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Engineering algae for biohydrogen and biofuel production

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There is currently substantial interest in utilizing eukaryotic algae for the renewable production of several bioenergy carriers, including starches for alcohols, lipids for diesel fuel surrogates, and H_2 for fuel cells. Relative to terrestrial biofuel feedstocks, algae can convert solar energy into fuels at higher photosynthetic efficiencies, and can thrive in salt water systems. Recently, there has been considerable progress in identifying relevant bioenergy genes and pathways in microalgae, and powerful genetic techniques have been developed to engineer some strains via the targeted disruption of endogenous genes and/or transgene expression. Collectively, the progress that has been realized in these areas is rapidly advancing our ability to genetically optimize the production of targeted biofuels.

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Introduction

Unicellular microalgae are at the forefront of research efforts aimed at developing technologies and model systems for the renewable production of H_2 and other biofuels [1–4,5•]. Relative to terrestrial plants, microalgae are more efficient at converting sunlight into chemical energy, and require a smaller footprint and less water for cultivation [2]. Many species of algae thrive in salt water, are able to grow year round in diverse conditions, and do not accumulate recalcitrant lignocellulosic biomass [2]. Importantly, genetic manipulation techniques have been developed for some species, and are increasingly being applied to optimize biofuel production in several algal systems. In contrast to traditional nutrient manipulation approaches, metabolic engineering improves control over metabolic pathways, increases the diversity of available phenotypes, and results in a more reproducible and predictable system [6].

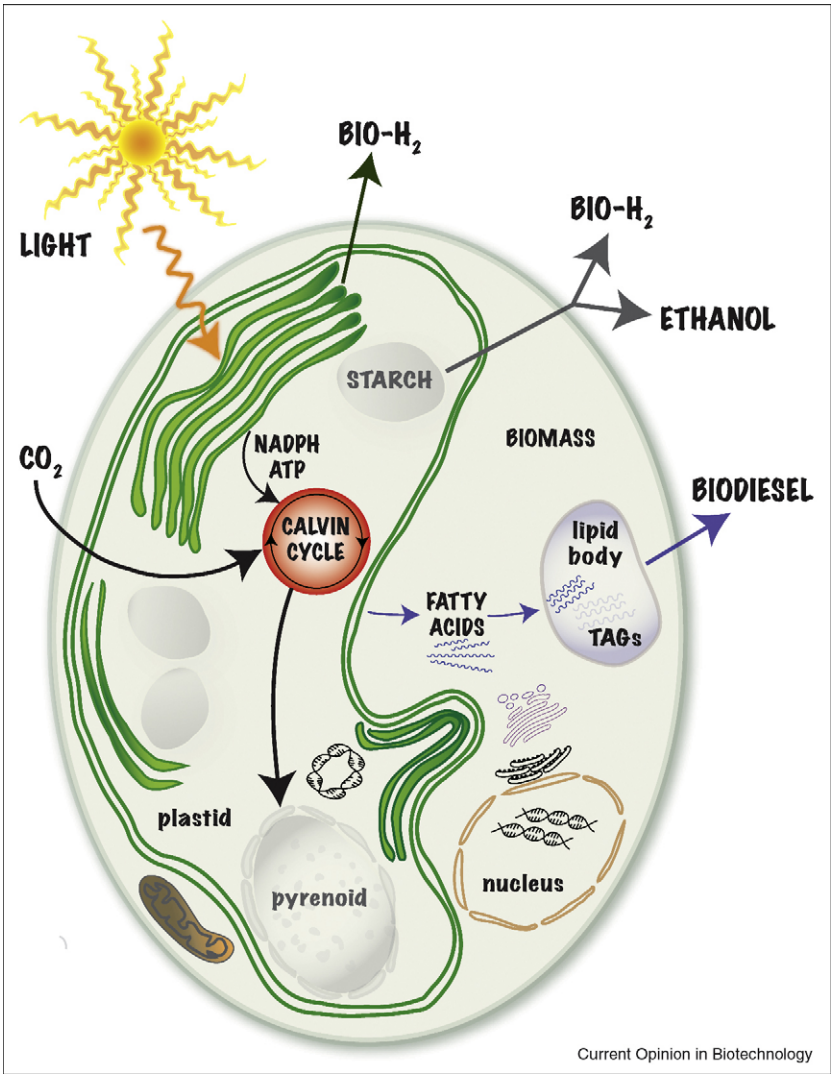
Photosynthesis is the fundamental driving force that supports all biofuel synthetic processes, converting solar energy into biomass, carbon storage products (e.g. carbohydrates and lipids), and/or H_2 (Figures 1 and 2). The integration of metabolic pathways is coordinated through complex mechanisms that regulate photosynthetic output to the distribution of reductant for the synthesis of proteins, nucleic acids, carbohydrates, lipids, and H_2. A comprehensive understanding of the biosynthesis and degradation of precursors, intermediates, and metabolic end products, and the identification of the regulatory networks that control metabolic flux is central to establish informed engineering strategies for optimizing biofuel production in microalgae. Several recent studies have used 'omics'-based strategies to begin unraveling the regulation and integration of these networks [7–10]. The insights gained from these studies and the discovery/generation of novel proteins that are potentially better suited for bioenergy applications are providing promising targets for genetic manipulation to enhance the accumulation of bioenergy carriers. In combination with increasingly refined genetic manipulation tools, the ability of scientists to engineer algae for the accumulation of specific metabolites is entering a new era.

