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Research review paper

Genetic engineering of microalgae for enhanced lipid production

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ABSTRACT

Microalgae have the potential to become microbial cell factories for lipid production. Their ability to convert sunlight and CO_2 into valuable lipid compounds has attracted interest from cosmetic, biofuel, food and feed industries.

In order to make microalgae-derived products cost-effective and commercially competitive, enhanced growth rates and lipid productivities are needed, which require optimization of cultivation systems and strain improvement. Advances in genetic tool development and omics technologies have increased our understanding of lipid metabolism, which has opened up possibilities for targeted metabolic engineering. In this review we provide a comprehensive overview on the developments made to genetically engineer microalgal strains over the last 30 years. We focus on the strategies that lead to an increased lipid content and altered fatty acid profile. These include the genetic engineering of the fatty acid synthesis pathway, Kennedy pathway, polyunsaturated fatty acid and triacylglycerol metabolisms and fatty acid catabolism. Moreover, genetic engineering of specific transcription factors, NADPH generation and central carbon metabolism, which lead to increase of lipid accumulation are also reviewed.

Abbreviations: ACBP, acyl-CoA-binding protein; ACC, acetyl-CoA carboxylase; ACP, acyl carrier protein; ACS, acetyl-CoA synthetase; ALA, α-linolenic acid; ALE, adaptive laboratory evolution; AP2-type TF, Apetala 2-type transcription factor; ARA, arachidonic acid; ATMT, Agrobacterium tumefaciens-mediated transformation; ATPase, ATP synthase; BCR, breakpoint cluster region protein; BCR1, biotin carboxylase; bHLH, basic helix-loop-helix; bZIP, basic leucine zipper; CAO, chlorophyllide a oxygenase; CBB cycle, Calvin-Benson- Bassham cycle; CBP-like protein SN03, SN = chitin binding protein-like protein stress-nitrogen 03; CHT7, Compromised Hydrolysis of TAG 7 (protein); DAG, diacylglycerol; DAGK, diacylglycerol kinase; DGAT, diacylglycerol acyltransferase; DGTS, diacylglycerol-trimethylhomoserine; DGTT1, type 2 diacylglycerol acyltransferase; DHA, docosahexaenoic acid; DHAP, dihydroxyacetone phosphate; DOF-type TF, DNA-binding with one finger type transcription factor; DPA, docosapentaenoic acid; ELO, elongase; EMS, ethyl methanesulfonate; ENR, enoyl-ACP reductase; EPA, eicosapentaenoic acid; F2BP, fructose 2,6 bisphosphatase; FA, fatty acids; FAD, fatty acid desaturase; FAS, fatty acid synthesis; FAT1, acyl carrier protein thioesterase; FNR, ferredoxin NADP+ oxidoreductase; G3P, glycerol 3-phosphate; G3PDH, glyceraldehyde 3-phosphate dehydrogenase; G6P, glucose 6-phosphate; G6PD, glucose 6-phosphate dehydrogenase; gdcw, gram dry cell weight; GK, glycerol kinase; GPAT, glycerol 3-phosphate acyltransferase; GPAT2, glycerol-3-phosphate acyltransferase 2; HD, 3-hydroxyacyl-ACP dehydrase; HE, heterologous expression; HES, heterologous expression strain; IDH, isocitrate dehydrogenase; KAR, 3-ketoacyl-ACP reductase; KAS, 3-ketoacyl-ACP synthase; KD, knockdown; KDS, knockdown strain; KO, knockout; KOS, knockout strain; LA, linoleic acid; LC-FACS, long-chain fatty acyl-CoA synthetase; LC-PUFA, long-chain polyunsaturated fatty acid; LD, lipid droplets; LPAT and LPAAT, lysophophatidic acid acyltransferase; MAGL, monoacylglycerol; MAT, malonyl-CoA ACP transacylase; MAT, malonyl-CoA: ACP transacylase; MCFA, medium-chain fatty acid; ME, malic enzyme; MGDG, monogalactosyldiacylglycerol; MUFA, monounsaturated fatty acid; NTG, N′-nitro-N-nitrosoguanidine; OA, oleic acid; OE, overexpression; OES, overexpression strain; oxPPP, oxidative pentose phosphate pathway; PA, phosphatidic acid; PAP, phosphatidic acid phosphatase; PC, phosphatidylcholine; PDAT, phospholipid diacylglycerol acyl transferase; PE, phosphatidylethanolamine; PFK2, 6-phosphofructo-2-kinase; PI, phosphatidylinositol; PNPLA3, Patatin-like phospholipase domain-containing protein 3; PPDK, pyruvate phosphate dikinase; PS, parental strain; PSI, photosystem I; PSII, photosystem II; PSR1, phosphorus starvation response 1 (protein); PUFA, polyunsaturated fatty acids; RNAi, RNA interference; ROS, reactive oxygen species; SFA, saturated fatty acid; SOD1, superoxide dismutase; SQD1, UDP-sulfoquinovose synthase; StLDP, Stramenopile lineage-specific lipid droplet protein; TAG, triacylglycerol; TAGL, triacylglycerol lipase; TE, thioesterase; TF, transcription factor; TFA, total fatty acids; UGDH, UDP-glucose 6-dehydrogenase; WT, wild-type.

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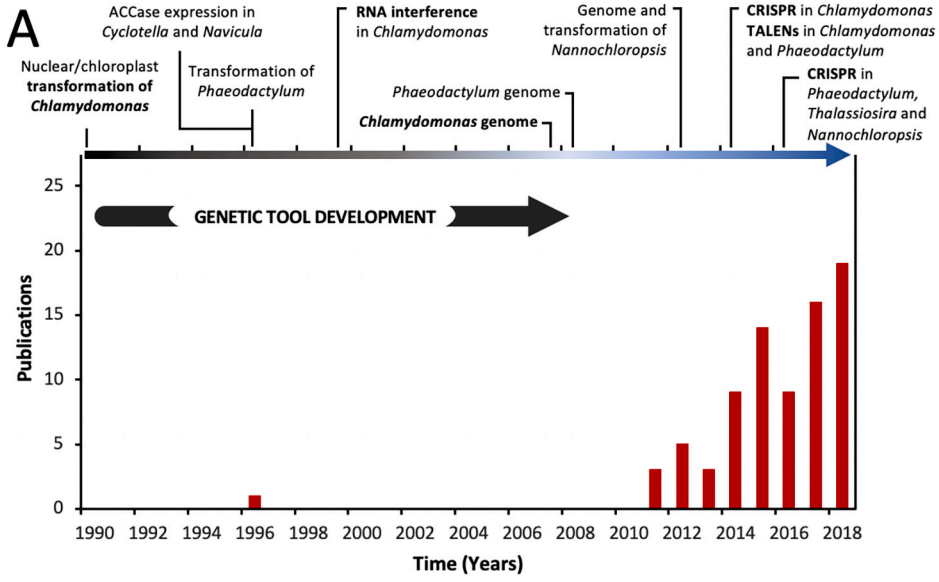
1. Introduction

Microalgae are a large polyphyletic group of unicellular eukaryotic photosynthetic microorganisms that are considered as promising platforms for sustainable production of bioproducts due to their ability to convert solar energy and carbon dioxide into organic compounds of commercial interest. Their role in carbon-sequestration and their ability to grow on freshwater, seawater or wastewater at high growth rate present the prospect to mitigate environmental issues and to reduce greenhouse gas emissions. Moreover, microalgae cultivation does not compete with agricultural food production, as it does not require arable land. The vast range of bioactive compounds naturally present in microalgal biomass, including antioxidants, carotenoids, proteins, polysaccharides, polyunsaturated fatty acids (PUFAs), triacylglycerols (TAGs), sterols and vitamins has attracted the attention of biotechnological, pharmaceutical, cosmetic, biofuel, food and feed industries (Chisti, 2007; Christaki et al., 2011; Draaisma et al., 2013; Pulz and Gross, 2004).

Therefore, microalgae have become an important subject of study in the last 50 years. During 1978–1996 the U.S Department of Energy's Aquatic Species Program analyzed over 3000 microalgae species for their potential use for biofuel production (Sheehan et al., 1998). Extensive research and development were conducted to identify species with desirable traits including high lipid content and productivity, competitiveness in outdoor cultivation and tolerance to fluctuations in temperature and salinity. Furthermore, evaluation and optimization of large-scale cultivation systems, harvesting, extraction and refinery techniques were performed. Despite the 20-year research period, the Aquatic Species Program and Biofuels Program did not yield any strain with suitable characteristics for cost-effective large-scale lipid production. Nevertheless, bioprospecting continues and researchers are characterizing new microalgae species in terms of growth and lipid composition (Barkia et al., 2019; Breuer et al., 2012; Duong et al., 2015; Ferreira et al., 2019; Gim et al., 2014; Lim et al., 2012).

