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Review

Solar energy conversion efficiencies in photosynthesis: Minimizing the chlorophyll antennae to maximize efficiency

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ABSTRACT

The theoretical maxima of solar energy conversion efficiencies and productivities in oxygenic photosynthesis are evaluated. These are contrasted with actual measurements in a variety of photosynthetic organisms, including green microalgae, cyanobacteria, C4 and C3 plants. Minimizing, or truncating, the chlorophyll antenna size of the photosystems can improve photosynthetic solar energy conversion efficiency and productivity up to 3-fold. Generation of truncated light-harvesting chlorophyll antenna size (**tla**) strains, in all classes of photosynthetic organisms would help to alleviate excess absorption of sunlight and the ensuing wasteful dissipation of excitation energy, and to maximize solar-to-product energy conversion efficiency and photosynthetic productivity in high-density mass cultivations. The **tla** concept may find application in the commercial exploitation of microalgae and plants for the generation of biomass, biofuels, chemical feedstocks, as well as nutraceuticals and pharmaceuticals.

Contents

1. Photon yield of O_2 evolution (CO_2 fixation)

In the unicellular green alga, *Chlorella vulgaris* [1], the minimum quantum requirement for photosynthesis, measured under light-limiting conditions, was equal to 9.7 photons absorbed per oxygen evolved, or carbon dioxide converted to biomass (equivalent to 0.103 O_2 evolved per photon absorbed). This quantum requirement was fairly constant and independent of the acclimation status of the cell, offering testimony for the ability of the photosynthetic apparatus to adjust and optimize under different growth conditions [2]. Similarly, the photon yield of O_2 evolution (CO_2 fixation) among several vascular plants of diverse origins was fairly constant and equal to 0.107

Abbreviations: Chl, chlorophyll; dcw, dry cell weight; LHC, light harvesting complex; PS, photosystem.

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O_2 evolved per photon absorbed (9.3 photons utilized per O_2 evolved) [3].

The Z-scheme of linear electron transport in oxygenic photosynthesis [4] requires cooperation of two photosystems (the O_2-evolving photosystem-II and the ferredoxin-reducing photosystem-I) and utilization of a minimum of 8 photons per each O_2 evolved or CO_2 fixed. The actual (average) measurement of 9.5 photons utilized per oxygen evolved, translates into an overall efficiency of absorbed photon utilization of about 84%. Such exceedingly high (absorbed) photon conversion efficiencies are attained only under limiting intensities of illumination, i.e., under conditions when light absorption and the light reactions, rather than the carbon reactions of photosynthesis are limiting the overall process. Under ambient conditions, however, the prevailing sunlight intensities are much higher than those required for the saturation of photosynthesis, resulting in dissipation and loss of the excess absorbed energy either as heat or fluorescence. This is evidenced in the "light saturation curve of photosynthesis" (Fig. 1), where the rate of photosynthesis, measured as oxygen evolution (mmol O_2 evolved [mol chlorophyll]$^{-1}$ s^{-1}) is plotted as a function of the incident light intensity (μmol photons m^{-2} s^{-1}). At zero light intensity (in the dark), there is O_2 uptake, as respiration is the only cellular bioenergetic activity, consuming about 3 mmol O_2 [mol Chl]$^{-1}$ s^{-1} (Fig. 1). The rate of O_2 evolution increases linearly with light intensity (low-light intensity range), corresponding to conditions when light absorption and the light reactions, rather than the carbon reactions, are limiting the overall process of photosynthesis. It is in this range of light intensities when photosynthesis operates with the maximum solar energy conversion efficiency. At higher light intensities, however, O_2 evolution and the rate of photosynthesis reach a plateau, of about 40 mmol O_2 [mol Chl]$^{-1}$ s^{-1} (Fig. 1), referred to as the light-saturated rate of photosynthesis (P_{max}), and reflecting the fact that brighter sunlight and a faster rate of absorption of incoming photons does not translate into a greater productivity by the cells. Limitation in the rate of photosynthesis is imposed upon by the carbon reactions (slow rate of catalysis by Rubisco) and by the bottleneck of electron transport through the Cytochrome b_6–f complex in the thylakoid membrane. Additional limitations may be imposed by the relatively slow rate of turnover of the Mn-containing H_2O-oxidation complex. In the example of Fig. 1, saturation of photosynthesis is reached at ~400 μmol photons m^{-2} s^{-1} (I_S). Since incident intensities of sunlight can reach up to 2500 μmol photons m^{-2} s^{-1}, it may be concluded that more than 80% of absorbed solar irradiance would be wasted in the photosynthetic apparatus during the course of a sunny day. Fig. 2 shows that, in the course of a sunny day in late spring to early summer, photosynthesis would saturate at about 7 a.m. and remain saturated until 5 p.m., when the solar intensity crosses the 400 μmol photons m^{-2} s^{-1} intensity level once again (Fig. 2). This estimate of non-photochemical quenching applies to cells and chloroplasts directly facing the sun. However, there would be cells and chloroplasts deeper in the high-density foliage, tissue, or culture, where the light-saturation effect of photosynthesis would be mitigated. Integrating over the course of the day and across the depth of a mass culture or canopy, it was estimated that photosynthesis would over-absorb and wastefully dissipate only about 60% of the daily irradiance [5].

