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**Molecular Genetic Tools and Emerging Synthetic Biology
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Molecular Genetic Tools and Emerging Synthetic Biology Strategies to Increase Cellular Oil Content in *Chlamydomonas reinhardtii*

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Abbreviation: amiRNA, artificial microRNA; CRISPR, clustered regularly interspaced short palindromic repeats; FA, fatty acid; FP, fluorescent protein; gRNA, guide RNA; N, nitrogen; RNAi, RNA interference; RNPs, ribonucleoproteins; S, sulfur; TAG, triacylglycerol; TALEN, transcription activator-like effector nuclease; TF, transcription factor; ZFN, zinc finger nuclease

Abstract:

Microalgae constitute a highly diverse group of eukaryotic and photosynthetic microorganisms that have developed extremely efficient systems for harvesting and transforming solar energy into energy-rich molecules such as lipids. Although microalgae are considered to be one of the most promising platforms for the sustainable production of liquid oil, the oil content of these organisms is naturally low, and algal oil production is currently not economically viable. *Chlamydomonas reinhardtii* (*Chlamydomonas*) is an established algal model due to its fast growth, high transformation efficiency, and well-understood physiology and to the availability of detailed genome information and versatile molecular tools for this organism. In this review, we summarize recent advances in the development of genetic manipulation tools for *Chlamydomonas*, from gene delivery methods to state-of-the-art genome-editing technologies and fluorescent dye-based high-throughput mutant screening approaches. Furthermore, we discuss practical strategies and toolkits that enhance transgene expression, such as choice of expression vector and background strain. We then provide examples of how advanced genetic tools have been used to increase oil content in *Chlamydomonas*. Collectively, the current literature indicates that microalgal oil content can be increased by overexpressing key enzymes that catalyze lipid biosynthesis, blocking lipid degradation, silencing metabolic pathways that compete with lipid biosynthesis, and modulating redox state. The tools and knowledge generated through metabolic engineering studies should pave the way for developing a synthetic biological approach to enhance lipid productivity in microalgae.

Key words: *Chlamydomonas reinhardtii*, Genome Editing, Metabolic Engineering, Oil content, Transgene Expression, Triacylglycerol

Introduction

Microalgae represent a polyphyletic group of photosynthetic eukaryotic microorganisms, and are sources of a number of value-added products, including proteins, pigments, carbohydrates, carotenoids, vitamins, anti-oxidants, and polyunsaturated very long chain fatty acids (Chew et al. 2017). Due to their high biomass productivity and diverse ecological habitats, microalgae have recently been considered an alternative sustainable resource for biofuels. However, the sustainable industrial production of oil, i.e., triacylglycerols (TAGs), from any microalgal species has yet to be realized, due to bottlenecks at both the biological and biotechnological levels. One of the most challenging issues is to increase oil productivity, because most microalgae accumulate large amounts of oil only when subjected to stress conditions (e.g., nutrient depletion), when cell growth is usually impaired (Merchant et al. 2012, Siaut et al. 2011). Therefore, current research is intensively focused on deciphering the molecular mechanisms underlying lipid biosynthesis and the relationship between lipid biosynthesis and biomass growth (Hu et al. 2008, Kong et al. 2018a).

Among microalgal species, the unicellular eukaryotic green alga *Chlamydomonas reinhardtii* (hereafter, *Chlamydomonas*) is a well-established algal model, due to its haploid genotype, fast growth, and sequenced genome (Merchant et al. 2007). *Chlamydomonas* is commonly used to study a number of biological processes, including photosynthesis and flagella-mediated motility. Over the past decade, spurred on by a renewed interest in microalgal oil, *Chlamydomonas* has emerged as a model organism for studies of lipid metabolism (Du and Benning, 2016, Li-Beisson et al. 2015).

Furthermore, the emergence of synthetic biology provides an opportunity to expedite the development of *Chlamydomonas* and other microalgal species as a useful industrial biotechnology platform. Synthetic biology, which is based on a combination of standardized biological components (e.g., promoters, terminators, and selection markers), predictive modeling, and iterative design and testing, was first used in bacterial systems and has since advanced to eukaryotic systems, including algae (Crozet et al. 2018). A modular cloning toolkit, comprising 119 functionally validated genetic parts, was developed for *Chlamydomonas* based on Golden Gate cloning with standard syntax (Crozet et al. 2018). This toolkit has established *Chlamydomonas* as the next chassis for sustainable synthetic biology.

Here, we present an overview of molecular approaches, such as metabolic engineering and gene disruption strategies, that enable the genetic manipulation of microalgae with the

aim of identifying metabolic pathways and elucidating physiological mechanisms. We provide examples of successful efforts to genetically manipulate *Chlamydomonas* to increase oil content, and discuss future challenges and directions in microalgal synthetic biology.

Gene delivery tools and selection systems for *Chlamydomonas*

Transformation methods

Various techniques have been established to deliver DNA into the *Chlamydomonas* nucleus, including biolistics, electroporation, *Agrobacterium*-mediated transformation, or even simply vortexing with glass beads, silicon carbide whiskers, or 3-aminopropyl-functionalized magnesium (reviewed by Jinkerson and Jonikas 2015). Biolistic transformation was the first method developed for this purpose and was mediated by microprojectiles, such as tungsten powder and gold particles, and, more recently, by size-controlled mesoporous TiO₂ nanoparticles, which are effective at lower density and are less prone to aggregation than are tungsten or gold particles (Hou et al. 2013). Biolistic methods can be used to transform nuclear, chloroplastic, and mitochondrial genomes. This method is efficient but expensive due to the requirement for specialized equipment. Consequently, cheaper and simpler methods (e.g., *Agrobacterium*-mediated transformation and electroporation) have been developed. *Agrobacterium*-mediated transformation yields a slightly lower proportion of transgene-silenced transformants than does electroporation, but does not provide significant advantages over electroporation-mediated transformation in *Chlamydomonas*, due to its labor-intensive nature and large number of false positives (Mini et al. 2018). Thus, electroporation is currently one of the most efficient methods for delivering DNA into the nuclei of cells (Shimogawara et al. 1998, Yamano et al. 2013).

