Synthetic biology is essential to unlock commercial biofuel production through hyper lipid-producing microalgae: a review

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ABSTRACT
The multiple drivers of increasing global energy demand, diminishing fossil fuel reserves and the urgent need to mitigate greenhouse gas emissions from non-renewable energy sources have led to a growing international interest in supplementary energy, and invigorated research into alternative and renewable fuel sources. To date, the majority of biofuels are derived from terrestrial crops which compete for freshwater and arable land resources in the face of a rising population lacking food or access to potable water. This means that photosynthetic organisms such as microalgae are a promising alternative, capable of growing in saltwater and a diverse range of environments. Furthermore, the cultivation process can take place on non-arable land, and the accumulated biomass can cleanly generate biofuel precursors. Despite their promise, none of the currently known strains of microalgae are biologically productive enough to produce commercially viable biofuels. At the forefront of this research is the development of 4th generation feedstocks for biofuel production. This development focuses on the use of synthetic biology to produce genetically modified industrial microalgal strains that hyper-accumulate neutral lipids. This review examines the most promising approaches in this rapidly evolving field, for example, direct upregulation of the storage lipid metabolic pathway, shunting of competing pathways such as the starch storage pathway, and improving photosynthetic efficiency. The most recent advances in gene editing technologies are also discussed. Though successful, these individual strategies do not yet offer enough of a biological improvement to achieve commercially viable biofuels, requiring, in our opinion, future research to focus on the combination of strategies to achieve the synergistic boost in lipid yield needed for commercial viability. In this review, we propose a strategy based on this principle which would theoretically increase the lipid yield of *Chlamydomonas reinhardtii* 10-fold, with minimal effect on growth rate.

Introduction

Since the first oil crisis in 1973, and the subsequent “second oil shock” of 1979, there has been a growing global interest in alternative and renewable energy sources (Garavini, 2011; Boyle, 2004). This interest is compounded by the dual demand of a predicted 30% rise in global energy requirement in the next 10 years alone, and crude oil reserves that are estimated to be depleted within the next 50 years (Shuba & Kifle, 2018; Central Intelligence Agency, 2019). Development of renewable energy sources such as solar and aeolian have seen great advancements in recent years (Boyle, 2004), but economies’ efforts to support the transition to “green energy”, such as widespread availability of electric-based transportation and its required infrastructure (extensive charging networks, specialized repair chains, lightweight batteries, etc.), are lagging behind and thus cannot yet be relied upon to replace the role of traditional non-renewable fuel. Consequently, there is a clear imperative for alternatives such as biofuels to help offset the vast global use of non-renewable energy sources. Though not as “environmentally friendly” as pure energy sources (e.g. solar, hydrothermal), biofuels are carbon neutral, at least in principle, and thus could serve a key role in mediating the transition to a truly clean-energy society.

Of the various iterations of biofuels that have been examined, arguably the most promising is 4th generation microalgal biodiesel. The 1st generation biofuels were envisioned around affordable and readily available terrestrial crops, such as corn and sugarcane. Their use is advantageous in part due to the extensive infrastructure and experience in growing and harvesting these feedstocks, but they suffer the major disadvantage of competing with food crops for freshwater and arable land resources, and therefore pose a significant threat to global food security (Lü, Sheahan, & Fu, 2011). The 2nd generation biofuels were developed to avoid this “fuel vs food” dilemma by using ligno-cellulosic materials, such
as straw, corn husks and other inedible agricultural biomass as a feedstock (Lü et al., 2011). However, this approach has been plagued by relatively poor conversion efficiencies from biomass to biofuel in part due to the need for extensive bioprocessing (Figure 1).

Oleaginous microorganisms comprise 3rd generation biofuel feedstocks, and arguably, the most promising are microalgae. Microorganisms are classified as oleaginous if they produce oils in excess of 20% of their dry weight biomass (Meng et al., 2009). Well known examples include microalgae such as Chlamydomonas reinhardtii and Microchloropsis gaditana (formerly Nannochloropsis gaditana), of which the latter can produce up to 60% lipids (ash-free dry weight) (Ajawi et al., 2017; Table 1). Microalgae have a number of advantages as feedstock for biodiesel, including their rapid growth cycles, high biomass yields, photoautotrophy, and their tolerance of and adaptability to grow in a range of diverse environments including brackish and saltwater (Chen et al., 2019; Fu et al., 2019; Shin, Lee, Jeong, Chang, & Kwon, 2016). The phototrophic growth of microalgae sets them apart from alternatives, such as bacteria and fungi which, as heterotrophs, require organic input to generate the biodiesel precursors. Moreover, the per hectare lipid production potential of microalgae is an order of magnitude higher than that of the best terrestrial crops (Chisti, 2007; Hu et al., 2008; Li et al., 2010a). In spite of this, the native lipid productivity of these microalgae is still too low for 3rd generation biodiesel to be cost competitive with fossil fuels. Encouragingly, lipid production from industrial scale microalgal cultures is still far from their theoretical maximum yield (Hu et al., 2008; Li et al., 2010a), implying that there are gains to be made.

4th generation biofuel research revolves around genetic and metabolic modification, whereas 3rd generation biofuels primarily bioprospected for the right species of microalgae, optimizing culture media and identifying the most apposite stressors to stimulate storage lipid production (Jagadevan et al., 2018). Research into 4th generation biofuels has been enabled by rapid advancements in genomics, biotechnology, and the rising effectiveness of genetic modification technologies in microalgae, which have elucidated key areas for improvement (Gimpel, Specht, Georgianna, & Mayfield, 2013; Lei et al., 2012).

| Table 1. The oil content of various microalgae with commercial potential (≥20% dry weight). This includes mixed heterotrophic and autotrophic growth values (≥2%), and where a range has been reported, the highest value was taken. Potential enhancements are listed in descending expected significance with respect to phylum, and greyed points reflect unclear significance in relevant phyla. |
Naduthodi, Barbosa, & van der Oost, 2018; Sizova et al., 1996).

