Available online at www.sciencedirect.com

SciVerse ScienceDirect

Current Opinion in Chemical Biology

Advances in microalgae engineering and synthetic biology applications for biofuel production

Javier A Gimpel, Elizabeth A Specht, D Ryan Georgianna and Stephen P Mayfield

Among the technologies being examined to produce renewable fuels, microalgae are viewed by many in the scientific community as having the greatest potential to become economically viable. Algae are capable of producing greater than 50,000 kg/acre/year of biomass [1]. Additionally, most algae naturally accumulate energy-dense oils that can easily be converted into transportation fuels. To reach economic parity with fossil fuels there are still several challenges. These include identifying crop protection strategies, improving harvesting and oil extraction processes, and increasing biomass productivity and oil content. All of these challenges can be impacted by genetic, molecular, and ultimately synthetic biology techniques, and all of these technologies are being deployed to enable algal biofuels to become economically competitive with fossil fuels.

Addresses
The San Diego Center for Algae Biotechnology, Division of Biological Sciences, University of California San Diego, La Jolla, CA 92093, USA

Corresponding author: Mayfield, Stephen P (smayfield@ucsd.edu)

Current Opinion in Chemical Biology 2013, **17**:xx–yy

This review comes from a themed issue on **Energy**

Edited by **Stephen Mayfield** and **Michael D Burkart**

1367-5931/$ – see front matter,

http://dx.doi.org/10.1016/j.cbpa.2013.03.038

Introduction

Recent advances in the development of genetic tools and *in silico* predictive capacity have facilitated the characterization and manipulation of algal genomes in the effort to develop designed algal biofuel production strains. Several strategies for increasing biofuel productivity from microalgae are now being examined, from optimizing light utilization or altering carbon flow pathways for increased biomass accumulation, to modifying lipid production. Depending on the properties of fuel desired, microalgae have potential for producing lipids for conversion into biodiesel, for hydrogen production, and even as a source of ethanol [1]; and several advances have been made to improve yields in each of these approaches. Algae can also produce complex hydrocarbons, for example terpenoids, which are useful both as biofuel additives and as valuable coproducts that could bolster the economic feasibility of the emerging algal biofuels industry (Figure 1). First, advances in genomic analysis will be covered, followed by improvements in light utilization, carbon flow manipulation, and lipid engineering. Finally, we will discuss advances in biohydrogen and terpenoid production in microalgae.

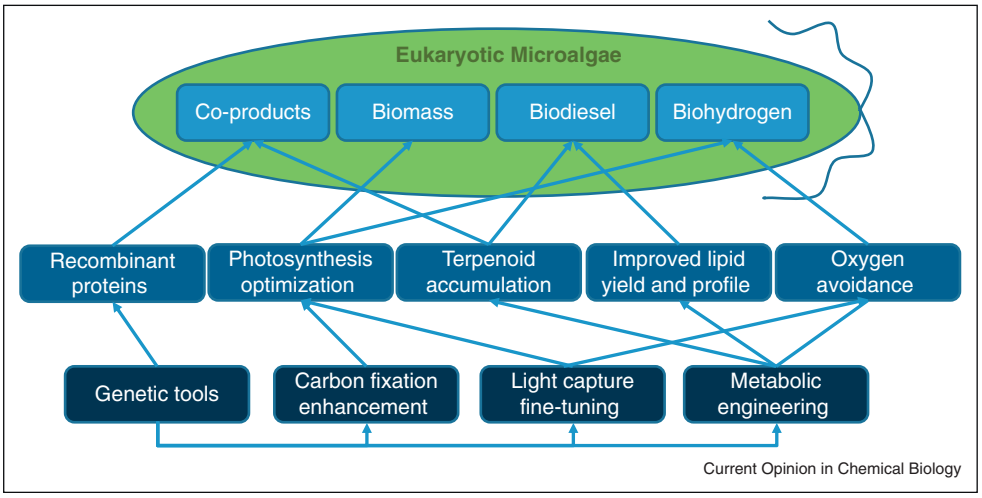
Advances in genetic and bioinformatic tools for manipulating microalgae

Since the early 2000s, the green alga *Chlamydomonas reinhardtii* has re-emerged as one of the dominant single-celled photosynthetic model organisms. This emergence has resulted in advances in genetic tools, a fully annotated genome, and a number of methods for molecular and genetic manipulation. On the basis of these advances, molecular toolkits are now being developed for a range of diverse algal species including strains more suitable for bioenergy production [2]. In *Chlamydomonas*, a number of selectable markers have been specifically developed for use in either the nuclear or chloroplast genome, and transformation methods for both genomes have been optimized [3]. Although we are currently limited to the chloroplast genome for targeted integration and gene knockout, artificial microRNAs have been engineered to create functional knockdowns of several nuclear genes in *Chlamydomonas* [4–7], in *Dunaliella salina* [8], and in diatoms [9] (see [10] for a review of microRNAs in algae). Naturally high rates of homologous recombination have been reported in other algal species including *Nannochloropsis* [11], showing promise for reverse genetics and targeted gene knockouts.

