Chapter 18
Omics in *Chlamydomonas* for Biofuel Production

Hanna R. Aucoin*, Joseph Gardner*, and Nanette R. Boyle

Abstract In response to demands for sustainable domestic fuel sources, research into biofuels has become increasingly important. Many challenges face biofuels in their effort to replace petroleum fuels, but rational strain engineering of algae and photosynthetic organisms offers a great deal of promise. For decades, mutations and stress responses in photosynthetic microbiota were seen to result in production of exciting high-energy fuel molecules, giving hope but minor capability for design. However, '-omics' techniques for visualizing entire cell processing has clarified biosynthesis and regulatory networks. Investigation into the promising production behaviors of the model organism *C. reinhardtii* and its mutants with these powerful techniques has improved predictability and understanding of the diverse, complex interactions within photosynthetic organisms. This new equipment has created an exciting new frontier for high-throughput, predictable engineering of photosynthetically produced carbon-neutral biofuels.

Keywords Nutrient limitation • Triacylglycerols • Biohydrogen • Stress response • Gene discovery

Abbreviations

Enzymes

ACCase	Acetyl-CoA carboxylase, E.C. 6.4.1.2
ACK1	Non-specific protein-tyrosine kinase, E.C. 2.7.10.2
ACK2	(Acetyl-CoA carboxylase) kinase 2, E.C. 2.7.11.27
ADH1	Alcohol dehydrogenase 1/Formaldehyde dehydrogenase (FDH1), E.C. 1.1.1.1

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ADH2	Alcohol dehydrogenase (NADP+), E.C. 1.1.1.2
ADH3	Alcohol dehydrogenase Isoform, E.C. 1.1.1.1
ADP-Glc PPase	ADP-glucose pyrophosphorylase, E.C. 2.7.7.27
DGAT	Diacylglycerol acyltransferase, E.C. 2.3.1.20
FAS	Fatty acid synthase, E.C. 2.3.1.85
GPAT	Glycerol-3-phosphate acyltransferase, E.C. 2.3.1.15
HYD1	Ferredoxin hydrogenase, E.C. 1.12.7.2
HYD2	Ferredoxin hydrogenase Isoform, E.C. 1.12.7.2
ICL	Isocitrate lyase, E.C. 4.1.3.1
LDH	Lactate dehydrogenase, E.C. 1.1.1.27
LPAT	Lysophosphatidic acid acyltransferase, E.C. 2.3.1.51
MCT	Malonyl acyl carrier protein transferase, E.C. 2.3.1.39
MLDP	Major lipid droplet protein
PAT1	Phosphate acetyltransferase, E.C. 2.3.1.8
PAT2	Phosphate acetyltransferase Isoform, E.C. 2.3.1.8
PDC3	Pyruvate decarboxylase, E.C. 4.1.1.1
PDAT	Phospholipid diacylglycerol acyltransferase, E.C. 2.3.1.158
PDH	Pyruvate dehydrogenase, E.C. 1.2.1.51
PFL1	Formate C-acetyltransferase, E.C. 2.3.1.54

Transcripts

DGAT1	Diacylglycerol acyltransferase
DGTT1	Diacylglycerol acyltransferase
GPD2	Glycerol-3-phosphate dehydrogenase
GPD4	Glycerol-3-phosphate dehydrogenase
LHCBM9	Light Harvesting Complex Chlorophyll a-b binding protein
LHCSR2	Light Harvesting Complex stress-related 2
LPAT1	Lysophosphatidic acid acyltransferase
MLDP1	Major lipid droplet protein
PDAT1	Phospholipid diacylglycerol acyltransferase

Molecules

DAG	Diacylglycerol
FA	Fatty acid
FDox	Ferredoxin (oxidized)
FDred	Ferredoxin (reduced)
G3P	Glycerol-3-phosphate
PFR1ox	Pyruvate ferredoxin (oxidized)
PFR1red	Pyruvate ferredoxin (reduced)
SQDG	Sulfoquinovovosyl diacylglycerol
TAG	Triacylglycerol

Structures and Proteins

$Cytb_6$	Cytochrome b6 (small subunit of cytochrome b6f complex)
Cytf	Cytochrome f (large subunit of cytochrome b6f complex)
Fd	Ferredoxin
FNR	Ferredoxin – NADP(+) reductase
LHC	Light Harvesting Complex
NDH	Plastidial NAD(P)H dehydrogenase complex
P680	Chlorophyll a P680 (680 nm)
P700	Chlorophyll a P700 (700 nm)
Pc	Plastocyanin
$PQ(H)_2$	Plastoquinone (reduced)
PSI	Photosystem I
PSII	Photosystem II
PQ_0	Plastoquinone (oxidized)

Miscellaneous

2-DE	2-Dimensional Gel Electrophoresis
GC/MS	Gas Chromatography/Mass Spectroscopy
HS	Heat Shock
LC/MS	Liquid Chromatography/Mass Spectroscopy
MALDI	Matrix-Assisted Laser Desorption/Ionization
NMR	Nuclear Magnetic Resonance
MFA	Metabolite Flux Analysis
TOF	Time of Flight

Introduction

For long term sustainability, there is a pressing need to develop renewable sources of fuels which are efficient and compatible with current infrastructure. There are a number of alternative energy sources including wind, solar and hydroelectric which can be used to replace our dependence on fossil fuels for electricity; however, the ability to convert these energy sources to a form easily used for transportation is neither straightforward nor energy efficient. Biomass derived biofuels are particularly well-suited to displace some (or all) of our dependency on fossil fuels for the transportation sector because metabolites in the cell, such as starch or lipids, can easily be converted into fuels using current technologies. Advances in molecular biology and synthetic biology have allowed researchers to redesign metabolism and tailor-make molecules for fuel production (Oliver et al. 2013, 2014; Yim et al. 2011; Li and Liao 2013; Nakamura and Whited 2003; Kurian 2005). Most currently

available biofuels are produced in *E. coli* or yeast using starch or cellulosic sugars as feedstocks. Another alternative, which has the potential to be carbon neutral, is the production of biofuels from photosynthetic microorganisms. Microalgae naturally accumulate lipids and starch or produce hydrogen when certain nutrients are depleted in the media (Miller et al. 2010; Li et al. 2010a; Moellering and Benning 2010; Blaby et al. 2013; Boyle et al. 2012; Timmins et al. 2009), making them particularly attractive as sources of biofuels. The exact mechanisms that control the accumulation or production of these important fuel precursors are still being investigated but great strides have been made to understand these mechanisms using '-omics' technologies.