**Advances in gene-editing technologies for microalgae**

A paradigm in synthetic biology is the Design-Build-Test-Learn (DBTL) cycle; this is a bio-engineering principle that seeks to minimize cost by maximizing efficiency at the ultimate level. Relevant to metabolic engineering, a gene construct may be designed (D-stage) and assembled from constituent parts via restriction and ligation (B-stage) and expressed in a model organism (T-stage). The cycle can be restarted at either the B- or T-stage to iteratively optimize the pipeline by learning (L-stage) from previous failings. The DBTL cycle is becoming increasingly relevant due to advancements in affordable and high-throughput gene synthesis (Kosuri et al., 2010; Rogers & Church, 2016) and the development of efficient gene assembly, such as Golden Gate cloning, and the related standardized Modular Cloning (MoClo) toolkit, that enable one pot and one-step synthesis of multi-gene constructs from standardized constituent parts (Engler et al., 2008; Crozet et al., 2018). Also, rapid advancements in mutagenic and transgenic efficiencies (Ferenczi, Pyott, Xipnitou, & Molnar, 2017; Serif et al., 2018; Angstenberger et al., 2018; Picariello et al., 2020), increasing understanding of regulatory elements (Baier, Jacobebbinghaus, Einhaus, Lauersen, & Kruse, 2020; Mehrshahi et al., 2020; Rose, 2019), coupled with high-performance screening techniques enabled by optimization of microfluidics (Nouemssi et al., 2020; Saad et al., 2019; Südfeld, Hubáček, D’Adamo, Wijffels, & Barbosa, 2020; Wheeler et al., 2003), have cumulatively enabled the automation of DBTL workflow (Carbonell et al., 2018). Most notably, the integration of machine learning has exponentially increased productivity in recent years (Carbonell, Le Feuvre, Takano, & Scrutton, 2020; Opgenorth et al., 2019; Radivojević, Costello, Workman, & Garcia Martin, 2020).

Through the previous decades, the most effective approaches to genetically modify eukaryotic organisms have been zinc-finger nucleases (ZFN), homologous recombination (HR) and transcription activator-like effector nucleases (TALEN) (Naduthodi et al., 2018; Shin et al., 2016). However, application of these techniques is time-intensive, complex and expensive (Naduthodi et al., 2018; Shin et al., 2016). Additionally, microalgae are notoriously difficult to engineer, due in part to low transformation efficiencies, off target cleavage, background mutations, and the fact that many microalgae are only available as diploid cell lines (Kim & Kim, 2014; Serif et al., 2018; Shin et al., 2016).

Development of a genome editing technique based on clustered regularly interspaced short palindromic repeats (CRISPR) and associated nuclease proteins (Cas9 primarily) has quickly become a very effective technique in a variety of organisms. Originally it was an immunity system found in bacteria acting against viral and mobile DNA.
elements, where the nuclease activity is guided by RNA fragments (crRNA) to cleave invading DNA. CRISPR-Cas9 is advantageous over TALEN and ZFN due to its simplicity, versatility, lower cost and improved specificity (Shin et al., 2016). Despite this, it has achieved only limited success in even the best understood model, the green microalga C. reinhardtii (Baek et al., 2016; Chen et al., 2019; Naduthodi et al., 2018). Specific issues with use of CRISPR-Cas9 in algae have been that expression of Cas9 can be cytotoxic in many microalgae (Baek et al., 2016), and CRISPR is relatively inefficient at introducing few specific nucleotides by non-homologous end joining (NHEJ) (Anzalone et al., 2019; Cohen, 2019).

A number of advances have increased the effectiveness of CRISPR-Cas9 in microalgae, many of which have come from applying developments in editing of diverse cell systems, such as plants and animals. For example, the use of pre-synthesized crRNA complexed with RNA-binding proteins (RNP) has been shown to noticeably decrease both cytotoxicity and off-target mutagenesis (Doudna & Charpentier, 2014; Kim & Kim, 2014; Shin et al., 2016). The use of RNP s in C. reinhardtii resulted in a 100-fold increase in targeted mutagenic efficiency in comparison with the first reported use of CRISPR-Cas gene editing (Shin et al., 2016). Another study reported the use of CRISPR-Cas9 RNP s in Phaeodactylum tricornutum to achieve double knock-out mutants with 65–100% efficiency (Serif et al., 2018).

A novel method termed targeted insertional mutagenesis (TIM), that utilizes Cas9 RNP s and double-stranded oligodeoxynucleotides, has recently been developed for C. reinhardtii (Picariello et al., 2020). TIM was used to achieve mutation efficiencies of 40–95%, though double knock-out efficiencies did not exceed 15%. Highlighting the rapid rate of advancement in terms of single-gene knock-in mutation efficiency in C. reinhardtii, TIM improves upon the 37% that had been previously achieved through optimization of RNP complex in C. reinhardtii, which in turn more than doubled that of previous efforts (Kim, Park, Cho, & Hwang, 2013).

A further issue is that mutagenic efficiencies suffer in diploid organisms, including many microalgal strains, due to the intrinsic issue of having to target multiple alleles across homologous genomes (Mertens et al., 2019). This is being addressed through multiplexing, where either a single locus is targeted multiple times throughout the genome (cis-multiplexing) or multiple copies of multiple genes are modified simultaneously (trans-multiplexing) (Adiego-Perez et al., 2019; Ryan et al., 2014).

Addressing such issues is CRISPR-Cas12a/Cpf1, a promising alternative to Cas9. This system is very similar in function to Cas9 despite being the result of independent evolutionary pathways (Paul & Montoya, 2020), but has several advantages over Cas9. Specifically, Cas12 recognizes a longer protospacer adjacent motif (PAM), 5’-TCTT-3’, which results in significantly increased on-target mutation efficiency due to its length increasing specificity and its composition decreasing the chance of it being misread on GC-rich genomes (Yang, Edwards, & Xu, 2020). After PAM recognition, Cas12a cleaves the target DNA far enough downstream of the PAM site that it is preserved during non-homologous end joining; as opposed to Cas9 in which the PAM site’s proximity to the cleavage site usually results in its destruction, precluding future edits (Yang et al., 2020). This allows the CRISPR-Cas12a editing stream to re-target the locus for repeated editing, thus increasing on-target efficiency. While multiplexing is possible with Cas9, Cas12a has been successfully used for both cis- and trans-multiplexing in polyploid organisms (Adiego-Perez et al., 2019). Recently, a CRISPR-Cas12a editing pipeline demonstrated efficient, multiplexed editing in the aerobic yeast Yarrowia lipolytica, achieving up to 83% editing efficiency for duplex loci targeting and 41.7% efficiency for triplex mutations (Yang et al., 2020). Cas12a has also been effectively employed in C. reinhardtii, enhanced by the use of single stranded oligodeoxynucleotides (ssODNs) (Ferenczi et al., 2017).