Recently, a viral sequence known as the 2A peptide was shown to function in algae. The 2A peptide allows for genetic linkage of a marker and a gene of interest while yielding discrete protein products. In algae this technology decreases the number of transformation steps and selectable markers required to integrate multicomponent biosynthetic pathways for metabolic engineering, and increases the transgene protein product accumulation when transcriptionally fused to an appropriate selectable marker gene, like the *ble* resistance gene [12•]. Advances in our understanding of chloroplast gene regulation are also showing potential for achieving higher expression levels of transgenes in the plastid, including characterization of chimeric promoter/UTRs

Please cite this article in press as: Gimpel JA, et al.: Advances in microalgae engineering and synthetic biology applications for biofuel production, Curr Opin Chem Biol (2013), http://dx.doi.org/10.1016/j.cbpa.2013.03.038

Figure 1



Strategies for improving the feasibility of algae-based biofuels.

[13] and designed synthetic UTRs that evade negative regulatory mechanisms [14]. Taking advantage of the chloroplast's minimal genome, an *ex vivo* genome assembly has been used to transfer genes for core photosystem subunits from *Scenedesmus* into multiple loci in the *Chlamydomonas* plastid genome [15], demonstrating a synthetic biology approach for engineering complex photosynthetic traits from diverse algae into more tractable production strains.

A host of novel bioinformatic tools are also coming online that enable researchers to characterize gene regulatory pathways, forecast outcomes of metabolic shifts, and functionally annotate genomes of diverse algal species. Detailed *in silico* models of metabolic networks have been used to identify rate-limiting steps in starch metabolism [16] and to predict the light-induced metabolic response to various wavelengths in a metabolic reconstruction that includes more than 1000 genes across ten cellular compartments [17••]. These algal metabolic models have been deposited into the BioModels Database accessible at www.ebi.ac.uk/biomodels-main. New approaches for assembling and annotating *de novo* algal genomes facilitate predictions of biosynthetic pathways based on genome sequence alone [18] using KEGG assignments (www.genome.jp/kegg), vastly increasing the utility of diverse algal genomic data sets for identifying lipid synthesis or metabolic pathways of interest for bioenergy production [2]. Thus far, six annotated chlorophyte genomes are available on Phytozome (www.phytozome.net), and NCBI hosts nearly two dozen green algae genomes either in full, as scaffolds, or with sequencing currently underway (www.ncbi.nlm.nih.gov/genomes). Figure 2 demonstrates the enormous amount of genetic diversity among microalgae, much of which remains untapped.

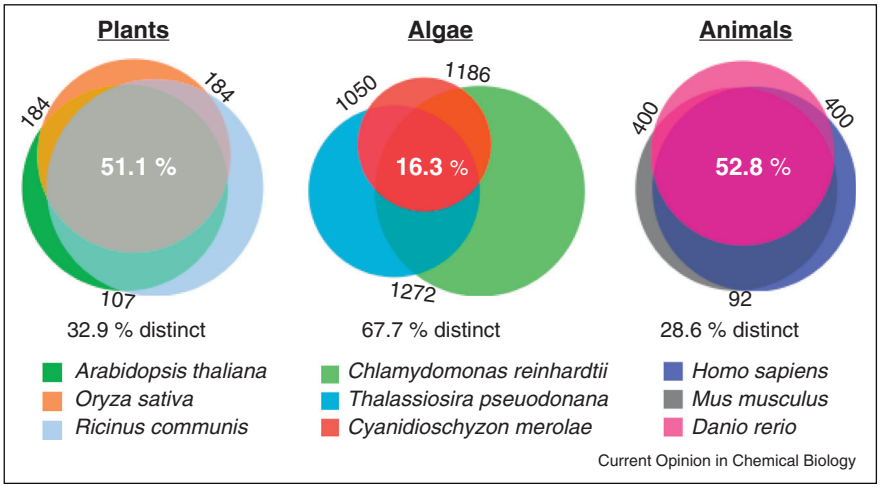
Improving light utilization and photosynthetic efficiency

Microalgae have evolved large light-harvesting complexes (LHCs) for maximizing light absorption in low-light environments in which they often live. Under artificial culture conditions (saturating light) excess energy is dissipated through heat and fluorescence quenching in the LHCs. Energy that cannot be dissipated results in direct photodamage and the production of reactive oxygen species (photoinhibition). The large size of the LHCs also limits light penetration into the culture media, therefore reducing the achievable cell density [21•]. To overcome this, a single RNAi construct has been effectively used for silencing all twenty LHC protein isoforms of *C. reinhardtii*. These cells have lower LHC mRNA (0.1–26% relative to the control) and protein accumulation for all LHC genes and 68% less chlorophyll than the parental strain, resulting in 290% higher light transmittance in the culture [22]. Furthermore they present less fluorescence quenching, which leads to an increase in photosynthetic quantum yield. Under high-light conditions, transformed cells were less susceptible to photoinhibition and grew at a faster rate; however, they did not reach a higher cell density [22]. The same group achieved similar results by downregulating LHC expression by using a redox-dependent translational repressor of the LHC protein family, NAB1. A constitutively activated version of NAB1 (2 amino acid mutations) was overexpressed in *C. reinhardtii*, resulting in a similar phenotype to the RNAi strain. Nonetheless, the effects were less dramatic, having a chlorophyll/cell reduction of 20% compared to that of 68% using RNAi [23].

