measurements provided a comparison of performance versus the wild type and validation of the TLA concept. **Table 1** below offers a summary and a temporal view of the microalgal technical targets, milestones and progress.

Table 1. Microalgal technical targets, milestones and progress.

Sunlight utilization efficiency is shown as % of incident solar energy that is translated into chemical energy at the photosystem level (not biomass). Per the MYPP, Utilization Efficiency of Incident Solar Light Energy was measured as E_0xE_1, %. According to these units, maximum possible energy conversion efficiency based on photosynthetically active radiation (PAR) is 30%.

	2000	2003	2005	2007	2008	2010	2011	2012	2015
Targets (Light utilization efficiency, E_0xE_1, %)	**3%**	**10%**				**15%**			**20%**
Efficiency E_0xE_1 (%) of TLA strain and when isolated	**3% (Wild type)**	**10% *TLA1***	**15% *TLA2***		**25% *TLA3***				
Gene cloning, functional analysis of corresponding protein, and when achieved.				**TLA1 Mov34 MPN**			**TLA2 FTSY**	**TLA3 SRP43**	

Highlights of the Work

***TLA1 project*: TLA1, a DNA insertional transformant of the green alga Chlamydomonas reinhardtii with a truncated light-harvesting chlorophyll antenna size.**

DNA insertional mutagenesis and screening of the green microalga *Chlamydomonas reinhardtii* was employed to isolate *tla1*, a stable transformant having a truncated light-harvesting chlorophyll antenna size. Molecular analysis showed down-regulation of a novel gene (*TLA1*) that encodes a protein of 213 amino acids (Tetali et al. 2007 Pub#3; Mitra and Melis 2010 Pub#6; Mitra et al. 2012 Pub#13) caused the antenna phenotype (Mitra et al. 2012

Pub#15). Biochemical analyses showed the TLA1 mutant to be chlorophyll deficient, with a functional chlorophyll antenna size being about 50% of that in the wild type (Polle et al Pub#1; Mitra et al 2012 Pub#13). It contained a correspondingly lower amount of light-harvesting proteins than the wild type. The TLA1 strain required a higher light intensity for the saturation of photosynthesis and showed greater solar energy conversion efficiencies and a higher by 1.5-fold photosynthetic productivity than the wild type under mass culture conditions (Polle et al 2003 Pub#1).

Pioneering contributions from this work included the first-time identification of a gene (the *TLA1* gene) that conferred a Truncated Light-harvesting Antenna size in green microalgae. Also novel was the development and application of methods by which to assess the mass culture productivity of microalgae (and later cyanobacteria) under ambient sunlight conditions. This is depicted in the results of Fig. 3 below.