Preliminary use of ssODNs for gene targeting in C. reinhardtii showed that they can significantly reduce off-target mutations, as illegitimate integration at non-homologous loci occurs almost exclusively with double stranded DNA (Zorin et al., 2005). It has been shown that the combined use of ssODNs as repair templates and Cas12a RNP s may increase nucleotide-level editing efficiencies up to 500-fold (Ferenczi et al., 2017).

Another very promising technique is known as prime editing (Anzalone et al., 2019). It is based and built on the same fundamental components as CRISPR-Cas9, but rather than introducing a double-stranded break, it only cuts one strand and the prime editing guide RNA (pegRNA) guides reverse transcriptase to introduce the desired changes. Prime editing has not yet been tested in microalgae, but preliminary experiments by the authors suggest the technique could be one of the most effective gene-editing tools to date (Anzalone et al., 2019).

When a double-strand break (DSB) occurs, such as caused by CRISPR-Cas9, either NHEJ or HR repair mechanisms can be triggered. In terms of transformational efficiency, HR is the desired repair pathway
because, unlike NHEJ, it does not induce indels (random insertions and deletions) which can disrupt the target sequence and impede accurate editing (Bae, Kweon, Kim, & Kim, 2014; Ferenczi et al., 2017; Yang et al., 2015). It has been shown that NHEJ/HR expression is correlated with cell stage, for example, in eukaryotes NHEJ is the dominant repair mechanisms in the G1 phase, and it has been proposed that transforming cells in HR dominant phases may result in higher transformation efficiencies (Hinz, Yamada, Salazar, Tebbs, & Thompson, 2005; Angstenberger et al., 2018). HR-mediated repair is often suppressed and is mainly associated with the S and M phases of the cell cycle, suggesting that this is when genetic editing attempts may be most successful (Frit, Baroule, Yuan, Gomez, & Calsou, 2014; Lin, Staahl, Alla, & Doudna, 2014). Furthermore, it has been hypothesized that endogenous DNA may present a more accessible target when it is in a relaxed chromatin state, and therefore highest transformation efficiencies may be achieved during interphase preceding mitotic chromatin condensation (Angstenberger et al., 2018; Buffaloe, 1958).

A limitation of this approach is that cells in a culture are naturally in disparate stages of the cell cycle, so cells must be highly synchronized for a significant effect of cell-stage HR proclivity.

Significant enhancement of Cas9-mediated transformation efficiencies has recently been demonstrated in C. reinhardtii by using synchronized cells to optimize HR (Angstenberger et al., 2018). Cell-cycle synchronization was achieved by long-term cultivation in alternating conditions of 12 h light at 28°C, followed by 12 h dark at 18°C. This resulted in a 130% increase in mutation efficiency over unsynchronized cells. This study highlights the potential of synchronization as a method to increase genome editing efficiencies using DSB approaches by circumventing NHEJ in favour of HR. Furthermore, this effect may be further aided by NHEJ pathway inhibition, which has seen limited success in other organisms; the effect of cell-cycle synchronization of NHEJ-inhibited cells, therefore, should be further studied in C. reinhardtii (Ishibashi, Suzuki, Ando, Takakura, & Inoue, 2006; Schorsch, Köhler, & Boles, 2009; Zhu, Mon, Xu, Lee, & Kusakabe, 2015; Angstenberger et al., 2018).

In addition to editing genes or introducing transgenes, it is also necessary to be able to regulate the expression of endogenous genes or transgenes. Introns are non-coding nucleotide regions flanking exons that code for peptide production. They function to modify an mRNA sequence, for example, by providing supplementary transcription factor binding sites, modifying mRNA stability and enabling the rearrangement of exons (Kolkman & Stemmer, 2001; LaRoche-Johnson et al., 2020; Le Hir, 2001; Wei et al., 2006). Inclusion of introns in transgene constructs can enhance expression – a technique known as Intron-Mediated Enhancement (IME) (Rose, 2019). Very recently, IME has been applied in microalgae by using introns from C. reinhardtii ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) small subunit 2 (RBCS2) and major light-harvesting complex 1 (LHCBM1) genes, that led to increases in expression levels of 5.5 and 8-fold, respectively (Baier et al., 2020).

Within introns, there are regions known as riboswitches that regulate gene expression in response to a specific ligand – such as metal ions and amino acids in prokaryotes, but in eukaryotes, riboswitches respond exclusively to thiamine pyrophosphate (TPP) (Mehrshahi et al., 2020). The binding of the ligand to the aptamer region causes a conformational change which ultimately results in alternative exon splicing and thus translation of a distinct protein. Recently, the aptamer region of the C. reinhardtii TPP riboswitch was disentangled from the rest of the complex, enabling modular modification (Mehrshahi et al., 2020). Splicing this riboswitch into transgene constructs enabled fine control of gene expression by regulating exogenous ligand concentration. Moreover, the modular design should enable the aptamer region to be swapped for other riboswitches and the potential to tune the expression of separate genes to different non-toxic ligands, enabling independent gene regulation. This could be an important technique in the development of multi-gene mutants, as the expression of transgenes could be simultaneously and independently regulated to determine the cross-effect of changing metabolic resource flows.