Metabolic engineering in algae

Although routine genetic manipulation remains limited to a few select algal laboratory models (e.g. *Chlamydomonas reinhardtii*, *Volvox carteri*, and the diatom *Phaeodactylum tricornutum*), the expanding interest in algal biofuels will likely lead to the development of techniques in other organisms and the establishment of new model systems. Algal transgenics has been previously reviewed [11]; however, the 'molecular toolkit' has since expanded because of recent seminal studies. Significant advances include: (a), the efficient expression of transgenes [12]; (b), a novel mechanism for gene regulation in algae using riboswitches [13]; (c), inducible nuclear promoters and luciferase reporter genes [12,14], and (d) inducible chloroplast gene expression [15].

To date, the generation of stable nuclear transformants in microalgae has relied primarily on random genomic integration, intensive screening, and the subsequent isolation of knockout mutants. The identification of disruptions in target loci typically requires the screening of tens of thousands of transformants using suitable activity assays and/or extensive DNA analysis. The ability to generate

Figure 1

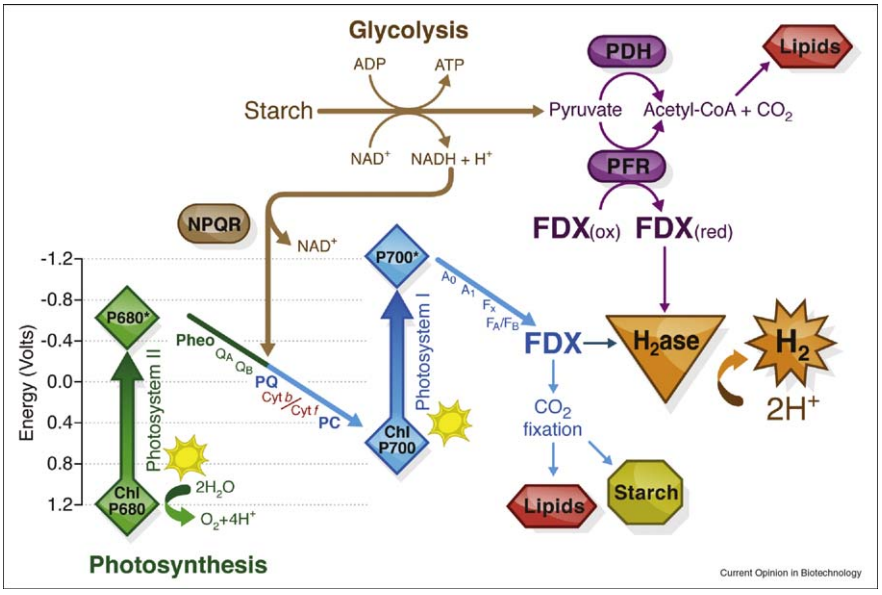


Metabolic pathways in green algae related to biofuel and biohydrogen production. In green algae, the light-harvesting complex bound to chlorophyll and carotenoids capture light energy as photons. This energy is used by photosystem II in the catalytic oxidation of water, forming protons, electrons, and molecular O_2. Low-potential electrons are transferred through the photosynthetic electron transport chain leading to the reduction of ferredoxin for the formation of NADPH. An electrochemical gradient is formed because of the release of protons after water oxidation into the thylakoid lumen, which is used to drive ATP production via ATP synthase. The photosynthetic products NADPH and ATP, are substrates for the Calvin–Benson cycle where inorganic CO_2 is fixed into 3-C molecules that are assimilated into the sugars, starch, lipids, or other molecules required for cellular growth. The substrates for hydrogenases, H^+ and e^-, are supplied via either the photosynthetic electron transport chain or from fermentation of stored carbohydrates (starch) via fermentation.

targeted gene knockouts through homologous recombination (as in yeast and cyanobacteria) has been difficult to achieve in algae. However, substantial research efforts in this area have led to steady progress and nonhomologous recombination to homologous recombination ratios of 100:1 have been reported in some *C. reinhardtii* strains [16•]. Thus, the ability of *C. reinhardtii* to undergo homologous recombination has been clearly demonstrated at a ratio suitable for many applications [16•], and although the general application of this method will require further development, progress is likely in the coming years.