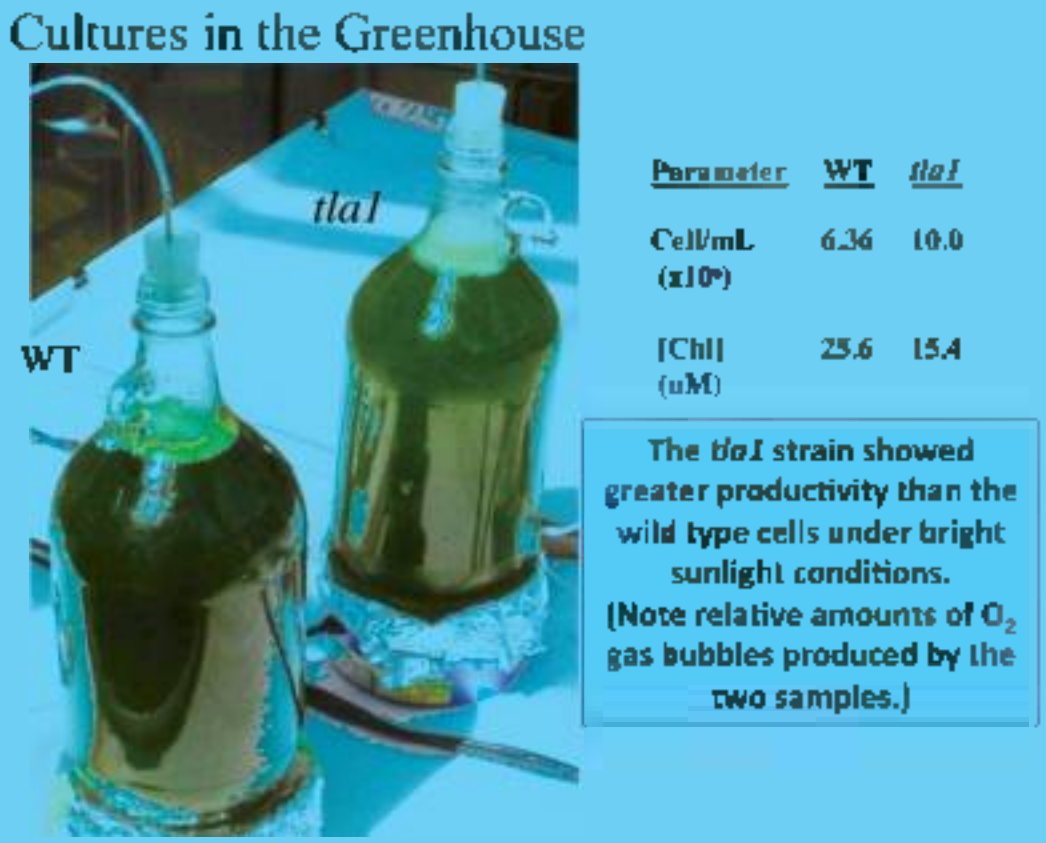

Fig. 3. A mass-culture setup of *Chlamydomonas reinhardtii* for measurements of photosynthetic productivity under ambient conditions. The wild type (WT; CC125, a CW+ and Arg+ strain) and the tla1 CW+ mutant were grown in 2.5-L bottles having an internal diameter of about 15 cm. This geometry simulates the photobioreactor configuration for the production of hydrogen under outside, commercial production conditions. Photosynthetic gases were drained through a syringe (inserted in the middle of the silicone stopper) and, through Teflon tubing, collected in upside-down graduated cylinders, where the volume of oxygen gas (in this case) was measured by the method of water displacement. Culture characteristics: wild type reached a biomass of 6.4×10^6 cells/ml and 25.6 µM Chl, whereas the culture of the tla1 strain reached 10×10^6 cells/ml and 15.4 µM Chl.

TLA2 project: Assembly of the light-harvesting chlorophyll antenna in the green alga Chlamydomonas reinhardtii requires expression of the TLA2-CpFTSY gene

The *tla2* mutant of *Chlamydomonas reinhardtii* showed a lighter green phenotype, had a lower Chl per cell content and higher Chl *a* / Chl *b* ratio than corresponding wild type strains. Physiological analyses revealed a higher intensity for the saturation of photosynthesis and greater photosynthetic productivity on per chlorophyll basis in the *tla2* mutant than in the wild type. Biochemical analyses showed that the *tla2* strain was deficient in the Chl *a* and Chl *b* light harvesting complex (LHC), and had a Chl antenna size of the photosystems that was only about 65% of that in the wild type. The *TLA2* gene, causing the *tla2* phenotype, was cloned by mapping the insertion site and upon complementation with each of the genes that were deleted. We identified the *Chlamydomonas reinhardtii TLA2-CpFTSY* gene, whose occurrence and function in green microalgae has not hitherto been investigated (Kirst et al. 2012 Pub#12). Functional analysis showed that the nuclear-encoded and chloroplast-localized CrCpFTSY protein specifically operates in the assembly of the peripheral components of the Chl *a* and Chl *b* light-harvesting antenna (Kirst et al. 2012 Pub#12). In higher plants, a *cpftsy* null mutation inhibits assembly of both the LHC and PS complexes, thus resulting in a seedling lethal phenotype (Asakura et al. 2008). The work showed that *cpftsy* deletion in green algae, but not in higher plants, can be employed to generate viable ***tla*** mutants. Accordingly, green algae with a *cpftsy* deletion exhibited improved solar energy conversion efficiency and photosynthetic productivity under mass culture and bright sunlight conditions.

TLA3 project: Truncated photosystem chlorophyll antenna size in the green microalga Chlamydomonas reinhardtii upon deletion of the TLA3-CpSRP43 gene

The *tla3* DNA insertional transformant of *Chlamydomonas reinhardtii* is a chlorophyll deficient mutant with a lighter green phenotype, a lower Chl per cell content and higher Chl *a* / Chl *b* ratio than corresponding wild type strains (Kirst et al 2012 Pub#14). Functional analyses revealed a higher intensity for the saturation of photosynthesis, greater per chlorophyll productivity in the *tla3* mutant than in wild type, and a Chl antenna size of the photosystems that was only about 40% of that in the wild type. SDS-PAGE and Western blot analyses showed that the *tla3* strain was deficient in the Chl *a* and Chl *b* light-harvesting complex (LHC). Molecular and genetic analyses revealed a *C. reinhardtii* homolog of the chloroplast-localized SRP43 signal recognition particle was interrupted, and this was responsible for the tla3 mutation. Occurrence and function of the CrCpSRP43 gene and protein in green microalgae has not hitherto been investigated. Biochemical analysis showed that the nuclear-encoded and chloroplast-localized CrCpSRP43 protein specifically operates in the assembly of the peripheral components of the Chl *a* and Chl *b* light-harvesting antenna (Kirst et al 2012 Pub#14). The work demonstrates that *cpsrp43* deletion in green microalgae can be employed to generate *tla* mutants with a substantially diminished Chl antenna size. The latter exhibit improved solar energy conversion efficiency and photosynthetic productivity under mass culture and bright sunlight conditions.

A review article on the chloroplast signal recognition particle (CpSRP) pathway as a tool to minimize chlorophyll antenna size and maximize photosynthetic productivity was published (Kirst and Melis 2014 Pub#16).