Oleaginous microalgal species belonging to genera *Acutodesmus*, *Phaeodactylum*, *Dunaliella* and *Nannochloropsis* have attracted interest in the last two decades (Fu et al., 2019). When exposed to nutrient limitation or starvation, these species can accumulate neutral lipids in the form of TAGs, which can be converted into renewable fuels of third generation via transesterification (Chisti, 2007; Wijffels and Barbosa,



B

Genetic modification	Publications
Kennedy pathway	31
Transcription factors	12
NADPH generation	12
PUFAS metabolism	10
FAS metabolism	14
Central carbon metabolism	3
Others	5
Total	**87**

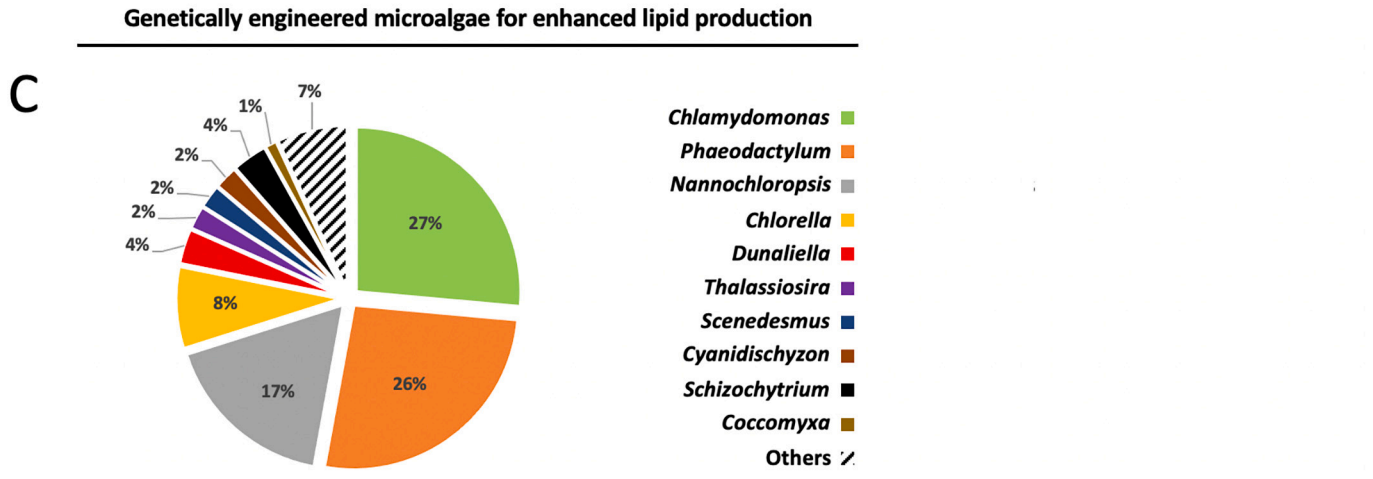


Fig. 1. Overview of genetic engineering studies for enhancing lipid production in microalgae. (A) Timeline of genetic tool development (top x-axis) and number of publications describing targeted genetic engineering of microalgae to enhance lipid production (bottom x-axis) (Apt et al., 1996; Bowler et al., 2008; Daboussi et al., 2014; Dunahay et al., 1996; Gao et al., 2014; Hopes et al., 2016; Jiang et al., 2014; Kindle, 1990; Kindle et al., 1989; Merchant et al., 2007; Nymark et al., 2016; Radakovits et al., 2012; Schroda et al., 1999; Wang et al., 2016). (B) Publications presented in this review. (C) Frequency of engineered microalga species.

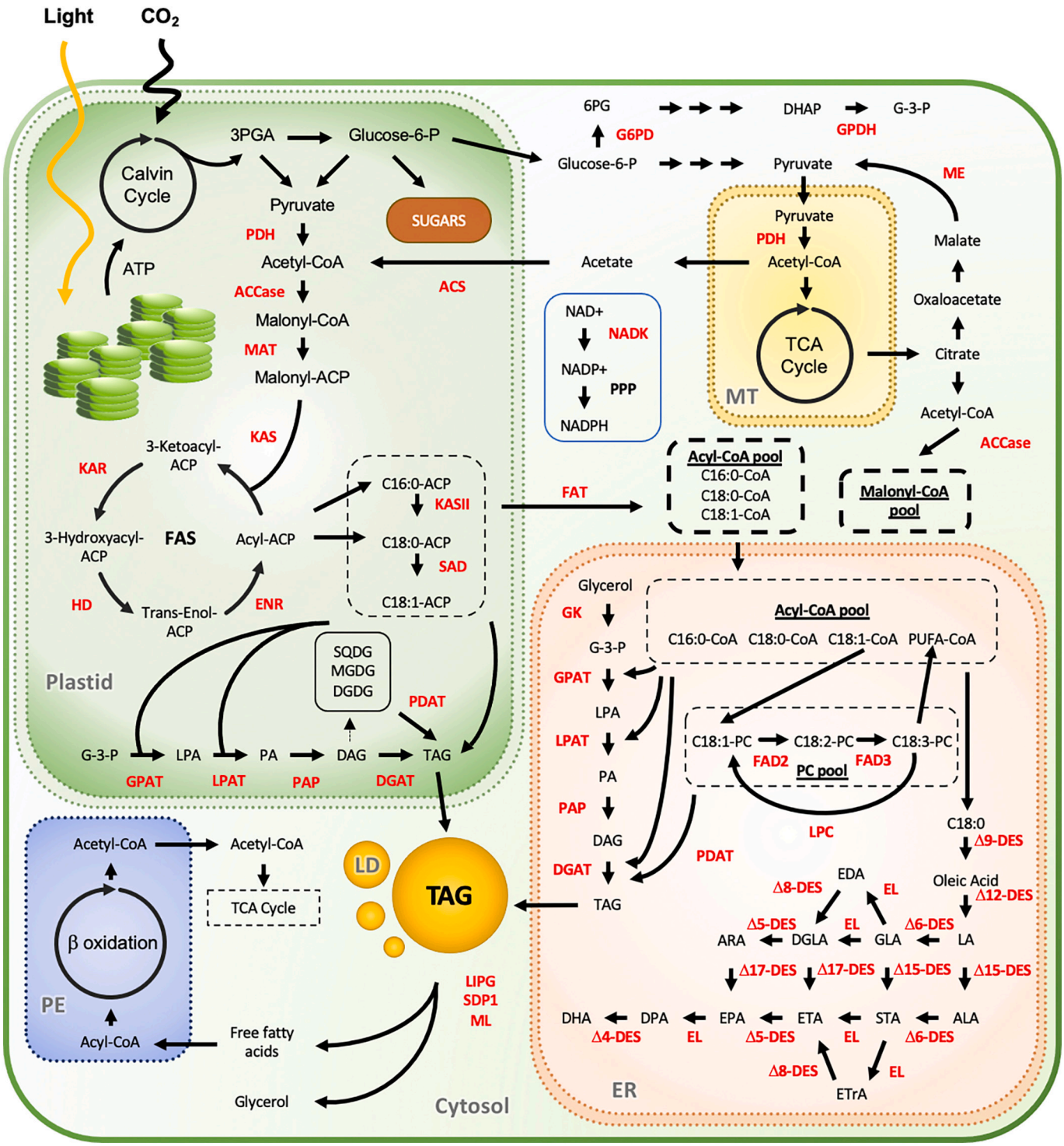


Fig. 2. Microalgal lipid biosynthesis pathways. Cellular organelles: plastid; ER, endoplasmic reticulum; PE, peroxisome; MT, mitochondria; LD, lipid droplets.

2010; Aratboni et al., 2019). Microalgal lipids are, furthermore, receiving growing interest from the food industry as an edible plant and fish oil substitute and as an alternative source of PUFAs such as omega-3 and omega-6 fatty acids, which provide health benefits when used as dietary supplement (Ghiffary et al., 2019).

Despite the industrial relevance and environmental benefits of microalgae compared to land plants, commercial-scale production of microalgae-derived commodity chemicals is not yet economically feasible due to high production costs. Recent studies have estimated that the production of one kilogram of biomass containing 24% of TAG in a 100 ha facility located in the south of Spain would cost 3.4 € kg^{-1} of biomass using flat panel photobioreactors (Ruiz et al., 2016). This study indicates the potential of implementing microalgae as efficient microbial cell factories for lipid production at large scale, using state-of-the-art cultivation systems. Improving microalgal strains could contribute to cost reduction and bring industrial production of microalgal lipids within reach. Remmers et al. (2018) have determined that the maximum theoretical lipid yield is about five times higher than what is currently achieved in outdoor settings, clearly indicating room for improvement (Benvenuti et al., 2016; Remmers et al., 2018; Ruiz et al., 2016).