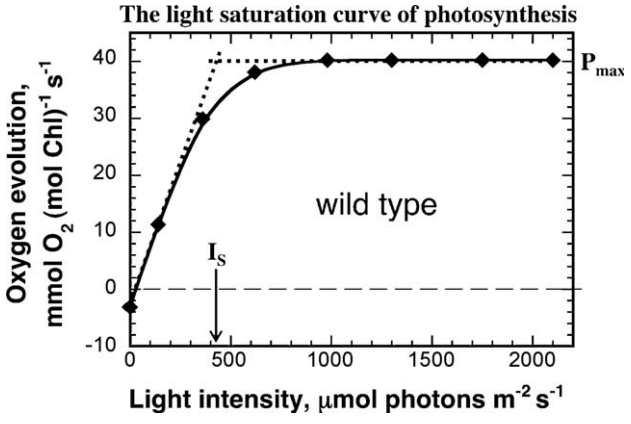

Fig. 1. The light-saturation curve of photosynthesis obtained with wild type microalgae. Plotted is the rate of photosynthesis (O_2 evolved per mol Chl per s) as a function of light intensity. Note the initial linear increase in the rate, the slope of which provides a measure of the quantum yield of photosynthesis. Also note the light-saturated P_{max} rate, and the light intensity I_S (about 400 μmol photons m^{-2} s^{-1}) at which photosynthesis saturates (full sunlight intensity = 2200–2500 μmol photons m^{-2} s^{-1}).

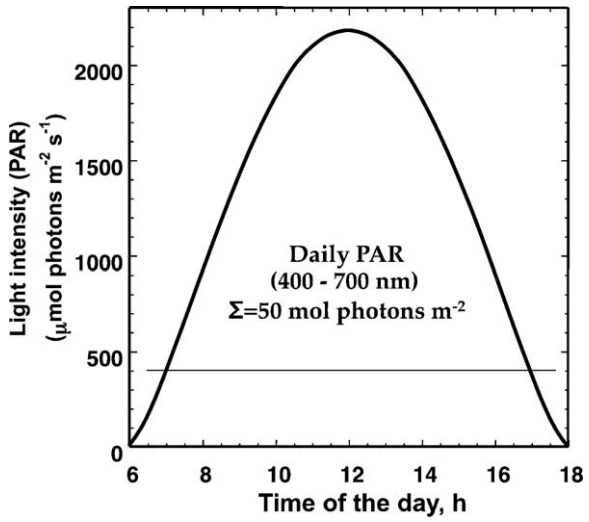

Fig. 2. Profile of the daily solar photosynthetically active radiation at sea level. Note that solar intensity will exceed that required to saturate photosynthesis at about 7 a.m. and will go below the photosynthesis saturation point at about 5 p.m. Excess photons absorbed between 7 a.m. and 5 p.m. will be wasted via non-photochemical quenching. Adapted from Melis et al. [5].

2. Solar energy conversion efficiencies in the photosynthetic apparatus

Based on the Z-scheme of electron transport and the mean energy of visible light (photosynthetically active radiation, PAR), maximal efficiencies of solar energy conversion during the process of photosynthesis were calculated. A maximum of about 45% of the energy of incident sunlight (5 kWh m^{-2} d^{-1} average insolation in the US) can be absorbed by the photosynthetic apparatus of oxygen evolving organisms (Fig. 3). A maximum of two thirds of the absorbed energy can be stored into chemical energy as a result of the charge separation reaction in the two photosystems, translating into solar-to-chemical energy conversion efficiency equal to 30%. In a temporal sequence of events, molecular hydrogen (H_2) is the first product that can be generated via photosynthesis [6], with electrons from reduced ferredoxin via the [Fe]-hydrogenase [7] in green microalgae [8]. This reaction occurs at the end of the Z-scheme of electron transport [9], entailing losses of potential (chemical) energy due to the "downhill" flow of electrons and translating into a solar-to-hydrogen energy conversion efficiency of 12–14%. Chloroplast and cellular metabolism for the generation of biomass entails additional losses in energy, further lowering the solar energy conversion efficiency. Thus, the best-case solar-to-biomass energy conversion efficiency was estimated to be 8–10% (Fig. 3, see also [10,11]).

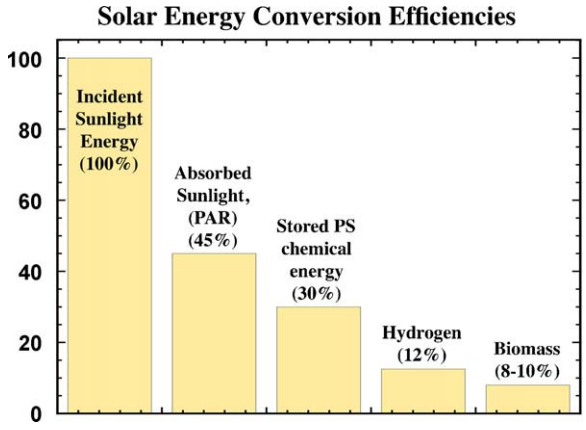

Fig. 3. Bar diagram of theoretically maximum solar energy conversion efficiencies in oxygenic photosynthesis. Average incident sunlight energy is assumed to be ~5 kWh m^{-2} (full solar spectrum) or ~35 mol photons m^{-2} (PAR).

3. Productivity of photosynthesis and biomass accumulation

The theoretically maximum productivity of photosynthesis was calculated on the basis of the overall reaction of CO_2 fixation and reduction:

$$CO_2 + H_2O + 9.5\mathbf{hv} \rightarrow (1/6)C_6H_{12}O_6 + O_2 \quad (1)$$

based on the following parameters and considerations:

- Energy requirement in the form of 9.5 mol photons (**hv**) for the conversion of 1 mol CO_2 into 30 g biomass equivalent (CH_2O) [1,3].
- A daily average delivery of about 35 mol photons m^{-2} (Fig. 2, adjusted for the annual average in the US) translating into the direct photosynthetic generation of 110 g biomass equivalent $m^{-2}\ d^{-1}$.
- Losses of 10% of the primary biomass in the course of the day due to cellular respiration (respiration/photosynthesis ratio = 0.1:1, Fig. 1, see also [12]).
- Losses of an additional 20% of the primary biomass due to photorespiration and other cellular metabolic activity [11]. Losses due to photorespiration are probably lower for microalgae and cyanobacteria because of the function of a carbon concentrating mechanism (CCM) in these aquatic photosynthetic microorganisms [13]. Losses due to photorespiration are also lower in C4 plants [14] but greater for C3 plants [15], especially under low CO_2/high O_2 partial pressures [14,15].