Cyanobacteria Δcpc-TLA project: Maximizing photosynthetic efficiency and culture productivity in cyanobacteria upon minimizing the phycobilisome light-harvesting antenna size.

A phycocyanin-deletion mutant of *Synechocystis* (cyanobacteria) was generated upon replacement of the *CPC*-operon with a kanamycin resistance cassette (Kirst et al. Pub#17). The *Δcpc* transformant strain (*Δcpc*) exhibited a green phenotype, compared to the blue-green of the wild type (WT), lacked the distinct phycocyanin absorbance at 625 nm, signifying absence of phycocyanin, had a lower Chl per cell content and a lower photosystem-I to photosystem-II reaction center ratio compared to the WT. Biochemical analyses showed absence of the phycocyanin α- and β-subunits from the *Δcpc*. Physiological analyses revealed a higher, by a factor of about 2, intensity for the saturation of photosynthesis in the *Δcpc* compared to the WT. Culture productivity analyses under simulated bright sunlight and high cell-density conditions showed that biomass accumulation by the *Δcpc* was 1.57-times greater than that achieved by the WT (Kirst et al. Pub#17). Thus, the work provides first-time direct evidence of the applicability of the **T**runcated **L**ight-harvesting **A**ntenna (TLA)-concept in cyanobacteria, entailing substantial improvements in the photosynthetic efficiency and productivity of mass cultures upon minimizing the phycobilisome light-harvesting antenna size.

Highlights of the Work

A summary of the green microalgal strains generated, their genotype characterization, Chl antenna size measured, and productivity measured under mass culture and bright sunlight conditions is given in **Table 2**. A summary of the properties in the cyanobacterial strain generated is given in **Table 3**.

Table 2.

Summary of TLA results in green microalgae. Genes and mutations that confer TLA genotype in green microalgae and the effect of the TLA phenotype on the biomass productivity of the cultures.

Strain	Genotype	Antenna size, Chl (a+b)	Measured productivity (rel. units)
Wild type	**Normal**	**Up to 600**	**1-fold**
tla1	***TLA1* gene down-regulation**	**300**	**1.7-fold**
tla2	***TLA2-ΔCpFTSY* deletion**	**220**	**N/A**
tla3	***TLA3-ΔCpSRP43* deletion**	**145**	**2-fold**
Minimal	**Core Chl antenna**	**130**	**N/A**

Table 3.
Summary of TLA results in cyanobacteria. Genes and mutations that confer TLA genotype in cyanobacteria and the effect of the TLA phenotype on the productivity of the cultures.

Strain	Genotype	Antenna size, Phycocyanin and Chlorophyll	Measured productivity (rel. units)
Wild type	**Normal**	**850**	**1-fold**
Δcpc-TLA	**Phycocyanin deletion**	**520**	**1.6-fold**
Minimal	**Core Chl antenna**	**130**	**N/A**

Green Microalgal TLA Project Deliverables

The following green microalgal *tla* strains have been generated during the course of the supported work. They have been deposited and are available to the public from the library of strains at the *Chlamydomonas* Resource Center (<chlamycollection.org>).

CC-4169 tla1 cw15 sr-u-2-60 mt+ Chromosome: 05Locus: TLA1
CC-4170 tla1 nr-u-2-1 mt- Chromosome: 05Locus: TLA1
CC-4473 tla3 mt+
CC-4474 tla4 mt+
CC-4475 tla5 mt+
CC-4472 tla2-ΔFtsY (cw15) mt+
CC-4476 tla2-ΔFtsY (CW15+) mt+
CC-4561 tla3-Δcpsrp43 (cw+) mt+
CC-4562 tla3-Δcpsrp43 (cw+) mt-

Maximizing sunlight utilization in cyanobacteria by minimizing the size of the native phycobilisome light-harvesting antenna

The work provided first-time direct evidence of substantial improvements in the biomass productivity of mass cultures upon minimizing the phycobilisome light-harvesting antenna size in cyanobacteria. The *Δcpc-TLA* strain is now maintained in this lab, and is available to interested parties. Details of the project are given below.

Solar-to-biomass energy conversion efficiency under otherwise optimal physiological growth conditions in cyanobacteria is about 1-2% (Melis 2009 Pub#5). The primary consideration for this low performance is the sizable light-harvesting antenna of cyanobacteria, the phycobilisome (PBS), which has the capacity to absorb sunlight far in excess of what photosynthesis can utilize. The consequence of this imbalance is that, under bright sunlight, more than 90% of absorbed irradiance can be wastefully dissipated in the form of heat (Melis et al. 1999). PBSs are biliprotein super-complexes peripherally associated with thylakoid membranes and anchored onto the chlorophyll-proteins CP43 and CP47 of photosystem-II (**Fig. 5**). Biliproteins form hexamer discs with their associated linker polypeptides, with the discs stacked against one another in the form of either allophycocyanin-containing core-cylinders (**Fig. 5, AP**), or peripheral phycocyanin-containing rods (**Fig. 5, PC**) (Watanabe and Ikeuchi 2013).