One of the main targets of photodamage is photosystem II (PSII), the multiprotein complex that performs the

Please cite this article in press as: Gimpel JA, et al.: Advances in microalgae engineering and synthetic biology applications for biofuel production, Curr Opin Chem Biol (2013), http://dx.doi.org/10.1016/j.cbpa.2013.03.038

Figure 2



Algae are a highly diverse set of organisms with largely unexplored genetic potential. The number of novel genes identified among different species indicates that although microalgae may be morphologically similar single-celled photosynthetic organisms, the functional genetic diversity is very large. This functional diversity is being exploited to develop elite algal strains for biofuels production. All ortholog data are from OrthoMCL [19]. Numbers at the intersection of circles are estimated divergence in millions of years obtained from the TimeTree of life project [20].

light-driven oxidation of water. Degradation of the D1 subunit of PSII is significantly increased when light is in excess [24•]. Rea *et al.* [25•] show that it is possible to select for mutant versions of the algal D1 protein that can evolve up to ~4.5-fold more oxygen *in vivo* under high-light conditions (50% midday sunlight) compared to the control. They achieved this by transforming error-prone PCR-amplified D1 coding sequences followed by selection under ionizing radiation. Unfortunately the mutant strains perform slightly worse under laboratory light conditions (10% midday sunlight) [25•], suggesting that this strategy may not translate to increased biomass yield for commercial biofuel production. Gimpel and Mayfield [26] have also used the gene coding for the D1 protein (*psbA*) to test the feasibility of using heterologous genes as interchangeable parts (biobricks) for synthetic biology in *C. reinhardtii* chloroplasts. This study showed that heterologous genes are not the best approach for engineering the chloroplast, but it also proved that heterologous coding sequences driven by endogenous regulatory regions are a viable way for modifying the algal photosynthetic machinery [26]. Furthermore, two isoforms of the D1 proteins from *Synechoccocus* sp. PCC 7942 (cyanobacteria) expressed in *C. reinhardtii* reconstituted the low-light and high-light phenotypes associated with each D1 isoform. Interestingly, *C. reinhardtii* expressing the cyanobacterial low-light isoform yielded 11% more dry weight biomass than the strains expressing the high-light isoform or the endogenous D1 protein, which is a highly desirable trait for biofuel production (Vinyard *et al.*, unpublished).

Modifying carbon assimilation and trophic conversion pathways

In microalgae grown under phototrophic conditions, all newly produced biomass, including lipids, derive from the fixation of CO_2 into ribulose-1,5-biphosphate (RuBP) to form 3-phosphoglycerate, catalyzed by the enzyme RuBP carboxylase/oxygenase (Rubisco). Several other enzymes are also required to regenerate RuBP in a process named the Calvin cycle. Considerable amounts of ATP and NADPH are also required for this process, which are supplied by the light-driven activity of photosystems I and II [27]. Several studies have shown that the activity of Rubisco is the major bottleneck for carbon flux through the Calvin cycle when CO_2 is not enriched in the media, or under high-light or high-temperature conditions (which are all present in commercial scale ponds for algae biomass production in desert areas) [27,28].

RuBP carboxylase/oxygenase is regarded as a 'slow and confused' enzyme [29•], because large amounts of Rubisco are required for achieving a sustainable carboxylation rate and it has an affinity for oxygen, which is used in a counter-productive reaction. *C. reinhardtii* is an ideal host for engineering Rubisco, since there are Rubisco deficient strains that can complete their life cycle heterotrophically, unlike plants [29•]. The small subunit of Rubisco genes (*rbcS*) of *Arabidopsis* and sunflower have been transformed into an *rbcS* deficient strain of *Chlamydomonas*, while preserving the endogenous large subunit gene (*rbcL*). The *in vitro* CO_2/O_2 specificity factor (Ω) was improved by up to 11% while maintaining the V_{max} of carboxylation (V_c). Nonetheless the cells

Please cite this article in press as: Gimpel JA, et al.: Advances in microalgae engineering and synthetic biology applications for biofuel production, Curr Opin Chem Biol (2013), http://dx.doi.org/10.1016/j.cbpa.2013.03.038

displayed lower growth rates and lacked pyrenoids, presumably due to mis-targeting of Rubisco caused by the heterologous small subunits [30]. *Chlamydomonas rbcL* has also been subjected to PCR-based gene shuffling with oligonucleotides representing the natural diversity of this gene. Three rounds of gene shuffling and three rounds of strain selection resulted in a Rubisco with up to 20% and 56% increase in Ω and V_c, respectively. Some of the enriched mutations were then incorporated into the tobacco *rbcL* and resulted in 14% Ω and 15% V_c increments [31]. Another interesting strategy would be to tune Rubisco abundance according to the environmental culture conditions in order to optimize the utilization of energy, carbon and nitrogen. *Chlamydomonas* strains with different amounts of Rubisco have been engineered by expressing the *rbcL* mRNA maturation factor MRL1 at different levels from the nuclear genome of an MRL1 deficient strain. Rubisco could be lowered up to 15% compared to that of wild-type, while maintaining phototrophic growth. An inducible promoter for MRL1 could potentially be used to tune Rubisco accumulation according to culture conditions, such as light intensity or CO_2 concentration [32].