One of the most significant advances in algal genetics is the development of improved gene silencing strategies in *C. reinhardtii*. High-throughput artificial miRNA (amiRNA) techniques for gene knockdown, which are highly specific and stable, were recently reported [17••,18••]. The targeted downregulation of gene expression in *C. reinhardtii* using RNAi had been previously

Figure 2



Photosynthetic and glycolytic pathways in green algae related to biofuel and biohydrogen production. Simplified illustration of the pathways used for lipid, starch, and H_2 production in *Chlamydomonas reinhardtii*. Electrons originating from either **(a)** water oxidation by photosystem II activity or **(b)** the nonphotochemical reduction of PQ are excited by photosystem I and used to reduce ferredoxin. Reduced ferredoxin subsequently reduces ferredoxin-NADPH oxidoreductase for the production of NADPH used in CO_2 fixation, leading to starch and lipid synthesis. Ferredoxin can also reduce H_2ase for the production of H_2 during anaerobic acclimation. The oxidation of pyruvate during glycolysis, catalyzed by either the pyruvate dehydrogenase complex (PDH) under aerobic conditions or pyruvate-ferredoxin oxidoreductase (PFR) under anaerobic conditions, can be used to generate acetyl-CoA for lipid biosynthesis. Other pathways leading to acetyl-CoA from pyruvate (e.g. from the activity of pyruvate formate lyase) are not shown for simplicity. Reduced ferredoxin resulting from the activity of PFR can be used to reduce H_2ase. Adapted from Posewitz *et al*. [4].

established; however, transcriptional silencing of the heterologous expression constructs was common and resulted in variable silencing efficiencies. Moreover, the large constructs used often affected other nontargeted transcripts [19]. The newly developed amiRNA techniques are likely to emerge as the method of choice for functional genomics studies in *C. reinhardtii*, and be applied to other species in order to elucidate general metabolic pathways, including those specifically related to biofuel production.

High-throughput sequencing and 'omics' technologies

Systems level technologies including genomics, transcriptomics, proteomics, and metabolomics are unraveling metabolic pathway regulation and integration, and are providing targets to optimize biofuel production [5•]. The *C. reinhardtii* genome sequence [20] revealed several unexpected pathways that are involved in fundamental metabolic processes, such as inorganic carbon fixation, fermentation, selenoprotein expression, and vitamin biosynthesis [21], each of which can be exploited to improve the accumulation of targeted bioenergy carriers. High-throughput DNA sequencing provides a new set of technologies for genomics and transcriptomics. In fact, the application of the 454 (Roche) pyrosequencing platform [22] resulted in the discovery of noncoding RNAs in *C. reinhardtii* and led to the first reports from *any* single-celled eukaryote of miRNAs [23•,24•,25]. High-throughput sequencing has become a pivotal technology for transcriptome analyses in plants [26] and is well suited for the identification of the regulatory genes that direct algal metabolism toward biofuel production. However, the full potential of transcriptomics can only be realized if the genome sequence for the target organism is available. Since relatively few algal genomes have been sequenced, a concerted effort from the community must be initiated to sequence relevant strains, and to develop the appropriate bioinformatic tools to exploit these strains for biofuels applications.

Progress in metabolic engineering toward biofuel production

Several approaches using random insertional mutagenesis and targeted gene disruption have been applied in the context of bioenergy applications in algae [27••,28]. Photosynthetic efficiency is directly related to the size of light-harvesting antennae complexes (LHC) and antennae size is optimized in response to light intensity [29]. Random insertion libraries were used to identify a mutant (*tla1*) that exhibited a truncated LHC, and

TLA1 has been verified as the first gene known to play a regulatory role in controlling antennae size in *C. reinhardtii* [30].

Additional *C. reinhardtii* mutants have been identified that produce high levels of H_2. In the *stm6* *s*tate *t*ransition *m*utant, starch over accumulates, rates of cellular respiration are increased, and cyclic electron transfer around PS I is inhibited leading to increased H_2-production rates [27••]. Hydrogen production has been further enhanced in the *stm6* mutant through heterologous expression of the hexose/H^+ symporter (*HUP1*), enabling *C. reinhardtii*, which lacks extracellular glucose uptake transporters, to couple glucose oxidation to H_2ase activity [31•]. *C. reinhardtii* mutants with starchless phenotypes have also been shown to influence H_2 production [32]. Starch synthesis and catabolism in *C. reinhardtii* has been thoroughly reviewed [33]; however, further research is required to better understand the partitioning of fixed carbon toward the increased production of starch for subsequent fermentation into H_2 or ethanol, or the redirection of photosynthate from starch into lipids for conversion to diesel fuels.

Metabolic engineering toward enhanced carbon storage

As illustrated in Figures 1 and 2, unicellular algae are capable of synthesizing a range of biofuels. Lipids and carbohydrates represent the main energy storage molecules in algae, and a broad understanding of primary metabolism is necessary to manipulate electron flux toward these products or H_2 for bioenergy applications. Complicating these efforts are the distinct metabolic processes that occur within algal organelles and the numerous enzyme isoforms present in a cell. For example, two isoforms of phosphoenolpyruvate carboxylase (PEP-C), the enzyme that carboxylates PEP, have been identified in *C. reinhardtii*, both of which are responsive to inorganic carbon and nitrogen levels [34]. Additionally, six pyruvate kinase homologs, six malic enzyme homologs, and five malate dehydrogenase homologs are present in the *C. reinhardtii* genome [20], some of which are differentially expressed under fermentative conditions in a *C. reinhardtii* mutant lacking hydrogenase activity [10]. These findings underscore the complexity of algal metabolism and emphasize the need to characterize the systems on an enzymatic level.