**Genes of interest and strategies to effect hyper-lipid production**

To date, there have been several general approaches to increase the commercial viability of fourth generation biofuels, including direct upregulation of the lipid metabolic pathway (Chen et al., 2019), inhibiting competing pathways such as the starch for storage pathway (Li et al., 2010a; Li, Han, Hu, Sommerfeld, & Hu, 2010b), and improving photosynthetic efficiency (Fu et al., 2017; Perrine, Negi, & Sayre, 2012; Shin et al., 2016; Vinyard, Gimpel, Ananyev, Mayfield, & Dismukes, 2014). The most promising of these approaches (Table 2) are discussed in detail below.
Table 2. Genes of interest that have been explored in microalgae which have a positive effect on lipid yield.

| DRAWBACKS | SOURCE | SPECIES | EXPRESSION | BENEFITS |
|-----------|--------|---------|------------|----------|
| 25% lower quantum yield of photosynthesis | Polle, 2002 | C. reinhardtii | GTPS3 | 3-fold increase in maximum photosynthetic rate, 2-fold higher oxygen evolution. |
| 30% decrease in number of ETCs | Polle et al., 2003 | C. reinhardtii | GTPS3 | 2-fold higher oxygen evolution, slight reduction in growth. |
| 1.29-fold increase in lipid content | Li et al., 2012 | C. reinhardtii | STA6 | 24.4% increase in lipid content, 1.29-fold higher growth rate. |
| None identified | Chen et al., 2019 | C. reinhardtii | STA6 | Not studied. |

**Direct upregulation of lipid production**

**Upregulating acetyl-CoA carboxylase**

Directly modifying the lipid synthesis pathway is one area that has seen moderate success in increasing lipid productivity. As opposed to the terrestrial crops used for 1st generation biofuels, microalgae can produce and store a wide range of lipids within their cell. Microalgae produce both storage and structural lipids (Chen et al., 2019), including mono- and polyunsaturated fatty acids (MUFA and PUFA, respectively) and triacylglycerides (TAG). TAGs, comprised of a glycerol backbone and three fatty acids, are the main form of storage lipid in the cell (Liang & Jiang, 2013), and under optimal conditions may account for up to 80% of cell lipid content (such as observed in *Nannochloropsis* sp. QII) (Hu et al., 2008; Suen, Hubbard, Holzer, & Tornabene, 1987; Tonon, Harvey, Larson, & Graham, 2002). TAGs are of particular interest because they can be converted into fatty acid methyl esters (FAME) through a simple, one step, transesterification reaction, making them an ideal precursor for 4th generation biodiesel (Shuba & Kifle, 2018).

Acetyl-CoA carboxylase (ACCase; EC 6.4.1.2) is known to be an essential enzyme in the first rate-limiting step of fatty acid biosynthesis, and thus TAG accumulation (Davis, Solbiati, & Cronan, 2000; Hasan et al., 2018; Klaus, Ohlrogge, Neuhaus, & Dörmann, 2004; Roesler, Shintani, Savage, Boddupalli, & Ohlrogge, 1997). ACCase overexpression was first explored in the oleaginous diatom *Cyclotella cryptica* by random recombinant DNA integration using chimeric plasmid vectors, introducing multiple copies of the gene (Dunahay, Jarvis, Dais, & Roessler, 1996). While this resulted in an observed 2- to 3-fold increase in ACCase activity, no increase in fatty acid synthesis was reported. However, *C. cryptica* has since been attributed with high genetic potential for hyper-lipid production, as genomic analysis revealed elevated expression of key lipogenesis genes and a significantly increased TAG biosynthesis enzyme inventory when compared with the analogous diatom *Thalassiosira pseudonana* (Traller et al., 2016). Additionally, ACCase manipulation in the closely related *T. pseudonana*, increased ACCase activity by 3.3-fold and resulted in a 1.77-fold increase in lipid content to reach 40.8% dry weight.

Previously, 60Co γ irradiation mutagenesis of the green microalgae *Scenedesmus* sp. MC-1 generated a mutant strain that exhibited a 113% increase in lipid productivity and a 73% increase in lipid content, upwards of 28% lipids (dry weight) (Liu et al., 2015). The cause was hypothesized to be due to mutations in
ACC genes, as ACCase expression increased 5-fold compared to the progenitor strain. This was later confirmed by genomic analysis of the two strains which revealed differentiation of ACCase and phosphoenolpyruvate carboxylase (PEPC), which when retrotransformed into the parental MC-1 resulted in a 28.6% increase in lipid content (Ma et al., 2019a, 2019b).

The first reported overexpression of ACCase in C. reinhardtii, achieved via introduction of an overexpression vector, has shown that this strategy can result in increased ACCase activity leading to improved fatty acid biosynthesis (Chen et al., 2019). The ACCase mutant and wild type (WT), when grown with optimized nitrogen and phosphorous sources and sampled every 2 days for 12 days, revealed the ACCase-overexpression mutants to have 21.6% and 28.4% higher lipid-related fluorescence than the WT. MUFA and PUFA were also significantly enriched in the mutant strains to yields of 16% more than in the WT.

This research strongly implies that ACCase regulation is essential in the fatty acid metabolism of C. reinhardtii and that its overexpression is an effective strategy to increase lipid accumulation (Chen et al., 2019). A further benefit of this approach was that the mutant strains had an up to 1.29-fold increase in growth rate compared to the wild type, showing that the modified metabolism of these cultures was not adversely affecting growth.

Genetically simulating a nitrogen-starved cell state

It is known that Microchloropsis hyper-accumulates TAGs when subjected to nitrogen (N) starvation (Boussiba, Vonshak, Cohen, Avissar, & Richmond, 1987; Li et al., 2014), however a lack of N can also severely impede growth which would potentially negate the effect of increased lipid yields per cell. Previous research had failed to solve this issue, until recent work with M. gaditana used a strategy to identify genes expressed in M. gaditana during N starvation, and then to apply a CRISPR-Cas9 reverse genetics pipeline to identify and modify a key lipid accumulation transcriptional regulator, Zn(ii)₂Cys₆ (ZnCys) (Ajjawi et al., 2017). Using a novel high-efficiency Cas9-expressing editor line (Ng-Cas9+) developed for M. gaditana, the authors were able to knock-out ZnCys to produce mutants that would accumulate lipids as though they were in N-deprived conditions when grown in standard and N-optimized media, and importantly, without any major negative effects on biomass productivity (Ajjawi et al., 2017). This strain of M. gaditana was reported to double its lipid accumulation compared to the WT while retaining growth and CO₂ fixation rates comparable to unaltered cultures. The authors also reported that mutant strains had an increased carbon flux to the lipid pathway of 40–55%, compared to the nominal 20% flux (Ajjawi et al., 2017). Even so, this increase is still insufficient to achieve cost parity with fossil fuels.