Advances in research fields such as synthetic biology and genetic engineering can complement current efforts to achieve an economically feasible process.

Strain improvement via direct or indirect genetic modification has been proposed as a methodology to enhance growth and lipid productivities in promising microalgal strains (Radakovits et al., 2010). Wild type microalgal strains accumulate lipids mainly under nutrient-limited conditions, which limits biomass productivities. Genetic engineering is a potential strategy to generate strains that accumulate lipids without growth impairment. This review provides an overview of genetic engineering strategies implemented in microalgae for improving the lipid content and fatty acid profile over the last 30 years.

2. Genetic tool development and metabolic approach

In the last decades, considerable progress has been made in the development of genetic tools and generation of genetically improved strains (Korkhovoy et al., 2016; Kumari et al., 2015; Lin et al., 2019). Genetic modification has been achieved by adaptive laboratory evolution (ALE), random mutagenesis and direct genetic engineering. ALE and physical mutagens such as UV light, gamma radiation and X-rays as well as chemical mutagens such as N′-nitro-N-nitrosoguanidine (NTG) and ethyl methanesulfonate (EMS) have been successfully applied on microalgae for introduction of random mutations. However, efficient genetic engineering strategies that could generate specific insertions, deletions or substitutions in the host genome are required to make targeted modifications while avoiding unpredictable results. Advances in sequencing technology, development of fast, accurate and efficient DNA delivery systems and development of high throughput genome editing tools have become a crucial component for the generation of genetically improved microalgal strains.

The first major breakthrough in microalgal biotechnology was the nuclear transformation of *Chlamydomonas reinhardtii* in 1990 (Fig. 1). Kindle (1990) performed agitation of cells in the presence of glass beads coated with DNA which allowed the creation of micro-pores in the cell membrane due to friction, causing the passage of DNA molecules into the cell. Although this development was made three decades ago, the DNA delivery into microalgal cells is still a major bottleneck due to the variation in cell sizes, cell wall structures and composition among the several genera of microalgae. Several transformation methods have been developed for the delivery of exogenous DNA into different microalgal species. The most common and successful techniques are electroporation, *Agrobacterium tumefaciens*-mediated transformation, particle bombardment and agitation with glass beads (Apt et al., 1996; Chow and Tung, 1999; Dunahay, 1993; Economou et al., 2014; Guo et al., 2013; Kindle, 1998, 1990; Ramesh et al., 2011; Shimogawara et al., 1998; Suttangkakul et al., 2019; Tan et al., 2005). Most of these methods are restricted to a limited number of species and require laborious optimization when applied to other microalgae. Moreover, most of the transformation methods can lead to high cell death because harsh treatments are required to overcome recalcitrant microalgal cell barriers (Muñoz et al., 2018).

Recent advances in omics technology and the development of genome scale metabolic models have increased our understanding of microalgal metabolism (Fig. 1). However, the lack of data available on genome sequencing and functional annotation for microalgal species limits the possibility of exploiting omics technologies (Lauritano et al., 2019; Lin et al., 2019; Zhang et al., 2019). While genome-scale metabolic models are powerful tools for predicting metabolic engineering strategies, they could be further improved by experimental characterization of enzymes, kinetic analyses, and identification of gene regulation patterns. An increase in genetic data availability and the validation of metabolic models will allow effective and strategic design of genetic approaches and will improve the accuracy and reliability of bioinformatic tools.

Although omics data for microalgae are in a developing stage, they have allowed the identification of regulatory elements necessary for the development of genetic engineering strategies. Identification of promoters, splicing signals, terminators, selection markers and reporter genes have facilitated the expression of heterologous genes in microalgae. This has accelerated the development of molecular tools including state-of-the-art genome editing systems such as Clustered Regularly Inter-Spaced Palindromic Repeats and associated proteins (CRISPR-Cas), Transcription Activator-Like Effector Nuclease (TALEN), Zinc Finger Nulease (ZFN) and RNA interference (RNAi). Most of these genetic tools have been used throughout the last decades in several publications (Kumari et al., 2015; Lin et al., 2019). Fig. 1 summarizes the collective efforts and advancements made in the last 30 years on genetic engineering of microalgae, including tool development and strategies aimed at enhancing lipid production. Recent research has demonstrated that this goal can be achieved by tuning fatty acid synthesis (FAS), Kennedy pathway, PUFA and TAG metabolism, transcription factors and nicotinamide adenine dinucleotide phosphate (NADPH) generation. Nevertheless, most of the studies focused on the genetic modification of model microalgal strains such as *Chlamydomonas reinhardtii*, *Phaeodactylum tricornutum* and more recently *Nannochloropsis spp*., due to the lack of a universal genetic toolbox for all microalgal species (Fig. 1).

2.1. Fatty acid synthesis (FAS) metabolism

Fatty acid synthesis (FAS) has been a logical target for improving the lipid production as it is the first step towards *de novo* lipid formation in microalgae. FAS occurs in the chloroplast with acetyl-CoA as the main precursor, which is converted to malonyl-CoA by an acetyl-CoA carboxylase (ACC) enzyme. Malonyl-CoA is then converted by malonyl-CoA ACP transacylase (MAT) to form malonyl-ACP by transferring a malonyl-CoA to the acyl carrier protein (ACP). Subsequently, malonyl-ACP undergoes a series of repeated rounds of condensation, reduction, dehydration and again reduction steps. These reactions are catalyzed by the enzymes 3-ketoacyl-ACP synthase (KAS), 3-ketoacyl-ACP reductase (KAR), 3-hydroxyacyl-ACP dehydrase (HD), and enoyl-ACP reductase (ENR) to finally form saturated fatty acids (SFA) such as C14:0, C16:0 and C18:0, whose length is determined by specific acyl-acyl carrier protein thioesterases (TEs) (Fig. 2).

Malonyl-CoA formation is catalyzed by acetyl-CoA carboxylase, and it is the major rate-limiting step for the *de novo* biosynthesis of fatty acids (Li-Beisson et al., 2019). Therefore, microalgal ACC genes have been overexpressed in multiple species, albeit with limited effect on lipid accumulation. The first attempt in this line of research was made by Dunahay et al. (1996) by overexpressing the native ACC protein in the microalgae *Cyclotella cryptica* and *Navicula saprophila*. Although they showed that overexpression resulted in a significant increase in ACC activity, no differences were observed regarding intracellular lipid content. On the other hand, overexpression of a yeast-derived ACC gene in *Scenedesmus quadricauda* resulted in 1.6-fold increase in total fatty acids (Gomma et al., 2015).

Fatty acid biosynthesis requires a continuous supply of acetyl-CoA. One of the main enzymes responsible for increasing intracellular acetyl-CoA pools is the acetyl-CoA synthetase (ACS). This enzyme catalyzes the conversion of acetate into acetyl-CoA and it has been a promising target for genetic engineering. An *Escherichia coli* acetyl-CoA synthetase was overexpressed in the marine microalga *Schizochytrium sp*., resulting in 11.3% increase in fatty acids and 29.9% increase in biomass content, improved carbon utilization and reduced intracellular acetate concentration (Yan et al., 2013). Moreover, overexpression of ACS in *C. reinhardtii* has increased intracellular acyl-CoA pools leading to 2.4-fold increase in TAG content when mutants were grown under nitrogen starvation conditions (Rengel et al., 2018).

Other recent studies have met with success by overexpressing the enzyme malonyl-CoA ACP transacylase (MAT) in *N. oceanica* and *Schizochytrium* sp., increasing the total lipid content by 36 and 10.1% compared to wild type strains, respectively (Chen et al., 2017; Li et al.,

Table 1
Genetic engineering of FAS metabolism for enhanced lipid production.