Selectable markers and reporter genes

Several antibiotic resistance genes have been developed for nuclear or chloroplast transformation in *Chlamydomonas*. Among these, *aadA*, encoding an aminoglycoside 3'-adenylyltransferase (spectinomycin and streptomycin resistance), is widely used in both nuclear and chloroplast transformation (Meslet-Cladière and Vallon 2011). The GC-rich *ble* gene, conferring resistance to zeocin, is an effective selectable marker for efficient transgene expression when fused to the target gene (Fuhrmann et al. 1999, Plucinak et al. 2015), but the underlying mechanism is not well understood. It is possible that bleomycin resistance proteins (BLEs) function by sequestering zeocin in a one-to-one ratio, but not through enzymatic

inactivation (Rasala et al. 2013). However, the use of *ble* as a selectable marker is limited, because zeocin is light sensitive and may function as a mutagen when applied at high concentrations (Lin et al. 2017). This prompted the development of other selectable markers, such as *aphVIII* and *aph7"*, which confer paromomycin and hygromycin-resistance, respectively. *AphVIII* is currently one of the most widely used selectable marker in *Chlamydomonas*, because it confers a stable drug-resistant phenotype (Meslet-Cladière and Vallon 2011).

In addition to selectable markers, reporter genes have also become important tools in microalgal genetic engineering. For instance, to monitor gene expression and protein localization *in vivo*, various fluorescent proteins covering most fluorescent ranges (i.e., yellow YFP, blue mTagBFP, cyan mCerulean, green CrGFP, yellow Venus, orange tdTomato, and red mCherry) have been adapted for optimal expression in *Chlamydomonas* (Neupert et al. 2009, Rasala et al. 2013).

Forward genetics in *Chlamydomonas*

Mutagenesis

Mutagenesis mediated by mutagens (e.g. ethylmethanesulfonate) has been used to generate a library of *Chlamydomonas* mutants that was subjected to forward genetic screens for altered oil content (Xie et al. 2014, Lee et al. 2014). However, a major drawback of using these mutagens is that point mutations are randomly produced, making it challenging to identify the causative gene(s) for the phenotype.

Random insertional mutagenesis of *Chlamydomonas* using drug resistance cassettes, which randomly integrate exogenous DNA into the nuclear genome, has been used to generate tens of thousands of transformants in numerous forward genetics studies. Insertional mutants have been generated at large scale and screened for phenotypes related to lipid metabolism, motility, and photosynthesis (Cagnon et al. 2013, Cheng et al. 2017, Dent et al. 2015). An advantage of this approach is that insertional mutations can be mapped without the need for extensive genetic crosses.

*Examples of medium-to-high-throughput screening developed for *Chlamydomonas**

Oil content can be estimated by staining with fluorescent dyes (e.g., Nile red and BODIPY) and can be combined flow cytometry analysis for high-throughput studies. This strategy has

been used to screen for *Chlamydomonas* mutants with altered oil content (Cagnon et al. 2013, Cheng et al. 2017, Tsai et al. 2014). Fluorescence-activated cell sorting of cells that have been stained with Nile red allows for the efficient screening of tens of thousands of *Chlamydomonas* mutants (Terashima et al. 2015, Xie et al. 2014). This method also allows for quantification of lipid content while preserving cell viability, as Nile red staining does not hinder growth, and requires only five weeks from mutagenesis to mutant isolation (Terashima et al. 2015).

Another approach commonly used to study lipid metabolism is a screen for mutants with altered FA composition. Differences in FA composition can be analyzed using a gas chromatography-flame ionization detector (GC-FID) after lipids are converted to their fatty acid methyl esters. Direct transmethylation of cell pellets by acid-catalyzed transmethylation coupled with a robotized GC-FID was developed for high-throughput screening for *Chlamydomonas* mutants with altered FA composition (Nguyen et al. 2013). Using this method, we previously isolated a mutant defective in the ω -3 FA desaturase (named *crfad7*) in a screen of 2,000 insertional mutants (Nguyen et al. 2013). CrFAD7 is localized to plastids and contributes to the formation of all ω -3 fatty acid-containing plastidic and extraplastidic glycerolipid species (Nguyen et al. 2013).

Genetic mapping of insertional mutants

Various PCR-based techniques have been developed to identify insertion sites within the nuclear genome of *Chlamydomonas*, including Genome Walker, restriction enzyme site-directed amplification-PCR (RESDA-PCR), thermal asymmetric interlaced-PCR, 3'-rapid amplification of cDNA ends, SiteFinding-PCR, and hairpin-PCR (reviewed by Li and Jonikas 2016). Among these methods, RESDA-PCR is the most commonly used and offers a high probability of identifying the insertion sites (González-Ballester et al. 2005, Kong and Li-Beisson 2018). However, this approach is labor-intensive and only identifies a handful of insertion sites at once, rendering it unsuitable for the simultaneous mapping of tens of thousands of *Chlamydomonas* insertional mutants. Additionally, the success of these PCR-based approaches is determined largely by the PCR product size, and requires intact PCR primer binding sites in the cassette, which are occasionally disturbed by cassette truncation, concatemerization after integration, and the presence of "junk DNA" from a distant locus (Li et al. 2016).

Prompted by these challenges, a method for mapping insertion cassettes in pools of

tens of thousands of *Chlamydomonas* mutants, named Mme1-based insertion site sequencing (ChlaMmeSeq), was developed (Zhang et al. 2014). Approximately >100,000 insertions were successfully mapped using ChlaMmeSeq (CLiP:<https://www.chlamylibrary.org/>). In this method, a type II restriction enzyme site (MmeI) that facilitates subsequent high-throughput sequencing is included at both ends of the transformation cassette. However, in some instances, upon fragmentation of the cassette, the binding sites for sequencing primers are eliminated, making it difficult or impossible to use ChlaMmeSeq to map that insertion site. Due to recent decreases in the cost of sequencing technologies, whole-genome re-sequencing has been used to identify point mutations and map target genes. Using this approach, we previously identified the site of genetic lesion for a *Chlamydomonas* mutant named constitutive-oil-accumulation 1 (*coal1*), which was found to harbor a >200 kb deletion (Goold et al. 2016).