Lipid secretion

The process of extracting lipids at an industrial scale is a significant economic hurdle, generally involving energy-intensive steps of dewatering and intracellular lipid extraction (Chaudry, Bahri, & Moheimani, 2018; Molina Grima, Belarbi, Acien Fernández, Robles Medina, & Chisti, 2003). An attractive way to obviate these cost-sinks could be to develop microalgae that extrude their lipids into the media (Li et al., 2019a). A natural example of this is the chlorophyte Botryococcus braunii, which produces up to 75% lipids (dry weight; Figure 2) that can be stored in an extracellular matrix (Jackson, Bahri, & Moheimani, 2017) allowing repeated and non-destructive extraction of the lipids (Chaudry et al., 2018; Jackson et al., 2017). Milking lipids in this way was shown to be sustainable for up to 70 days without the addition of supplementary nutrients, partially overcoming the main limitation of B. braunii – its slow growth rate. In a suitable continuous milking bioreactor (CMB), a high-volume dense culture may be grown, and then milked over time to minimize the significance of growth rate as a factor, replaced instead with the challenge of offsetting contamination-related crashes (Griehl et al., 2004).

Ideally, this concept should be applied to a commercial lipogenic species with a higher native growth rate. Recently, a transcription factor (NobZIP1) was identified in Nannochloropsis oceanica which both upregulates lipogenic genes and downregulates those involved in cell wall metabolism. NobZIP1 overexpression resulted in simultaneous lipid production increase and secretion (Li et al., 2019a). This has yet to be tested in other model species, but it may have the potential to save costs at the lipid extraction stages of biofuel production (Chaudry et al., 2018).

Inhibiting starch synthesis

It is known that in green microalgae starch and lipid synthesis utilize common carbon precursors, and are therefore competing pathways (Li et al., 2010b). Starch is a major storage molecule in many microalgae including green microalgae; consequently, a logical and promising approach to increase TAG content is to inhibit
starch biosynthesis. ADP-glucose pyrophosphorylase (EC 2.7.7.27) has been identified as a key enzyme in the production of starch for storage, and its disruption can lead to inhibition of starch biosynthesis. Starchless mutants of *C. reinhardtii* have been studied since ca. 1991, and several strains have since been isolated (Ball et al., 1991; Colleoni et al., 1999; Wattebled et al., 2003; Zabawinski et al., 2001). Two studies have reported TAG accumulation in *C. reinhardtii* starchless mutants (Li et al., 2010a, 2010b), establishing the efficacy of shunting the starch pathway as a means of increasing the effectiveness of microalgae as a biodiesel feedstock. The *C. reinhardtii* starchless mutant BAFJ5 was reported to accumulate 47% of its dry weight in (total) lipid compared to 13% in the WT and achieved slightly over 10 times (20.5% vs 2% WT) the TAG content (Li et al., 2010a). This is notable as the improved TAG accumulation is the largest reported for any transgenic algae, as well as for crop plants (Li et al., 2010a). The authors also noted that due to differences in mutant and

Figure 2. This figure summarizes the methodology and results of Li et al. (2010a), and Perrine et al. (2012) and illustrates these two approaches may be combined to yield a potentially commercial *Chlamydomonas* strain. Li et al.’s approach (left) resulted in 10x TAG synthesis compared to wild type (WT), incurring a 30% decrease in growth rate, while Perrine et al.’s approach yielded a strain which had similar composition to WT cells but a 30% augmented growth rate. The color change of the DNA strands reflects the mutated gene (green for CR-113 – and blue for STA6-1); note the green and blue both appear in the hypothetical double mutant. The lighter color of the CR-113 mutant’s chloroplast results from lower Chl-b levels. The central bar graph is a visual representation of the effect of each approach on growth rate.
WT cultures grown under heterotrophic conditions, it could be concluded that *C. reinhardtii* TAG accumulation is dependent on photosynthesis. Importantly, it is hypothesized that this is because TAG synthesis utilizes primarily photosynthetically produced carbon.

It was observed that when the starchless mutant cells were hyper-accumulating lipids (grown under N-starvation) their growth was impaired and they had a malformed cellular morphology; this was not unexpected considering such a radical redirection of metabolic resources. Under mixotrophic conditions with acetate as a carbon source (light and TAP medium), the BAFJ5 mutant exhibited a 12.3% reduction in growth, which was further exacerbated when grown autotrophically (which as mentioned above is more conducive to the desired TAG synthesis) (Li et al., 2010a). However, the significant increase in TAG productivity of the mutant was greater than this growth impairment, and thus the BAFJ5 mutant had a net 4–8.7-fold increase in TAG productivity compared to the WT.

The impairment in growth seen in the starchless mutants may be caused by the shifting of carbon flux away from the starch pathway leading to a negative effect on photosynthesis (Li et al., 2010b). Studies have shown that the incapability to produce starch may disrupt energy partitioning at photosystem II (PSII), as well as RuBisCO regulated carboxylation connected to a decrease in the regeneration of D-ribulose-1, 5-bisphosphate (Lytovchenko, Bieberich, Willmitzer, & Fernie, 2002; Peterson & Hanson, 1991; Sicher & Kremer, 1996). However, given the advancement and availability of metabolic engineering toolkits for *C. reinhardtii*, there are methods by which this discrepancy may be overcome (Mayfield et al., 2007; Rosenberg, Oyler, Wilkinson, & Betenbaugh, 2008), such as the generation of a synthetic pathway to supplement the regeneration of D-ribulose-1, 5-bisphosphate.

This genetic engineering strategy can potentially be applied to further species of microalgae (Li et al., 2010a), providing they have a similar starch/lipid metabolism. Therefore, it could conceivably be applied to species with native lipid contents higher than that of *Chlamydomonas*, such as *M. gaditana* (Ajawi et al., 2017), for a magnified effect. Altogether, this indicates that redirecting carbon partitioning from starch synthesis in favour of TAG synthesis is likely to be a more effective approach than directly manipulating the TAG biosynthesis pathway.