On the basis of these considerations, the theoretically maximum photosynthetic biomass generation of about 110 g $m^{-2}\ d^{-1}$ would be lowered by about 30%, i.e., the equivalent of 33 g $m^{-2}\ d^{-1}$. Accordingly, the theoretical maximum net biomass accumulation (plant productivity) would be expected to be 77 ± 5 g dry cell weight (dcw) per square meter cultivation area per day (Table 1).

The above calculations provide theoretical maxima of solar energy conversion efficiencies in photosynthesis (e.g. 8–10% solar-to-biomass) and productivity yield (77 ± 5 g dcw $m^{-2}\ d^{-1}$). There are reports in the literature of maximal microalgal biomass production rates exceeding 50 g dcw $m^{-2}\ d^{-1}$, and as high as 79 dcw $m^{-2}\ d^{-1}$ ([16] and references therein). These high values may represent peak performance in geographical locations of high insolation and not year-round averages. On the contrary, lab or small-scale sustained green microalgal productivity has not exceeded 20–40 g dcw $m^{-2}\ d^{-1}$ under otherwise optimal growth conditions (Table 1, see also [10,17–20]). Similarly, commercial rates of annual production have been reported for *Chlorella* [21], averaging 60–75 ton dcw $ha^{-1}\ y^{-1}$ (17–20 g dcw $m^{-2}\ d^{-1}$); *Spirulina* [21], averaging 12–24 ton dcw $ha^{-1}\ y^{-1}$ (equivalent of 3.3–6.6 g dcw $m^{-2}\ d^{-1}$); *Miscanthus* [22,23] averaging 30–60 ton dcw $ha^{-1}\ y^{-1}$ (8–17 g dcw $m^{-2}\ d^{-1}$); and switchgrass [24], averaging the 5–10 ton dcw $ha^{-1}\ y^{-1}$ (1.4–2.8 g dcw $m^{-2}\ d^{-1}$). These productivities are far from the 77 ± 5 g dcw $m^{-2}\ d^{-1}$ theoretical maximum that was calculated. The best-case scenario (lab and small-scale green microalgal productivities) has achieved only about 40% (or less) of the theoretical maximum productivity. Similarly, the best-case solar-to-biomass energy conversion efficiency reported with green microalgae did not exceed the 3% value [10,18]. As evidenced by the results of Table 1, solar-to-biomass energy conversion efficiencies for *Spirulina* and switchgrass are lower than 1%, i.e., substantially lower than that reported for *Chlorella* and *Dunaliella*. *Miscanthus giganteus* approaches the productivity characteristics of *Chlorella* (Table 1, see also [22,23]). Biomass productivity is much lower for traditional C3 crop [25] and wild land plants, with solar-to-biomass conversion efficiency values below 0.1% (not shown).

Productivity and solar-to-biomass conversion efficiency comparisons between microalgae/cyanobacteria and land plants in this work are made on an annual basis. It is implicit in this analysis that microalgae/cyanobacteria operate and produce throughout the year, whereas land plants have a limited growth season. Readers of the literature in this field are cautioned to delineate between the averaged annual performance of a strain, and the peak performance measured during the middle of the growth season. It is obvious that peak performance would be substantially greater than annual average values.

4. Evolution of light harvesting, competition in the natural ecotype, and effect on mass culture productivity

The above considerations clearly suggest that solar-to-biomass energy conversion efficiency and photosynthetic productivity can be substantially improved. For many of the C3 crop plants (e.g. soybean), less than full foliage coverage of a given surface area (low plant density, also referred to as the "carpeting effect"), and a limited growth season account for the lower per surface area productivity and lower solar-to-product energy conversion efficiency. Long et al. [15] presented an authoritative analysis,

Table 1
Photosynthetic productivities and solar conversion efficiencies. Productivity numbers in parentheses have been converted from the units given in the actual measurement. Note that yields were reported in g dcw $m^{-2}\ d^{-1}$ or metric ton dcw $ha^{-1}\ y^{-1}$.

Species/process	Productivity	Productivity, % of theoretical maximum	Solar-to-product energy conversion efficiency
Theoretical productivity of photosynthesis	77 g dcw $m^{-2}\ d^{-1}$ (280 ton dcw $ha^{-1}\ y^{-1}$)	100%	8–10%
Lab or small-scale green microalgal productivity [10,17–19]	20–40 g dcw $m^{-2}\ d^{-1}$ (73–146 ton dcw $ha^{-1}\ y^{-1}$)	27–54%	3%
Chlorella (commercial, average rates of production) [19,21]	60–75 ton dcw $ha^{-1}\ y^{-1}$ (17–20 g dcw $m^{-2}\ d^{-1}$)	22–27%	2%
Spirulina (*Arthrospira*) (commercial, average rates of production) [20,21]	12–24 ton dcw $ha^{-1}\ y^{-1}$ (3.3–6.6 g dcw $m^{-2}\ d^{-1}$)	4.5–9%	0.5%
Miscanthus giganteus (field trial) [22,23]	30–60 ton dcw $ha^{-1}\ y^{-1}$ (8–17 g dcw $m^{-2}\ d^{-1}$)	11–22%	1–2%
Switchgrass (field trial) [22–24]	5–10 ton dcw $ha^{-1}\ y^{-1}$ (1.4–2.8 g dcw $m^{-2}\ d^{-1}$)	1.8–3.6%	0.2%

summarizing limitations and suggesting potential improvements in photosynthesis leading to increase in crop yields. They identified six potential routes ranging from altered canopy architecture to improved regeneration of RuBP for CO_2 fixation. Collectively, a 50% improvement to productivity could be achieved by these changes [15]. In all photosynthetic systems, however, 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 300%.