With advances in analytical technologies, it is now possible to collect large amounts of data to characterize both model and non-model organisms. These data sets are coined '-omics'; meant to imply "quantification of a whole" (Jamers et al. 2009), '-omics' are divided into observing cellular behavior at different levels of regulation: predominantly at the genomic (DNA), transcriptomic (RNA), proteomic (protein/enzyme), and metabolomic (metabolism) levels. By comparing data sets from different growth conditions, environmental stimuli, or genetic backgrounds, a more complete picture of how the cell responds to different stimuli can be gained. Since little is known about the genes and regulatory mechanisms which control the pathways involved in fuel-relevant molecule production, '-omics' analyses are needed. In the discussion that follows, we will describe how '-omics' technologies have enabled advances in the understanding of biofuel production in algae, particularly *Chlamydomonas reinhardtii*.

Chlamydomonas reinhardtii *as a Model Organism*

Chlamydomonas reinhardtii, a soil-dwelling, unicellular green alga, has served as a model organism since its discovery and isolation 70 years ago. *C. reinhardtii* is particularly interesting because it can function photoautotrophically, mixotrophically, or heterotrophically on acetate while maintaining its photosynthetic apparatus (Kempa et al. 2009; Chen and Johns 1996). Its flexible metabolism is complemented by a complex structure reminiscent of higher order plants: it has multiple mitochondria, a chloroplast, flagella (Harris and Stern 2009), and a cell wall of extensin-like hydroxyproline-rich glycoproteins (Woessner and Goodenough 1994). There is also an emerging toolbox of molecular techniques for genetic manipulation of *C. reinhardtii* and libraries of knock-out mutants are available (Gonzalez-Ballester et al. 2011). The availability of knock out libraries enables further research into gene/function relationships. One of the most significant mutants to be isolated is the cell wall-less mutation as it demonstrates higher permeability to exogenous DNA (Harris 2001) and is therefore more genetically tractable. Likewise, knowing the genes participating in cell walls and vacuolization can help *C. reinhardtii* model organism acclimation to hyperosmotic environments. *C. reinhardtii* has also been studied to understand flagella dysfunction in complex eukaryotes, especially for

heritable disease research in cilia and other filamentous structures (Cole 1999; Pazour et al. 1999a, b, 2000). *C. reinhardtii* can thus be used to model structural elements to determine mechanical and chemical stress responses in simpler, more controlled situations.

While understanding of the cellular machinery is crucial to strain design, deconvolution of metabolism has been possibly the largest goal in rational biosynthetic design. *C. reinhardtii* has been at the forefront of this research not only because it has metabolic flexibility, but because its metabolic flexibility is relatively well understood. As such, subjecting *C. reinhardtii* to nutrient stress has been a consistent tactic for investigation: nutrient deplete phenotypes and phenotype rescues are used as controls for conclusive, rigorous experimentation. Conclusions can be drawn from these experiments by using knowledge and connecting it to observed phenotypes; for example, removing copper – a cofactor in plastocyanin (Li et al. 1996) – resulted in a remodeling of the photosynthetic apparatus and identification of the *Crd1* gene product as a response to oxygen deficiency (Moseley et al. 2000). Carbon dioxide, sulfur deprivation, and other nutrient studies have demonstrated observable metabolic remodeling through starch accumulation, hydrogen accumulation, and various transcriptional changes, respectively (Buléon et al. 1997; Melis et al. 2000; Navarro et al. 2000; Quesada et al. 1998; Zhou et al. 2000; Quiñones et al. 1999). These stresses are now being studied at molecular levels to draw even deeper conclusions about stress response.

C. reinhardtii may not be the optimal organism for large-scale production of biofuels, but its research has been extremely valuable to the field as a whole (Merchant et al. 2007). In a lot of ways, the diversity of mutants and biological characterization afforded by the community has outpaced the sophistication of characterization techniques. As '-omics' analytical methods become more powerful, the links between phenotypes and genes can be further unveiled, resulting in concise, predictable genetic targets for strain and bioprocess design. Not only that, but the burgeoning molecular toolbox (Beer et al. 2009), including high-fidelity transgene expression (Neupert et al. 2009), gene regulation manipulation through riboswitches (Croft et al. 2007), inducible promoters (Neupert et al. 2009, Shao and Bock 2008), and chloroplast manipulation (Surzycki et al. 2007), can improve the experimental power in *C. reinhardtii*. Overall, the move towards carbon neutral fuels has been aided by *C. reinhardtii* and its degree of characterization, and further analysis into the precise molecular mechanisms is becoming available.

'-OMICS' Approaches

Genomics and Transcriptomics

Knowledge of the DNA and RNA sequences within a cell provides a picture of the cells capabilities and gene expression under different conditions. Genomics and transcriptomics can, then, be used to determine conserved behaviors across organisms, to infer transcript functions, or to determine gene function from phenotypes.

Since genomics is the analysis of the whole information reservoir for an organism, it establishes the boundaries of the cellular landscape. Transcriptomics augments this analysis by cooperatively restricting the landscape through finding which parts of the genome are expressed and by elucidating complex gene interactions. By varying conditions, both changes and constancy in transcript levels can provide insight into regulatory pathways and the mechanisms by which fitness is maintained. Understanding this passage of information aids in determining and assessing the viability of genetic targets while establishing the boundaries of the cellular landscape.