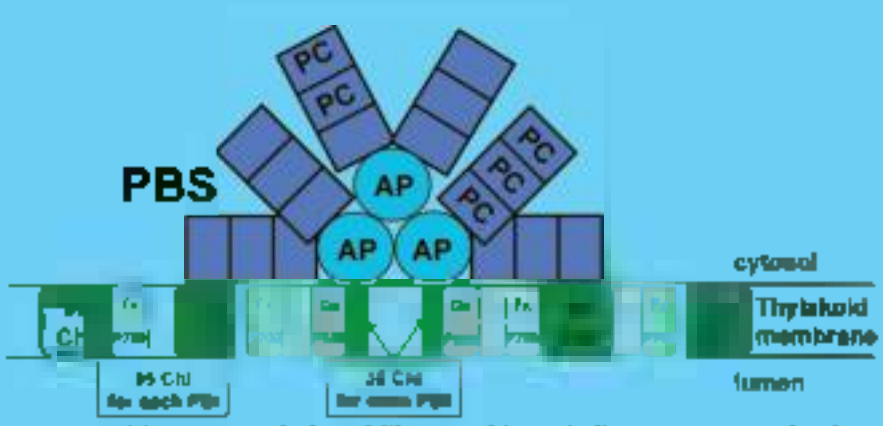

Fig. 5. Photosystem stoichiometry and phycobilisome-chlorophyll antenna organization in the thylakoid membrane of cyanobacteria. Cyanobacteria may possess up to 850 phycocyanin (PC), allophycocyanin (AP), and chlorophyll (Chl) molecules per unit photosynthetic apparatus. Phycobilisome (PBS) schematic adapted from Glazer and Mells (1987).

The research aimed to improve the solar-to-biomass, and solar-to product energy conversion efficiency of photosynthesis in high-density cyanobacterial cultures, under ambient sunlight conditions, from the current 1-2%. This was achieved by minimizing the size of the auxiliary PBS light-harvesting antenna, e.g. by genetically removing the phycocyanin peripheral rods (**Fig. 5, PC**), in an effort to prevent over-absorption and wasteful dissipation of excitation energy. *Synechocystis* with a truncated light-harvesting antenna (TLA-strains) was tested in mass culture under bright sunlight conditions to evaluate the efficiency of sunlight utilization.

Cyanobacterial strains with a TLA property were generated upon deletion of the genes encoding for the phycocyanin-encoding genes from the phycobilisome (PBS) light-harvesting antenna in *Synechocystis* (Kirst et al. 2014 Pub#17). This was implemented upon deletion and replacement of the *cpc* DNA operon that encodes most of the phycocyanin peripheral antenna rods in these cyanobacteria (**Fig. 5**). A blueprint for the generation of cyanobacterial TLA-strains is offered below (**Fig. 6**). Deletion of the *cpc* operon, encoding the phycocyanin genes in *Synechocystis*, resulted in the removal of the peripheral rods from the PBS and in a truncated phycobilisome possessing only the core cylinders (**Fig. 7**).

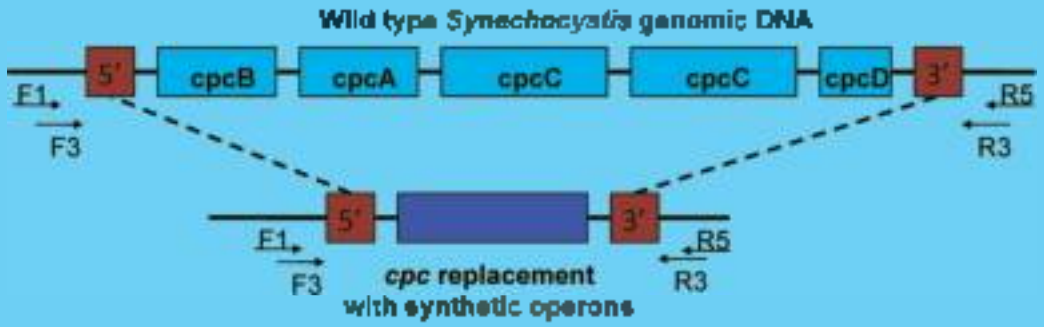

Fig. 6. The *cpc* operon in *Synechocystis*. *cpcB* (sll1577), gene for phycocyanin β-subunit; *cpcA* (sll1578), gene for phycocyanin α-subunit; *cpcC* (sll1579; sll1580), gene for phycobilisome rod linker polypeptide C; *cpcD* (ssl3093), gene for phycobilisome small rod linker polypeptide D. The replacement construct could be a simple antibiotic resistance cassette or a full transgenic operon. Locations of forward (F) and reverse (R) primers for insert map analysis are indicated.