Reverse genetics in *Chlamydomonas*

A successful reverse genetic approach relies on the selection of suitable target gene(s) for genetic manipulation. There are two major ways to identify and select target genes in *Chlamydomonas*. First, *Chlamydomonas* genes that are homologous to ones already known to increase seed oil content in terrestrial plants or in other organisms can be identified. This case requires selection of the genes and rigorous confirmation of the activity of the proteins encoded by the genes. However, many genes in the *Chlamydomonas* database are not well annotated or characterized. In general, the *Chlamydomonas* genome most closely resembles those of plants, followed by those of animals and fungi. This is particularly the case for genes involved in lipid metabolism, as *de novo* FA synthesis occurs in the chloroplast, an organelle common to plants and algae. However, the flagellar and basal body proteins tend to show greater similarity to those of animals (Merchant et al. 2007). To select suitable target genes for reverse genetics, one can search for genes homologous to those already reported as being effective in model plant databases, such as Arabidopsis TAIR (<https://www.arabidopsis.org>). Further steps in selecting a target gene may include constructing phylogenetic trees; aligning the amino acid sequences of the predicted proteins; performing a subcellular localization analysis, using PredAlgo (Tardif et al. 2012); and verifying the expression profiles, using the Chlamy Network Portal (<http://networks.systemsbiology.net/chlamy-portal/>) or AlCOdb (<http://alcoodb.jp/coexpression/search>). Second, candidate genes can be identified in comparative transcriptomic studies, gene co-expression analyses, or proteomics studies.

Acyltransferases and lipid droplet-associated proteins are of particular interest to many scientists working in the field of lipid production.

Strategies to improve nuclear transgene expression

Codon optimization

Although foreign genes are easily integrated into the nuclear genome, transgene expression levels are notoriously poor in *Chlamydomonas* (Kong et al. 2014, Lumbreras et al. 1998, Neupert et al. 2009). The efficiency of gene expression partially depends on the nucleotide composition (particularly the GC content) of the inserted DNA fragment and on its consistency with the codon-usage of the host organism (Barahimipour et al. 2015, Neupert et al. 2009). *Chlamydomonas* coding sequences have an average GC content of 68% (Merchant et al. 2007). An unfavorable GC content affects gene expression at the level of chromatin structure. Recently, codon usage was reported to be more critical than GC content for gene expression efficiency in *Chlamydomonas* (Barahimipour et al. 2015). Codon optimization through gene re-synthesis improves transgene expression, even if *Chlamydomonas* contains a high GC content.

Choice of promoters

Both constitutive and inducible promoters have been developed for *Chlamydomonas*. Established constitutive promoters present in *Chlamydomonas* include the *Hsp70A*, *RbcS2*, and *PsaD* promoters and the *Hsp70A-RbcS2* chimeric promoter, and the inducible promoters include those present in the copper-*(Cyc6)* and alcohol-inducible (*AlcR-P_{alcA}*) promoter systems (Fischer and Rochaix 2001, Lee et al. 2018, Quinn and Merchant 1995). The *AlcR-P_{alcA}* system is composed of a regulatory protein (*AlcR*) and a promoter controlling *alcA* expression (*P_{alcA}*), which has previously been used for inducible transgene expression in plants, and was also recently used in *Chlamydomonas* (Lee et al. 2018). In addition to the aforementioned traditional promoters, Scranton et al. (2016) generated a set of synthetic algal promoters (e.g., *sap11*) that contain a new DNA motif that is essential for promoter function and highly conserved in *Chlamydomonas*. The synthetic algal promoters were shown to drive higher expression of transgenes in *Chlamydomonas* than the strongest known endogenous promoter, the chimeric *Hsp70A-RbcS2* promoter. Endogenous gene flanking sequences in *Chlamydomonas* have also been shown to enhance the expression level of a transgene. For

example, a 1,425-bp upstream region of the endogenous gene *ARG7* can drive the expression of the *ARS2* reporter as strongly as the *Hsp70A-RbcS2* promoter (Specht et al. 2015). López-Paz et al. (2017) recently reported that *RPL23* flanking sequences (including promoters, terminators, and UTRs) resulted in stable expression of luciferase or *sh-ble*, at levels much higher than *Hsp70A-RbcS2* or the *PsaD* promoter in *Chlamydomonas*.

Synthetic intron

Inclusion of a native intron in the cDNA of a transgene can improve expression level. For example, when the first intron of *Chlamydomonas RbcS2* (*RbcS2i1*) is introduced into the coding region of *ble*, it dramatically increases the transformation frequency and the level of *ble* expression in a manner independent of orientation (Lumbreras et al. 1998). Interestingly, Eichler-Stahlberg et al. (2009) found that the addition of *RbcS2i1* (e.g., one to three copies) resulted in increased expression levels of the luciferase gene in *Chlamydomonas*. They also showed that a particular number and genomic placement of the inserted *RbcS2i1* had an over-proportionally stimulating effect on expression.

Recently, it was reported that the repetitive dispersal of *RbcS2i1* throughout the codon-optimized coding sequences (CDS) of transgenes enabled robust expression from the nuclear genome of *Chlamydomonas*. Using this strategy, the large sesquiterpene synthase gene *AgBs* as well as genes encoding two farnesyl pyrophosphate synthases, *ERG20* and *ispA*, were efficiently expressed (Lauersen et al. 2016a, Wichmann et al. 2018). The optimal *RbcS2i1* insertion site (...NG/GN...) in the codon-optimized transgene CDS for efficient splicing was also systematically determined (Baier et al. 2018). Possible reasons for the “intron-mediated efficient transgene expression” phenomenon could either be that introns contain enhancers or that introns facilitate transcript maturation and export from the nucleus; however, the mechanism remains to be elucidated.

Cleavable fusion peptide system

Nuclear transgene expression levels can be improved by incorporating resistance to a selective agent. Rasala et al. (2012) developed the *ble2A* system in *Chlamydomonas*, which enables efficient selection of transformants by transcriptional fusion of the heterologous gene to the *ble* selectable marker via self-cleavage of viral 2A peptides (20 amino acids). The effectiveness of this system was demonstrated by expressing various fluorescent proteins in *Chlamydomonas* (Rasala et al. 2013). A significantly improved *ble2A* system, either using an

extended 2A peptide (39 amino acids) or combining it with an improved ultraviolet-mutant strain (UVM4), was reported to drive the high-level expression of dicistronic nuclear genes (Kong et al. 2015, Plucinak et al. 2015). However, the ability to use only one selectable marker limits the utility of the ble2A system.