The similar structures for information storage in DNA and RNA correspond to similar methods of determining base pair order. Replacing classical Sanger sequencing (Sanger and Coulson 1975), the most common methods begin with shotgun which require DNA or RNA to be cut or sheared randomly into smaller fragments. From there, classic methods like the chain termination method or Maxam-Gilbert sequencing are used to determine the sequence of individual fragments for DNA (Sanger et al. 1977; Maxam and Gilbert 1977). Exciting advances in high-throughput, cost-controlled sequencing have resulted in next-generation sequencing methods like single-molecule real-time sequencing (Thompson and Steinmann 2010), ion semiconductor (Rusk 2011), pyrosequencing (Ronaghi et al. 1996), Applied Biosystems' SOLiD Sequencing (Valouev et al. 2008), 454 Life Science pyrosequencing (Rothberg and Leamon 2008), and sequencing by synthesis (Illumina) (Brenner et al. 2000). The main drawback of these methods is the high capital investment (Liu et al. 2012) but the cost to sequence DNA continues to fall rapidly (https://www.genome.gov/sequencingcosts). Sequencing of RNA (RNA-seq) is more challenging due to the nature of RNA: rapidly changing and easily degraded. Current methods allow for cheap, single, and simultaneous library generation reactions (Shishkin et al. 2015) and improved coverage of RNA functionality through separating different types of RNA (by size exclusion or similar routines) and consequent sequencing (Morin et al. 2008). Direct RNA Sequencing (without conversion to cDNA), is hypothetically less error-prone but is still in its infancy due to the instability of RNA (Ozsolak et al. 2009). Once the sequence of these small DNA or RNA sequences is known, computational methods need to be used to assemble sequences into longer pieces. Manual annotation of sequenced genomes is not possible given how quickly genomes can be sequenced today. Therefore, computational tools have been created which are capable of predicting gene structure and function, such as AUGUSTUS (now u10) (Specht et al. 2011). Genome browsers are available for a number of photosynthetic organisms through the Phytozome Genome Browser (Goodstein et al. 2012), which allows searching genomes for genes and supports further annotation efforts. A large amount of *Chlamydomonas reinhardtii* transcriptome data from various experiments is also available on the UCSC genome browser (https://genome.ucsc.edu/); this gives excellent snapshots of how transcript abundance varies in any given condition.

Base pairs sequences on their own offer little insight into cell behavior; additional computation is used to find patterns between genomic and transcriptomic data. While earlier studies were conducted slowly by referencing primary literature, software tools (Overbeek et al. 2014; Curwen et al. 2004; Zerbino 2010; Haas et al.

2013) in combination with genome and transcriptome databases (Kanehisa and Goto 2000; Caspi et al. 2008; Pruitt et al. 2012; Nordberg et al. 2014) have significantly streamlined the process. Most tools operate by automatically patching (if necessary) and correlating sequences of highly conserved genetic regions. This allows examination of the actual regions expressed in the genome and can be used to determine the structure of genes and possibly their function based on conserved sequences. These sequences can also be correlated across related strains to construct phylogenetic trees (Qi et al. 2004).

Proteomics

Proteomic analysis is conducted with the same shotgun approach as the previous two methods but with a different mean of quantifying the fragments. Similarly to genomics and transcriptomics, proteomics begins with deconstruction into peptides via proteolytic digesting. Once done, chromatography, electrophoresis, or similar separations tactics are used to separate proteins. Tandem mass spectrometry, Matrix-Assisted Laser Desorption/Ionization (MALDI) with time-of-flight (TOF) spectrometry, or gas chromatography/mass spectrometry are used to determine the peptide "fingerprints" (Karas et al. 1987). The peptide fragments can be sequenced using this method and analyzed by comparison to databases to identify concentrations and functions of the associated proteins. One drawback of this method is that degenerate peptides are indistinguishable with this technique (Alves et al. 2007). Computational and manual techniques can be used to link proteins to transcriptomics and genomics. This can be useful for several reasons: it identifies cell functions, protein conservation, and helps determine regulatory networks. In the first result, it builds on previous data from transcriptomics and genomics with either computational correlation (Förster et al. 2006) or more easily measured protein interactions. Furthermore, proteomics can be used in comparison to mutants or other organisms to determine conserved active sites and the effects of mutation (Morgan et al. 2014). Lastly, by determining which RNAs are translated into proteins, regulatory networks and RNAs can start to be decoded. Proteomics can build on other '-omics' analyses to determine further cellular behavior and parse the inner mechanisms contributing to it.

Metabolomics

Metabolomics, which measures the presence and/or concentration of metabolites within the cell, is the '-omics' method which is closest to measuring the phenotype of the cell. Metabolomics shows both conditional responses and the functional mechanisms in the cell which are not directly evident – simply implied – from protein concentrations and transcript levels. Methods in these studies are much different from those encountered in either of the other three '-omics': instead of

defragmenting and sensing individual compounds, metabolites are quantified in the cell using gas chromatography/mass spectrometry (GC/MS), liquid chromatography/mass spectrometry (LC/MS) or nuclear magnetic resonance (NMR) (Griffiths and Wang 2009). Knowledge of the intracellular metabolite concentrations can be used for metabolic modeling efforts, such as metabolic flux analysis (MFA) (Edwards et al. 1999; Stephanopoulos et al. 1998; Wiechert 2001), as well. As with the other '-omics' technologies, metabolomics can be used as a comparative tool to gain a better understanding of how the cell functions at all levels of control (DNA, RNA, protein and metabolism).

Understanding Biofuel Production Using '-OMICS' Analyses

C. reinhardtii has played a significant role in cataloguing cellular machinery and stress responses in photosynthetic organisms, but molecular characterization of these processes has lagged behind observable phenomena. A symptom of this stress is the accumulation of energy storage and/or potential fuel molecules. '-Omics' approaches have revolutionized the field by offering insight into these complex cellular processes and giving researchers targets for further engineering efforts to maximize production.

Production of High Energy Carbon Storage Molecules

Several studies have illustrated that *C. reinhardtii* accumulates carbon storage molecules (starch and triacylglycerols (TAGs)) during periods of macronutrient (sulfur, nitrogen, and phosphorus) (Matthew et al. 2009; Hu et al. 2008; Miller et al. 2010; Moellering and Benning 2010; Goodson et al. 2011; Weers and Gulati 1997) or micronutrient (zinc, copper, and iron) (Kropat et al. 2011) limitations (See Fig. 18.1 for TAG synthesis pathway). It has also been reported that by removing the ability to store starch, cells accumulate much higher levels of TAGs (Wang et al. 2009; Li et al. 2010b; Work et al. 2010). Unfortunately, one undesirable side effect of nutrient depletion is reduced production of biomass (Sheehan et al. 1998). In addition to micro- and macronutrient conditions, exposing *C. reinhardtii* cells to heat shock also induces increased lipid accumulation (Hemme et al. 2014). These nutrient limitations and environmental stresses used to induce TAG accumulation result in global changes within the cell which can be measured by evaluating changes in gene expression, protein concentrations and metabolite abundance. Information gathered from '-omics' studies in *C. reinhardtii* when exposed to unfavorable environmental conditions can be used to understand the regulatory mechanisms of lipid production in *C. reinhardtii*. This in turn can be used to direct genome editing of other algal species more amenable to genetic manipulation or possessing traits more desirable for large scale production to improve the production of biofuels while maintaining normal growth rates and optimal cellular health.