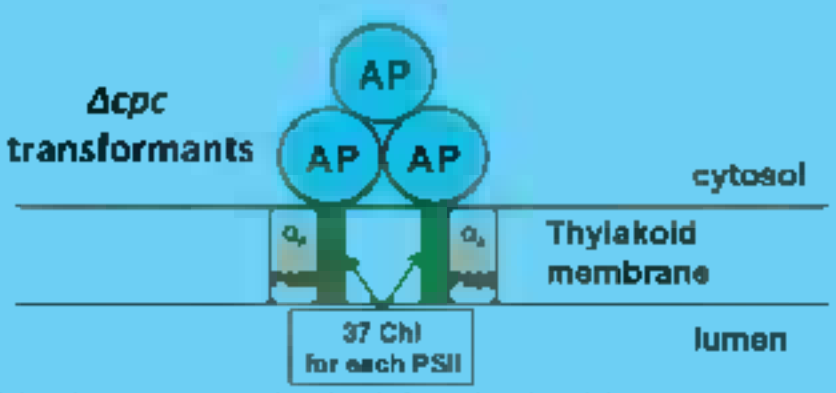

Fig. 7. Deletion of the phycocyanin peripheral rods from the phycobilisome structure, resulting in a TLA-cyanobacteria

Comparative light-penetration experiments through high-density wild type and Δcpc transformants, and the light-saturation curves of photosynthesis (Kirst et al. 2014 Pub#17) indicated that Δcpc transformants are promising in the application of the TLA technology concept. This was tested upon wild type and Δcpc growth under simulated bright sunlight conditions (e.g. 2000 µmol photons m^{-2} s^{-1}) in the laboratory, with cultures having optical density sufficient to absorb ~98% of the incident irradiance. These conditions ensured that light-energy input for the wild type and Δcpc transformant cultures was about the same. Biomass accumulation results of representative wild type and Δcpc transformant cultures are shown in **Fig. 8.**

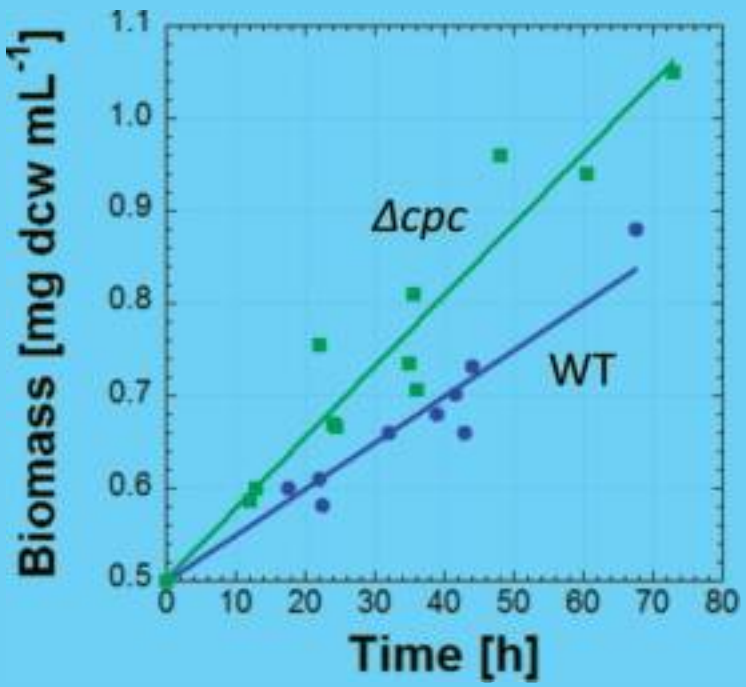

Fig. 8. Summary of biomass accumulation measurements from three batch cultures under simulated bright sunlight conditions (2000 µmol photons m−2 s−1). The linear regression of the points represents the average rate of biomass accumulation of wild type (WT) and Δcpc transformants. A 57% greater productivity of the Δcpc transformants, relative to that of the wild type, was measured (Kirst et al. 2014 Pub#17).

In this experiment, cultures were diluted with fresh growth media once they approached the end of the growth phase, i.e., at about 0.9–1.0 g dcw L^{-1}, to ensure that nutrient availability will not adversely affect growth, and to also permit for a continuous production process over a long growth period, during which to assess the effect of the TLA phenotype on the productivity of the culture. We compiled results from several such continuous growth experiments for wild type and Δcpc transformant cultures (Fig. 8). In this presentation, initial cell density of the cultures was about 0.5 g dcw L^{-1}. The average rate of biomass accumulation in this presentation (Fig. 8) was defined by the slopes of the linear regression of the points and was measured to be about 4.9 mg dcw L^{-1} h^{-1} for the wild type and 7.7 mg dcw L^{-1} h^{-1} for the Δcpc transformants. This analysis, therefore, showed that culture productivity of the Δcpc transformants exceeded that of the wild type by about 57% (Kirst et al. 2014 Pub#17).

Unexpected observations that could find application in transgene expression

Total protein extracts from wild type and Δcpc transformants provided further insight into the phenotype of the latter (**Fig. 9**). In the wild type, the phycocyanin alpha and beta subunits were visible as abundant low molecular weight proteins (**Fig. 9**, CPC-α and CPC-β). These protein bands were absent from the Δcpc transformants. The latter showed an unexpected pronounced band at around 27 kD, which was identified by Western blot analysis to be the NPTI protein, conferring the kanamycin resistance to the Δcpc transformants. Of interest in this respect is the high expression level of the kanamycin resistance protein, suggesting that the strategy of the codon use optimization of the *nptI* gene for expression in *Synechocystis* was highly successful.