Recently, Dong et al. (2017) developed the IFT25P-IFT25-2A^E system, which uses the *Chlamydomonas* endogenous intraflagellar transport 25 (*IFT25*) gene instead of *ble*. They showed that combining a more efficient endogenous gene that is expressed at high levels (e.g., *IFT25*) in-frame with the 2A peptide improves transgene expression levels (Dong et al. 2017). A viral 2A peptide-based toolbox has also been used in *Nannochloropsis salina* (Unkefer et al. 2017). A modified 2A peptide, P2A (60 amino acids), was found to be most efficient when placed in-frame with either the *ble* or *aph7*" marker for bicistronic expression in *Nannochloropsis oceanica* (Poliner et al. 2018). The viral 2A peptide system is a useful tool for transgene expression in *Chlamydomonas*; however, the self-cleavage efficiency of the 2A peptide varies based on the transgene used, and the mechanism by which the 2A system drives high expression of the transgene is unclear (Rasala et al. 2013, Kong et al. 2015).

Background strains

Mutants defective in gene silencing mechanisms are useful recipients for efficient transgene expression. Several *Chlamydomonas* mutant lines that show improved transgene expression levels have been generated (e.g., UVM4 and UVM11) (Neupert et al. 2009). These strains have been used as background strains for protein subcellular localization studies, as host strains for protein secretion, and for the production of terpene squalene (Kajikawa et al. 2015, Lauersen et al. 2013). However, these UVM strains are still subjected to transgene silencing, probably because the gene silencing mechanisms have not been completely knocked out (Kong et al. 2015, Kurniasih et al. 2016).

We previously found that the *met1* mutant, which is deficient in one of the key enzymes mediating epigenetic gene silencing, showed significantly improved expression levels of nuclear transgenes, similar to UVM strains (Kong et al. 2015). When the *met1* strain was further mutated by UV irradiation, we obtained *met1*(UVM)-47A and B mutants, which enable robust expression of nuclear transgenes regardless of the genomic position of the insertion, due to disruption of DNA methylation-dependent and -independent silencing systems. Moreover, *met1*(UVM) mutants were more capable of robustly expressing native,

fluorescent, or heterologous proteins than were other *Chlamydomonas* strains tested (Kurniasih et al. 2016).

Tools for targeted gene repression or disruption

In the case of essential genes, full disruption of the target gene results in cell lethality; therefore, RNAi-mediated gene repression is a more useful approach for assigning functions to these genes. RNAi approaches include expressing the antisense sequence or an inverted repeat of a gene targeted for knockdown (Schroda 2006). The Maa7/X-IR (NE-537) plasmid tool and web-based RNAi design software (e.g., THE RNAi WEB, <http://www.rnaiweb.com/>) facilitate the use of RNAi technology to modulate transgene expression in *Chlamydomonas* (Rohr et al. 2004). Despite their effectiveness, transgenes are often not stable in *Chlamydomonas*, and are subsequently silenced. For example, we and others have reported that RNAi silencing efficiency is reduced in *Chlamydomonas* after successive mitotic cell divisions (Schroda 2006, Yamasaki et al. 2008). Unstable RNAi effects, including off-target effects, limit applications of RNAi in genetic engineering of *Chlamydomonas*. To overcome this obstacle, a novel artificial microRNA (amiRNA) system based on ligation of DNA oligonucleotides was developed, because microRNA is normally not associated with transcriptional silencing (Molnar et al. 2009). To inactivate genes with higher accuracy and efficiency, CRISP (clustered regularly interspaced short palindromic repeats)-interference (CRISPi), which evolved from CRISPR/Cas9 (CRISPR associated nuclease 9) and uses the same type of guide RNA (gRNA), but nuclease-deficient Cas9 (Kao and Ng 2017), has recently been applied in *Chlamydomonas*.

Homologous recombination enables targeted gene integration, but under normal conditions, high-efficiency homologous recombination occurs only in a few algal species, such as *Nannochloropsis* sp. (Kilian et al. 2011). Although homologous recombination has been shown to occur during nuclear transformation in *Chlamydomonas* (Sodeinde and Kindle 1993), the frequency of homologous recombination is very low compared to that of random insertion (Zorin et al. 2005).

Site-specific nuclease-mediated genome editing technologies, such as the ZFN (zinc finger nuclease), TALEN (transcription activator-like effector nuclease), and CRISPR/Cas9 systems, are emerging as important alternative tools for gene knockout in microalgae. ZFN was initially used in a specially designed mutated *Chlamydomonas* strain, which is transformed by a non-functional *aphVIII* selectable marker interspaced with a short

channelrhodopsin gene (*COP3*). The nonfunctional *aphVIII* was restored through ZFN-mediated cleavage of *COP3*, when the cells were co-transformed with ZFNs and an *aphVIII* DNA template (Sizova et al. 2012). Recently, the same group optimized ZFN editing protocols for several *Chlamydomonas* strains (i.e., CC3403 and CC503), making ZFN-based editing a reliable and predictable tool for homologous recombination (Greiner et al. 2017). However, ZFN methodology is somewhat laborious, as a specific ZFN protein must be engineered for each target and the targeting efficiency and specificity are poor.

The TALEN system is composed of a variable number of four different DNA recognition domains, each specifically recognizing one of the four nucleotides. Gene activation induced by artificially designed TALE (dTALE) has been studied in multiple organisms including *Chlamydomonas*. For example, dTALE was used to target the promoters of periplasmic arylsulfatase (*ARS*) genes in *Chlamydomonas*, resulting in their overexpression at both the mRNA and protein levels (Gao et al. 2014). In another study, a putative inorganic carbon transporter (*HLA3*), proposed to be involved in inorganic carbon uptake, was overexpressed using dTALE in *Chlamydomonas* (Gao et al. 2015). Furthermore, TALE in the form of TALEN was also used to induce the targeted knockout of a gene involved in a carbohydrate storage pathway (e.g., the gene encoding UDP-glucose pyrophosphorylase) in *Phaeodactylum tricornutum*, resulting in a strain producing higher levels of TAG (Daboussi et al. 2014).