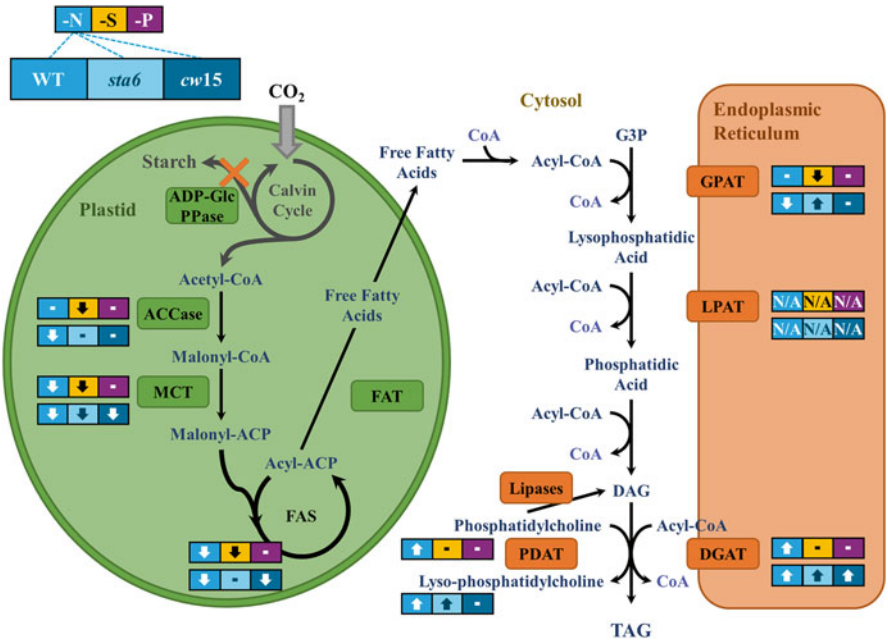

Fig. 18.1 Reported changes in transcript abundance in biosynthetic pathways of triacylglycerols in *Chlamydomonas reinhardtii*. Transcripts corresponding to enzymes involved in triacylglycerol (TAG) synthesis have varying transcript levels when starved of nitrogen (–N), sulfur (–S), or phosphorous (–P). In general, phosphorous starvation had little influence on TAG transcript levels, while sulfur starvation caused transcripts for MCT, ACCase, GPAT, and the subunits of FAS to decrease. Nitrogen starvation caused transcript levels in the plastid to decrease (ACCase, MCT and FAS), while PDAT and diacylglycerol acyltransferase DGAT transcripts increased. The *sta*6 mutant does not produce starch due to inactivation of ADP-Glc PPase in the starch synthesis pathway indicated by ×. ↑: 1-$\log_2$(fold change) transcript increase; ↓: 1-$\log_2$(fold change) transcript decrease; – : no transcript change. –N data from Schmollinger et al. (Schmollinger et al. 2014); –P and –S data from González et al. (González-Ballester et al. 2010)

Nitrogen Starvation

'-Omics' data of *C. reinhardtii* cells grown under nitrogen deplete conditions provide a fundamental understanding of the regulatory mechanics that induce TAG accumulation during stress conditions. Cells starved of nitrogen can no longer synthesize new nucleic acids or proteins and therefore they are forced to slow (or cease) growth; however, to take advantage of ample carbon supply they divert their metabolism into storage of carbon as lipids and starches. Blocking the assimilation of starches for energy storage forces *C. reinhardtii* to accumulate more lipids. Strains of *C. reinhardtii* have been developed that carry an insertional deletion of either the *sta1* or *sta6* gene encoding the large and small subunit of ADP-glucose pyrophosphorylase, respectively (ADP-Glc PPase). Critical to starch synthesis, ADP-Glc PPase converts glucose-1-phosphate to ADP-glucose, the precursor to starch

(Ballicora et al. 2003). Wang et al. showed that when the *sta6* mutant of *C. reinhardtii* is deprived of nitrogen for 48 h, the number of cytoplasmic oil bodies increased 30-fold, while the oil body content in the wild type only increased 15-fold under the same conditions (Wang et al. 2009). Several transcriptomic, proteomic, and metabolomic studies exist for this condition in both wild type as well as the starchless mutant. Information gathered at each level may be used congruently to shed light on the regulatory mechanism for lipid accumulation without hindering overall biomass production.

Overall Metabolism

Nitrogen limitation or deprivation drastically alters the overall metabolism of all *C. reinhardtii* strains as the cells are forced to reduce their nitrogen to carbon ratio, reduce photosynthetic activity, and increase respiration to acclimate to the reduced nitrogen conditions (Schmollinger et al. 2014). To determine the underlying causes of this fundamental response to nitrogen deprivation, Schmollinger et al. measured the '-omics' of three *C. reinhardtii* strains: CC-4348 (starchless *sta6* mutant), CC-4349 (cell wall-less, starch producing *cw15*), and CC-4532 (wild type). Each strain had different transcriptomes; however, amongst all three strains 27 % of the total transcripts were shared. As expected, *cw15* and *sta*6 shared 35 % of the transcripts measured. Unsurprisingly, all three strains increase transcripts for nitrogen transportation and assimilation; the proteins translated by the increased transcripts were also found to have increased concentrations. Additionally, proteins with low nitrogen content also increased in abundance during nitrogen starvation, while those of high nitrogen content decreased (Schmollinger et al. 2014). This coincides with observations made by Miller et al.: up-regulation of genes involved in nitrogen metabolism, uptake, or consumption; down-regulation of protein biosynthesis genes due to decreased availability of amino acids; and down-regulation of photosynthetic genes to regulate the metabolic state of the cell (Miller et al. 2010). Consistent with transcriptomic predictions, Wase et al. found that the abundance of proteins involved in nitrogen and amino acid metabolism, oxidative phosphorylation, as well as glycolysis/gluconeogenesis were increased, while the abundance of proteins involved with protein synthesis and photosynthesis decreased in nitrogen starved cells. Of those glycolysis/gluconeogenesis enzymes with increased abundance, pyruvate carboxylase, citrate synthase, and ATP-citrate lyase may assist lipid synthesis by providing acetyl-CoA. Interestingly, of the 18 metabolites observed whose levels changed, amino acids phenylalanine, tryptophan, and aspartic acid as well as the amino acid degradation product putrescine were among those whose abundance decreased, possibly to serve as nitrogen sources for other metabolic needs (Wase et al. 2014). This mechanism of adaptation is responsible for the reduced biomass and cell arrest observed in cells starved of nitrogen. Thus, the regulatory mechanism by which these genes interact with those involved with lipid synthesis should be further investigated to identify specific genes that can be changed without causing cell death.