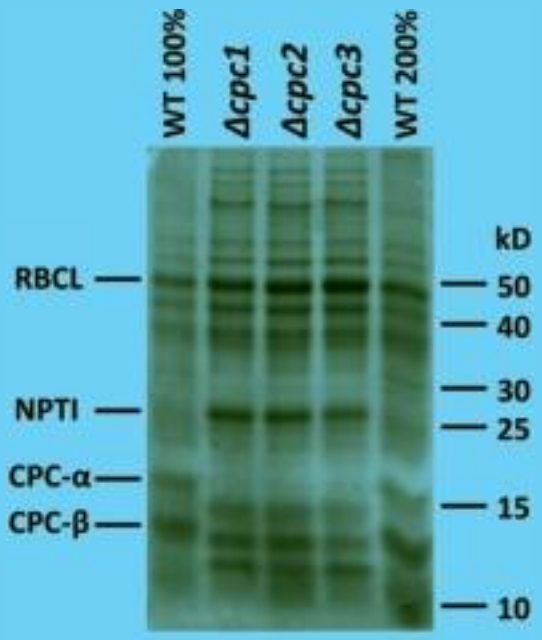

Fig. 9. SDS-PAGE analysis of total protein extracts from Synechocystis wild type and Δcpc transformants. Phycocyanin α-subunit (CPC-α) and phycocyanin β-subunit (CPC-β) are migrating at around 17 and 13 kDa, respectively, and are clearly absent from the Δcpc transformants. The NPTI protein with a molecular weight of 27 kDa is highly abundant in the Δcpc transformants.

The absence of the phycocyanin alpha and beta subunits from the Δcpc transformants corroborated the model shown in **Fig. 7**, and is further consistent with the notion of a severely truncated phycobilisome antenna size in the Δcpc transformants, one that contains only the core allophycocyanin component, as the auxiliary antenna of the PSII reaction center.

e-PAR: Expanding photosynthetically active radiation to the near-infrared

A most significant improvement in the existing natural photosynthetic system capability entails increasing the range of the solar spectrum that can be absorbed and utilized by microorganisms. This aspect of the work explored the design, development, and utilization of novel hybrid composite bioenergetic membranes based on cyanobacteria, which are driven by absorption/utilization of sunlight in the visible portion of the solar spectrum, augmented with the bacteriochlorophyll-containing photosystem of purple photosynthetic bacteria, which is driven by the near infrared portion of the spectrum (Melis and Melnicki 2006). The novel hybrid photosystem composition would permit expanding solar irradiance absorption and utilization from the visible to the near infrared of the solar spectrum, effectively permitting photosynthetic absorption and utilization of 60-65% of incident solar energy (**Fig. 10**)

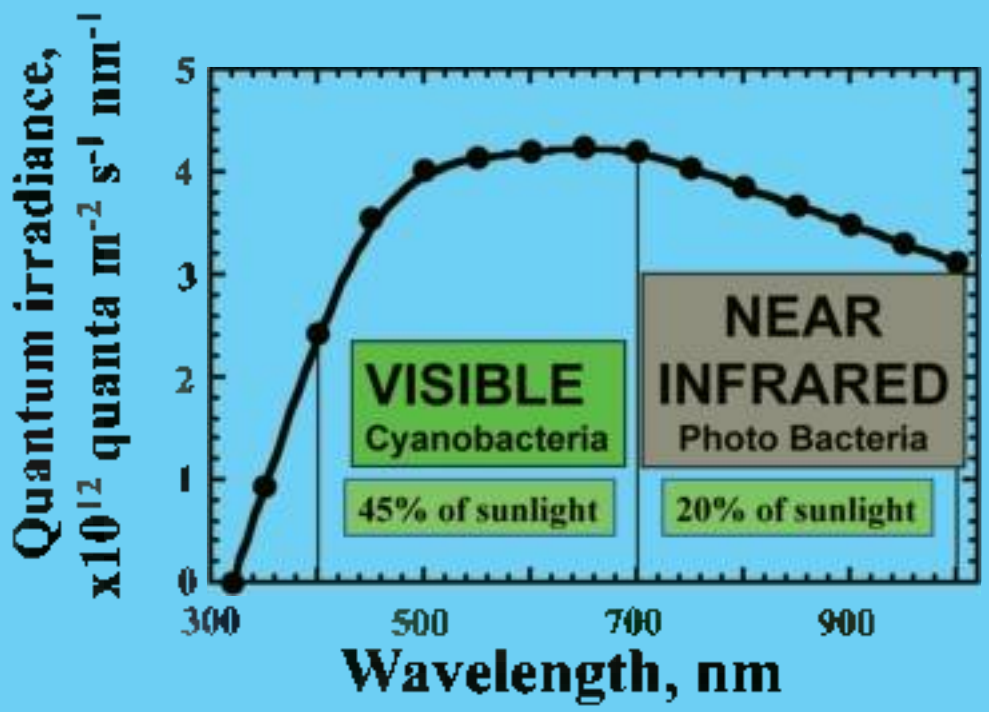

Fig. 10. Solar quantum irradiance distribution as a function of wavelength in the UV-A, visible, and near infrared regions of the spectrum. The visible portion of the solar spectrum (400–700 nm) contains about 45% of the total solar energy, whereas the near infrared region (700–1000 nm) contains an additional 20% of the total solar energy incident on the surface of the earth (Melis and Melnicki 2006).