Heterotrophic growth has some advantages over autotrophic growth, like higher cell densities, more controlled culture conditions in closed fermentors, higher overall lipid productivity per volume per day, and the ability to utilize more of the nutrients from wastewater [33]. Some algae are strict heterotrophs or are highly selective for their organic carbon source. Trophic conversion has been achieved, allowing heterotophy in previously obligate phototrophic species as a proof of concept for simple metabolic engineering. *Volvox carteri*, *C. reinhardtii*, *Cylindrotheca fusiformis* and *Phaeodactylum tricornutum* have been transformed with the *HUP1* hexose transporter gene resulting in glucose utilization [34–37]. However, adding hexoses to the culture media may not be optimal for biofuel production given the additional costs and increased risk of contamination.

Lipid synthesis metabolic engineering

Lipid metabolism in microalgae is highly complex, and it is clear that better characterization of the metabolic pathways is needed before high-production biodiesel strains can be engineered. For example, the fatty acids in *P. tricornutum* are composed primarily of 16-carbon chains, with C16:1 comprising about 50% of the total fatty acids, while C16:0 accounts for close to 25% and C20:5 and C14:0 make up most of the remainder [38]. In *C. reinhardtii*, the fatty acids are fairly evenly split between 16-carbon chains (two-thirds of which are C16:0) and 18-carbon chains (predominantly C18:3) [39]; neither of these organisms naturally provide optimal lipid profiles for biofuel feedstocks. Recent work has done much to elucidate the regulation underlying carbon accumulation into primary storage molecules like starch and lipids [40], and to identify the key players responsible for the well-documented nitrogen starvation-induced triacylglycerol (TAG) accumulation [41•,42]. Several reports indicate that mutants deficient in starch synthesis shuttle more carbon into lipid synthesis, especially under nitrogen starvation, at the expense of biomass accumulation [43,44]. However, others have suggested that there is little correlation between starch reserves and lipid content when mutants are compared to their progenitor strain, and that there is significant variability among various wild-type lab isolates [45•].

Studies in the diatom *P. tricornutum* produced a 72% increase in total fatty acid content without altering relative chain length compositions by overexpressing an endogenous thioesterase [46]. In an attempt to produce biodiesel fuel-stocks with shorter-chain fatty acids — and therefore better cold flow properties — two thioesterases from different terrestrial plants were introduced into *P. tricornutum* and increased the ratios of C12 and C14 fatty acids, most of which were incorporated into TAG [47]. However, when three diacylglycerol acyltransferases (DGATs) were overexpressed in *C. reinhardtii*, there was no significant difference in TAG composition or accumulation despite higher levels of transcript [48]. This supports the observation based on proteomic and transcriptomic data that diatoms may employ different carbon metabolism pathways than plants or green algae, at least in response to nitrogen starvation [49]. Still, previous success manipulating lipid synthesis in plants seems likely to translate into advances in green algae lipid metabolic engineering as a better understanding of algal lipid regulatory networks emerges [50]. Recent work in *C. reinhardtii* has shown that the fatty acid profile can indeed be altered by overexpressing the endogenous thioesterase, as predicted by *in vitro* cross-linking assays [51]. These methods are paving the way for a more thorough characterization of algal lipid biosynthetic enzymes, which appear to be distinct from those found in terrestrial plants or in diatoms.

Optimizing biohydrogen production

While other strains may be more suitable for lipid synthesis and biodiesel production, *C. reinhardtii* is an attractive candidate for hydrogen production due to its relatively high hydrogenase activity, determined to derive predominantly from the [FeFe]-hydrogenase HYDA1 [52]. Biohydrogen is an alluring fuel alternative because it produces no carbon dioxide byproducts and it is a superior fuel for electricity production by fuel cells. Although hydrogen is naturally produced by *C. reinhardtii* under sulfur starvation, and hydrogenase activity can be externally induced by adding DCMU (a PSII electron chain uncoupler), hydrogen production cannot be sustained while photosynthesis is actively occurring because oxygen inactivates hydrogenase [53•]. This necessitates a biphasic production strategy in which

Please cite this article in press as: Gimpel JA, et al.: Advances in microalgae engineering and synthetic biology applications for biofuel production, Curr Opin Chem Biol (2013), http://dx.doi.org/10.1016/j.cbpa.2013.03.038

cells grow photosynthetically to accumulate biomass, which is then exploited for H_2 production under anoxic conditions. Several approaches to overcome this limitation have recently shown promising results. Introducing leghemoglobin proteins, which sequester oxygen in the nitrogen-fixing root nodules of legumes, can facilitate a fourfold increase of H_2 production in *Chlamydomonas* [54]. In a truncated antenna mutant initially engineered for increased photosynthetic performance, an eightfold increase in H_2 production was observed under sulfur deprivation in high light [55]. Finally, a PSII protein D1 mutant exhibits increased carbohydrate storage and H_2 production [56]; however, the highest reported yields for this strain are still three to five times below a light-to-H_2 conversion that is considered competitive with alternative production strategies like direct photolysis [53•]. H_2 production pathways predicted from *in silico* reconstructions suggest that increased H_2 production can occur under conditions of inhibited cyclic electron flow [57], which is indeed observed in the high-H_2 producing mutant *Stm*6 [58], and may suggest future targets for metabolic engineering. For a comprehensive review of strategies to increase hydrogen production in microalgae, see Esquivel *et al.* [53•].