Photosynthetic activity is also reduced in nitrogen starved cells, which limits their growth and ability to fix carbon. Enzymes and pigments required for photosynthesis have relatively high nitrogen content, thus, under nitrogen deplete conditions, the *C. reinhardtii* cells are limited in how many of these proteins they can produce (Blaby et al. 2013). This limitation reduces the need to produce chloroplast ribosomes to synthesize these photosynthetic proteins and is apparent by the reduced chloroplast ribosome transcript levels (~75 % reduction) observed by Schmollinger et al. Transcripts associated with chlorophyll degradation however remained constant after N-deprivation, therefore cells preferentially maintained chlorophyll despite the high nitrogen content (Boyle et al. 2012). Additionally, transcripts for the Calvin-Benson cycle were reduced two to eightfold as well as a 20 % decrease in the corresponding proteins (Schmollinger et al. 2014). From this, we see that cells subjected to nitrogen starvation are severely hampered in their ability to grow normally and re-organize their metabolism to maintain growth as much as possible.

TAG Synthesis

Of great interest to biofuel production are the '-omics' data associated with TAG biosynthesis. Contrary to phenotypic results (TAG accumulation), most transcripts involved in TAG synthesis remain stable, and if anything, decrease; however, some of the reduced transcript levels returned to similar levels during nitrogen replete conditions. The exceptions to this trend were transcripts for the genes encoding two acyl-CoA dependent diacylglycerol acyltransferases *DGAT1* and *DGTT1* and two glycerol-3-phosphate dehydrogenase isozymes (*GPD2* and *GPD4*) whose transcript levels showed significant increase (8.2, 529.9, 146.0, and 20.1 fold respectively in *cw15*) (Schmollinger et al. 2014). Boyle et al. also identified the transcripts of *DGAT1* and *DGTT1* as being highly up-regulated (6.3 and 30.8 fold respectively) in wild type *C. reinhardtii* after exposure to nitrogen deplete conditions for 48 h. Transcripts for the gene *PDAT1* encoding another acyltransferase, phosphatidylcholine dependent acyltransferase (PDAT) were also reported to increase (2.9 fold) in nitrogen deplete conditions (Boyle et al. 2012). In the *sta6* mutant, the diacylglycerol acyltransferases, *DGTT2*, was reported by Blaby et al. to have ~4 times the number of transcripts than *cw15* during nitrogen deplete conditions. Of additional interest, transcript levels for the major lipid droplet protein gene (*MLDP1*) were also ~4 times higher in *st6* than in *cw15* (Blaby et al. 2013). The major lipid droplet protein (MLDP) is an important structural protein found in lipid bodies. Repression of *MLDP1* in *C. reinhardtii* using RNA interference has been shown to cause lipid bodies to increase in size, but not in TAG content (Moellering and Benning 2010). Although few major changes in TAG transcripts were observed in the aforementioned studies, TAG accumulation still occurs. Thus, proteomic studies are necessary to determine the amount of protein actually resulting from these transcripts.

Although transcript abundance for some TAG synthesis genes increased, their corresponding protein concentrations did not show corresponding changes in nitrogen

starved *C. reinhardtii*. Indeed, protein abundance levels of most enzymes involved with lipid synthesis either remained the same or decreased indicating different levels of regulation involved with lipid synthesis. Of those enzymes involved with lipid synthesis whose associated transcript levels increased identified above (Boyle et al. 2012; Schmollinger et al. 2014; Blaby et al. 2013), Wase et al. were unable to identify any corresponding change in protein abundance. Indeed, only the following proteins associated with lipid synthesis were found to have increased abundance during nitrogen starvation: long-chain acyl-CoA synthetase, plastid lipid associated protein, and triglyceride lipases (Wase et al. 2014). Similar results were observed by Schmollinger et al. in addition to the reduced abundance of the acetyl-CoA carboxylase (ACCase) subunits (Schmollinger et al. 2014). Of great interest is the decreased abundance of the AMP-activated protein kinase under nitrogen deplete conditions also observed by Wase et al. This kinase, responsible for inhibiting ACCase, could cause the observed TAG accumulation despite the reduced abundance of ACCase subunits (Wase et al. 2014). Such discrepancies between the transcript and protein abundance levels indicate that lipid synthesis is possibly regulated by variations in enzyme kinetics at the protein level caused by substrate deviations at the metabolite level and/or post-translational modifications of those enzymes to promote lipid storage under nitrogen deplete conditions.

Other Macronutrient Conditions: Phosphorus and Sulfur

It has been shown that phosphorous and sulfur deplete conditions result in similar genetic regulatory results. According to Mosely, et al., *C. reinhardtii* cells respond similarly when they are exposed to phosphorous deplete or sulfur deplete conditions. Interestingly, when cells acclimated to phosphorous deplete conditions are exposed to sulfur deplete conditions, the cellular responses associated with sulfur deficiency become inhibited. Thus, the regulatory responses involved in sulfur deplete, phosphorous deplete, and possibly other nutrient deplete conditions are intimately dependent on one another (Moseley et al. 2009). Additional sulfur deplete studies indicate that the accumulation of lipids is a result of the conversion of phospholipids in the membranes into neutral lipids. Sugimoto et al. showed that when *C. reinhardtii* cells are starved of sulfur, they degrade almost 85 % of the sulfoquinovovosyl diacylglycerol (SQDG) present in their chloroplast membranes to reallocate the sulfur to increase the sulfur content in proteins (Sugimoto et al. 2007). Further studies from the same group indicate that the degradation of SQDG is coupled to the synthesis of phosphatidylglycerol, another acidic lipid present in chloroplasts; however, the increase in phosphatidylglycerol synthesis is most likely due to the SQDG loss, not the sulfur deplete conditions (Sugimoto et al. 2008). This insight indicates an opportunity to modify SQDG content in the membranes via gene deletion of those enzymes involved with SQDG synthesis to induce phosphatidylglycerol synthesis.