The CRISPR-Cas9 system, consisting of two key molecules, cas9 protein and gRNA, is a faster and more accurate genome-editing tool than ZFN and TALEN. Since its discovery in 2012, the CRISPR-Cas9 system has been widely applied to land plants (Langner et al. 2018). The first trial of the CRISPR-Cas9 system in microalgae was carried out in *Chlamydomonas*, but resulted in a low targeting efficiency, with no surviving transformants. This is probably due to the toxicity of constitutive Cas9 expression (Jiang et al. 2014). Cytotoxicity was avoided by directly delivering Cas9 protein-gRNA ribonucleoproteins (RNPs) assembled *in vitro* into *Chlamydomonas*. However, the gene targeting efficiency (targeted colonies/preselected colonies) was still low, ranging from 0.17% to 1.4% (Shin et al. 2016). To disrupt genes in a cost-effective, routine manner, Greiner et al. (2017) optimized CRISPR/Cas9 protocols, improving the targeting efficiency (ranging from 5% to 15%). Using this modified method, the team disrupted eight photoreceptor genes in four different *Chlamydomonas* strains (Greiner et al. 2017). Delivering another pre-assembled RNP, *Lachnospiraceae* Cpf1 (an ortholog of Cas9), along with single-stranded DNA repair

templates, resulted in efficient editing and replacement of the targeted DNA at frequencies of as high as 10% in *Chlamydomonas*. Moreover, this method allows for transgene-free generation of sequence-specific mutations and for epitope tagging of endogenous genes (Ferenczi et al. 2017). Some examples of nuclease-directed genome editing in *Chlamydomonas* are provided in **Table 1**.

Plastidial genome engineering

The chloroplast genome of *Chlamydomonas* is a primary target in efforts to produce useful recombinant proteins, predominantly because it can be readily engineered via homologous recombination and lacks transgene silencing (Rasala and Mayfield 2011). Robust heterologous protein accumulation ($\geq 5\%$ total soluble protein) by *Chlamydomonas* chloroplasts was achieved by using the endogenous *psbA* promoter and the corresponding 5' UTR to drive expression of the target gene; however, the endogenous *psbA* gene had to be removed, rendering the cells non-photosynthetic (Minai et al. 2006, Rasala and Mayfield 2011). To address this problem, the 16S rRNA-*atpA* UTR hybrid promoter and synthetic regulatory elements (i.e., UTRs) have been developed to efficiently drive transgene expression in chloroplasts without having to remove endogenous *psbA* (Rasala et al. 2011, Specht and Mayfield 2013). Cleavable fusion of the target gene to the C-terminal of *rbcL* also increased foreign protein accumulation. Using this approach, the luciferase activity was increased up to 5-fold in the wild type and 33-fold in the *rbcL* knock-out background (Muto et al. 2009).

Modification of the N-terminus of a transgene can also enhance foreign protein accumulation in algae. Recently, fusion of the downstream box (the first 15 codons following the start codon) of the *TetC* gene to the N-terminus of a transgene was shown to enhance foreign protein accumulation in *Chlamydomonas* chloroplasts. Its effect is probably related to translation or to other post-transcriptional processes (Richter et al. 2018). Two intercistronic expression elements from *psbN-psbH* and *tscA-chlN* have recently been identified (Macedo-Osorio et al. 2018) that enable the expression of foreign genes (i.e., *aphA-6* and *gfp*) in *Chlamydomonas* chloroplasts in a synthetic operon-like manner. This approach could be useful for stacking genes for metabolic engineering or synthetic biology circuits in *Chlamydomonas* chloroplasts (Macedo-Osorio et al. 2018). However, chloroplasts are deficient in post-translational modifications, such as glycosylation of proteins, which limits chloroplasts as a robust platform for protein production.

In addition to being a suitable expression platform for efficient protein production, plastids can be used to produce lipids in *Chlamydomonas*. For example, plastid engineering through overexpressing plastid-localized 2-lysophosphatidic acid acyltransferase 1 (*CrLPAAT1*) (Yamaoka et al. 2016) can increase oil content in *Chlamydomonas*, as discussed below.

Examples of genetic approaches for increasing oil content in *Chlamydomonas*

Over the past decade, significant advances in metabolic engineering have been made to modify microalgal genomes to increase oil content. For instance, the genetic engineering tools detailed above have been used to modify lipid metabolism in *Chlamydomonas* through the following main approaches (**Figure 1**): (1) Enhancing TAG biosynthesis; (2) Increasing acetyl-CoA supply; (3) Manipulating transcription factors/regulators; (4) Blocking competing pathways; (5) Reducing lipid degradation; and (6) Modulating redox state. Some examples of engineering attempts are described below.

Enhancing TAG biosynthesis

Glycerolipids are one of the most abundant lipid classes in microalgae. Their assembly starts with the stepwise acylation of glycerol 3-phosphate (G3P) to phosphatidic acid (PA) using fatty acyl donors. The 2-lysophosphatidic acid acyltransferase (LPAAT) catalyzes the acylation at the *sn*-2 position of 2-lysophosphatidic acid to produce PA, the first common precursor of membrane and storage lipids. We reported that *Chlamydomonas* encodes a plastid-targeted LPAAT (*CrLPAAT1*) and that overexpression of *CrLPAAT1* in plastids led to a more than 20% increase in oil content under nitrogen (N)-deficient conditions (Yamaoka et al. 2016). Likewise, transgenic *Chlamydomonas* strains overexpressing *CrLPAAT2* showed greater accumulation of TAG than the parental strain (Kim et al. 2018). By contrast, RNAi-mediated suppression of *CrLPAAT2* reduced the TAG content of the RNAi lines under N deprivation (Kim et al. 2018).

G3P dehydrogenase (GPDH) catalyzes the reversible redox conversion of dihydroxyacetone phosphate to G3P. Recently, the codon-optimized *LPAAT* gene from *Brassica napus* and *GPDH1* from *Saccharomyces cerevisiae* were heterologously expressed in *Chlamydomonas* driven by the *Hsp70A-RbcS2* chimeric promoter, respectively. The *LPAAT* and *GPDH1* transgenic lines showed a 44.5% and 67.5% increase in lipid contents, respectively, after triple heat-shock treatments (Wang et al. 2018). These results are consistent

with those of an earlier transcriptome analysis in which *GPDH* and *LPAAT* were found to be significantly upregulated when high levels of lipids accumulated in *Chlamydomonas* (Lv et al. 2013).

Phospholipid:diacylglycerol acyltransferase (PDAT), an acyl-CoA-independent acyltransferase, catalyzes the transfer of an acyl group from the *sn*-2 position of a phospholipid to a diacylglycerol to make TAG. This PDAT pathway is considered to be another important source of TAG biosynthesis from DAG, in addition to the acyl-CoA-dependent pathway catalyzed by diacylglycerol acyltransferase (DGAT) (Li-Beisson et al. 2015). Heterologous expression of *Saccharomyces cerevisiae* PDAT in *Chlamydomonas* increased the contents of total fatty acids (FAs) and TAG by 22% and 32%, respectively, under N-replete conditions (Zhu et al. 2018).