It is important to note that increases in TAG accumulation in starchless mutants are not always found. High variability in TAG and starch content amongst *C. reinhardtii* strains has been observed, and blocking the starch synthesis pathway does not always result in apparent TAG accumulation (Siaut et al., 2011). Starchless mutations in *Chlorella sorokiniana* were also found to be ineffective at increasing TAG content, with mutants showing a significant increase in water-soluble polysaccharides instead (Vonlanthen, Dauvillé, & Purton, 2015). Nonetheless, a range of other studies do show significant increase in TAG accumulation in starchless mutants of *Scenedesmus obliquus* (Breuer et al., 2014; de Jaeger et al., 2014) and *Chlorella pyrenoidosa* (Ramazanov & Ramazanov, 2006). Taken together, these studies indicate complex interplays between starch and lipid pathways in green microalgae that exceed simple competition for carbon flux, and it cannot be assumed that TAG hyper-accumulation as a result of starch inhibition will translate between species, or even strains, of microalgae.

**Improving photosynthetic efficiency**

Rather than manipulating biosynthesis of storage lipid and TAG production, either through direct upregulation or modification of competing metabolic pathways, another approach to increase the economic viability of 4th generation biofuels is to increase photosynthetic efficiency, thereby generating more biomass for the same or less energy input. The relatively high photosynthetic efficiency of single-celled microalgae already distinguishes them as some of the most efficient autotrophic primary producers (Chisti, 2007; Mata, Martins, & Nidia. S, 2010; Mussungnug et al., 2007; Sayre, 2010), however their theoretical potential is reportedly up to 3-times higher than what they ordinarily exhibit (Melis, 2009; Ort, Zhu, & Melis, 2010), lending some credence to the feasibility of this approach. Recently, a *C. reinhardtii* mutant library (60 000 unique mutants) covering 83% of nuclear-protein coding genes has been made public and should enable rapid characterization of thousands of genes (Li et al., 2019b). The authors’ initial analysis has identified ~300 photosynthetic genes, including 21 novel genes with high engineering potential. This library and the rapidly advancing genetic resources summarized above, will accelerate the development of commercially viable strains.

**Reducing chlorophyll antenna size**

One of the more successful ways this strategy has been implemented in terrestrial crops is through the reduction in size of the light-harvesting chlorophyll antenna
complex (LHC) (Melis, 2009; Perrine et al., 2012; Polle, 2002; Polle, Benemann, Tanaka, & Melis, 2000; Shin et al., 2016). In nature, a large chlorophyll antenna can be advantageous in outcompeting rival photosynthetic species by capturing as many photons as possible and depriving competitors of photosynthetically active radiation (Müssnug et al., 2007). Furthermore, it allows a cell to maximize light capture in both high and low light conditions. However, in industrial monocultures where species competition is absent and light is abundant, the metabolic cost of these adaptations can be deleterious to the photosystem (Kok, 1953; Melis, 2009).

Outdoors, chlorophyll antennae in most photosynthetic organisms, including microalgae on a cell-per-cell basis, are considered saturated at approximately a quarter of full sunlight because above this light intensity the electron transport chain (ETC) becomes rate-limiting (Melis, 2009; Müller, Li, & Niyogi, 2001). At full sunlight intensities, energy flow from the LHC to the reaction centre can reach 100x the rate of electron flow in the ETC, resulting in losses of ca. 75–80% of captured energy, mainly in the form of heat dissipation (Perrine et al., 2012; Shin et al., 2016; Polle et al., 2003). This disparity and its role in energy loss is known as non-photocchemical quenching (NPQ; Müller et al., 2001). Moreover, these conditions may even cause a quantifiable decrease in photosynthetic efficiency as excess photon-excited electrons induce the breakdown of D1, a key protein in PSII (Nakajima et al., 2001).

Taken together, cells with large chlorophyll antennae in mass culture conditions invariably will either experience excess light intensities which can induce photoinhibition or will be shaded by superposed cells and not exposed to enough photosynthetic radiation. Thus, reducing the cross-sectional area of the LHC has been proposed to benefit the overall photosynthetic efficiency of a microalgal culture (Beckmann et al., 2009; Nakajima & Ueda, 1997; Perrine et al., 2012; Polle, 2002; Shin et al., 2016).

**Targeted reduction of chlorophyll b to tune peripheral light harvesting antenna**

Rather than random mutagenesis to generate truncated antennae, targeting chlorophyllide-α oxygenase is potentially more precise, as approaches such as inhibiting or eliminating Chl b synthesis or inducing under-expression of LHC genes can produce mutants with reduced antenna sizes (Müssnug et al., 2007; Negi et al., 2020; Polle et al., 2000). A demonstration of this approach used RNAi-mediated silencing of *C. reinhardtii* Chl b synthesis gene (Perrine et al., 2012). Through this method, the authors were able to achieve various sizes of chlorophyll antenna and to determine the optimal sized antenna for highest growth rates across a range of light intensities. They found that mutants with intermediate size of antennae were optimal, growing 15–35% denser than the WT under high light (500 µmol photons m⁻² s⁻¹), and had comparable growth rates to WT under low light (50 µmol photons m⁻² s⁻¹). Mutants showed a doubling in photosynthetic rate at high light intensities. Furthermore, photon flux density was retained to a much larger degree in penetrating the culture, meaning a decrease in both cell-shading and loss of energy through non-photocchemical quenching due to reduced absorption by any given cell (Perrine et al., 2012).

Altogether, the findings of these studies show that reducing the size of the light-harvesting chlorophyll antenna is an effective strategy to increase microalgal biomass productivity and thus the feasibility of producing marketable 4th generation biodiesel.

**Generating random mutants with truncated chlorophyll antennae**

*C. vulgaris* is a single-celled green microalgae which is one of the more promising species for mass cultivation and biodiesel production (Patnaik, Singh, Bagchi, Rao, & Mallick, 2019). To test the effectiveness of truncated chlorophyll antennae as a method of increasing biomass productivity in *C. vulgaris*, random mutants were generated via ethyl methane-sulphonate mediated mutagenesis, selecting for colonies with reduced pigmentation indicative of chlorophyll deficiencies (Shin et al., 2016). Colonies that showed the greatest increase in Chl a/b ratio were selected, as it has been shown that this is typical of truncated light-harvesting antennae (Hankamer et al., 1997; Melis, Neidhardt, & Benemann, 1999; Beckmann et al., 2009), and mutants were verified through western blot analysis.