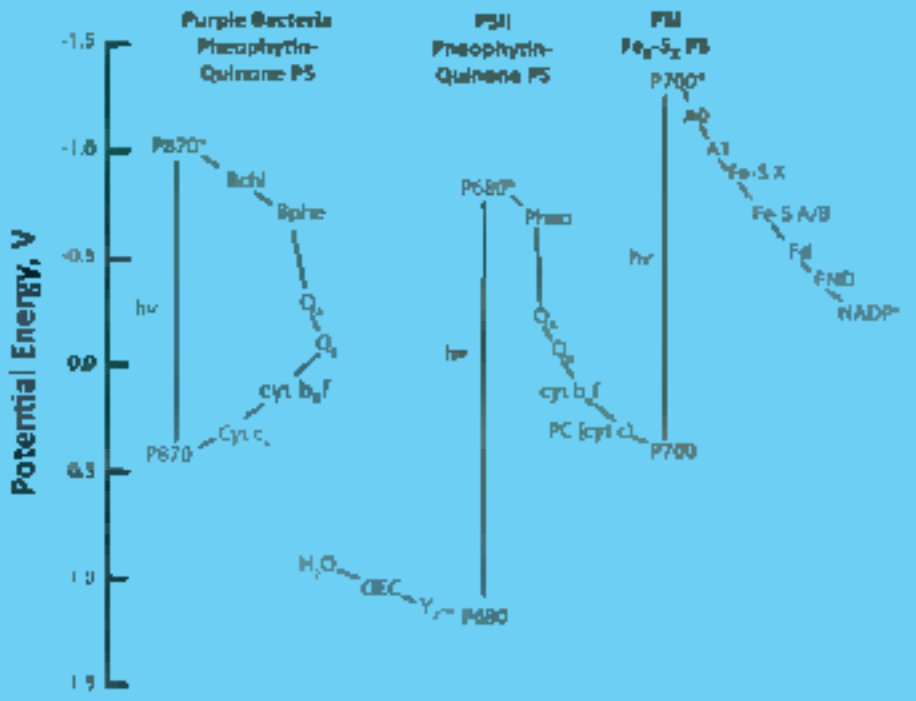

Fig. 11. Photochemical apparatus configuration in a hybrid thylakoid membrane including the purple bacterial pheophytin-quinone photosystem, operated by the infrared-utilizing P870 reaction center, the H_2O-oxidizing photosystem-II (PSII, operated by P680), and the ferredoxin/$NADP^+$ reducing photosystem-I (PSI, operated by P700). Utility of the same cytrochrome b_6-f complex in the electron transport process of the hybrid system is noted. Schematic modified from Blankenship (2002).

Thylakoid membranes with hybrid photosystems, when employed with a minimalized photosynthetic microorganism, may be suitable for all applications where sunlight provides the original input of energy (Melis and Happe 2001; Melnicki et al. 2009; Abed et al. 2009). Such composite bioenergetic membranes could help to improve solar-to-biomass energy conversion efficiency substantially above the 8-10% theoretical maximum of cyanobacteria. The putative bioenergetic and electron transport properties of such hybrid system are shown in **Fig. 11**, comprising both the purple bacterial pheophytin-quinone photosystem and the two photosystems (PSII and PSI) of the current oxygenic photosynthesis.

The purple bacterial photosystem superoperon

Methods that could be applied for the genetic engineering of such novel hybrid photosynthetic membranes in microorganisms include transformation of *Synechocystis* (Lindberg et al. 2010, Bentley et al. 2013) with synthetic operons derived from *Aerobic Anoxygenic Phototrophic* (**AAnP**) purple bacteria (Yurkov and Beatty 1998; Bauer et al. 1991; 1999; Beja et al. 2002; Fuchs et al. 2007; Spring et al. 2009), including genes that encode the protein constituents of the bacterial photosystem, as well as genes for the synthesis of accessory pigments. The advantage afforded by the selected **AAnP** purple bacteria is expression and function of the bacterial photochemical reaction center under ambient oxygen conditions (Spring et al. 2009). The complete superoperon organization from the aerobic purple bacterium KT71 is shown in **Fig. 12**.