The FAs used for TAG biosynthesis can also be derived from recycled membrane lipids (i.e., monogalactosyldiacylglycerol [MGDG] and digalactosyldiacylglycerol [DGDG]). It was reported that PGD1 (PLASTID GALACTOGLYCEROLIPID DEGRADATION 1) from *Chlamydomonas* can hydrolyze MGDG to produce free FAs and lyso-MGDG *in vitro*, and the *PGD1* knockout mutant had lower levels of TAG than the wild type following N deprivation (Li et al. 2012a).

Furthermore, perturbing lipid trafficking or altering membrane lipid composition has been shown to result in TAG over-accumulation. For example, in *tg2* (*trigalactosyldiacylglycerol*) mutants, which are defective in lipid trafficking, excess levels of MGDG are produced that are postulated to contribute to additional acyl-groups for TAG synthesis (Warakanont et al. 2015). Additionally, the *Chlamydomonas fdx5* mutant, which has a defect in the *ferredoxin5* gene, was shown to have changes in thylakoid membrane ultrastructure and lipid profiles in darkness (e.g., the ratio of MGDG to DGDG strongly declined), resulting in strains that hyper-accumulate TAG (Yang et al. 2015).

Increasing acetyl-CoA supply

Acetyl-CoA is an essential carbon precursor for *de novo* FA biosynthesis; therefore, increasing the acetyl-CoA supply by metabolic engineering could improve oil content. Phosphoenolpyruvate carboxylase (PEPC) is a key enzyme belonging to the family of decarboxylases in plants and microalgae. PEPC controls the carbon flux that enters the tricarboxylic acid (TCA) cycle, and plays an important role in carbon partitioning of substrates in competition with lipid synthesis (Deng et al. 2014, Izui et al. 2004). In

Chlamydomonas, inhibiting PEPC1 activity by RNAi or CRISPRi-based techniques increased the oil content by 20%, probably by enhancing the flow of carbon to FA synthesis (Deng et al. 2014, Kao and Ng 2017). Down-regulating both PEPC isoforms (*CrPEPC1* and *CrPEPC2*) by amiRNA resulted in a 29% to 48% increase in total FA levels, respectively, which was suggested to occur due to the flux of carbon away from the TCA cycle, and the subsequent increase in the availability of acetyl-CoA for FA biosynthesis (Wang et al. 2017). Consistent with this hypothesis, the expression levels of TAG biosynthesis-related genes, such as *DGAT2* and phosphatidate phosphatase (*PAP2*), were increased in *CrPEPC1* RNAi strains (Deng et al. 2014).

Manipulating transcription factors/regulators

Manipulating the expression of transcription factor (TF) genes usually affects multiple steps/genes of the same pathway, thereby upregulating the entire pathway rather than a specific enzymatic step. TF engineering is a promising strategy, because it can circumvent the secondary bottlenecks commonly observed when only a single gene is targeted (Courchesne et al. 2009). TFs showing positive or negative correlations with lipid biosynthetic genes at the RNA expression level could be potential targets for engineering of oil content (Hu et al. 2014). Dof-binding sequences are abundant in the promoter regions of many genes related to lipid biosynthesis (Wang et al. 2006), indicating that Dof genes might contribute to lipid biosynthesis by upregulating genes associated with FA biosynthesis. Indeed, a study in higher plants showed that overexpression of Dof-type TF genes (*GmDof1* and *GmDof14*) from soybean (*Glycine max*) enhanced TAG accumulation in the seeds of transgenic *Arabidopsis* plants (Wang et al. 2007). A codon-optimized soybean DOF-type TF gene (GenBank ID: DQ857261.1) was subsequently heterologously expressed in *Chlamydomonas*, and the resulting transgenic lines had an up to 2-fold increase in lipid content (Ibáñez-Salazar et al. 2014). Ngan et al. (2015) identified another TF named PSR1 (PHOSPHORUS STRESS RESPONSE 1), which triggers cytosolic lipid accumulation, as a pivotal switch in carbon storage metabolism in *Chlamydomonas*. Overexpression of *PSR1* increased TAG accumulation 2-fold (on a dry weight basis) in cells grown under normal culture conditions without growth inhibition (Ngan et al. 2015).

We recently isolated a *Chlamydomonas* mutant (*starch degradation 1, std1*) deficient in a dual-specificity tyrosinephosphorylation-regulated kinase (DYRK) (Schulz-Raffelt et al. 2016). The *std1* mutant accumulates higher levels of oil than the wild type under

photoautotrophic N starvation conditions. By contrast, another DYRK, named TAR1 (TAG ACCUMULATION REGULATOR 1), was found to positively regulate TAG accumulation under mixotrophic N and S deficiency, and the *tar1-1* mutant accumulated lower levels of TAG than did the wild-type strain (Kajikawa et al. 2015b). The effect on oil content has not yet been evaluated for *tar1* under photoautotrophic conditions. This plant-specific DYRK kinase (DYRKP), which has been proposed to act as a positive or negative regulator of sink capacity and thereby integrate nutrient and energy signals (Schulz-Raffelt et al. 2016), represents a promising target for improving energy density in microalgae.

Additionally, VIP1, an inositol hexakisphosphate/diphosphoinositol phosphate kinase involved in phosphatidylinositol signaling, has recently been reported to show synergism with target of rapamycin kinase signaling, which governs carbon metabolism and lipid accumulation in *Chlamydomonas*. The null *vip1-1* mutant exhibits increased levels of TAG and a higher content of lipid droplets in both N-replete and -deplete conditions (Couso et al. 2016). A comprehensive understanding of the underlying mechanism by which TFs regulate lipid alteration and the discovery of TFs regulating algal lipid metabolism will facilitate targeted engineering strategies to improve lipid production in microalgae.

Blocking competing pathways

Disrupting competing metabolic pathways of lipid biosynthesis has been routinely used to enhance lipid productivity. In addition to TAG, starch is another primary carbon sink in green microalgae. Previous work showed that knocking-out starch synthesis could increase oil content (Li et al. 2010, Work et al. 2010); however, this does not always seem to be the case (Siaut et al. 2011, Vonlanthen et al. 2015), and varied depending on the background strains and cultivation conditions. The relationship between starch synthesis and TAG accumulation merits further investigation.