Metabolic engineering toward enhanced lipid biosynthesis

Much of our current knowledge on fatty acid (FA) biosynthetic enzymes is inferred from genome databases and relatively few studies on algal FA biosynthesis have been published [35]. Fatty acid synthesis (FAS) occurs in the plastid of plants before translocation to the cytoplasm for further assembly into diacylglyceride (DAG) and triacylglyceride (TAG) molecules. Many FAS enzymes are encoded by single genes and are thought to also be targeted to the mitochondria where FA precursors are required to produce essential cofactors for mitochondrial enzyme activity [36]. In contrast to the FA and TAG profiles from oilseed crops, which are fairly consistent, the lipids isolated from microalgae are variable and frequently composed of TAGs and polyunsaturated FAs that are prone to undesirable oxidation reactions affecting downstream biofuel applications.

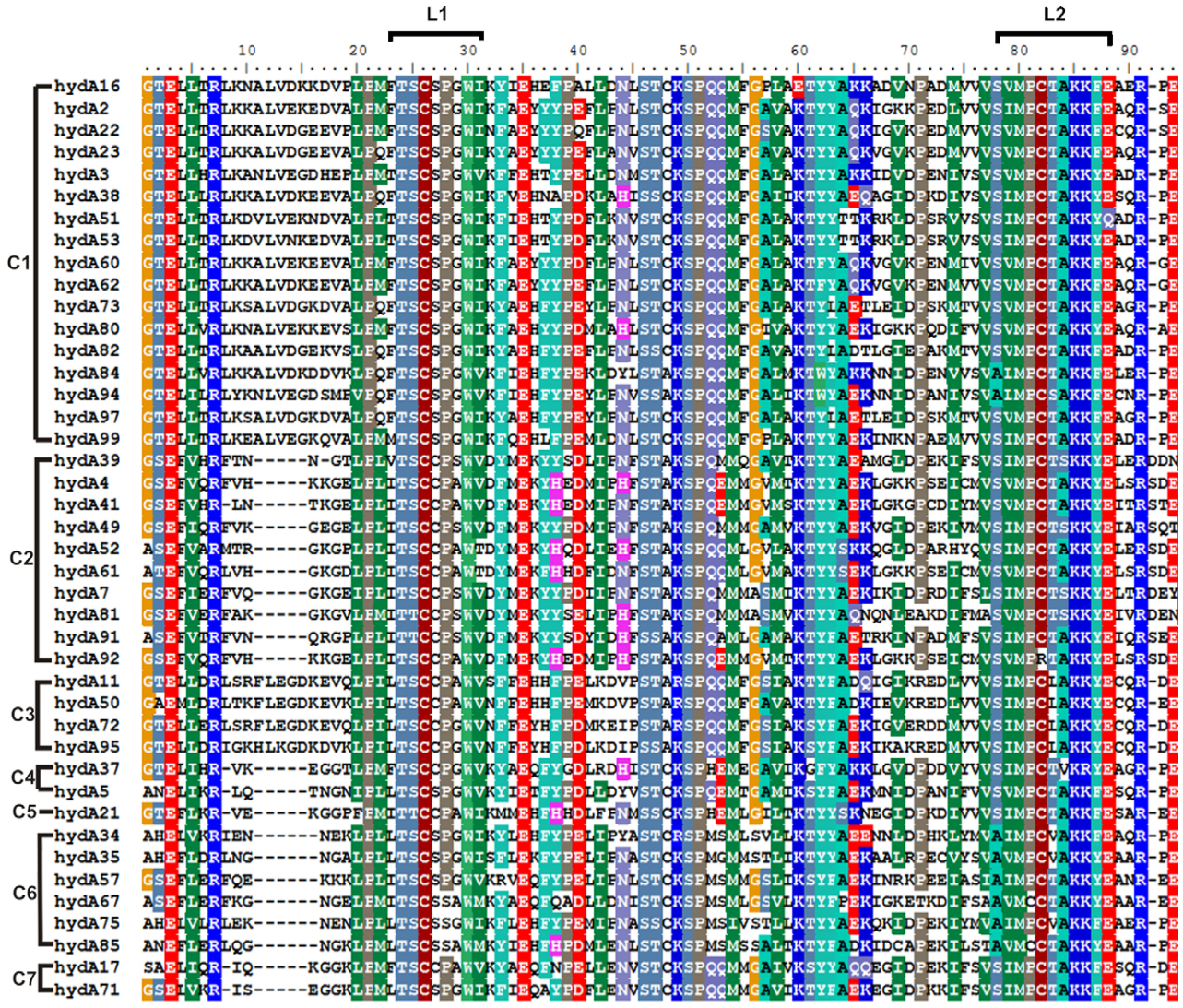
Historically, nutrient stress has been the method *de rigueur* for increasing lipid and starch accumulation in green algae and diatoms. Under nitrogen deplete conditions, some green algae accumulate high levels of lipids as TAGs [3], and phosphorus and sulfur deprivation induce the conversion of membrane phospholipids to neutral lipids, though the regulatory mechanisms in these systems are poorly understood [37–39]. Strategies to engineer FA biosynthesis toward more compatible lipid profiles are to: firstly, overexpress FA biosynthetic enzymes; secondly, increase the availability of precursor molecules, such as acetyl-CoA; thirdly, downregulate FA catabolism by inhibiting β-oxidation, or lipase hydrolysis; fourthly, alter saturation profiles through the introduction or regulation of desaturases; and fifthly, optimize FA chain length with thioesterases. The complexity of lipid metabolism in algae is illustrated by recent large-scale mutant screening in a *C. reinhardtii* insertional library, which identified 80 mutants with altered FAS activity (C Benning, unpublished).