Over-absorption of sunlight is attributed to the assembly of large arrays of chlorophyll (Chl) antenna molecules in the photosynthetic apparatus. Up to 600 Chl *a* and Chl *b* molecules can be found in association with the PSII and PSI reaction centers [5,26]. At high light intensities, the rate of photon absorption by the large Chl 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 [27,28]. Up to 80% of the absorbed photons could thus be wasted [5], minimizing solar-to-product conversion efficiencies and photosynthetic productivity to unacceptably low levels (Fig. 4). 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 [29,30], a phenomenon that further compounds losses in productivity [31]. Meanwhile cells deeper in the plant tissue or culture are deprived of much needed sunlight, as this is strongly attenuated due to the filtering [5,32,33] (see also Fig. 4). Alleviating this optical pitfall could improve photosynthetic productivity and solar-to-product conversion efficiency by up to 300% [5,34].

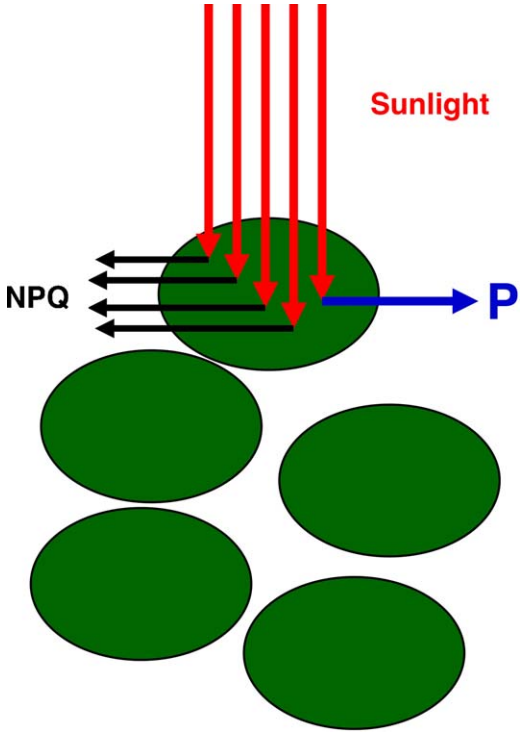

Fig. 4. Schematic presentation of incident sunlight absorption and processing by fully pigmented (dark green) microalgae in a high-density culture. Individual cells at the surface of the culture would over-absorb incoming sunlight (more than can be utilized by photosynthesis), and dissipate most of it via non-photochemical quenching (NPQ), thus limiting productivity (*P*). Note that a high probability of absorption by the first layer of cells would cause shading, i.e., prevent cells deeper in the culture from being exposed to sunlight.

To achieve the higher performance characteristics, it is necessary to minimize the absorption of sunlight by individual chloroplasts in the surface of the canopy or culture, so as to permit greater transmittance of irradiance through a high-density cultivation. This requirement was recognized long ago [35–37], but could not be satisfied because algae with a "truncated light-harvesting chlorophyll antenna size" are not fit to be competitive in the natural environment and, therefore, do not survive in the wild. Until recently, this problem could not be addressed and solved in the laboratory either, due to the lack of the necessary technologies by which to approach it. The advent of genetic engineering in combination with the application of sensitive absorbance-difference kinetic spectrophotometry for the precise *in situ* measurement of the Chl antenna size in photosynthetic systems now permit a genetic approach to minimizing the number of photosystem Chl antenna molecules, and a functional assay by which to test and verify such alterations.

A genetic tendency of photosynthetic organisms to assemble large arrays of light absorbing chlorophyll antenna molecules in their photosystems is a survival strategy and a competitive advantage in the wild, where light is often limiting [38]. Maximum competition in the wild requires capturing more light for self, even if wasted, and preventing light capture by competing neighbors. Obviously, this property is detrimental to the yield and productivity in dense culture. Earlier research [39] revealed that the minimum number of Chl molecules, needed for the assembly of the photosystem–core complexes, was 37 Chl for PSII and 95 for PSI (a combined of 132 Chl molecules for the two photosystems). This is the smallest Chl antenna size that permits assembly of the photosystems in the chloroplast [39]. If that were the mature Chl antenna size of the photosystems, it would compromise the competitive ability and survival of the cells in the wild. However, it would enable efficient solar-to-product conversion by the cells in a protected high-density mass cultivation, leading to improved solar-to-biomass energy conversion efficiency and photosynthetic productivity. Early proof-of-concept experiments [5,33] confirmed that a smaller Chl antenna size would result in a relatively higher light intensity for the saturation of photosynthesis in individual cells, while alleviating photoinhibition of photosynthesis [31] and permitting for an overall greater productivity by the mass culture [34,40,41]. Thus, elucidation of the molecular mechanism for the regulation of the Chl antenna size via genetic approaches is of fundamental importance to the field and of practical importance to the algal biotechnology, biofuels, and agricultural sectors.

The rationale for attempting a bioengineering approach to truncate the Chl antenna size in green microalgae is that such modification would minimize light absorption by individual cells and would, therefore, permit greater transmittance of sunlight deeper into the culture, thus enabling more cells to contribute to useful photosynthesis and culture productivity (Fig. 5). It has been shown that a fully truncated Chl antenna size will enable a ~3-fold greater solar-energy conversion-efficiency and photosynthetic productivity than could be achieved with fully pigmented cells [5]. Such bioengineering, therefore, would substantially enhance photosynthetic productivity in a high-density cultivation under bright sunlight conditions.