Reducing lipid degradation

Cellular lipid content is the end result of an equilibrium between synthesis and degradation. Therefore, lipid hydrolytic enzymes (i.e., lipases) are also potential targets for engineering efforts to increase TAG content. In *Chlamydomonas*, the lipase CrLIP1 was found to be a DAG lipase with broad substrate specificity. Silencing of *CrLIP1* resulted in an increase in oil content following N replenishment upon N starvation due to the arrest of TAG hydrolysis (Li et al. 2012). TAG degradation is also directly linked to the cell cycle in *Chlamydomonas*. The

insertional null mutant *cht7* (compromised hydrolysis of triacylglycerols 7) is severely impaired in TAG degradation, and the TAG level in this mutant remains 10-fold higher than that of wild-type cells upon N resupply after N starvation, due to a defect in exiting quiescence (Tsai et al. 2014).

Recently, we reported that functional FA β -oxidation is involved in lipid homeostasis during nutrient stress in *Chlamydomonas*. In the insertional knockout mutants for ACX2 (ACYL-COA OXIDASE 2), a key enzyme in peroxisomal FA β -oxidation, oil remobilization is severely blocked (by 60–80%) upon N resupply after N starvation and oil content is increased by 20% during N starvation (Kong et al. 2017). In *Chlamydomonas*, with the exception of isocitrate lyase (ICL), all other enzymes of the glyoxylate cycle are localized in peroxisomes (Lauersen et al. 2016b). The null *icl* mutant overaccumulated total FAs when cultivated mixotrophically, presumably due to a reduction in the β -oxidation of FAs (Plancke et al. 2014). Overall, reduced lipid turnover may enhance the net oil content; therefore, identifying and engineering genes involved in lipid degradation is a promising avenue toward increasing oil content in microalgae.

Modulating redox state

The FA synthase complex is activated by NADPH (Rawsthorne 2002), and a positive link between the level of NADPH and FA content has been established in microalgae (Osada et al. 2017, Xue et al. 2015). Malic enzyme (ME) catalyzes the conversion of malate to pyruvate and simultaneously reduces NADP⁺ to NADPH. The total lipid content and the neutral lipid content were increased 2.5-fold and 3.2-fold via transgenic overexpression of *PtME* in *Phaeodactylum tricornutum* and *Chlorella pyrenoidosa*, respectively (Xue et al. 2015, 2016). Recently, the enzyme glucose-6-phosphate dehydrogenase (G6PD), which is involved in the formation of NADPH in the pentose phosphate pathway, was reported to contribute to lipid synthesis in the oleaginous diatoms *Phaeodactylum tricornutum* and *Fistulifera solaris*. Overexpression of *G6PD* elevated lipid productivity 2.7-fold in *Phaeodactylum tricornutum* during the stationary phase, and 1.5-fold in *Fistulifera* under N depletion (Osada et al. 2017, Xue et al. 2017).

Malate dehydrogenase (MDH) catalyzes the reversible conversion of NAD(P)H to NAD(P)⁺ via the reduction of oxaloacetate to malate (Mettler and Beevers 1980). Recently, we isolated and characterized a *Chlamydomonas* mutant deficient in the peroxisomal NAD⁺-dependent *MDH2*. In its absence, *mdh2* mutant cells are compromised in TAG breakdown and

showed an increased capacity to make NAD(P)H, resulting in a >50% increase in storage lipids and a 200% increase in starch during photoautotrophic N deprivation (Kong et al. 2018b). Enhancing the NADPH supply using metabolic engineering techniques seems to be a promising strategy for boosting lipid accumulation in microalgae.

Conclusion and prospectives

Here we reviewed the essential techniques for improving the oil content of *Chlamydomonas*, from transformation methodologies to genome engineering strategies. The state-of-the-art CRISPR-Cas9 system shows great potential for precise genetic engineering, and has been successfully used in *Chlamydomonas*, diatoms, and *Nannochloropsis*. The continued progress of synthetic biology in *Chlamydomonas* will facilitate the development of the microalgal biotechnology sector.

We also described cases in which genetic tools were successfully used to increase oil content in *Chlamydomonas*. It is apparent that microalgae that accumulate oils have acquired not only divergent enzymes that produce these FAs, but also a large number of enzymes (e.g., acyltransferases) that can effectively channel these FAs into oil. Understanding and manipulating the regulatory processes of lipid metabolism may turn out to be even more effective than manipulating specific enzymes. Thus, boosting oil accumulation in microalgae may require a combination of strategies involving both genetic engineering tools and synthetic biology approaches employing many genes. A breakthrough in this technology is hopefully not far off.

Disclosures

The authors have no conflicts of interest to declare.

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Table 1. Summary of current uses of nuclease-directed genome editing in Chlamydomonas.

Editing method	Chlamydomonas species	Targeted gene/Marker gene	Transformation method	Comment	Reference
ZNF	CC4350	<i>COP3/aphVIII</i>	Glass beads	The first application of ZNF to knockout gene in Chlamydomonas	Sizova et al. (2012)
	CC3403, CC503	<i>COP3/ARG7, aphVIII</i>	Electroporation	ZNF mediated-targeting of photoreceptor genes	Greiner et al. (2017)
TALEN	CC849	<i>ARS1, ARS2/ble</i>	Electroporation	The first success case of TALEN mediated-gene expression activation in Chlamydomonas	Gao et al. (2014)
	CC849	<i>HLA3/ble</i>	Electroporation	TALE mediated gene activation	Gao et al. (2015)
CRISPR/Cas9	CC503	<i>FKB12/---</i>	Electroporation	First trail of CRISPR/Cas9 system in Chlamydomonas, but lack of surviving transformants	Jiang et al. (2014)
	CC124	<i>MAA7, CpSRP43, ChlM /aph7"</i>	Electroporation	Succeeded in obtaining stable Chlamydomonas transformants by delivering preassembled Cas9-gRNA RNPs	Shin et al. (2016)
	CC4349	<i>ZEP, CpFTSY/---</i>	Electroporation	Generation of two-gene knockout mutant by delivering Cas9-gRNA RNPs	Baek et al. (2016)
	CC3403	<i>COP1/2, COP5, PHOT, PSY1/aphVIII</i>	Electroporation	Targetting photoreceptor genes in Chlamydomonas via CRISPR/Cas9	Greiner et al. (2017)
	CC3403, CC125, SAG73.72	<i>aCRY, COP1/2, COP5, MAT3, PHOT, POLQ, KU80, UVR8, VGCC/ARG7, aph7", aphVIII</i>	Electroporation	Targetting photoreceptor genes by delivering exogenously supplied recombinant Cas9-gRNA RNPs	Greiner et al. (2017)
	CC1883	<i>FKB12, CpFTSY, CpSRP43, PHT7/---</i>	Electroporation	The first CRISPR/Cpf1 RNPs-mediated efficient genome editing in Chlamydomonas	Ferenczi et al. (2017)
	CC4349	<i>LTD/---</i>	Electroporation	Photosynthesis gene	Jeong et