Algal strain	Targeted genes	Strategy	Effect on lipid synthesis	Comments	References
C. cryptica and *N. saprophila*	*ACCase*	OE, HE	No effect on lipid content	Significantly increased ACCase activity	Dunahay et al. (1996)
S. quadricauda	*ACC1* *GPD1* *GUT1*	HE	ACC1, GPD1 and GUT1 increased lipid content by 1.6, 1.6 and 1.9-fold. Multi-gene expression increased lipid content by 1.45-fold	No significant effect on growth among all transgenic strains	Gomma et al. (2015)
Schizochytrium sp.	*ACS*	HE	11.3% increase in fatty acids	29.9% increase in biomass	Yan et al. (2013)
C. reinhardtii	*ACS2*	OE	2.4-fold increased TAG content under N-starvation conditions	2-fold increased starch content and 60% higher acyl-CoA in N-replete conditions	Rengel et al. (2018)
N. oceanica	*NoMCAT*	OE	36% increased TFA and 31% increased neutral lipid content. Increased C20:5 by 8%	Higher growth rate and photosynthetic efficiency compared to wild type strains	Chen et al. (2017)
Schizochytrium sp.	*MAT*	OE	10.1 and 24.5% increased TFA and PUFA content, respectively. TFA, DHA and EPA yields increased by 39.6, 81.5 and 172.5%, respectively	Carotene content was decreased and redirected toward PUFA synthesis	Li et al. (2018)
P. tricornutum	*UcFATB* *CcFATB*	HE	Increased accumulation of C12:0 of up to 6.2% of TFA and C14:0 of up to 15%. 75–90% of the shorter chain length fatty acids were incorporated into TAGs	No significant secretion of fatty acids was observed	Radakovits et al. (2011)
P. tricornutum	*PtTE*	OE	Overexpression did not alter fatty acid composition. Enhanced TFA by 72%	Overexpression in *E. coli* increased TFA content and composition in membrane lipid	Gong et al. (2011)
C. reinhardtii	*CrTE*	OE	No significant change in fatty acid content. 2.5-fold increased levels of short-chain fatty acids (C14:0)	Demonstration of protein-protein interactions between fatty acid acyl carrier protein (ACP) and thioesterase (TE)	Blatti et al. (2012)
C. reinhardtii	*DtTE*	HE	63–69% increase in neutral lipids and 56% improvement in TFA	No significant effect on growth	Tan and Lee (2017)
D. tertiolecta	*C14TE*	HE	C12:0 was increased from 0.32 to 0.43% and C14:0 was increased from 0.75 to 1.47% w/w of TFAs	Medium chain length fatty acid levels were negatively correlated with expression of fatty acid synthesis genes; KASII, Δ9D and Δ12D	Lin et al. (2018)
C. reinhardtii	*CrFAB2*	OE	oleic acid (C18:1) increased by 2.4-fold compared to wild-type strain. TFA increased by 28 %	CrFAB2 overexpression resulted in induction of CrFAD2 expression. No significant effect on growth	Hwangbo et al. (2014)
C. merolae	*acyl-ACP-reductase*	HE	3-fold increased TAG content, which led to an increase in the number and size of lipid droplets	Expression led to up-regulation of genes related to degradation of branched chain amino acids, fatty acid synthesis and degradation. No significant effect on growth	Sumiya et al. (2015)
P. tricornutum	*MaKCS*	HE	0.2% content of nervonic acid (C24:1) in total FAMEs	No presence of nervonic acid in wild type strain	Fan et al. (2018)

OE, overexpression; HE, heterologous expression.

2018). Chen et al. (2017) demonstrated that neutral lipid contents in mutant strains of *Nannochloropsis* was increased by 31% and they reported higher growth rates and photosynthetic efficiencies. Li et al. (2018) reported an increase of 24.5% in PUFA production of transgenic *Schizochytrium* strains, increasing DHA and EPA yields by 172.5 and 81.5%, respectively.

The mature fatty acid assembled on the ACP is hydrolyzed by an acyl-acyl carrier protein thioesterase, which determines the length and type of the fatty acid produced. Although microalgal TEs remain largely uncharacterized, studies suggest that these enzymes are distinct from their counterparts in plants. Plants have developed TEs for different acyl chain lengths (FatA and FatB), while microalgae use one thioesterase with broad specificity, Fat1 (Blatti et al., 2012). Several studies have attempted to express endogenous or exogenous TEs to manipulate length and type of fatty acids and to synthesize more saturated, medium-chain fatty acids (MCFAs, C8-14), which are preferable for biodiesel and other bulk chemical applications (Gong et al., 2011; Tan and Lee, 2017; Wang et al., 2021). In particular, a C12:0-biased FatB TE from *Umbellularia californica* and myristic acid (C14:0)-biased FatB TE from *Cinnamomum camphora* were used to redirect FA synthesis in the microalga *P. tricornutum*, obtaining a composition of 5% w/w C12:0 and 12% w/w C14:0 of TFAs, respectively (Radakovits et al., 2011). By using a C12-specific TE and an MCFA-specific KAS, it was possible to increase the accumulation of C12:0 and C14:0 in *Dunaliella tertiolecta* up to 0.4 and 1.4% w/w of TFAs, respectively (Lin et al., 2018; Lin and Lee, 2017) (Table 1).

2.2. Kennedy pathway

Microalgae can accumulate large amounts of neutral lipids when exposed to stress conditions such as nutrient limitation (Janssen et al., 2018). These lipids are mainly composed of saturated or monounsaturated fatty acyl groups, which are of great industrial interest for their potential use as a feedstock for biofuel production (Brennan and Owende, 2010). Among neutral lipids that can be found in microalgal cells, the most common saturated fatty acids are C14:0, C16:0 and C18:0 (Zulu et al., 2018). Moreover, the most abundant neutral lipids found in microalgae are monoacylglycerols (MAGLs), diacylglycerols (DAGs) and triacylglycerols (TAGs), the latter being a major lipid class in cellular storage compounds such as lipid bodies. Glycerolipids are synthesized in the Kennedy pathway, which begins with glycerol 3-phosphate (G3P). Acylation of glycerol-3-phosphate (G3P) is catalyzed by glycerol 3-phosphate acyltransferase (GPAT) to form lyso-phosphatidic acid, which is further converted into phosphatidic acid by lysophophatidic acid acyltransferase (LPAT). Phosphatidic acid is then dephosphorylated by phosphatidic acid phosphatase (PAP) and diacylglycerol (DAG) is generated. Diacylglycerol acyltransferase (DGAT) catalyzes the final step in TAG synthesis using diacylglycerol and acyl-CoA as substrates (Yu et al., 2011). It has been proposed that TAG biosynthesis takes place either in the chloroplast or in the endoplasmic reticulum (ER) (Fig. 2).

The first step of TAG synthesis is catalyzed by GPAT and is considered the rate-limiting reaction (Yu et al., 2018). Balamurugan et al. (2017) and Niu et al. (2016) reported that overexpression of endogenous GPAT in *Phaeodactylum tricornutum* increased neutral lipid content by 1.8 and 2-folds compared to wild type strains, respectively. Both studies showed significantly higher levels of unsaturated fatty acids and no effect on growth rates. Recently, a glycerol-3-phosphate acyltransferase 2 (GPAT2) isoform was identified and overexpressed in *Phaeodactylum tricornutum,* showing similar results (Wang et al., 2020). Wang et al. (2020) observed a reduced carbohydrate and protein content, and 2.9-

Table 2
Genetic engineering of Kennedy pathway for enhanced lipid production.