Metabolic engineering of terpenoids

D. salina and *Haematococcus pluvialis* are the main natural sources of the commercial carotenoids beta-carotene and astaxanthin, respectively. Under optimal culture conditions they can accumulate up to 13.5 mg L^{-1} d^{-1} of beta-carotene (*D. salina*) and 8.0 mg L^{-1} d^{-1} of astaxanthin (*H. pluvialis*) [59]. These carotenoids are used as animal feed additives and in supplements for human consumption, and could become valuable coproducts in the biofuel industry. Carotenoids are also a type of terpene, a large family of molecules which have great potential in biofuel formulations [60]. Recently, the phytoene synthase gene (*psy*), the enzyme for the committing step in carotenoid synthesis, has been transformed in *C. reinhardtii* resulting in increased carotenoid accumulation. Transgenic strains overexpressing *psy* from *D. salina* and *Chlorella zoofingensis* accumulated 2.6-fold and 2.2-fold more lutein (a commercially valuable carotenoid) than the controls, respectively [61,62]. RNA interference technology has also been used to modify the carotenoid profile of *C. reinhardtii* and *D. salina* through silencing of the phytoene desaturase gene (*pds*), the second step of carotenoid synthesis. Reduction of up to 93% and 72% of pds mRNA were achieved in *Chlamydomonas* and *Dunaliella*, respectively. However, the carotenoid content of *C. reinhardtii* did not change significantly and the one from *D. salina* was not measured [8,63].

Concluding remarks

Microalgae exhibit enormous biodiversity and the potential for producing large quantities of biomass that contains high concentrations of lipids. Advances in trait mining, genetic engineering and synthetic biology will facilitate the design of production strains suitable to specific environmental constraints and output requirements. On many fronts — from manipulating lipid and biomass accumulation to increasing biohydrogen production — algae are proving to be extremely malleable photosynthetic organisms suited to meet the growing need for bioenergy, bioproducts, and even food resources. However, there are critical challenges that still need to be addressed, like the engineering developments required for massive algae culture and harvest, the deployment of effective crop protection strategies, and the compliance with restrictions imposed to growing genetically modified algae in outdoor ponds.

References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Cuff DJ, Young WJ: *The United States Energy Atlas*. New York: Free Press/Collier-Macmillan; 1980, .

2. Georgianna DR, Mayfield SP: **Exploiting diversity and synthetic biology for the production of algal biofuels**. *Nature* 2012, **488**:329-335.

3. Specht E, Miyake-Stoner S, Mayfield S: **Micro-algae come of age as a platform for recombinant protein production**. *Biotechnol Lett* 2010, **32**:1373-1383.

4. Schmollinger S, Strenkert D, Schroda M: **An inducible artificial microRNA system for *Chlamydomonas reinhardtii* confirms a key role for heat shock factor 1 in regulating thermotolerance**. *Curr Genet* 2010, **56**:383-389.

5. Molnar A, Bassett A, Thuenemann E, Schwach F, Karkare S, Ossowski S, Weigel D, Baulcombe D: **Highly specific gene silencing by artificial microRNAs in the unicellular alga *Chlamydomonas reinhardtii***. *Plant J* 2009, **58**:165-174.

6. Zhao T, Wang W, Bai X, Qi Y: **Gene silencing by artificial microRNAs in *Chlamydomonas***. *Plant J* 2009, **58**:157-164.

7. Moellering ER, Benning C: **RNA interference silencing of a major lipid droplet protein affects lipid droplet size in *Chlamydomonas reinhardtii***. *Eukaryot Cell* 2010, **9**:97-106.

8. Sun G, Zhang X, Sui Z, Mao Y: **Inhibition of pds gene expression via the RNA interference approach in *Dunaliella salina* (Chlorophyta)**. *Mar Biotechnol (NY)* 2008, **10**:219-226.

9. De Riso V, Raniello R, Maumus F, Rogato A, Bowler C, Falciatore A: **Gene silencing in the marine diatom *Phaeodactylum tricornutum***. *Nucleic Acids Res* 2009, **37**:e96.

10. Cerutti H, Ma X, Msanne J, Repas T: **RNA-mediated silencing in Algae: biological roles and tools for analysis of gene function**. *Eukaryot Cell* 2011, **10**:1164-1172.

11. Kilian O, Benemann CS, Niyogi KK, Vick B: **High-efficiency homologous recombination in the oil-producing alga *Nannochloropsis* sp.**. *Proc Natl Acad Sci U S A* 2011, **108**:21265-21269.

12. • Rasala BA, Lee PA, Shen Z, Briggs SP, Mendez M, Mayfield SP: **Robust expression and secretion of Xylanase1 in *Chlamydomonas reinhardtii* by fusion to a selection gene and processing with the FMDV 2A peptide**. *PLoS ONE* 2012, **7**:e43349.