Understanding the enzymatics: H_2 as a model system

A major obstacle in the development of algal bioenergy systems is the heterologous expression of enzymes that have been optimized for the production and accumulation of biofuels under the environmental conditions expected to prevail in a scaled-up operation. Owing to limited freshwater resources, salt water is likely to be incorporated into any algal biofuel production process. Thus, it is imperative that suitable organisms and corresponding enzymes that function in saline systems be identified. Furthermore, the optimization of H_2 photoproduction will also require identification of an O_2-tolerant H_2ase as the active site metal cluster of HydA is O_2 labile.

One approach to address this problem is gene shuffling, which has been used to generate a diverse recombinant hydrogenase library to screen for enhanced O_2 tolerance and/or stability [40]. A more recent strategy has been to search natural diversity through the use of degenerate PCR primers specific for the large subunit of *hydA* [41••]. DNA extracted from microbial mats that inhabit saline environments and that are exposed to supersaturating concentrations of O_2 during peak photosynthesis contained a diversity of deduced HydA amino acid sequences, resulting in a near doubling of the known diversity of this protein-encoding gene. Further, many of the sequences exhibit novel substitutions in

Figure 3



Alignment of putative HydA sequences from Guerrero Negro (GN), Baja California Sur, Mexico. Various substitutions in the L1 and L2 sequence motifs and insertional domains upstream from the L1 motif in the rich and diverse HydA assemblage in the top 1 mm of the GN microbial mat are apparent. The presence of a rich and diverse assemblage of HydA with phylogenetically coherent substitutions and insertions suggests a strong selective pressure to maintain HydA in these microbial ecosystems. Cluster (C) designations for individual HydA sequences correspond to phylogenetic clusters as presented in Boyd *et al*. [41••]. Phylogenetic affiliations of individual sequence clusters are as follows: C1 and C5, *Moorella thermoacetica*; C2, *Opitutus terrae*; C3, *Bacteroides thetaiotaomicron*; C4, *Alkaliphilus oremlandii*; C6, *Heliobacillus mobilis*; C7, *Thermoanaerobacterium saccharolyticum*. Adapted from Boyd *et al*. [41••].

the L1 (FLI]**T**S**C**[C/S]**P**[GAS]W[VIQH]) and L2 ([IVLF]M**PC**x[ASRD]**K**[KQ]xE) (bold underline is used to indicate strictly conserved amino acids) sequence motifs [42] which may be involved in modulating the redox properties of the [4Fe–4S]-subcluster of the active site H-cluster. Other sequences contain insertions upstream of the L1 motif that are involved in coordinating the FeS cubane of the active site (Figure 3) [41••]. Such studies demonstrate that firstly, the diversity of H_2ases in nature is severely underrepresented and secondly, enzyme variants that exhibit desired properties may be discovered by examining the diversity of protein-encoding genes in microbial assemblages that have evolved in the presence of a particular environmental stress. Full-length sequences of genes of interest can ultimately be determined from environmental DNA through a variety of techniques such as thermal asymmetric interlaced PCR [43].

Recent advances with respect to the identification of genes involved in [FeFe] H_2ase [44,45••,46] and [NiFe] H_2ase [47,48] maturation and regulation, and the development of heterologous expression systems for both classes of enzyme [44,45••,49–51], make it feasible to

biochemically characterize H_2ases by heterologous expression in organisms that do not possess endogenous H_2ase machinery. Target genes identified through bioprospecting efforts can therefore be characterized genetically and biochemically using heterologous expression studies. Moreover, the natural diversity of genes encoding other key enzymes with relevance to bioenergy can be examined, and exploited using similar approaches.

Conclusions

Research over the past two years has led to significant improvements in our ability to manipulate gene expression in eukaryotic algae, primarily *C. reinhardtii*. This progress will allow the manipulation of algal metabolism with far more precision than was historically feasible. Moreover, progress in all aspects of developing informed models of integrated algal metabolism, and strides in algal transgenics have led to a better understanding of how fixed carbon is partitioned toward bioenergy related molecules. Transformative high-throughput 'omics'-based research will continue to identify targets relevant to bioenergy production pathways. These advances, combined with the ability to effectively probe the natural diversity of enzymes such as the H_2ases, will continue to dramatically expand our understanding of enzyme and pathway diversity. This untapped natural diversity, along with genetically optimized proteins, can be exploited through heterologous expression in hosts amendable to large-scale cultivation. Additional genome sequencing efforts are necessary, and research directed toward generating more universal/general genetic transformation tools and screening methods will facilitate the development of informed strategies to optimize the accumulation of targeted biofuels. Significant breakthroughs in the development of improved tools for genetic manipulation in eukaryotic algae, and the current level of interest in algal-based biofuels and phototroph basic research will undoubtedly provide further advances in the coming years.