In addition to nitrogen response analyses, Schmollinger et al. also compared transcript levels of *C. reinhardtii* under nitrogen deplete conditions with those transcript levels measured by González-Ballester et al. during phosphorus and sulfur deprivation (Schmollinger et al. 2014; González-Ballester et al. 2010). Despite similar physiological responses between sulfur and phosphorous deplete conditions, the transcriptome of *C. reinhardtii* in phosphorous deplete conditions experienced fewer changes than the transcriptome of sulfur deprived cells. This observation is intriguing as phosphorous and sulfur deplete conditions exhibit a similar response as discussed above. FA synthesis transcripts varied amongst the conditions as illustrated in Fig. 18.2. The only consistent changes were found *DGAT1* and *DGTT1* which increased in all macronutrient deplete conditions. Again, this discrepancy between varied transcript data and the resulting TAG accumulation over all of the macronutrient deplete conditions illustrates the need for further investigation of the regulatory mechanisms involved in lipid production during nutrient deplete conditions by gathering more information on these conditions at the proteomic and metabolomic levels.

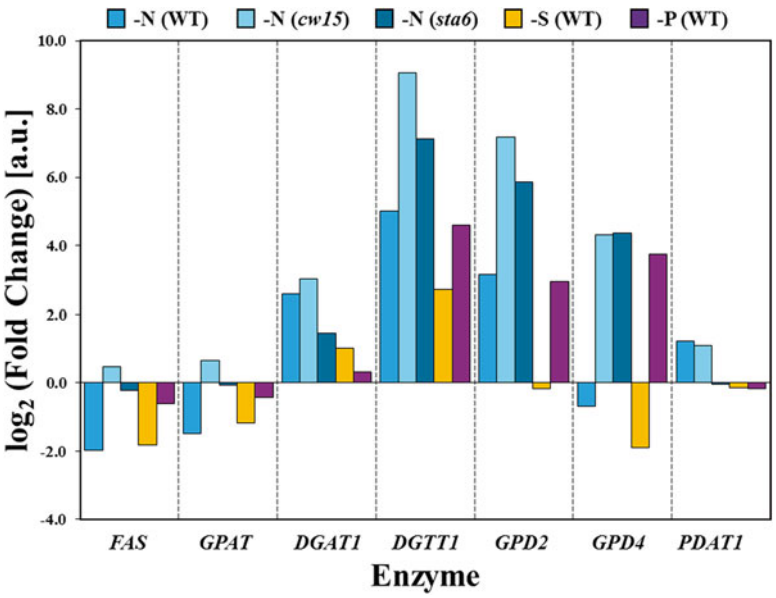

Fig. 18.2 Transcript fold changes of genes associated with enzymes involved in triacylglycerol synthesis of *Chlamydomonas reinhardtii* subjected to macronutrient deprivation. In general, most transcripts remain either unchanged or decrease when starved of nitrogen (-N), sulfur (-S), or phosphorus (-P). The response to -N causes relatively similar TAG synthesis transcript level changes in WT, *cw15*, and *sta6*. All three strains saw drastic transcript level increase for *DGAT1*, *DGTT1*, and *GPD2*. Even –S and –P conditions caused some transcript level changes in WT. Both –S and –P conditions caused *DGAT1* and *DGTT1* transcripts to increase. Only –P caused transcript levels for *GPD2* and *GPD4* to increase. –N data from Schmollinger et al. (2014); –P and –S data from González et al. (González-Ballester et al. 2010)

Heat Stress

Proteomic and metabolomic information from *C. reinhardtii* under heat stress (HS) conditions provides additional insight to lipid accumulation. Hemme et al. performed a large-scale proteomic and metabolomic analysis of cells before HS, during various times at 42 °C for 24 h, and during the 8 h recovery after HS conditions (Hemme et al. 2014). Although transcript levels were not analyzed, many predictions can be made about how cells accumulate lipids when heat stressed from the protein and metabolite variations observed. Like other stress conditions, *C. reinhardtii* experiences a restructuring of their central metabolism during and after HS that specifically induces cell arrest. Proteins critical to the Calvin-Benson cycle as well as those involved in gluconeogenesis and the TCA/glyoxylate cycle decreased, and their respective metabolites had a sharper decrease indicating reduced enzyme activity (Hemme et al. 2014). This decline in metabolic function illustrates that the cells undergo severe cell arrest, which may contribute to lipid accumulation. Similar to macronutrient deficient conditions, little variation in abundance was observed for *de novo* FA synthesis proteins during and after HS. Membrane lipid metabolism proteins did have a marked increase during HS and were reduced during recovery thus allowing the cells to restructure their membranes during and after HS. Specifically, polyunsaturated lipids decrease as they are replaced by saturated lipids and saturated diacylglycerol trimethyl homoserine accumulates during heat shock. As saturated FAs replace their unsaturated counterparts, the unsaturated FAs accumulate as DAGs and TAGs. With the accumulation of lipids, starch levels also decrease during HS (Hemme et al. 2014). This carbon source preference indicates that the starch is possibly being degraded to form glycerol and acetyl-CoA to synthesize the saturated FAs used in the membrane. By shifting the ratio of saturated to unsaturated FAs in the membrane in response to temperature fluctuations, the cells are able to regulate the viscosity of the membrane keeping membrane bound proteins, especially those of photosynthesis, functional in a process known as "homeoviscous adaptation" (Michael 1974). During recovery, the polyunsaturated FAs of TAGs in the oil bodies are exchanged with the saturated FAs in the lipid membrane. It is important to note that saturated FAs can only be produced from *de novo* biosynthesis in the chloroplast, while unsaturated FAs can be produced by *de novo* biosynthesis or by desaturases (Hemme et al. 2014). Because saturated FAs are most likely replacing unsaturated FAs in the lipids, these unsaturated FAs accumulate in the form of TAGs creating oil bodies as HS inhibits cell division. This FA exchange could be manipulated in conjunction with FA pathway modifications to optimize the yield of unsaturated TAGs.