The short-antenna mutants were shown to have 2.5-fold higher photosynthetic efficiency and 2.2-fold higher rate of electron transport in the chloroplast (Shin et al., 2016). Furthermore, as the authors hypothesized, the *C. vulgaris* mutant also exhibited decreased non-photochemical quenching and decreased photooinhibition compared to the WT. Overall, the short antenna mutants displayed a 44.5% increase in biomass productivity under high-light conditions, but it should be noted that lower light levels (50 µmol photons m⁻² s⁻¹) resulted in an 18.6% decrease in biomass productivity.
**Engineering PSII for different solar intensities**

D1 is a key protein in PSII that is essential for the proper functioning of the water-oxidizing complex, the source of photosynthetic oxygenesis (Vinyard et al., 2014). The approach to engineering PSII stems from the discovery of two isoforms of the D1 protein (D1:1 and D1:2) in the cyanobacterium *Synechococcus elongatus* (Bustos, Schaefer, & Golden, 1990). Environmental factors such as light intensity will modulate the expression of the isoforms coding for this protein (Schaefer & Golden, 1989a, 1989b); where D1:1 is expressed during nominal light intensities and D1:2 is expressed in high-light intensities (Bustos et al., 1990; Golden, Brusslan, & Haselkorn, 1986), where expression under their corresponding light conditions increases light utilization and biomass yield (Vinyard et al., 2014). It has been demonstrated that the native PSII-functionality is a compromise between photochemical productivity and photoprotection, and by expressing D1 isomorphs in *C. reinhardtii* this can be used to tune the strain for either high or low light intensities (Vinyard et al., 2014). This in turn confers a significant growth advantage by prioritizing photoprotection against photoinhibition and photochemical productivity (Vinyard et al., 2014).

**Intracellular spectral recomposition of light**

The use of special photobioreactors (PBRs) with innovative light configurations such as strobing light-emitting diodes (LEDs) and rapid mixing have been proposed to increase biomass yield in industrial-sized cultures (Heining and Buchholz, 2015). One of the most widely adopted PBR modifications has been integrating photovoltaic panels into enclosed bioreactors and open systems, which increases light utilization by reconfiguring the spectral irradiance to increase specific photosynthetically active wavelengths (Sforza, Barbera, & Bertucco, 2015; Wondraczek, Tyystjärvi, Méndez-Ramos, Müller, & Zhang, 2015; Delavari Amrei et al., 2014). This is not a new concept, nor is the addition of fluorescent dyes to culture media which absorb light in the ultra-violet to blue spectrum (300–400 nm) and re-emit radiance in the photosynthetically active yellow-red spectrum (550–700 nm; Prokop, Quinn, Fekri, Murad, & Ahmed, 1984). This strategy increased biomass yields by 15% in *C. sorokiniana* cultures grown using natural sunlight cultures. This principle has been taken a step further by engineering the diatom *P. tricornutum* to express an enhanced green fluorescent protein (eGFP; Fu et al., 2017). This conferred biogenic intracellular spectral recompositioning of light effectively converts excess blue to yellow-green wavelengths which can be readily absorbed by accessory photosynthetic pigments. This works on the principle that unabsorbed high-energy blue light is otherwise partially dissipated as heat in cells that are overexposed, before it can reach shaded cells in high-density regions of the cultures. Therefore, rather than reconfiguring the spectrum of light in the media (Prokop et al., 1984), expressing eGFP within the cell is more efficient by providing an intracellular light source (Gressel, Eisenstadt, Schatz, Einbinder, & Ufaz, 2010). Moreover, because diatoms absorb blue light more readily than the converted yellow-green light, the latter is less attenuated as it propagates further into the culture, and thus higher light penetration may be achieved in dense cultures (Kuczynska, Jemiola-Rzeminska, & Strzalka, 2015; Prokop et al., 1984).

Fu et al. (2017) tested their transgenic *P. tricornutum* in both open pond cultures under-simulated high-intensity sunlight and in enclosed PBRs and found substantial improvements in biomass production in both systems, proving the applicability of this approach for at least some industrial culturing techniques. Expressing eGFP in the chloroplast was considered the most appropriate strategy because the proteins are in closer proximity to the light-harvesting complexes, but Fu et al. (2017) reported significantly higher expression in the cytoplasm, making the latter the most efficient approach. The eGFP mutants displayed a quantifiably impressive 28% higher photosynthetic efficiency compared to the WT, and under-simulated outdoor sunlight conditions exhibited a 50% improvement in biomass production rate (Fu et al., 2017).

The authors explored chemical staining of cells using fluorophores, but while this resulted in improved yields, there were disadvantages to this approach, including the potentially prohibitive cost of dyes for industrial use, and decomposition of commercial dyes into potentially harmful derivatives (Fu et al., 2017). In either case, biogenic intracellular recompositioning of light is superior due to the stability of GFP (Tanaka, Nakatsuka, Harada, Ishida, & Matsuda, 2005) and the low cost of producing eGFP diatom transformants.

The applicability of this approach to other microalgae is largely dependent on these algae having appropriate accessory pigments such as the carotenoid fucoxanthin found in diatoms, that efficiently absorbs light in the yellow-green spectrum (Fu et al., 2017; Kuczynska et al., 2015). This should make this approach directly transferable to other species of brown algae (Ochrophyta) or to red algae (Rhodophyta) that use phycobiliproteins to similar effect (Thomas et al., 1999). Green algae contain β-
carotene which can absorb green light (albeit less efficiently), and so, this approach may still be of value in organisms such as *C. reinhardtii*, currently being considered for biofuel production. Furthermore, the high neutral lipid contents of *P. tricornutum* preferentially incorporate lipophilic fluorophores (Fu et al., 2017), and therefore this approach may be particularly effective in strains that are naturally high in lipids or are being engineered for higher neutral lipid content. Model algae could be concomitantly engineered to express higher levels of accessory pigments to further improve the feasibility of this approach in non-Ochro- and Rhodophyta strains, however, such a high degree of metabolic engineering may be more trouble than it is worth.