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References and recommended reading

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

- • of special interest
- •• of outstanding interest

1. Skjanes K, Knutsen G, Kallqvist T, Lindblad P: **H_2 production from marine and freshwater species of green algae during sulfur deprivation and considerations for bioreactor design**. *Int J Hyd Energy* 2008, **33**:511-521.

2. Dismukes GC, Carrieri D, Bennette N, Ananyev GM, Posewitz MC: **Aquatic phototrophs: efficient alternatives to land-based crops for biofuels**. *Curr Opin Biotechnol* 2008, **19**:235-240.

3. Hu Q, Sommerfeld M, Jarvis E, Ghirardi M, Posewitz M, Seibert M, Darzins A: **Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances**. *Plant J* 2008, **54**:621-639.

4. Posewitz MC, Dubini A, Meuser JE, Seibert M, Ghirardi ML: **Hydrogenases, hydrogen production and anoxia**. In *Organellar and Metabolic Processes,* edn 2. Edited by Stern DB. In *The Chlamydomonas Sourcebook*, Vol. 2. Edited by Harris EE. *The Chlamydomonas Sourcebook*. Vol. 2Academic Press; 2009: 217-246.

5. • Schenk P, Thomas-Hall S, Stephens E, Marx U, Mussgnug J, Posten C, Kruse O, Hankamer B: **Second generation biofuels: high-efficiency microalgae for biodiesel production**. *Bioenerg Res* 2008, **1**:20-43.

The review article provides an overview of second-generation biodiesel production systems using microalgae. Sustainability, photosynthetic conversion efficiencies, and the requirements, advantages and disadvantages of microalgal feedstocks are discussed.

6. Grossman AR: **Acclimation of *Chlamydomonas reinhardtii* to its nutrient environment**. *Protist* 2000, **151**:201-224.

7. Naumann B, Busch A, Allmer J, Ostendorf E, Zeller M, Kirchhoff H, Hippler M: **Comparative quantitative proteomics to investigate the remodeling of bioenergetic pathways under iron deficiency in *Chlamydomonas reinhardtii***. *Proteomics* 2007, **7**:3964-3979.

8. Nguyen AV, Thomas-Hall SR, Malnoe A, Timmins M, Mussgnug JH, Rupprecht J, Kruse O, Hankamer B, Schenk PM: **Transcriptome for photobiological hydrogen production induced by sulfur deprivation in the green alga *Chlamydomonas reinhardtii***. *Eukaryot Cell* 2008, **7**:1965-1979.

9. Mus F, Dubini A, Seibert M, Posewitz MC, Grossman AR: **Anaerobic acclimation in *Chlamydomonas reinhardtii*: anoxic gene expression, hydrogenase induction and metabolic pathways**. *J Biol Chem* 2007, **282**:25475-25486.

10. Dubini A, Mus F, Seibert M, Grossman AR, Posewitz MC: **Flexibility in anaerobic metabolism as revealed in a mutant of *Chlamydomonas reinhardtii* lacking hydrogenase activity**. *J Biol Chem* 2009, **284**:7201-7213.

11. Walker TL, Collet C, Purton S: **Algal transgenics in the genomic era**. *J Phycol* 2005, **41**:1077-1093.

12. Neupert J, Karcher D, Bock R: **Generation of *Chlamydomonas* strains that efficiently express nuclear transgenes**. *Plant J* 2008, **57**:1140-1150.

13. Croft MT, Moulin M, Webb ME, Smith AG: **Thiamine biosynthesis in algae is regulated by riboswitches**. *Proc Natl Acad Sci U S A* 2007, **104**:20770-20775.

14. Shao N, Bock R: **A codon-optimized luciferase from *Gaussia princeps* facilitates the in vivo monitoring of gene expression in the model alga *Chlamydomonas reinhardtii***. *Curr Genet* 2008, **53**:381-388.

15. Surzycki R, Cournac L, Peltier G, Rochaix JD: **Potential for hydrogen production with inducible chloroplast gene expression in *Chlamydomonas***. *Proc Natl Acad Sci U S A* 2007, **104**:17548-17553.

16. • Zorin B, Lu Y, Sizova I, Hegemann P: **Nuclear gene targeting in *Chlamydomonas* as exemplified by disruption of the PHOT gene**. *Gene* 2009, **432**:91-96.

The authors report the continued improvement of homologous recombination techniques in the green alga *Chlamydomonas reinhardtii*.

17. •• 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.

This article describes *C. reinhardtii* miRNAs that direct site-specific cleavage of target mRNA with miRNA-complementary motifs and, presumably, act as regulatory molecules in growth and development. A novel artificial miRNA system based on ligation of DNA oligonucleotides that can be used for specific high-throughput gene silencing in green algae is described.