Algal strain	Targeted genes	Strategy	Effect on lipid synthesis	Comments	References
C. reinhardtii	*CrDGAT2a* *CrDGAT2b* *CrDGAT2c*	OE	No change in lipid content	Enhanced mRNA expression of DGAT genes did not boost TAG accumulation and did not result in alterations of fatty acid profiles	La Russa et al. (2012)
C. reinhardtii	*CrDGAT2-1* *CrDGAT2-5*	OE	*DGAT2-1* and *DGAT2-5* increased TFA by 27.25% and 48% respectively, during replete conditions.	Silencing of *CrDGAT2-1* or *CrDGAT2-5* decreased lipid content by 16%–24% or 28%–37%, respectively	Deng et al. (2012)
P. tricornutum	*DGAT2*	OE	35% increased neutral lipids. EPA increased by 76.2%.	Growth rate of transgenic strains remained similar to WT	Niu et al. (2013)
C. reinhardtii	*CrDGTT4*	OE	2.5-fold increased TAG during P starvation	Enhanced TAG accumulation with a slight increase in C18:1 content, using a P starvation-inducible promoter	Iwai et al. (2014)
C. reinhardtii	*BnDGAT2*	HE	7% decrease in total saturated fatty acids. 7% increase in overall PUFA content	Growth rate of transformants similar to wild type strains	Ahmad et al. (2015)
T. pseudonana	*DGAT2*	OE	1.52–1.95-fold increased TAG content. Increased C16:1, C16:2, C20:5 and C22:6 fatty acid contents	No change in growth rate compared to wild type strains	Manandhar-Shrestha and Hildebrand (2015)
Nannochloropsis strain NIES-2145	*CrDGTT4*	HE	1.3–1.7-fold increased TAG content. The levels of C16:0, C16:1, C18:1 ω-9, and C20:3 ω-6 fatty acids increased, whereas C16:3, C18:2 ω-6, C20:4 ω-6 and C20:5 fatty acids decreased under P starvation conditions	No significant changes in TFA and total polar lipids were observed	Iwai et al. (2015)
N. oceanica	*DGAT2*	OE	69% and 129% increased TAG content during replete and deplete conditions. 74% and 53% decreased PUFA and MUFA content, respectively	DGAT2 overexpression did not show negative impact on algal growth	Li et al. (2016)
N. salina	*DGA1*	HE	18–38% increased TFA during replete conditions. 75% increased productivities in mid-exponential phase	Reduced growth rate in transgenic strains compared to wild type.	Beacham and Ali (2016)
S. obliquus	*DGTT1*	HE	2-fold increased TFA	29% higher biomass concentration than that of the wild type	Chen et al. (2016)
P. tricornutum	*DGAT2D*	OE	1.6–2-fold higher total lipid content	50–100-fold higher DGAT2D mRNA levels and 30–50-fold increased enzyme abundance. 15% decrease in growth rate	Dinamarca et al. (2017)
N. oleoabundans	*NeoDGAT2*	OE	1.6–2.3-fold increased TFA and 1.6–3.2-fold increased total lipid productivity. 1.8–3.2-fold increased TAG and 1.6–4.3-fold increased total TAG productivity	No significant effect on growth	Klaitong et al. (2017)
N. oceanica	*DGAT1A*	OE	39% increase in TAG content under nitrogen-depletion. 2.4-fold increased TAG under nitrogen-replete. TAG yield 47% greater than for the wild type strain	NoDGAT1A overexpression did not show negative impact on growth. NoDGAT1A knockdown caused 25% decline in TAG content during nitrogen depletion.	Wei et al. (2017a)
N. oceanica	*NoDGTT5*	OE	1.75-fold increased TAG under N-replete conditions	50% decreased growth rate in transgenic strains compared to wild type	Zienkiewicz et al. (2017)
T. chui	*EpDGAT1* *ScDGAT2*	HE	40–115% increase in TAG content	No significant effect on growth	Úbeda-Mínguez et al. (2017)
P. tricornutum	*ScDGA1* *AtOLEO3*	HE	ScDGA1 and AtOLEO3 increased TAG content 2.3- and 1.4-fold, respectively. Co-expression resulted in 3.6-fold increased TAG content. TAG productivity increased by 2-folds under N-stress	Fatty acid composition remained unchanged in TFA and TAG	Zulu et al. (2017)
Coccomyxa sp.	*cDGAT2d* *cFAT1*	OE	FAT1 and DGAT2 increased TFA content 1.1- and 1.1-fold, respectively. Co-expression resulted in 1.12-fold increased TFA content and 1.4-fold increased TFA productivity	Single expression did not impair growth rates. Co-expression increased growth rate by 1.27-folds	Kasai et al. (2018)
C. reinhardtii	*CrLPAAT1*	OE	20% increase in TAG content under nitrogen-deficient conditions	CrLPAAT1 was localized to the plastid membrane in *C. reinhardtii* cells	Yamaoka et al. (2016)
C. reinhardtii	*CrPAP2*	OE	7.5–21.8% increased TFA content	Silencing of the CrPAP2 gene resulted in 2.4–17.4% decrease in total lipid content	Deng et al. (2013)
P. tricornutum	*GPAT*	OE	1.34–2-fold increase in TAG content under N-deplete conditions. 35%, 12% and 45% decrease in SFA and MUFAs, respectively. 41% increased PUFA content	Overexpression did not show significant effect on growth and slight increase in photosynthetic efficiency	Niu et al. (2016)
C. reinhardtii	*LiGPAT*	HE	50% increase in TAG content. Increase in C18:1 n-9 and 1C6:0, decrease in C18:3 n-3 and C16:4 n-3	Overexpression did not have significant effect on growth	Iskandarov et al. (2016)
P. tricornutum	*AGPAT1*	OE	1.81-fold increase in TAG content and 2.04-fold higher lipid yield	No effect on growth. Decreased protein and carbohydrate content	Balamurugan et al. (2017)
C. merolae	*CmGPAT1*	OE	No significant effect on TFA. 19-fold increased TAG content and 56.1-fold increase in TAG productivities	Overexpression did not show significant effect on growth	Fukuda et al. (2018)
P. tricornutum	*GPAT2*	OE	2.9-fold increase in TAG content. Altered fatty acid profile in TAGs with increase of C16:0	No effect on growth and photosynthetic efficiency. Enhanced tolerance to hyposaline and chilling conditions	Wang et al. (2020)
C. reinhardtii	*LPAAT* *GPD1*	HE	LPAAT and GPD1 increased TFA content by 44.5% and 67.5%. Increase in long-chain	No effect on growth. Decreased protein content	Wang et al. (2018a)

(*continued on next page*)

Table 2 (*continued*)

Algal strain	Targeted genes	Strategy	Effect on lipid synthesis	Comments	References
			saturated fatty acids and decreased unsaturated fatty acids		
P. tricornutum	*GPAT1* *LPAAT1*	OE	Dual expression resulted in 2.3-fold increased TAG content under N-replete conditions. 2.7 and 3-fold increase in TFA and TAG productivities, respectively	Dual expression resulted in 1.5-fold increase in growth rate during mid-log phase	Wang et al. (2018b)
P. tricornutum	*DGAT2* *GPAT*	OE	2.6-fold increased TFA compared to WT, reaching up to 57.5% DCW. Increased MUFA and PUFA content	No effect on growth. Slight increase in photosynthetic efficiency	Zou et al. (2018)
N. oleoabundans	*LPAAT1* *DGAT2*	OE	Co-expression resulted in 1.6 and 2.1-fold increased TFA and TAG content, respectively. 1.9 and 2.1-fold increased TFA and TAG productivities, respectively	Co-expression did not affect growth	Chungjatupornchai and Fa-aroonsawat (2020)
N. oleoabundans	*GPAT* *LPAAT* *DGAT*	HE	Single-gene expression resulted in 1.3 and 1.4-fold increased TFA and TAG content, respectively. Multi-gene expression resulted in 1.2-fold increase in TFA and TAG	Single expression did not affect growth and photosynthetic efficiency. Multi-gene expression decreased growth rate, photosynthetic efficiency, carbohydrates and protein content	Muñoz et al. (2019)
C. minutissima	*G3PDH* *GPAT* *LPAAT* *PAP* *DGAT*	HE	Quintuple-gene expression resulted in 2-fold increased TAG content and 1.8-fold increased TAG productivity. 2-fold increased TFA	Single-gene constructs showed little effect on enhancing TAG production	Hsieh et al. (2012)
Chlorella sp.	*GPAT* *LPAAT* *PAP* *DGAT*	HE	Quadruple-gene expression resulted in 2.3-fold increased TAG content	Similar growth and TAG productivity	Chien et al. (2015)

OE, overexpression; HE, heterologous expression.

fold increased TFA and TAG content in transgenic strains. GPAT2 overexpressing strains showed no growth impairment, and an altered lipid composition with an increase of C16:0 and a decrease of both C18:0 and unsaturated fatty acid content.

Furthermore, Fukuda et al. (2018) demonstrated that endogenous overexpression of GPAT in the red alga *Cyanidioschyzon merolae* can influence lipid composition. Although the TFA content was not significantly changed in comparison to WT strains, relative abundance of C18:2 in the sn-1/sn-3 positions of TAGs were significantly increased without a negative impact on growth, and thus leading to 56.1-fold increase in maximum TAG productivities in mutant strains. Additionally, heterologous expression of GPAT in microalgae has shown a successful increase in lipid accumulation. Overexpression of GPAT from *Saccharomyces cerevisiae* and *Lobosphaera incise* in *Chlamydomonas reinhardtii* resulted in an increase of 67.5% and 50% for TFA and TAG, respectively. Additionally, an increase in long-chain saturated fatty acids, and decrease in PUFAs without any inhibition on growth were observed in these mutants (Iskandarov et al., 2016; Wang et al., 2018a).

Overexpression of LPAT, the next enzyme in the Kennedy pathway, has also been reported to improve the lipid content in *C. reinhardtii*. Yamaoka et al. (2016) investigated the effect of the endogenous overexpression of a plastidial LPAT and showed an increase of 20% in TAGs under nitrogen-deficient conditions. Likewise, Wang et al. (2018a) reported that heterologous expression of LPAT from *Brassica napus* in *C. reinhardtii* increased lipid content by 44.5% without affecting growth.

Furthermore, Deng et al. (2013) have studied the biological activity of PAP in *C. reinhardtii* and its impact on lipid biosynthesis. Phosphatidic acid phosphatase dephosphorylates phosphatidic acid into DAG, which is the main precursor for TAG synthesis. Overexpression and knockdown of *PAP* resulted in 7.5–21.8% increase and 2.4–17.4% decrease in lipid content, respectively, demonstrating its importance in the lipid biosynthesis pathway (Deng et al., 2013).