5. Improvements in solar energy conversion efficiency and photosynthetic productivity

A systematic approach to this problem is to identify genes that determine and/or regulate the development of the Chl antenna size of photosynthesis and, further, to manipulate such genes so as to confer a permanently truncated Chl antenna size. Identification of such genes in a model organism, e.g., the model green alga *Chlamydomonas reinhardtii,* will permit a subsequent transfer of

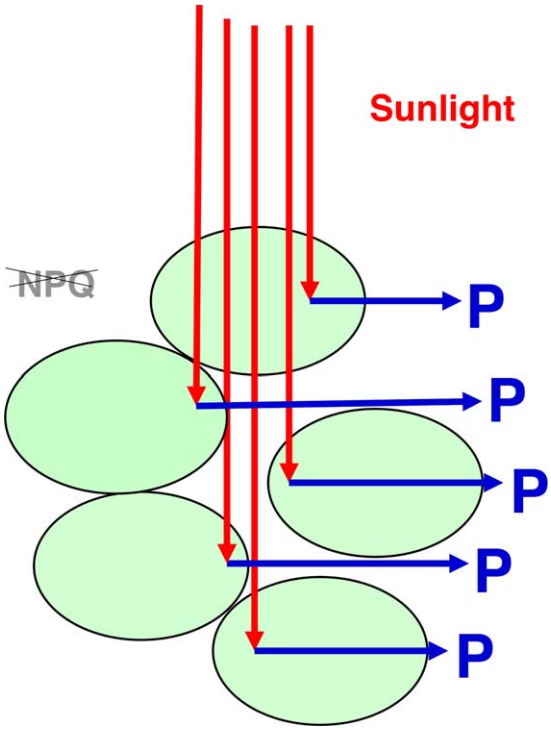

Fig. 5. Schematic presentation of incident sunlight absorption and processing through cells with a truncated chlorophyll antenna size. Individual cells have a diminished probability of absorbing sunlight, thereby permitting greater penetration and a more uniform distribution of irradiance through the culture. This alleviates NPQ and enhances photosynthetic productivity (*P*) by the culture as a whole.

this trait to other microalgae of interest to the alga biotechnology, biofuels, and agricultural sectors. The *t*runcated *l*ight-harvesting chlorophyll *a*ntenna size (**tla**) property may specifically find application in the commercial exploitation of microalgae for the generation of biomass, biofuel, chemical feedstocks, nutraceutical, and pharmaceutical products [42–47]. This objective has been approached in the laboratory of the author upon application of DNA insertional mutagenesis techniques [48–50], followed by stringent screening, biochemical/molecular/genetic and absorbance-difference kinetic spectrophotometry analyses [51] of *C. reinhardtii* cells. An evaluation of the methods employed in this and other labs for the isolation of **tla** mutant strains is offered.

6. Mutagenesis, screening protocols, functional analyses, and expertise required

The developmental regulation of the light-harvesting Chl antenna assembly and step-wise increase is highly conserved in all photosynthetic organisms. However, the molecular mechanism of this phenomenon is not known. A random tag mutagenesis approach, coupled with a stringent screening procedure, can be applied to isolate transformants in which this developmental process of the Chl antenna size increase is interfered with [52]. Such tagged mutants can serve two purposes: (i) used to clone genes that determine the development of the Chl antenna size, and (ii) test the prediction of increased photosynthetic productivity under bright illumination, either in the laboratory or in outdoor pilot scale-up experiments. Interference with the developmental program for the assembly and expansion of the light-harvesting Chl antenna size is one possible approach by which to achieve a truncated light-harvesting Chl antenna (**tla**) in photosynthesis. This approach entails fundamental research and discovery leading to practical application (see below).

Screening for the isolation of truncated light-harvesting Chl antenna (**tla**) mutants requires a number of steps to ensure that putative strains have a smaller than wild type Chl antenna size, without any additional undesirable side effects. This is an important consideration, as recent mutagenesis effort in the lab of the author showed a very high proportion of false positives. For example, out of a library of 6500 DNA insertional transformants, a first screening step resulted in the isolation of 129 putative **tla** mutants [53]. A second screening analysis step narrowed this list to only six putative antenna size strains. Of those, only one strain, termed **tla1** [34] proved to be a truly positive **tla** mutant.

A stringent screening protocol was developed and coupled with functional analysis measurements (outlined below). It offers example of an approach that could be undertaken in the development of **tla** strains. The method was developed for unicellular green algae and successfully applied to *Chlamydomonas reinhardtii*. However, it can easily be adapted to fit other systems of oxygenic photosynthesis. The rationale of this approach is based on the understanding that genetic determinants and a regulatory mechanism defining the size of the Chl antenna size of the photosystems are highly conserved and function in all chloroplasts [54–59]. The approach seeks to generate and isolate mutants with aberrant developmental regulation of the Chl antenna size in microalgae. In the process, mutants with a permanently truncated Chl antenna size will be derived.

Random DNA insertional mutagenesis [48,49], related biochemical, genetic, and molecular analyses [52,60–62] are necessary to isolate positive **tla** strains of *Chlamydomonas reinhardtii,* having a permanently truncated chlorophyll antenna size of PSII and PSI, but without any other structural or functional adverse effect due to the mutagenesis procedure. The advantage of using *C. reinhardtii* in this DNA insertional mutagenesis approach is that:

- Thousands of transformants can easily be generated.
- Vegetative *C. reinhardtii* cells are haploid, allowing the immediate phenotypic expression and identification of mutations;
- Photosynthesis mutants can be rescued upon cell cultivation in the presence of acetate.
- Genetic and molecular techniques, including crosses, tetrad analysis, and gene cloning, are routine in *C. reinhardtii*, so that mutations caused by the integration of exogenous plasmid DNA can readily be analyzed.
- *C. reinhardtii* is amenable to biophysical, biochemical and functional analyses of its photosynthetic apparatus.
- The nuclear, chloroplast and mitochondrial genomes of *C. reinhardtii* have all been sequenced.