18. •• Zhao T, Wang W, Bai X, Qi Y: **Gene silencing by artificial microRNAs in *Chlamydomonas***. *Plant J* 2009, **58**:157-164.
The researchers have developed an artificial miRNA-based strategy to silence gene expression in *Chlamydomonas*. The authors demonstrated that two amiRNAs could be expressed from a dimeric amiRNA precursor to knockdown the transcript levels of two unrelated genes simultaneously.

19. Rohr J, Sarkar N, Balenger S, Jeong BR, Cerutti H: **Tandem inverted repeat system for selection of effective transgenic RNAi strains in *Chlamydomonas***. *Plant J* 2004, **40**:611-621.

20. Merchant SS, Prochnik SE, Vallon O, Harris EH, Karpowicz SJ, Witman GB, Terry A, Salamov A, Fritz-Laylin LK, Marechal-Drouard L *et al.*: **The *Chlamydomonas* genome reveals the evolution of key animal and plant functions**. *Science* 2007, **318**:245-250.

21. Grossman AR, Croft M, Gladyshev VN, Merchant SS, Posewitz MC, Prochnik S, Spalding MH: **Novel metabolism in *Chlamydomonas* through the lens of genomics**. *Curr Opin Plant Biol* 2007, **10**:190-198.

22. Margulies M, Egholm M, Altman WE, Attiya S, Bader JS, Bemben LA, Berka J, Braverman MS, Chen Y-J, Chen Z *et al.*: **Genome sequencing in microfabricated high-density picolitre reactors**. *Nature* 2005, **437**:376-380.

23. • Zhao T, Li GL, Mi SJ, Li S, Hannon GJ, Wang XJ, Qi YJ: **A complex system of small RNAs in the unicellular green alga *Chlamydomonas reinhardtii***. *Genes Dev* 2007, **21**:1190-1203.
This research showed that *Chlamydomonas* contains numerous microRNAs (miRNAs) that can regulate the transcript levels of endogenous genes.

24. • Molnar A, Schwach F, Studholme DJ, Thuenemann EC, Baulcombe DC: **miRNAs control gene expression in the single-cell alga *Chlamydomonas reinhardtii***. *Nature* 2007, **447**:1126-1129.
This research showed that *Chlamydomonas* contains many microRNAs (miRNAs) that can downregulate expression of endogenous genes.

25. Eamens A, Wang MB, Smith NA, Waterhouse PM: **RNA silencing in plants: yesterday, today, and tomorrow**. *Plant Phys* 2008, **147**:456-468.

26. Baerenfaller K, Grossmann J, Grobei MA, Hull R, Hirsch-Hoffmann M, Yalovsky S, Zimmermann P, Grossniklaus U, Gruissem W, Baginsky S: **Genome-scale proteomics reveals *Arabidopsis thaliana* gene models and proteome dynamics**. *Science* 2008, **320**:938-941.

27. •• Kruse O, Rupprecht J, Bader KP, Thomas-Hall S, Schenk PM, Finazzi G, Hankamer B: **Improved photobiological H_2 production in engineered green algal cells**. *J Biol Chem* 2005, **280**:34170-34177.
The authors describe the isolation of the *C. reinhardtii stm6* mutant that has an increased H_2-production phenotype compared to the parental strain. The mutant is blocked in cyclic e-transport, has a modified respiratory metabolism, and large starch reserves.

28. 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.

29. Tanaka A, Melis A: **Irradiance-dependent changes in the size and composition of the chlorophyll a–b light-harvesting complex in the green alga *Dunaliella salina***. *Plant Cell Physiol* 1997, **38**:17-24.

30. Tetali SD, Mitra M, Melis A: **Development of the light-harvesting chlorophyll antenna in the green alga *Chlamydomonas reinhardtii* is regulated by the novel Tla1 gene**. *Planta* 2007, **225**:813-829.

31. • 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 *C. reinhardtii*: impacts on biological H_2 production**. *J Biotechnol* 2007, **131**:27-33.
This article reports the successful heterologous expression of HUP1 (hexose uptake protein) from *Chlorella kessleri* in the *C. reinhardtii stm6* mutant, resulting in the utilization of externally supplied glucose. The process resulted in 150% greater H_2-production capacity.

32. Posewitz MC, Smolinski SL, Kanakagiri S, Melis A, Seibert M, Ghirardi ML: **Hydrogen photoproduction is attenuated by disruption of an isoamylase gene in *Chlamydomonas reinhardtii***. *Plant Cell* 2004, **16**:2151-2163.

33. Ball SG: **The intricate pathway of starch biosynthesis and degradation in the monocellular alga *Chlamydomonas reinhardtii***. *Aust J Chem* 2002, **55**:49-59.

34. Moellering ER, Ouyang Y, Mamedov TG, Chollet R: **The two divergent PEP-carboxylase catalytic subunits in the green microalga *Chlamydomonas reinhardtii* respond reversibly to inorganic-N supply and co-exist in the high-molecular-mass, hetero-oligomeric Class-2 PEPC complex**. *FEBS Lett* 2007, **581**:4871-4876.