**Challenges of GMO cultivation**

As with any work directly involving the culture of genetically modified organisms (GMOs), it is imperative to adhere to the legal and environmental framework that is intrinsic to practical GMO work. A ubiquitous issue is that of containment; in this case how it can be ensured that any GM algae produced in the endeavour for 4th generation biofuels will not proliferate in the surrounding environment. This is critical because of a GMO’s potential to outcompete endemic species and possibly alter the local ecosystem (Henley et al., 2013; Snow et al., 2012).

Though the importance of containment measures cannot be understated, responsibly it should be assumed that given enough time a breach in containment is inevitable. Thus, it is potentially equally important to detect and neuter any GM microalgae such that they either cannot survive or cannot proliferate in a natural environment. There are in theory as many of these “biocontainment strategies” as there are ways to genetically modify microalgae in the first place, with some more ingenious than others and, for most, feasibility is dependent on the situation. For example, it has been proposed that the elimination of inorganic carbon pumps may allow for normal growth in CO₂ enriched cultures and impaired growth at atmospheric concentrations (Henley et al., 2013), conferring an ecological advantage to natural algal strains. This approach would be ideal for a cultivation site using power plant flue gas as a CO₂ source, where the additional CO₂ required would not impact production cost. Alternatively, it has also been proposed that including conditional lethality traits in GM microalgae could be a possible solution. Barnase is a cytotoxic enzyme which causes apoptosis via the degradation of cellular RNA in the absence of its inhibitor, barstar (Henley et al., 2013). An effective biocontainment strategy could be to engineer an algal GM strain to express barnase, coupled with addition of barstar in the culture media as a xenobiotic (Clark and Maciej, 2020; Henley et al., 2013). Such measures would trigger cell death upon containment break, however, as with many biocontainment strategies, it is unclear whether or not the metabolic cost of barnase synthesis, combined with the monetary cost of supplementing media with barstar at large scales would nullify any cost-benefit conferred from the algae being transgenic.

**Future directions**

Biofuels from microalgae are some of the most promising alternative fuels being investigated to simultaneously mitigate the adverse effects of fossil fuel use on the environment and compensate the rising energy demand of an increasing global population. So far, a true 4th generation microagal biofuel feedstock has not been applied in large-scale biodiesel production; however, the approaches discussed in this review show an accelerating rate of progress as synthetic biology tools and techniques are applied to this goal. Several promising strategies have proven their standalone efficacy, such as ACCase over-expression, shunting starch biosynthesis, truncating photosynthetic antennae or expressing eGFP. However, if biodiesel is to be cost-competitive with fossil fuels, future synthetic biology research must focus on combining both direct and indirect hyper-lipid production, as well as incorporating strategies to increase light utilization and the intrinsic photosynthetic efficiency of microalgal species.

For example, *C. reinhardtii* expressing disrupted AGP pyrophosphorylase has been shown to increase TAG content by 10-fold but was growth-inhibited by 30%. We propose that this growth deficit could be redressed by supplementary reduction of Chl b to generate truncated chlorophyll antenna, which independently has been shown to increase growth by 30% (also in *C. reinhardtii*). In this way, strains which hyper-accumulate TAG on par with the highest recorded levels whilst maintaining a normal growth rate may be achievable (Figure 2). Such a mutant would begin to tackle some of the major intrinsic issues with 4th generation biofuel production such as insufficient cell lipid content and poor natural photosynthetic efficiency, but also introduces an additional problem – and a potential solution to industrial GMO use.

A significant issue with the synthetic biology approach is that the additional permits and infrastructure (containment) needed to grow GM microalgae at an industrial scale may counteract the financial benefits offered by the mutations in the first place (Beacham,
Sweet, & Allen, 2017). Considering this, studies show that truncated LHC mutants (depending on the extent of truncation) grow less effectively than WT under low light (<50 μmol photons m⁻² s⁻¹) for the same reason that they grow more effectively under high light (>500 μmol photons m⁻² s⁻¹) (Perrine et al., 2012; Shin et al., 2016). Under low light the significantly reduced chlorophyll antenna cross-section results in insufficient photon flux to the electron transport chain, manifesting lower growth rates. It may be advantageous for commercial biodiesel production to use this double mutant because the reduction in growth rate in the natural environment conditions is sufficient to enable the endemic microalgal population to consistently out-compete the mutants. An industrial biofuel plant could therefore consider forgoing the strict GM containment protocols and infrastructure that would otherwise be necessary, thus reducing costs.

Concluding remarks

As accessible and low-cost gene editing toolkits such as CRISPR-Cas9 RNPs and prime editing continue to permeate and enable molecular and biotechnology on a larger scale, the barriers hindering the engineering of non-model algae become less onerous. The recent developments of CRIPSR-Cas12a multiplexing may be especially useful for creating advanced multi-mutants at lower costs by supplanting iterative rounds of mutagenesis. Altogether, this will allow efforts to shift towards engineering new species with higher native lipid contents than model species, as they are made tractable for genetic engineering. Additionally, many microalgae produce value-added products such as terpenoids that could be recovered via downstream processing to subsidize the cost of 4th generation biodiesel and thus increase market competitiveness.

In conclusion, a strong case can be made for engineered hyper-lipid producing microalgae being the most apposite approach to achieve commercially viable biofuels. We would also suggest that such synthetic biology should go hand-in-hand with engineering of cultivation strategies to extract the greatest synergies and concomitantly address the important issue of GMO containment.

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The authors have nothing to disclose.

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Highlights

- Increasing population and global energy demands may be mitigated by developing renewable microalgal-based biofuels (3rd generation), however these are not yet cost competitive with fossil fuels.
- Synthetic biology is being employed to create genetically modified microalgae (4th generation) with higher biomass productivity and storage lipid accumulation (such as TAGs), however while significant progress is being made efforts continue to fall short.
- Future research in synthetic biology of microalgal-based biofuels must focus on concomitant implementation of multiple gene editing approaches to unlock the commercial viability of 4th generation biofuels.

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