Figure 12. Schematic of the photosynthesis superoperon containing 40 genes present in the aerobic purple bacterium KT71 (Fuchs et al. 2007) and presumably required for the assembly and function of the O_2-tolerant purple bacterial photosystem. **blue**: *hem* genes needed for heme synthesis; **gray**: genes of unknown function; **red**: *bch* genes needed for BChl *a* synthesis; **orange**: *crt* genes encoding for proteins of carotenoid biosynthesis; **green**: *bch* genes needed for chlorophyllide *a* synthesis; **purple**: *puc* genes encoding for the assembly of the pheophytin-quinone-type reaction center and the light harvesting complex 1 needed for reaction center function; **black**: *ipi* gene encoding for isopentenyl diphosphate isomerase.

Design of minimized partial operons for the assembly and function of the purple bacterial photosystem

This putative task would assemble two partial operons. The first is designed to encode structural and functional proteins of the purple photosynthetic bacterial reaction center, including proteins of the auxiliary light-harvesting complex I (**Fig. 13, purple**). This construct would also include those proteins encoded by unknown genes, which are conserved in the photosynthesis superoperon of purple bacteria (**Fig. 12 and Fig. 13, gray**) (Fuchs et al. 2007). Inclusion of the latter was deemed to be important, as they likely play essential roles in the correct assembly of the reaction center and LH-I complexes. Double-homologous recombination will be used to replace the wild type *cpc* operon in *Synechocystis* (**Fig. 6**) with the purple photosynthetic bacteria reaction center operon, thus generating *Δcpc* transformants that encode the pheophytin-quinone reaction center of purple photosynthetic bacteria instead.

Fig. 13. Schematic of the reaction center operon for the assembly of the pheophytin-quinone reaction center of purple photosynthetic bacteria in *Synechocystis*. **purple**: *puc* genes encoding for the assembly of the pheophytin-quinone-type reaction center and the light harvesting complex I needed for reaction center function; **gray**: genes of unknown function found in the superoperon of Fig. 13.

The second partial operon is designed to encode proteins required for the synthesis of bacteriochlorophyll *a* (BChl *a*) molecules, i.e., the light-harvesting pigments that have the ability to absorb the near infrared portion of the solar spectrum and transfer the excitation energy to the P870 reaction center (**Fig. 14**). Double-homologous recombination could be used to insert this operon in the slr0168 neutral site or in the *psbA2* site (Lindberg et al. 2010, Bentley and Melis 2012) of the *Synechocystis* genomic DNA.

Fig. 14. Schematic of the purple bacterial pigment operon for the synthesis of BChl *a* in *Synechocystis* (**BChl *a* operon**). red: *bch* genes original to the BChl *a* synthesis pathway (Chew and Bryant 2007).

Successful implementation of this project would have enhanced and expanded the solar energy conversion efficiency of photosynthesis to new levels. Such engineered composite bioenergetic membranes, and their carrier microorganisms, can be licensed and would serve in all future applications, where the efficient and cost-effective production of biomass or fuel is at issue (Melis 2012 Pub#11). In addition, they will find a variety of other applications (Abed et al. 2009) in fuel and chemical product generation as advocated by the PI.

In silico work was conducted to advance exploration of the "*extended Photosynthetically Active Radiation*" (e-PAR) concept. Proprietary preliminary information on the molecular genetic design was arrived at but not implemented or disclosed. Successful implementation of the e-PAR concept is a long-term project, one that could not be further pursued under the auspices of this contract.

Overall Accomplishments, Comments and Conclusions

1. Work on the development and application of the Truncated Light-harvesting Antenna (TLA)-concept in green microalgae and cyanobacteria was successfully completed. The work provided first-time evidence of the applicability of the TLA-concept in these photosynthetic microorganisms, entailing improvements in the photosynthetic efficiency and productivity of mass cultures upon minimizing the light-harvesting antenna size.
2. A new field of science and technology was created from the execution of this project. The *TLA technology* promises to enhance the photosynthetic productivity of microalgae, plants, and cyanobacteria by up to 300% over currently achieved yields. It is beginning to find application in the commercial sector.
3. Four novel genes were identified for the first time in green microalgae, acting as determinants of the light-harvesting antenna size in these microorganisms, manipulation of which resulted in the generation of TLA strains.
4. TLA strains of green microalgae were deposited in a national library (The *Chlamydomonas* Center) and are available to the field.
5. Twenty four highly-cited peer-reviewed papers were published.
6. Three different pieces of intellectual property and associated patents resulted from this work.

Publications emanating from this work **(listed chronologically)**

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Patents and Patent Applications

Melis A and Mitra M (2010) Suppression of *Tla1* gene expression for improved solar energy conversion efficiency and photosynthetic productivity in plants and algae. United States Patent 7,745,696 (issued 29-June-2010)

Melis A and Kirst H (patent pending, priority date: October 24, 2011) Suppression of *TLA2-CpFTSY* gene expression for improved solar energy conversion efficiency and photosynthetic productivity in algae.

Melis A and Kirst H (patent pending, priority date: May 21, 2014) Decreased light-harvesting antenna size in cyanobacteria.

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