Demonstrates a technique for successfully enhancing transgene protein accumulation from nuclear expression, which has previously been difficult to achieve.

Please cite this article in press as: Gimpel JA, et al.: Advances in microalgae engineering and synthetic biology applications for biofuel production, Curr Opin Chem Biol (2013), http://dx.doi.org/10.1016/j.cbpa.2013.03.038

13. Rasala BA, Muto M, Sullivan J, Mayfield SP: **Improved heterologous protein expression in the chloroplast of *Chlamydomonas reinhardtii* through promoter and 5′ untranslated region optimization**. *Plant Biotechnol J* 2011, **9**:674-683.

14. Specht EA, Mayfield SP: **Synthetic oligonucleotide libraries reveal novel regulatory elements in *Chlamydomonas* chloroplast mRNAs**. *Am Chem Soc Synth Biol* 2012, **1**:34-36.

15. O'Neill BM, Mikkelson KL, Gutierrez NM, Cunningham JL, Wolff KL, Szyjka SJ, Yohn CB, Redding KE, Mendez MJ: **An exogenous chloroplast genome for complex sequence manipulation in algae**. *Nucleic Acids Res* 2012, **40**:2782-2792.

16. Nag A, Lunacek M, Graf PA, Chang CH: **Kinetic modeling and exploratory numerical simulation of chloroplastic starch degradation**. *BMC Syst Biol* 2011, **5**:94.

17. •• Chang RL, Ghamsari L, Manichaikul A, Hom EF, Balaji S, Fu W, Shen Y, Hao T, Palsson BO, Salehi-Ashtiani K *et al.*: **Metabolic network reconstruction of *Chlamydomonas* offers insight into light-driven algal metabolism**. *Mol Syst Biol* 2011, **7**:518.
In silico modeling of carbon metabolism under various light conditions accurately predicts algal response to growth conditions, verified experimentally by transcript level and growth rate measurements.

18. Rismani-Yazdi H, Haznedaroglu BZ, Bibby K, Peccia J: **Transcriptome sequencing and annotation of the microalgae *Dunaliella tertiolecta*: pathway description and gene discovery for production of next-generation biofuels**. *BMC Genomics* 2011, **12**:148.

19. Chen F, Mackey AJ, Stoeckert CJ Jr, Roos DS: **OrthoMCL-DB: querying a comprehensive multi-species collection of ortholog groups**. *Nucleic Acids Res* 2006, **34**:D363-D368.

20. Hedges SB, Dudley J, Kumar S: **TimeTree: a public knowledge-base of divergence times among organisms**. *Bioinformatics* 2006, **22**:2971-2972.

21. • Ort DR, Melis A: **Optimizing antenna size to maximize photosynthetic efficiency**. *Plant Physiol* 2011, **155**:79-85.
Explains the biophysical parameters that limit photosynthetic efficiency and how light-harvesting antenna size modifications could impact them.

22. Mussgnug JH, Thomas-Hall S, Rupprecht J, Foo A, Klassen V, McDowall A, Schenk PM, Kruse O, Hankamer B: **Engineering photosynthetic light capture: impacts on improved solar energy to biomass conversion**. *Plant Biotechnol J* 2007, **5**:802-814.

23. Beckmann J, Lehr F, Finazzi G, Hankamer B, Posten C, Wobbe L, Kruse O: **Improvement of light to biomass conversion by de-regulation of light-harvesting protein translation in *Chlamydomonas reinhardtii***. *J Biotechnol* 2009, **142**:70-77.

24. • Keren N, Krieger-Liszkay A: **Photoinhibition: molecular mechanisms and physiological significance**. *Physiol Plant* 2011, **142**:1-5.
Brief review that presents the different points of view in the controversial field of photoinhibition.

25. • Rea G, Lambreva M, Polticelli F, Bertalan I, Antonacci A, Pastorelli S, Damasso M, Johanningmeier U, Giardi MT: **Directed evolution and in silico analysis of reaction centre proteins reveal molecular signatures of photosynthesis adaptation to radiation pressure**. *PLoS ONE* 2011, **6**.
Generated high-light resistant strains of *C. reinhardtii* by mutating the key D1 protein from PSII. Previous studies using random mutagenesis have not been able to identify the modifications that confer this phenotype.

26. Gimpel JA, Mayfield SP: **Analysis of heterologous regulatory and coding regions in algal chloroplasts**. *Appl Microbiol and Biotechnol* 2012 http://dx.doi.org/10.1007/s00253-012-4580-4.

27. Raines CA: **Increasing photosynthetic carbon assimilation in C3 plants to improve crop yield: current and future strategies**. *Plant Physiol* 2011, **155**:36-42.

28. Ducat DC, Silver PA: **Improving carbon fixation pathways**. *Curr Opin Chem Biol* 2012, **16**:337-344.

29. • Whitney SM, Houtz RL, Alonso H: **Advancing our understanding and capacity to engineer nature's CO_2-sequestering enzyme, Rubisco**. *Plant Physiol* 2011, **155**:27-35.
Examines the structural and enzymatic properties of Rubisco, along with its regulation and evolutionary history. At the same time it indicates the key aspects that might benefit from genetic engineering for improving carbon capture efficiency.