The last step of the TAG biosynthesis pathway is catalyzed by diacylglycerol acyltransferase (DGAT). Overexpression of genes encoding DGAT has so far been the most frequently employed strategy for increasing TAG contents in microalgae. Although the first attempt performed in the model microalga *C. reinhardtii* (La Russa et al., 2012) did not lead to significant change in lipid content, later studies demonstrated an increase of TFA between 1.5- to 2.5-fold in the same species (Ahmad et al., 2015; Deng et al., 2012; Iwai et al., 2014). Moreover, overexpression of *DGAT* in *Nannochloropsis* strains has been reported to increase the TAG content between 1.3- to 2.4-fold or to enhance the TFA content (Beacham and Ali, 2016; Iwai et al., 2015; Li et al., 2016; Wei et al., 2017a; Zienkiewicz et al., 2017). The same strategy has been used for the diatom *Phaeodactylum,* resulting in 1.3- to 2.3-fold increases in TAG content (Dinamarca et al., 2017; Niu et al., 2013; Zulu et al., 2017). While most of the research focused on species from the genus of *Chlamydomonas*, *Nannochloropsis* and *Phaeodactylum*, similarly promising results were achieved for microalgae belonging to *Thalassiosira*, *Scenedesmus*, *Neochloris*, *Tetraselmis* and *Coccomyxa* genera (Chen et al., 2016; Kasai et al., 2018; Klaitong et al., 2017; Manandhar-Shrestha and Hildebrand, 2015; Muñoz et al., 2019; Úbeda-Mínguez et al., 2017).

Several studies have further shown promising results when using a multi-gene expression approach. Wang et al. (2018b) have demonstrated that dual expression of *LPAT* and *GPAT* in *P. tricornutum* leads to 2.4- and 2.3-fold increase in TFA and TAG content, respectively. Likewise, dual expression of *DGAT* and *GPAT* was reported to increase TFA by 2.6-fold in the same strain (Zou et al., 2018). Furthermore, simultaneous expression of *DGAT*, *GPAT* and *LPAT* was reported to increase TFA and TAG content by 1.2-fold in *N. oleoabundans* (Muñoz et al., 2019). Similarly, the overexpression of endogenous LPAT1 and DGAT2 in the same species lead to 1.6- and 2.1-fold increase of TFAs and TAG, respectively (Chungjatupornchai and Fa-aroonsawat, 2020). Studies have shown that overexpression of the entire pathway including *DGAT*, *GPAT*, *LPAT* and *PAP* genes can also have a significant impact on lipid biosynthesis by increasing TAG content between 2 and 2.3-fold in *Chlorella* species (Chien et al., 2015; Hsieh et al., 2012) (Table 2).

2.3. Polyunsaturated fatty acids (PUFAs) metabolism

Long-chain polyunsaturated fatty acids (LC-PUFAs) are aliphatic carbon chains consisting of 18, 20 or 22 carbons. They are classified into two main groups based on the position of the first double bond at the 3rd (ω-3) or 6th (ω-6) carbon atom counting from the methyl end. In nature,

Table 3
Genetic engineering of PUFA metabolism for enhanced lipid production.

Algal strain	Targeted genes	Strategy	Effect on lipid synthesis	Comments	References
C. reinhardtii	*CrΔ4FAD*	OE	No significant effect on TFA. Increase in C16:4 and total MGDG	CrΔ4FAD knockdown decreased C16:4 and C18:3 in TFA	Zäuner et al. (2012)
P. tricornutum	*PtD5b*	OE	75% and 64% increased MUFA and PUFA content. 58% and 65% increased EPA and neutral lipids, respectively	No significant effect on growth	Peng et al. (2014)
P. tricornutum	*OtElo5* *OtD6N*	HE	OtElo5 expression increased DHA content 8-fold. Co-expression of OtElo5 and OtD6N increased DHA up to 11.4% of TFA	Accumulation of DHA in TAGs	Hamilton et al. (2014)
P. tricornutum	*OtElo5* *Ppglut1*	HE	32.2% PUFA per TFA produced under mixotrophic conditions. Under phototrophic conditions, up to 36.5% and 23.6% DHA and EPA in TFA, respectively	*Ppglut1* allowed growth in the dark if medium supplemented with glucose	Hamilton et al. (2016)
N. oceanica	*NoD12*	OE	50–75% increased AA under N-starvation conditions. 32.6% increased LA in PC	Overexpression under the control of stress-inducible endogenous lipid droplet surface protein (LDSP) promoter, (Higher expression under N-starvation conditions)	Kaye et al. (2015)
P. tricornutum	*D6FAD*	OE	47.66% increased EPA content, reaching up to 38.101 mg/g dcw. 16.4–18.64% increased TFA compared to wild type strain	Slight reduction in specific growth rate	Zhu et al. (2017)
D. salina	*TpFADS6* *DsFADS6*	OE, HE	Enhanced EPA up to 21.3 mg/L, compared to 1.6 mg/L in the WT. Up to 91, 193 and 554 mg/L EPA in TFA when supplemented with myoinositol, CO_2 and PeSM, respectively	Use of myoinositol, CO_2 and PeSM to promote growth	Shi et al. (2018)
C. vulgaris	*ω-3 FAD*	OE	7% increased TFA when grown under N-deficient conditions. 2.8% increased ALA in TFA	No significant effect on growth	Norashikin et al. (2018)
N. oceanica	*Δ12-FAD* *Δ9-FAD* *Δ5-FAD*	OE	Single and co-expression resulted in 25% increased EPA. Decreased TFA content in transformants	Cell growth increased in all transformant lines	Poliner et al. (2018)
P. tricornutum	*PtDGAT2B* *OtElo5*	OE, HE	Co-expression resulted in 37-fold increased TAG from N-replete to starved conditions, compared to 1.8-fold in wild type, respectively. High DHA and DPA content in TAG	No significant effect on growth	Haslam et al. (2020)

OE, overexpression; HE, heterologous expression.

LC-PUFAs are found mainly in fish oils and they are essential in human diet. In addition to the benefits of physical and mental wellbeing, LC-PUFAs are also reported to promote the development of infant health and prevention of diseases (Zárate et al., 2017). Although they can be obtained from marine fish oils, increased pollution of the oceans, climate variations and overfishing are causing a decline in wild fish stocks and thereby reduce the supply required to meet the global demand (Nations, 2016).

Microalgae are the primary producers of LC-PUFAs in aquatic environments. Such lipids serve as building blocks and are mainly present in photosynthetic membranes (thylakoids), but they can also be found in lipid bodies as a response to unexpected environmental changes or during nutrient starvation (Solovchenko, 2012). Commercially relevant LC-PUFAs such as eicosapentaenoic (EPA), docosahexaenoic (DHA) and arachidonic acid (ARA) can be found predominantly in marine or salt-tolerant microalgae species such as *Phaeodactylum tricornutum* and *Nannochloropsis oceanica* (Boelen et al., 2013).

Genetic engineering of various microalgae species has enhanced the accumulation of industrially relevant PUFAs, by regulating the expression of genes encoding fatty acid desaturases (FADs) and elongases (ELOs). These enzymes catalyze a series of desaturation and elongation steps of aliphatic carbon chains that lead to PUFA formation (Fig. 2) (Khozin-Goldberg et al., 2011). For instance, Zäuner et al. (2012) have demonstrated that overexpression of Δ4-FAD in *C. reinhardtii* (CrΔ4FAD) increased the total MGDG content from 9 fmol/cell to 15 fmol/cell compared to the WT. This modification did not alter TFA content and resulted in 12% increase of C16:4 and 8% increase of C18:3. On the other hand, the CrΔ4FAD knockdown decreased the fraction of C16:4 and C18:3 in TFAs (Zäuner et al., 2012).

An endogenous Δ5-FAD was overexpressed in *P. tricornutum* CCMP2561 (Peng et al., 2014), resulting in an increase of neutral lipid content up to 65% with no effect on growth. An increase of 75% of MUFAs and 64% PUFAs was observed with a 16% decline in saturated fatty acids (SFAs), indicating redirection of lipid flux towards PUFA synthesis. EPA was increased by 58% and 0.44 mg/g dcw of DHA was produced (Peng et al., 2014). Similarly, Hamilton et al. (2014) found that DHA content of *P. tricornutum* was substantially increased by heterologous expression of Δ5-ELO from *Ostreococcus tauri*. In this case, DHA was increased by 8-fold and accounted for 10.4% of the TFAs during the stationary phase, while EPA levels almost halved compared to the WT. Moreover, co-expression of Δ5-ELO and Δ6-FAD from *O. tauri* further increased DHA levels to 11.4% of TFA (Hamilton et al., 2014). Furthermore, Hamilton et al. (2016) co-expressed the Δ5-ELO and a glucose transporter from *Physcomitrella patens* in *P. tricornutum*, enabling glucose uptake in a phototrophic microalga that naturally does not grow on glucose (Hamilton et al., 2016). Under heterotrophic conditions, transformants produced 7.3% of DHA per TFAs and up to 9.1% of docosapentaenoic acid (DPA), with no significant variations in the EPA content. Moreover, after transferring the cultures from heterotrophic to phototrophic conditions, both EPA and DHA levels further increased, reaching 19% and 9.2% of TFAs, respectively. Under mixotrophic cultivation, EPA and DHA levels increased 1.7- and 1.1-fold, respectively, compared to strains expressing only a Δ5-ELO (Hamilton et al., 2016).