6.1. Mutagenesis

Tagged transformants of plants and algae, generated by DNA insertional mutagenesis, have proved to be valuable in the search and identification of novel genes, leading to elucidation of their function. This technology holds promise in opening-up the box of the developmental regulation of the Chl antenna size in photosynthesis. DNA insertional mutagenesis was successfully applied for the first-time identification of genes that impact the development of the Chl antenna size in the model organism *Chlamydomonas reinhardtii* [34,52,63]. Briefly, *C. reinhardtii* mutants were generated by genetic transformation with a linearized plasmid carrying a suitable selectable marker [48–50,60,61]. Linearizing the plasmid is a simple but important step because genetic analyses of mutants, in which the co-segregation of the transforming phenotype (selectable marker) with the mutant phenotype (e.g., truncated Chl antenna size) is scored in backcrosses, indicated that linkage of the phenotypes is seen more often when linear rather than circular DNA is used. DNA analyses also

indicated that integrations of transforming plasmids in *C. reinhardtii* tend to cause deletions or rearrangements within the plasmid [50], and often deletions of genomic DNA [52,64]. The incidence of these undesirable phenomena is significantly lower in transformants generated by linear plasmid DNA. The exogenous DNA tag provides a method of cloning the genomic DNA flanking the insertion, leading to identification of the gene(s) affected by the mutagenesis and, therefore, identification of the genes that play a role in the developmental control of the light-harvesting Chl antenna size.

In *C. reinhardtii*, integration of the exogenously added transforming DNA occurs almost exclusively by nonhomologous recombination within the parental genome and results in transformants carrying integrated plasmid DNA at random locations in the *Chlamydomonas* genome [65]. Thousands of such transformants can easily be generated and isolated upon growth in Petri plates under selective conditions. Such mutants are subsequently grown and tested through a stringent screening protocol.

6.2. Screening procedures

An appreciation of the labor intensive and delicate approach in the identification of proper mutants is given by enumeration of the screening procedure in *C. reinhardtii*. In a *first screening step*, transformant colonies were visually scanned for pigmentation under photosynthetically active radiation and, separately, by fluorescence imaging analysis [53]. The criterion for selection in the visual screening was to pick colonies that have a low density of Chl and/or appear yellow-green to the eye. This is the visual phenotype expected in mutants with a highly truncated Chl antenna size [2]. The criterion for selection in the fluorescence imaging analysis was to pick colonies that display low or no red Chl fluorescence upon pulsed actinic illumination. This is the fluorescence phenotype expected of mutants with a highly truncated Chl antenna size [34].

Low-chlorophyll pigmentation and low-fluorescence yield transformants were isolated and subjected to a *second screening step*. Cell lines were grown in liquid culture, and an aliquot was suspended in the presence of the electron-transport inhibitor diuron (DCMU). The variable (Fv), non-variable (Fo) and maximal (Fm) Chl fluorescence parameters were measured. The Fv/Fm ratio provides a direct measure of the photochemical charge separation efficiency of PSII [66]. This value is normally about 0.8 or higher, in agreement with the high quantum yield of photosynthesis. It should remain high in selected putative antenna transformants, as this would offer indication that the mutagenesis procedure did not have unintended adverse affects on the efficiency and function of PSII. Registration of the variable chlorophyll fluorescence induction kinetics under weak green actinic excitation and measurement of the halftime of the variable fluorescence yield increase is then required. This parameter is inversely proportional to the PSII light-harvesting Chl antenna size [51]. The chlorophyll fluorescence induction kinetics measurement is completed within about 2 min per colony, enabling a relatively high throughput screening of many cell lines.

Independent of the Chl fluorescence yield and induction kinetic measurements, the Chl *a*/Chl *b* ratio of the putative **tla** transformants has also been used as an indirect measure of the Chl antenna size. It is qualitatively known that the smaller the auxiliary light-harvesting Chl antenna, the larger the Chl *a*/Chl *b* ratio would be [67]. However, a high Chl *a*/Chl *b* ratio is not always unequivocal indication of a small Chl antenna size. For example, a Chl *b*-less pigment mutant of *C. reinhardtii* [68], and a lutein, violaxanthin and neoxanthin *minus* pigment mutant [69] both had elevated Chl *a*/Chl *b* ratios and a smaller PSII Chl antenna size relative to the wild type, but a substantially larger PSI antenna size. The latter was countering the **tla** effect.

The above summary presentation of the second screening step shows how a large number of cell lines can be tested and provides a measure of the labor-intensive screening that is required to narrow-down the number of putative Chl antenna transformants. Cell lines with a putative smaller Chl antenna size can be isolated and analyzed further to determine in greater detail the functional characteristics of their photosynthetic apparatus.

6.3. Functional analyses

Selected transformants from the second screening step were tested in a *third screening step* for reaction center (PSII and PSI) concentration and for the absolute Chl antenna size that services each photosystem.

The concentration of the photosystems can be measured spectrophotometrically from the light *minus* dark absorbance difference at 700 nm (P700) for PSI, and 320 nm (Q_A) for PSII [51]. Extinction coefficients applied for the calculation of the amount of P700 (PSI) and Q_A (PSII) are 64 $mM^{-1}\,cm^{-1}$ [70] and 13 $mM^{-1}\,cm^{-1}$ [71], respectively.

The absolute Chl antenna size of PSI and PSII can be reliably measured with the so-called kinetic/spectrophotometric method [51,72,73]. This method is based on the premise that the functional size of the Chl antenna of PSII and PSI can be determined *in situ* from the known ratio of total Chl to a reaction center (statistical Chl/PSI and Chl/PSII ratios, determined spectrophotometrically) upon partitioning of this total Chl into distinct PSI and PSII antenna components. The method assigns functional Chl molecules to each photosystem in direct proportion to the kinetics of Q_A photoreduction (for PSII) and of P700 photooxidation (for PSI) [51].