35. Riekhof WR, Benning C: **Glycerolipid biosynthesis**. In *The Chlamydomonas Sourcebook Organellar and Metabolic Processes,* edn 2. Edited by Stern DB. In *The Chlamydomonas Sourcebook*, Vol. 2. Edited by Harris EE. *The Chlamydomonas Sourcebook*. Vol. 2Academic Press; 2009:41-68.

36. Riekhof WR, Sears BB, Benning C: **Annotation of genes involved in glycerolipid biosynthesis in *Chlamydomonas reinhardtii*: discovery of the betaine lipid synthase BTA1(Cr)**. *Eukaryot Cell* 2005, **4**:242-252.

37. Moseley JL, Gonzalez-Ballester D, Pootakham W, Bailey S, Grossman AR: **Genetic interactions between regulators of *Chlamydomonas* phosphorus and sulfur deprivation responses**. *Genetics* 2008, **181**:889-905.

38. Sugimoto K, Midorikawa T, Tsuzuki M, Sato N: **Upregulation of PG synthesis on sulfur-starvation for PS I in *Chlamydomonas***. *Biochem Biophys Res Commun* 2008, **369**:660-665.

39. Sugimoto K, Sato N, Tsuzuki M: **Utilization of a chloroplast membrane sulfolipid as a major internal sulfur source for protein synthesis in the early phase of sulfur starvation in *Chlamydomonas reinhardtii***. *FEBS Lett* 2007, **581**:4519-4522.

40. Nagy LE, Meuser JE, Plummer S, Seibert M, Ghirardi ML, King PW, Ahmann D, Posewitz MC: **Application of gene-shuffling for the rapid generation of novel [FeFe]-hydrogenase libraries**. *Biotechnol Lett* 2007, **29**:421-430.

41. •• Boyd ES, Spear JR, Peters JW: **[FeFe]-hydrogenase genetic diversity provides insight into molecular adaptation in a saline microbial mat community**. *Appl Environ Microbiol* 2009, in press.
This report details the development and application of robust primers for the highly specific amplification of novel [FeFe]-hydrogenases from environmental genomic DNA samples isolated from a saline system with high microbial diversity.

42. Meyer J: **[FeFe] hydrogenases and their evolution: a genomic perspective**. *Cell Mol Life Sci* 2007, **64**:1063-1084.

43. Liu YG, Whittier RF: **Thermal asymmetric interlaced PCR: automatable amplification and sequencing of insert end fragments from P1 and YAC clones for chromosome walking**. *Genomics* 1995, **25**:674-681.

44. Girbal L, von Abendroth G, Winkler M, Benton PMC, Meynial-Salles I, Croux C, Peters JW, Happe T, Soucaille P: **Homologous and heterologous overexpression in *Clostridium acetobutylicum* and characterization of purified clostridial and algal Fe-only hydrogenases with high specific activities**. *Appl Environ Microbiol* 2005, **71**:2777-2781.

45. •• Posewitz MC, King PW, Smolinski SL, Zhang L, Seibert M, Ghirardi ML: **Discovery of two novel radical *S*-adenosylmethionine proteins required for the assembly of an active [Fe] hydrogenase**. *J Biol Chem* 2004, **279**:25711-25720.
The authors report the initial identification of the [FeFe]-hydrogenase maturases, and demonstrate the first heterologous expression of an [FeFe]-hydrogenase in a host lacking an endogenous [FeFe]-hydrogenase.

46. McGlynn SE, Shepard EM, Winslow MA, Naumov AV, Duschene KS, Posewitz MC, Broderick WE, Broderick JB,

Peters JW: **HydF as a scaffold protein in [FeFe] hydrogenase H-cluster biosynthesis**. *FEBS Lett* 2008, **582**:2183-2187.

47. Schubert T, Lenz O, Krause E, Volkmer R, Friedrich B: **Chaperones specific for the membrane-bound [NiFe]-hydrogenase interact with the Tat signal peptide of the small subunit precursor in *Ralstonia eutropha* H16**. *Mol Microbiol* 2007, **66**:453-467.

48. Ludwig M, Schubert T, Zebger I, Wisitruangsakul N, Saggu M, Strack A, Lenz O, Hildebrandt P, Friedrich B: **Concerted action of two novel auxiliary proteins in assembly of the active site in a membrane-bound [NiFe] hydrogenase**. *J Biol Chem* 2009, **284**:2159-2168.

49. Sybirna K, Antoine T, Lindberg P, Fourmond V, Rousset M, Mejean V, Bottin H: ***Shewanella oneidensis*: a new and efficient system for expression and maturation of heterologous [Fe–Fe] hydrogenase from *Chlamydomonas reinhardtii***. *BMC Biotechnol* 2008, **8**:73.

50. Lenz O, Gleiche A, Strack A, Friedrich B: **Requirements for heterologous production of a complex metalloenzyme: the membrane-bound [NiFe] hydrogenase**. *J Bacteriol* 2005, **187**:6590-6595.

51. King PW, Posewitz MC, Ghirardi ML, Seibert M: **Functional studies of [FeFe] hydrogenase maturation in an *Escherichia coli* biosynthetic system**. *J Bacteriol* 2006, **188**:2163-2172.