Biohydrogen Production

Industrial hydrogen production occurs via steam reforming of small hydrocarbons and carbon monoxide, providing a potentially more efficient fuel source through use of low-energy molecules with a water. However, as indicated by the requirement

for hydrocarbons and steam, this is not a carbon neutral process; in fact, it has been shown to have even more of a net negative impact than fossil fuels (Fishtik et al. 2000). Biological sources of hydrogen offer an advantage: the hydrogenase enzyme functions cooperatively with photosynthesis to convert protons into hydrogen gas as an electron sink instead of oxygen. '-Omics' has demonstrated that stress conditions affecting the photosynthetic apparatus cause activation of the hydrogenase enzyme. In particular, acclimation to sulfur deplete and anoxic environments result in hydrogen gas being formed to compensate for cellular overprotonation (acidification through proton accumulation) and electron evolution. Since these environments are suboptimal for *C. reinhardtii*, a corresponding loss of cellular fitness is observed. Analysis of these phenomena is crucial to eliminating the stress-induced cellular inefficiencies while maintaining the desired increase of biohydrogen.

Anoxic Conditions

Anoxic conditions in photosynthetic organisms result in the more hydrogenase enzyme to provide an electron sink and resist overprotonation of the thylakoid space. Transcriptome level studies support this, showing an increase in the concentration of *HYD1* and *HYD2* transcripts – coding for hydrogenase enzymes (Mus et al. 2007). Knockout studies indicate that absence of hydrogenase genes in anoxia display a diversion of resources to relieving oxidative stress via other pathways such as succinate production (Dubini et al. 2009) which can potentially provide targets for maintenance of cellular fitness during anoxia (Fig. 18.3).

In dark anaerobiosis, responses to the electron sink void are further exacerbated by the requirement to mitigate toxic byproduct formation while still providing cellular energy. These result from the inability to fully oxidize carbon substrates to CO_2, diverting glycolysis toward 2-carbon molecules (acetate and ethanol) and toward hydrogen from pyruvate. The transcriptomic and metabolomic studies display an increase in transcripts encoding proteins associated with fermentation as well as increases in ethanol and hydrogen production (Mus et al. 2007). This fermentative behavior is coupled with a slight remodeling of carbon metabolism to remove bottlenecks and conserve energy. Notably, the Calvin Cycle is subject to slowing due to accumulation of cycle intermediates and electron dense molecules in anoxia while upregulating pentose phosphate transcripts in order to adjust. Meanwhile, glyoxylate-related enzymes are synthesized to aid in conversion from acetyl-CoA – resulting from lipid catabolism – to organic acids (Mus et al. 2007). Isocitrate lyase (*ICL*) is one enzyme which bypasses α-ketoglutarate, a key precursor for chlorophyll biosynthesis, and has been implicated in cell progression towards senescence (Terashima et al. 2010). Reducing activity of this enzyme may aid in reducing senescent activity while retaining increased cellular growth rates. Glyoxylate related enzymes could also be downregulated to retain lipids, which represent much more valuable commodities than sugars and organic acids. Fermentation pathways upregulate important metabolites while providing insight into maintenance routines that can be exploited to maintain high growth rates (Fig. 18.4).

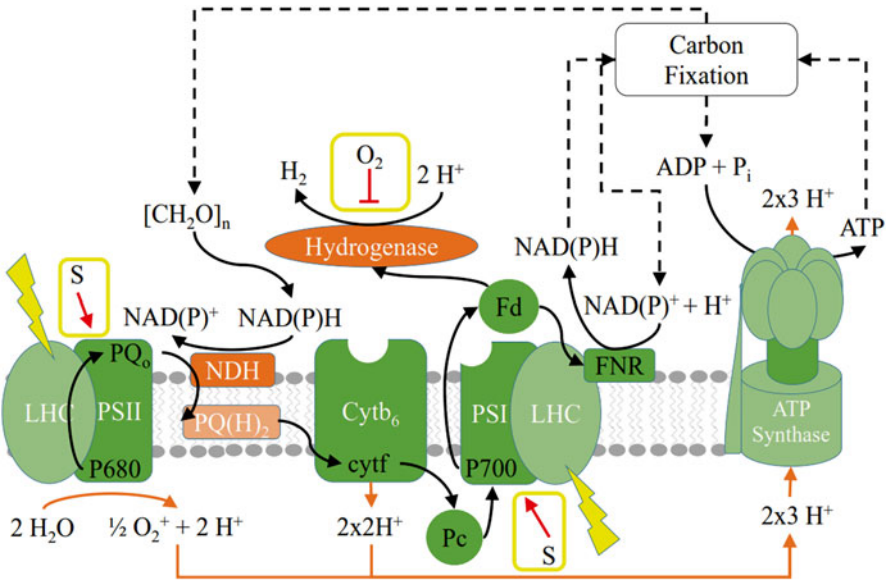

Fig. 18.3 Photosynthetic response to anoxia in light replete conditions to induce hydrogen formation and maintain photosynthesis. Biohydrogen in *Chlamydomonas reinhardtii* is generated via photosynthetic diversion of electrons into hydrogen protons. Sulfur deplete conditions reduce function of the photopigments as repair and protein synthesis are stunted. Oxygen deplete conditions upregulate hydrogenase related transcripts to ensure function of the hydrogenase enzyme and limit accumulation of protons. *Orange arrows* indicate molecular flow; *black arrows* indicate electron flow; *black* labeled structures are electron transporters; *white* labeled structures are photosynthetic macrostructures; *orange* structures are bound proteins. *Yellow* boxes are conditions leading to biohydrogen generation

Not only do energy metabolism cycles change, transcripts and proteins associated with amino acid synthesis show an accumulation of serine and isocitrate amino acids in conjunction with an upregulation of genes encoding enzymes associated with the glycine decarboxylase system. Proteomics imply that, by induction of glycolytic enzymes, intracellular nitrogen reorganization occurs via amino acid degradation as opposed to traditional methods of assimilation (Terashima et al. 2010). This determines that lower protein synthesis is present in these conditions and likewise a remission of cellular fitness. Adjustment of these pathways to more efficiently retain nitrogen may help in maintaining cellular fitness within biohydrogen production endeavors.