These measurements are essential to ensure that selected transformants have a specific decrease in the number of accessory pigments per photosystem but otherwise retain the number of photosynthetic units and electron transport chains in the chloroplast. The light saturation curve of photosynthesis should then be measured for each of these selected transformants to determine the efficiency (quantum yield) and productivity of photosynthesis, thus ensuring that the functionality of the photosynthetic apparatus has not been compromised. Further "proof-of-concept" can be attained by mini scale-up productivity measurements under ambient sunlight and mass culture conditions [34].

6.4. Genetic and molecular analyses

Linkage of the transformants with a "truncated Chl antenna" to the selectable marker can be tested in organisms having a sexual reproduction cycle by analysis of the progeny from the backcross of the putative "truncated Chl antenna" strains with a suitable wild type. In *C. reinhardtii,* this analysis includes scoring of the "truncated Chl antenna" and the selectable marker phenotypes [74]. Mutants were selected on the basis of co-segregation of the "truncated Chl antenna" and selectable marker phenotypes. In our experience, such co-segregation occurred in about 50% of the transformants [62]. Co-segregation is an important prerequisite for the subsequent cloning of the gene interrupted by the exogenous DNA insertion because it signals the presence of the "tag" in the position of the lesion.

Genomic DNA of cells transformed with the plasmid DNA can independently be subjected to Southern blot analysis with a probe specific for the selectable marker and, separately, with the cloning vector. Such comparative Southern blot analyses provide a way to visualize the number of independent insertions of plasmid/

selectable marker genes in the host genome and complements the genetic analysis outlined above.

6.5. Gene cloning and mutant complementation

The presence of the transforming DNA in the locus of the gene of interest helps in the cloning of the genomic DNA that flanks the insertion. Briefly, digestion of the genomic DNA with restriction enzyme(s) that do not cut within the vector DNA create many different fragments, only one of which will contain the vector DNA with the *E. coli* origin of replication (*ori*) and the gene for antibiotic resistance (*bla*). These fragments are ligated to form circular DNA suitable for transformation of *E. coli*. Only those fragments carrying *ori* and *bla* will replicate as a plasmid and yield antibiotic resistant *E. coli* colonies. The flanking DNA is then isolated from the plasmid and used to screen a wild type genomic library. DNA sequence analysis can be performed to identify the gene(s) and protein(s) involved in the regulation of the Chl antenna size. In turn, the wild type clones thus obtained can be tested for their ability to complement the "truncated Chl antenna" mutant [52]. Complementation of **tla** mutants with a wild type copy of the cloned putative gene is a last step in the process. Recovery of the wild type phenotype serves as evidence that the right and full-size gene has been isolated [52,64].

6.6. Quality control

To avoid pitfalls, and to ensure that putative **tla** strains truly operate with improved solar energy conversion efficiency, it is imperative to measure and demonstrate a number of parameters, including:

(a) The number of functional PSII and PSI reaction centers per chloroplast or cell, which must remain unaffected;
(b) The functional PSII and PSI Chl antenna size, which must be smaller in the **tla** strains compared to the corresponding wild type;
(c) The quantum (or photon) yield of photosynthesis, which must remain unchanged in the **tla** strains and equal to 0.105 mol O_2 evolved per mol absorbed; and
(d) The light-saturated rate of photosynthesis (P_{max}, measured as mmol O_2 [mol Chl]$^{-1}$ s^{-1}), which should be inversely proportional to the measured Chl antenna size.

Measurements (a–d) are an absolute requirement to ensure that putative **tla** strains would operate with improved solar energy conversion efficiency, and that improved productivity would result upon cultivation under mass culture and bright sunlight conditions. Parameters (a–d) were met with the *C. reinhardtii* **tla1** mutant [34,52]. The Chl content, reaction center concentration, photosystem Chl antenna size, quantum yield of photosynthesis, and P_{max} values in wild type and *tla1* mutant are compared in Table 2. This detailed characterization supports the notion of improved solar energy conversion efficiency and productivity of the **tla1** strain, over that of the wild type, and was experimentally verified [34]. A nearly 2-fold increase in **tla1** productivity over that of the wild type was seen in high-cell density cultures in the greenhouse.

An advantage of the DNA insertional mutagenesis and screening approach is that it affords gains in fundamental knowledge by contributing to the elucidation of genes and regulation of assembly of the Chl light-harvesting antenna. However, deletion or down-regulation of the *Lhcb* and *Lhca* gene families, encoding for the Chl *a*–*b* light harvesting complexes (LHC) of PSII and PSI, respectively, can also lead to **tla** strains. There are six such subunits for PSII (Lhc b1–b6) and four for PSI (Lhc a1–a4) [75–77]. Their deletion or down-regulation should result in a **tla** property for the plant. These peripheral Lhc subunits are not essential for the process of photosynthesis. Indeed, when chloroplast development is limited, stable assembly of the PSII–core and PSI–core complexes takes place in the absence of any Lhc proteins [39]. In principle, the corresponding 10 genes (*Lhc b1–b6* and *Lhc a1–a4*) could be deleted in order to limit the size of the Chl antenna. In practice, this gene deletion approach met with mixed results because of the possible existence of multiple copies of each of these genes, all of which would have to be deleted. An additional difficulty was that in the absence of one of the Lhc subunits, microalgae were able to recruit another Chl-protein subunit for the assembly of the fully pigmented Chl antenna [78,79].

Powerful gene silencing RNA interference technologies can be applied in the model microalga *C. reinhardtii* to generate **tla** mutants. The RNAi-generated *stm3LR3* mutant had a significantly reduced content in both LHCII and LHCI proteins [80]. This *stm3LR3* strain exhibited lower levels of *in vivo* Chl fluorescence, a higher photosynthetic quantum yield, and a reduced sensitivity to photoinhibition, resulting in an elevated efficiency of cell cultivation under high irradiance conditions [80].