In an attempt to increase DHA levels, Haslam et al. (2020) characterized and overexpressed native *P. tricornutum* DGAT2B, which showed preferences for C16 and LC-PUFA acyl groups (Haslam et al., 2020). In the same study, the combined expression of DGAT2B and Δ5-ELO led to an increased incorporation of PUFAs into TAGs. The overexpression of endogenous Δ6-FAD in *P. tricornutum* increased EPA contents by 48% (Zhu et al., 2017). Although transformants did not show any change in DHA content, SFA content was increased by 28% and MUFA content was slightly reduced. Similarly, overexpression of Δ6-FAD from *Thalassiosira pseudonana* (TpFAD6) in *D. salina* enhanced EPA up to 21.3 mg/L compared to 1.6 mg/L in the WT (Shi et al., 2018). Moreover, by improving the carbon availability during cultivation, EPA content was further enhanced by 25-fold to 554 mg/L.

An endogenous Δ12-FAD was overexpressed in *N. oceanica*, which caused a decrease in OA (substrate for Δ12-FAD) and increase in LA in TAGs and other lipid classes of transgenic strains (Kaye et al., 2015).

Furthermore, ARA levels were increased in different lipid classes, indicating an increase in carbon flux towards the omega-6 PUFA biosynthesis pathway. Poliner et al. (2018) observed a 25% increase in EPA in *N. oceanica* strains overexpressing a Δ12-FAD or Δ5-FAD. Co-expression of Δ9 and Δ12-FADs or Δ9, Δ5 and Δ12-FADs did not improve the EPA content compared to the previously attained 25%. Although cell growth was increased in all transformant lines, the total fatty acid content per cell was decreased compared to the wild type strain (Poliner et al., 2018).

Norashikin et al. (2018) reported an effect of overexpressing the endogenous ω-3-FAD in *C. vulgaris*. A significant increase in TFA from 40% to 47% was observed in transformants when grown under nitrogen-deficient conditions. Transformants also showed a slight increase in α-linolenic acid (ALA) content from 8% to 10.8% of TFA compared to the WT (Norashikin et al., 2018).

Other strategies such as increasing fatty acid precursors, enhancing the TAG biosynthesis pathway, optimizing the supply of cofactors and downregulating lipid catabolism have also shown promising results in enhancing LC-PUFAs and they are reported in other sections of this review (Table 3).

2.4. Transcription factors

Transcription factors (TFs) are DNA-binding proteins that function as key regulators of gene expression. There are several types of TFs that are distinct in their mechanism of recognizing and binding DNA and influencing transcription (Inukai et al., 2017). A single TF can control the expression of multiple genes simultaneously, which makes them an ideal target to shift metabolic fluxes towards lipid biosynthesis. In microalgae, several TFs have been identified as potential targets for genetic engineering, mostly in species of the genera *Nannochloropsis* and *Chlamydomonas*.

The first report by Yohn et al. (2011) has shown that overexpression of the chitin binding protein (CBP-like protein SN03) in *C. reinhardtii* resulted in a 60% increased TFA content under replete conditions without affecting growth. Tsai et al. (2014) have found the protein "Compromised Hydrolysis of TAG 7" (CHT7) to be a repressor of cellular quiescence in *C. reinhardtii*. *CHT7* knockout caused a differential gene expression under replete conditions, partially resembling that of wild type cells under deplete conditions. Photosynthesis and flagellum assembly-related functions were observed among the regulated genes. Resupply of nutrients in starved *CHT7* knockout cells showed a severely hampered remobilization of storage compounds. Although, *CHT7* knockout cells did not display an increase in lipid content, regulation of CHT7 may rewire cellular metabolism under replete conditions.

Ngan et al. (2015) systematically assayed chromatin states and gene expression under nitrogen and sulphur (S) depletion in *C. reinhardtii*. Upregulated expression of "phosphorus starvation response 1" (*PSR1*) gene showed significant correlation with lipid content during stress condition. PSR1 had already been investigated before as a regulator of phosphorus metabolism (Wykoff et al., 1999) however, it was not reported to be involved in lipid metabolism. Knockout of *PSR1* had no effect on accumulation of TAG under N, S, and P stress, but *PSR1* overexpression increased lipid contents and cell size by 100% with only minor effects on growth (Ngan et al., 2015). Bajhaiya et al. (2016) have reported that *PSR1* knockout in *C. reinhardtii* caused differential gene expression of 900–1000 genes upon P_i starvation, most of which are involved in P_i homeostasis, starch and lipid metabolism, confirming the findings of Ngan et al. (2015). Under deplete conditions, starch and lipid contents decreased in knockout strains by ~60% and ~70%, respectively. Overexpression of *PSR1* in a cell wall-less strain resulted in ~60% increased starch content and 25% decreased lipid content. PSR1 seems to be a key regulator of nutrient stress response, however, further investigation is necessary to better understand the effect of this regulator on lipid and starch contents due to the contradictory results.

Recently, Yamaoka et al. (2019) have found that the TF basic leucine zipper 1 (BZIP1) from *C. reinhardtii* (CrBZIP1) is involved in ER stress response. The protein promotes expression of genes involved in the unfolded protein response. During ER stress, CrBZIP1 knockdown strains showed a reduced expression of genes implicated in DGTS and pinolenic acid biosynthesis, while the expression of the type II DGAT gene *DGTT1* was increased. Accordingly, levels of DGTS and pinolenic acid were decreased and TAG content was increased 5.8–9.4-fold compared to the WT.

In recent years, species from the genus *Nannochloropsis* are emerging as model organisms, shifting TF engineering from the green algae to the heterokonts. The first effort was taken by Kang et al. (2015) who overexpressed a basic helix-loop-helix (bHLH) motif TF in *N. salina* and found a 55% increased growth rate in the mutant strain with no relevant change in TFA content. While *NsbHLH2* overexpression resulted in an overall 43% increased lipid productivity, growth rates were relatively low compared to values reported in other studies (Poliner et al., 2018; Vieler et al., 2012).

While the bHLH TF type commonly regulates growth, nutrient uptake and stress response in plants and animals, basic leucine zipper (bZIP) TFs are often involved in abiotic stress, developmental responses and lipid metabolism in plants (Agarwal et al., 2019; Song et al., 2013). Studies have suggested that complex bZIP TFs in plants have evolved from algal ancestors, facilitating the transfer of fundamental knowledge about TF function from plants to microalgae (Corrêa et al., 2008; Peviani et al., 2016). For instance, NsbZIP1 from *Nannochloropsis* shows homology to type C bZIP TFs present in plants. Moreover, an *in silico* study based on TF binding sites prediction identified a homologue of NsbZIP1 in *N. oceanica* as a putative positive regulator of lipid metabolism related genes (Hu et al., 2014). Recently, Kwon et al. (2018) have achieved the constitutive overexpression of a bZIP TF in *N. salina*. *NsbZIP1* overexpressing strains had increased expression levels of enzymes involved in the Kennedy pathway and fatty acid synthesis causing a simultaneous improvement in growth rate and lipid content. Neutral lipid and TFA contents were increased by 33% and 21%, respectively, compared to the wild type. Under N limitation, TFAs and TAGs were further increased by 39% and 88%, respectively, and cultivation under high salinity stress resulted in 60% and 203% higher TFA and TAG contents, respectively, with similar increases in growth rates. A recent study of the NsbZIP1 homologue in *N. oceanica*, NobZIP1, corroborates the importance of this TF (Li et al., 2019). Although NobZIP1 and the previously described CrbZIP share 73% sequence identity, they possess very different functions in microalgal metabolism. Consistent with the observations for *NsbZIP1*, *NobZIP1* overexpression resulted in a 65–100% increase in lipid content and a ~60% decrease in carbohydrate and protein contents without affecting growth or photosynthetic performance. Intriguingly, a substantial part of lipids (up to 40% on dry cell weight basis) were present in the growth media in mutant cultures (Li et al., 2019). The authors attribute this finding to thinning of the cell wall in the mutants and hypothesized that this would promote lipid secretion. Overexpression of the TF not only led to an increased expression of *KAS1*, *LCFACS*, *ACBP* and *LPAAT*, but it also decreased the expression of *UGDH* (encoding UDP-glucose 6-dehydrogenase) which plays a key role in cell wall polymer metabolism. Indeed, *UGDH* is an attractive target for redirecting carbon flux from carbohydrates to lipids (Li et al., 2017; Oka and Jigami, 2006), as the authors further found that *UGDH* knockdown strains displayed the same phenotype regarding cellular composition as *NobZIP1* overexpressing strains. Uncertain consequences on the cell wall thinning remain to be investigated. It has been reported that strains with reduced cell wall thickness possess higher mortality rates in industrial scale cultivation system due to shear stress, as previously demonstrated for cell wall-lacking mutants (Barbosa et al., 2003; Wang and Lan, 2018).