Sulfur Deplete Conditions

Sulfur depletion in *C. reinhardtii* and other photosynthetic organisms has been indicated to decrease photosystem II (PSII) activity and increase in hydrogenase activity in a similar way to light anoxia. It does this by halting the methionine repair

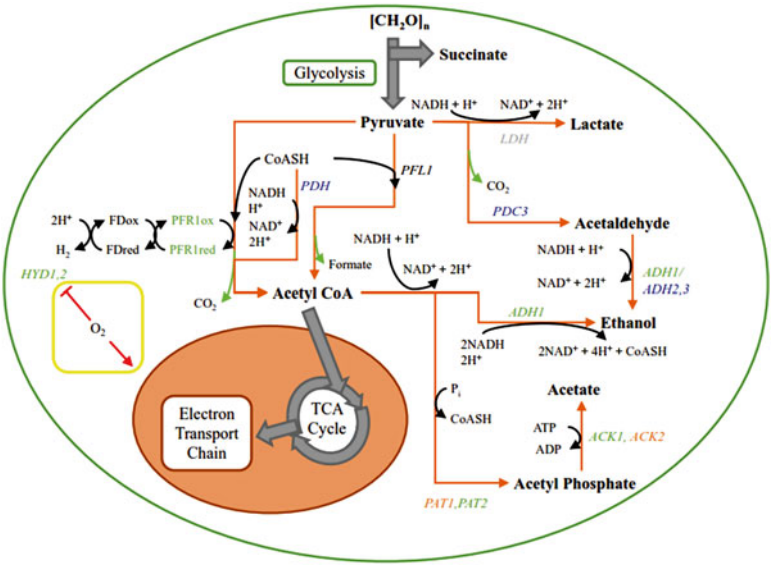

Fig. 18.4 Dark anaerobiosis to induce hydrogen and lipid accumulation in *C. reinhardtii* to maintain energy generation and reduce overprotonation. Biohydrogen accumulates via fermentation in anoxic/dark environments. The lack of oxygen cooperatively ceases function of the electron transport chain while halting inhibition of hydrogenase enzymes. Hydrogen is reduced to diatomic gas for lack of oxygen as an electron sink. Green enzymes are expressed in the chloroplasts, orange in the mitochondria, blue in the cytoplasm, black in both chloroplast and mitochondria, and gray is undetermined. *Orange arrows* indicate metabolite flow, *black arrows* indicate electron flow, *green arrows* indicate exported metabolites, and large *gray arrows* indicate summary reactions. The orange structure is the mitochondrion, the *green* circle is the cell, and the *yellow* box is the exterior conditions

system that responds to photo-damage in *C. reinhardtii*, causing the photosynthetic apparatus to remodel, protein synthesis to slow, and intracellular scavenging activities to gain more importance. These cellular diversions cause significant remodeling of PSII proteins and reapportioning of metabolites through different carbon pathways in nearly identical ways to those observed in anoxia (Zhang et al. 2004). The 2-DE coupled MALDI-TOF and MALDI-TOF/TOF-MS and 2-D gel maps displayed proteome and transcriptome changes including an overall decrease in PS related transcripts except *LCHBM9* and declined proclivity carbon-fixation. Meanwhile, metabolomic surveys determine that acetate is increased with hydrogen as an energy spilling mechanism without sulfur (Matthew et al. 2009). This correlates to the hypothesis that photosynthesis repair is no longer a feasible activity for energy harvesting and cell biosynthesis except for the proteins related to photoprotection and singlet oxygen reduction (Grewe et al. 2014). Protein repair operations, especially methionine dependent repair proteins, indicate a lower level of synthesis and the increase of chaperone proteins indicates more care taken to synthesize the required proteins for photosynthesis (Nguyen et al. 2008). These deficits cause

oxygen to become unavailable for assimilation, so many events that occur during anoxia are consistent with sulfur deplete conditions (Timmins et al. 2009). Stress-response proteins show a high-fold increase in activity and an upregulation in transcripts like *LHCSR2* and contribute to the formation of hydrogen. However, sulfur-depletion induces a strong suppression of glyoxylate proteins and a heightened level of succinate synthesis which aids in continuation of energy cycles and protein synthesis (Timmins et al. 2009). Returning the cell to higher levels of protein synthesis and balancing redox stress may help create more valuable hydrogen producing processes.

Conclusion

'-Omics' provide a platform on which to build genetic re-design strategies by identifying key points of regulation in response to stressful conditions. These regulatory targets can be identified at the genomic level (knockout, promoter variation, etc.), the transcript level (promoter binding sites), or at the proteomic level (protein engineering). '-Omics' can then affirm these targets at the metabolomic level. Although directed genetic manipulation of *C. reinhardtii* is difficult to achieve, the insights provided by '-omics' can be used to predict the regulatory responses of other more genetically tractable algae. Combining these multi-level regulatory targets could pave the way towards creation of an optimized algal strain able to grow at operational capacities and maximize flux through biofuel pathways.

There are still shortcomings in knowledge and execution of these systems. For example, these '-omics' techniques are primarily diagnostic: they provide the means to determine enzymatic regulation and cellular tactics for maintaining homeostasis. Moreover, there have not been forward-genetic studies that seek to improve cellular performance in response to these mechanisms. That is to say, studies need to be done on how genetic modification for chlorophyll upregulation in concert with limiting oxygen might affect cellular dynamics and biohydrogen formation. In this regard, the knowledge that has been accumulated needs to be put to the test by inducing feasible cellular modifications. Computational methods are also helping spur this field forward but still have a lot of room for development. Current techniques hinge on the conservation of proteins between species, but rely on regression between similar DNA or peptide sequences. Predictive modeling of structural motifs could go a long way toward use of computational genomics as viable discovery platforms. DNA, RNA, and protein reconstitution in sequencing can be improved to augment throughput and accuracy of those studies. Other concerns involve studying these organisms in scaled-up environments. It has been demonstrated consistently that behaviors change in industrial size processes and this is a major concern; since these '-omics' studies are only performed in a lab-controlled environment, steps have to be taken to ensure application to industry. For this reason, nutrient fluctuations, combinations of stressful conditions, and larger population studies should be conducted to understand how microalgae respond to scale-up.

Fortunately, '-omics' approaches have shown a wide variety of applications and are appealing especially as the technological prowess of the field progresses. Even though there are shortcomings in the discoveries to this point, '-omics' for biofuel production in *C. reinhardtii* has given an incredible amount of targets for microbial development. Since the hardest part of creative discovery is asking the right questions, it can be considered that '-omics' in *C. reinhardtii* is a major victory for the industry as a whole.

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