30. Genkov T, Meyer M, Griffiths H, Spreitzer RJ: **Functional hybrid rubisco enzymes with plant small subunits and algal large subunits: engineered rbcS cDNA for expression in *Chlamydomonas***. *J Biol Chem* 2010, **285**:19833-19841.

31. Zhu G, Kurek I, Liu L: **Engineering photosynthetic enzymes involved in CO_2 — assimilation by gene shuffling**. In *The Chloroplast: Basics and Applications.* Edited by Rebeiz CA, Benning C, Bohnert HJ, Daniell H, Hoober JK, Lichtenthaler HK, Portis AR, Tripathy BC. Netherlands: Springer; 2010:307-322.

32. Johnson X: **Manipulating RuBisCO accumulation in the green alga, *Chlamydomonas reinhardtii***. *Plant Mol Biol* 2011, **76**:397-405.

33. Chen CY, Yeh KL, Aisyah R, Lee DJ, Chang JS: **Cultivation, photobioreactor design and harvesting of microalgae for biodiesel production: a critical review**. *Bioresour Technol* 2011, **102**:71-81.

34. Hallmann A, Sumper M: **The *Chlorella* hexose/H^+ symporter is a useful selectable marker and biochemical reagent when expressed in Volvox**. *Proc Natl Acad Sci U S A* 1996, **93**:669-673.

35. Fischer H, Robl I, Sumper M, Kroger N: **Targeting and covalent modification of cell wall and membrane proteins heterologously expressed in the diatom *Cylindrotheca fusiformis* (Bacillariophyceae)**. *J Phycol* 1999, **35**:113-120.

36. Zaslavskaia LA, Lippmeier JC, Shih C, Ehrhardt D, Grossman AR, Apt KE: **Trophic obligate conversion of an photoautotrophic organism through metabolic engineering**. *Science* 2001, **292**:2073-2075.

37. Doebbe A, Rupprecht J, Beckmann J, Mussgnug JH, Hallmann A, Hankamer B, Kruse O: **Functional integration of the HUP1 hexose symporter gene into the genome of *reinhardtii*: impacts on biological H-2 production**. *J Biotechnol* 2007, **131**:27-33.

38. Griffiths M, Hille R, Harrison SL: **Lipid productivity, settling potential and fatty acid profile of 11 microalgal species grown under nitrogen replete and limited conditions**. *J Appl Phycol* 2012, **24**:989-1001.

39. James GO, Hocart CH, Hillier W, Chen H, Kordbacheh F, Price GD, Djordjevic MA: **Fatty acid profiling of *Chlamydomonas reinhardtii* under nitrogen deprivation**. *Bioresour Technol* 2011, **102**:3343-3351.

40. Fan J, Yan C, Andre C, Shanklin J, Schwender J, Xu C: **Oil accumulation is controlled by carbon precursor supply for fatty acid synthesis in *Chlamydomonas reinhardtii***. *Plant Cell Physiol* 2012, **53**:1380-1390.

41. • Boyle NR, Page MD, Liu B, Blaby IK, Casero D, Kropat J, Cokus SJ, Hong-Hermesdorf A, Shaw J, Karpowicz SJ *et al.*: **Three acyltransferases and nitrogen-responsive regulator are implicated in nitrogen starvation-induced triacylglycerol accumulation in *Chlamydomonas***. *J Biol Chem* 2012, **287**:15811-15825.
This RNA-seq study makes significant advances toward a mechanistic understanding of the long-observed effect of nitrogen starvation on TAG accumulation in algae.

42. Msanne J, Xu D, Konda AR, Casas-Mollano JA, Awada T, Cahoon EB, Cerutti H: **Metabolic and gene expression changes triggered by nitrogen deprivation in the photoautotrophically grown microalgae *Chlamydomonas reinhardtii* and *Coccomyxa* sp., C-169**. *Phytochemistry* 2012, **75**:50-59.

43. Work VH, Radakovits R, Jinkerson RE, Meuser JE, Elliott LG, Vinyard DJ, Laurens LM, Dismukes GC, Posewitz MC: **Increased lipid accumulation in the *Chlamydomonas reinhardtii* sta7-10 starchless isoamylase mutant and increased carbohydrate synthesis in complemented strains**. *Eukaryot Cell* 2010, **9**:1251-1261.

44. Wang ZT, Ullrich N, Joo S, Waffenschmidt S, Goodenough U: **Algal lipid bodies: stress induction, purification, and biochemical characterization in wild-type and**

Please cite this article in press as: Gimpel JA, et al.: Advances in microalgae engineering and synthetic biology applications for biofuel production, Curr Opin Chem Biol (2013), http://dx.doi.org/10.1016/j.cbpa.2013.03.038

starchless *Chlamydomonas reinhardtii*. *Eukaryot Cell* 2009, **8**:1856-1868.

45. • Siaut M, Cuine S, Cagnon C, Fessler B, Nguyen M, Carrier P, Beyly A, Beisson F, Triantaphylides C, Li-Beisson Y *et al.*: **Oil accumulation in the model green alga *Chlamydomonas reinhardtii*: characterization, variability between common laboratory strains and relationship with starch reserves**. *BMC Biotechnol* 2011, **11**:7.
Illustrates the strain-specific variability of starch and lipid accumulation under various culture conditions, highlighting the importance of strain background for experimental comparison.