A "state transitions" "mutant" (*stm3*) of *C. reinhardtii*, had increased levels of LHCII subunits and a lower Chl *a*/Chl *b* ratio, indicating a larger than wild type Chl antenna size [63]. This mutant is also of interest in the context of this work, as it suggested aberrant development of the Chl antenna size. The affected nuclear gene in the *stm3* strains encodes the RNA binding protein NAB1 (nucleic acid binding protein). NAB1 binds and stabilizes the LHCII mRNA at the pre-initiation stage via sequestration and thereby represses translation of the LHCII protein. Thus, over-expression of

Table 2
Chlamydomonas reinhardtii wild type and **tla1** photochemical apparatus organization and performance characteristics. Measurements show the Chl per cell, Q_A (PSII) and P700 (PSI) content, functional Chl antenna size, i.e., the number of Chl (*a* and *b*) molecules specifically associated with each photosystem, as determined by sensitive absorbance-difference kinetic spectrophotometric analysis. The PSII and PSI Chl antenna size values given have a ±10% SD of the mean (the minimum Chl antenna size (limiting values of the Chl antenna size) that can be assembled is 37 Chl for PSII and 95 Chl for PSI, representing the minimal photosystem core Chl antenna.) The quantum yield Φ of O_2 evolution under limiting intensity of illumination, the light saturated rate of photosynthesis (P_{max}), and the light intensity needed to reach the 50% rate of P_{max} ($P_{max}/2$) are also given. Mass culture productivity measurements were conducted in the greenhouse under ambient conditions [34].

Parameter measured	Wild type	*tla1* Mutant	% Change
mol Chl/cell ($\times 10^{-15}$)	2.4 ± 0.5	0.9±0.06	−62.5%
$\times 10^{-18}$ mol Q_A/cell	6.9	4.8	−30%
$\times 10^{-18}$ mol P700/cell	3.9	2.2	−44%
PSII/PSI (mol:mol)	1.8/1	2.2/1	N/A
PSII Chl antenna size	230	115	−50%
PSI Chl antenna size	240	160	−33%
Φ, Mol O_2 evolved per mol photon absorbed	0.105	0.105	0%
P_{max}, mmol O_2 [mol Chl]$^{-1}$ s^{-1}	44	82	+86%
Half-saturation ($P_{max}/2$) intensity, μmol photons m^{-2} s^{-1}	300	650	+117%
Mass culture productivity at 1500 μmol photons m^{-2} s^{-1} and 6–10×10^6 cells/mL	11 mL O_2 L^{-1} culture	16 mL O_2 L^{-1} culture	+45%

the *NAB1* gene in *C. reinhardtii* can lead to a smaller chlorophyll antenna size for PSII. These results showed progress toward identification of genes and elucidation of processes that define the chlorophyll antenna size of oxygenic photosynthesis.

Chemical (ethylmethylsulfonate) and UV mutagenesis of wild type strains [81–83] for the purpose of generating **tla** mutants have also been successfully employed. Importantly, this was achieved both with *Chlamydomonas reinhardtii* [81] and with strains that may not be amenable to genetic transformation, i.e., *Chlamydomonas perigranulata* [82] and *Cyclotella* sp. [83]. The *Chlamydomonas* **tla** mutants showed improvement in photosynthetic CO_2 fixation at high light intensity [81], and improved productivity under mass culture conditions [82], attributed to the smaller chlorophyll antenna size. Two different *Cyclotella* sp. **tla** strains were also isolated and tested [83]. These pigment mutant strains showed a two-to-three times greater light intensity at which photosynthesis saturates (I_S) than that in the wild type. This is consistent with a smaller Chl antenna size. However, these strains failed to show greater productivity under mass culture and bright sunlight conditions. It was pointed out that chemical and UV mutagenesis may introduce many mutations in the genome, most of which are unrelated to the **tla** phenotype. As such, they might adversely affect the overall fitness of the organism [83].

7. Concluding remarks

Theoretically maximum solar energy conversion efficiencies and productivities in oxygenic photosynthesis are contrasted with actual measurements in a variety of photosynthetic organisms, including green microalgae, cyanobacteria, C4 and C3 plants. Improvements in photosynthetic solar energy conversion efficiency and productivity can be achieved upon minimizing, or truncating, the chlorophyll antenna size of the photosystems. Specific examples are offered on how this could be experimentally achieved. Generation of truncated light-harvesting chlorophyll antenna size *(tla)* strains in all classes of photosynthetic systems will help to maximize solar-to-product energy conversion efficiency and photosynthetic productivity under high-density cultivation and bright sunlight conditions. Further, the DNA insertional mutagenesis approach, outlined in this article, can help to identify currently unknown genes that determine the development of the Chl antenna size in photosynthetic organisms, and demonstrates that a truncated Chl antenna size decreases excess absorption and wasteful dissipation of sunlight by individual cells, resulting in better light utilization efficiency and greater photosynthetic productivity under mass culture conditions. To achieve these objectives, a promising approach was employed, based on DNA insertional mutagenesis, screening, biochemical and molecular analyses for the isolation of "truncated Chl antenna size" strains in the green alga *C. reinhardtii.*

The state of the art in this field suggests that a truncated chlorophyll antenna size alleviates the over-absorption of incident sunlight by individual cells in a high-density culture, and minimizes the wasteful dissipation of irradiance [5,34,40,41]. A truncated light-harvesting chlorophyll antenna size in such mutants diminishes the severe cell shading that occurs in mass culture with normally pigmented wild type. This permits a more uniform illumination of the cells in the culture, and results in a greater solar-to-product conversion efficiency and photosynthetic productivity under high cell density and bright sunlight conditions. Accordingly, the truncated light-harvesting chlorophyll antenna size (**tla**) property may find application in the commercial exploitation of microalgae and plants for the generation of biomass, biofuel, chemical feedstock, as well as nutraceutical and pharmaceutical products.

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