			targeting by delivering Cas9-gRNA RNPs	al. (2018)
CC503, CC4530	<i>FKB12</i> , <i>ALS</i> , <i>ARG7-8/---</i>	Electroporation	The Cas9/intron-sgRNA hybrid construct (i.e., gene-within-a-gene) enables gene editing in Chlamydomonas	Jiang and Weeks. (2018)
CC4349	<i>PLA2/aph7"</i>	Electroporation	The first success case of increased overall lipid productivity from Chlamydomonas mutant generated by CRISPR/Cas9 RNPs	Shin et al. (2019)

Abbreviations:

aCRY, animal-like cryptochrome; *aph7"*, hygromycin B resistance gene; *aphVIII*, aminoglycoside 3'-phosphotransferase (paromomycin resistance gene); *ALS*, acetolactate synthase; *ARG*, argininosuccinate lyase; *ARS*, arylsulfatase; *ble*, bleomycin resistance gene; CRISPR/Cas9, clustered regularly interspersed palindromic repeats associated protein 9; *COP*, light-gated proton channel rhodopsin; *CpSRP*, chloroplast signal recognition particle; *ChlM*, Mg-protoporphyrin IX S-adenosyl methionine O-methyl transferase; *CpFTSY*, signal recognition particle receptor; *FKB12*, peptidyl-prolyl cis–trans isomerase; *HLA3* (also known as *MRP1*), a putative inorganic carbon transporter; *KU80*, ATP-dependent DNA helicase 2 subunit 2; *LTD*, light-harvesting chlorophyll- and carotenoid-binding proteins translocation defect gene; *MAT3*, retinoblastoma; *MAA7*, beta subunit of tryptophan synthase; *PSYI*, PHYTOENE SYNTHASE 1 ; *PLA2*, phospholipase A2; *POLQ*, DNA polymerase theta; *PHOT*, photoreceptor phototropin; *PHT7*, a putative phosphate transporter; TALEN, transcription activator-like effector nuclease; *UVR8*, a homomultimeric UV-B photoreceptor; *VGCC*, voltage-gated calcium channel; *ZEP*, zeaxanthin epoxidase, ZNF, zinc finger nuclease. “---” indicates selectable marker gene was not used.

Legends to figure

Figure 1. The main approaches of genetic engineering of *Chlamydomonas* for altered oil content.

Abbreviations: amiRNAi, artificial microRNA interference; CRISPRi, clustered regularly interspaced short palindromic repeats interference; *cht7*, compromised hydrolysis of triacylglycerols 7; *CrPEPC1/2*, *Chlamydomonas* phosphoenolpyruvate carboxylase1/2; *CrLPAAT1* and 2, *Chlamydomonas* lysophosphatidic acid acyltransferase 1 and 2; *CrLIP1*, *Chlamydomonas* lipase 1; *cracx2*, *Chlamydomonas* acyl-CoA oxidase 2; *DOFs*, Dof-type transcription factor genes; ER, endoplasmic reticulum; FA, fatty acid; *fdx5*, ferredoxin5; *icl*, isocitrate lyase; *lcs2*, long-chain acyl-CoA synthetase; LD, lipid droplet; *mldp*, major lipid droplet protein; *mdh2*, malate dehydrogenase 2; *nrr1*, nitrogen-responsive regulator 1; OE, over-expressing strains; *PSR1*, phosphorus stress response 1; *pgd1*, plastid galactoglycerolipid degradation 1; *pdat1*, phospholipid:diacylglycerol acyltransferase 1; RNAi, RNA interference; *std1*, starch degradation 1; *sta6* and 7; starch-less mutant 6 and 7; TAG, triacylglycerol; *tgd2*, trigalactosyldiacylglycerol 2; *tar1*, triacylglycerol accumulation regulator 1; *vip1*, a predicted diphosphoinositol pentakisphosphate kinase.

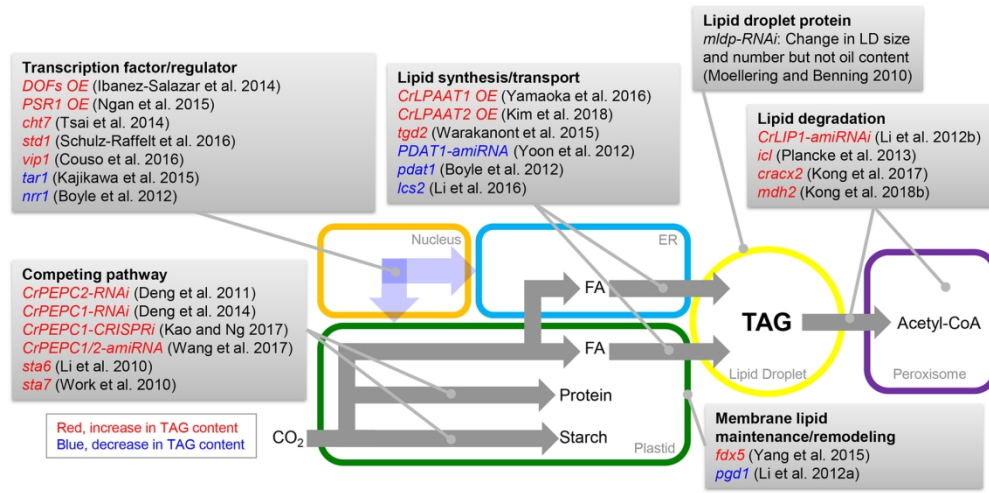


Figure 1. The main approaches of genetic engineering of *Chlamydomonas* for altered oil content.

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