46. Gong Y, Guo X, Wan X, Liang Z, Jiang M: **Characterization of a novel thioesterase (PtTE) from *Phaeodactylum tricornutum***. *J Basic Microbiol* 2011, **51**:666-672.

47. Radakovits R, Eduafo PM, Posewitz MC: **Genetic engineering of fatty acid chain length in *Phaeodactylum tricornutum***. *Metab Eng* 2011, **13**:89-95.

48. La Russa M, Bogen C, Uhmeyer A, Doebbe A, Filippone E, Kruse O, Mussgnug JH: **Functional analysis of three type-2 DGAT homologue genes for triacylglycerol production in the green microalga *Chlamydomonas reinhardtii***. *J Biotechnol* 2012, **162**:13-20.

49. Hockin NL, Mock T, Mulholland F, Kopriva S, Malin G: **The response of diatom central carbon metabolism to nitrogen starvation is different from that of green algae and higher plants**. *Plant Physiol* 2012, **158**:299-312.

50. Yu WL, Ansari W, Schoepp NG, Hannon MJ, Mayfield SP, Burkart MD: **Modifications of the metabolic pathways of lipid and triacylglycerol production in microalgae**. *Microb Cell Fact* 2011, **10**:91.

51. Blatti JL, Beld J, Behnke CA, Mendez M, Mayfield SP, Burkart MD: **Manipulating fatty acid biosynthesis in microalgae for biofuel through protein–protein interactions**. *PLoS ONE* 2012, **7**:e42949.

52. Meuser JE, D'Adamo S, Jinkerson RE, Mus F, Yang W, Ghirardi ML, Seibert M, Grossman AR, Posewitz MC: **Genetic disruption of both *Chlamydomonas reinhardtii* [FeFe]-hydrogenases: insight into the role of HYDA2 in H(2) production**. *Biochem Biophys Res Commun* 2012, **417**:704-709.

53. • Esquivel MG, Amaro HM, Pinto TS, Fevereiro PS, Malcata FX: **Efficient H2 production via *Chlamydomonas reinhardtii***. *Trends Biotechnol* 2011, **29**:595-600.
Concise review describing all strategies attempted to date to increase hydrogen production in *Chlamydomonas*.

54. Wu S, Xu L, Huang R, Wang Q: **Improved biohydrogen production with an expression of codon-optimized hemH and lba genes in the chloroplast of *Chlamydomonas reinhardtii***. *Bioresour Technol* 2011, **102**:2610-2616.

55. Kosourov SN, Ghirardi ML, Seibert M: **A truncated antenna mutant of *Chlamydomonas reinhardtii* can produce more hydrogen than the parental strain**. *Int J Hydrogen Energy* 2011, **36**:2044-2048.

56. Scoma A, Krawietz D, Faraloni C, Giannelli L, Happe T, Torzillo G: **Sustained H(2) production in a *Chlamydomonas reinhardtii* D1 protein mutant**. *J Biotechnol* 2012, **157**:613-619.

57. Dal'Molin CG, Quek LE, Palfreyman RW, Nielsen LK: **AlgaGEM — a genome-scale metabolic reconstruction of algae based on the *Chlamydomonas reinhardtii* genome**. *BMC Genomics* 2011, **12(Suppl 4)**:S5.

58. Kruse O, Rupprecht J, Bader KP, Thomas-Hall S, Schenk PM, Finazzi G, Hankamer B: **Improved photobiological H2 production in engineered green algal cells**. *J Biol Chem* 2005, **280**:34170-34177.

59. Guedes AC, Amaro HM, Malcata FX: **Microalgae as sources of carotenoids**. *Mar Drugs* 2011, **9**:625-644.

60. Peralta-Yahya PP, Ouellet M, Chan R, Mukhopadhyay A, Keasling JD, Lee TS: **Identification and microbial production of a terpene-based advanced biofuel**. *Nat Commun* 2011, **2**.

61. Cordero BF, Couso I, Leon R, Rodriguez H, Vargas MA: **Enhancement of carotenoids biosynthesis in *Chlamydomonas reinhardtii* by nuclear transformation using a phytoene synthase gene isolated from *Chlorella zofingiensis***. *Appl Microbiol Biotechnol* 2011, **91**:341-351.

62. Couso I, Vila M, Rodriguez H, Vargas MA, Leon R: **Overexpression of an exogenous phytoene synthase gene in the unicellular alga *Chlamydomonas reinhardtii* leads to an increase in the content of carotenoids**. *Biotechnol Prog* 2011, **27**:54-60.

63. Vila M, Couso I, Leon R: **Carotenoid content in mutants of the chlorophyte *Chlamydomonas reinhardtii* with low expression levels of phytoene desaturase**. *Process Biochem* 2008, **43**: 1147-1152.

Please cite this article in press as: Gimpel JA, et al.: Advances in microalgae engineering and synthetic biology applications for biofuel production, Curr Opin Chem Biol (2013), http://dx.doi.org/10.1016/j.cbpa.2013.03.038