Ajjawi et al. (2017) have systematically studied TF expression in *N. gaditana* under N stress conditions and found 20 putative negative regulators of lipid production. Knock out of a homologue of fungal Zn $(II)_2Cys_6$ TFs, *NgZnCys,* was found to improve carbon partitioning

Table 4
Genetic engineering of transcription factors for enhanced lipid production.

Algal strain	Targeted genes	Strategy	Regulated genes	Effect on lipid synthesis	Comments	References
C. reinhardtii	*SN03* (CREB binding Zn-finger protein)	OE	ND	60% increase in TFA during replete	KD impaired in lipid accumulation during deplete	Yohn et al. (2011)
C. reinhardtii	*CHT7*	KO	2968 differentially regulated genes, enrichment in flagellum assembly & photosynthesis	-	Repressor of cellular quiescence, severely delayed TAG degradation upon nutrient resupply	Tsai et al. (2014)
C. reinhardtii	*PSR1*	OE	ND	100% increased TAG under replete condition	KO strain significantly lower TAG during N, S & P stress	Ngan et al. (2015)
C. reinhardtii	*PSR1*	OE	Starch & lipid metabolism genes under Pi stress	>50% decrease in lipids during –P in KOS. ~20% decrease in OES	Starch also decreased in KOS but increased in OES	Bajhaiya et al. (2016)
C. reinhardtii	*CrBZIP1*	KD	Pinolenic acid & DGTS biosynthesis	480–860% increased TAG, decreased DGTS & pinolenic acid	-	Yamaoka et al. (2019)
N. salina	*NsbHLH2*	OE	ND	43% increased lipid productivity	No change in lipid content. Growth rate increased by 55%	Kang et al. (2015)
N. salina	*NsbZIP1*	OE	*KAS1*, *LC-FACS*, *ACBP* & *LPAAT*	21% increased TFA during late replete, 39% during late stress. Neutral lipids increased 33% during replete, 88% during stress. Osmotic stress markedly increased TFA and neutral lipids in mutant by 60% and 203% respectively	Increased QY growth rate in mutants especially under stress conditions	Kwon et al. (2018)
N. oceanica	*NobZIP1*	OE	Lipid metabolism genes↑ (ia. *KAS1*, *LC-FACS*, *ACBP* & *LPAAT*) *UGDH*↓	65–100% increased lipids in OE strain. Further, 40% lipids per dcw present in medium. 40% decreased lipid content in KD strain	Main mode of action likely regulation of *UGDH*. No change in growth rate but thinning of cell wall	Li et al. (2019)
N. gaditana	*NgZnCys*	KD	Desaturases, elongases, lipases, acyltransferases & lipid droplet surface protein↑ in KD strain	100% increased TFA during growth phase in KD strain 100–175% increase in TFA in KO strain	KO strain grows poorly, KD strain unaffected in growth	Ajjawi et al. (2017)
N. oceanica	*NoAP2*	KO	Chloroplastic fatty acid biosynthesis, glycolysis and CBB cycle genes	40% increased neutral lipids during growth phase	No effect on growth	Südfeld et al. (2021)
C. reinhardtii	*GmDOF11*	HE	Lipid synthesis related (*BCR1*, *SQD1* and *FAT1*) ↑	140% increased TFA during stationary phase	No effect on growth	Ibáñez-Salazar et al. (2014)
Chlorella ellipsoidea	*GmDOF4*	HE	1076 genes differentially expressed in mutant. 22 genes related to lipid synthesis (i.e. *ACCase*↑)	49–53% increased lipids during early stationary phase	No effect on growth. Decreased carbohydrate and protein in mutant	Zhang et al. (2014)
N. salina	*AtWRI1*	HE	i.e. *TAGL*, *DAGK* and *PPDK*↑	32% increased TFA, 21–70% increased TAG during replete phase. 64% higher TFA yield	Increased growth rate, especially under N limitation, but no increase in TFA during –N.	Kang et al. (2017)

OE, overexpression; HE, heterologous expression; KO, knock-out; KD, knock-down.

towards lipids during exponential growth. *NgZnCys* mutants showed 100–175% increased TFAs (mainly TAGs), resulting in a significant reduction in growth rate, and thus overall lipid productivity. On the other hand, knockdown of *NgZnCys* did not affect the growth rate while causing a 100% increase in TFA content. Cells were substantially bigger, and had 15% decreased total cellular proteins, whereas carbohydrate content was unaffected. Moreover, *NgZnCys* knockdown mutants showed upregulation of six fatty acid desaturases, elongases, lipases and acyltransferases as well as proteins localized on the surface of lipid droplets. However, the relative PUFA contents decreased by ~73%, despite the increase in desaturase expression. The function of the fungal Zn $(II)_2Cys_6$ TF may be conserved among the genus *Nannochloropsis*, as *N. oceanica* mutants carrying an insertion cassette in the 3'-UTR of a *NgZnCys* homologue had an increased neutral lipid content (Südfeld et al., 2021). The authors of this study further found that knockout of an APETALA2-like transcription factor *NoAP2* elevated neutral lipid contents and productivity by ~40% in *N. oceanica*, concomitant with an increased expression of genes related to chloroplastic glycolysis, fatty acid biosynthesis and the Calvin-Benson-Bassham cycle. A different approach was reported by Ibáñez-Salazar et al. (2014). They investigated the effect of heterologous expression of codon-optimised DNA-binding with one finger type transcription factor (DOF-type TF) *GmDOF11* from soybean in *C. reinhardtii*. Overexpression of this TF induces lipid synthesis in seeds of *A. thaliana* (Wang et al., 2007). Ibáñez-Salazar et al. (2014) have found that GmDOF11 promotes expression of lipid synthesis-related genes in *C. reinhardtii* such as *BCR1*, *SQD1* and *FAT1*. Mutant strains showed a 140% increase in TFA content during the stationary phase compared to the wild type while the FA profile and growth rate were unaffected. Moreover, Zhang et al. (2014) also showed that heterologous expression of another soybean DOF-type TF *GmDOF4* in *C. vulgaris* resulted in differential expression of 1076 genes in the transgenic strain, 22 of which are related to lipid synthesis. Mixotrophically cultivated mutant strains had a 49–53% increase in lipids, a 9–14% decrease in protein and a 15–19% decrease in carbohydrate content with unchanged growth or fatty acid profile. Another case of heterologous expression of a plant TF was reported for *Nannochloropsis salina*. Kang et al. (2017) expressed the *A. thaliana* Apetala 2-type transcription factor (AP2-type TF) *Wrinkled1* (*AtWRI1*) which is a master regulator of lipid synthesis during seed maturation. In *A. thaliana, AtWRI1* binds to promoter regions (AW-boxes) of several genes involved in lipid biosynthesis related genes (Maeo et al., 2009). In *N. salina* these promoter regions are present in 475 promoters. Overexpression of *AtWRI1* in *N. salina* led to altered regulation of genes including triacylglycerol lipase (*TAGL*), diacylglycerol kinase (*DAGK*) and pyruvate phosphate dikinase (*PPDK*), among others. Growth rates of mutant strains were significantly higher during nitrogen replete conditions, compared to the wild type. Moreover, transformants showed 21–70% and 32% increase in TAG and TFA content, respectively, which reportedly resulted in a 64% higher TFA yield.

TFs can be used as versatile and practical tools for metabolic

engineering. Overexpression or knockout of a single TF can lead to up- or downregulation of metabolic pathways or outright rewire the entire cellular metabolism. However, fundamental research needs to be done to elucidate the role of individual TFs in the complex regulatory network of microalgal cells. To this end, predicting relevant TFs with *in silico* approaches can be an effective way of combining systems biology and metabolic engineering (Hu et al., 2014; Kwon et al., 2018; Li et al., 2019) (Table 4).

2.5. Photosynthesis and NADPH generation

2.5.1. Photosynthetic electron transport, redox homeostasis and energy balancing

In eukaryotic microalgae, photosynthesis occurs in the thylakoid membranes of the chloroplast. A highly complex and conserved mechanism transfers electrons from H_2O to $NADP^+$ in a series of redox reactions from redox couples with lower potentials to those with higher potentials. Such reactions are driven by light energy and take place in chlorophylls P680 in the photosystem II (PSII) reaction center and P700 in photosystem I (PSI). The electron transfer from PSII to PSI is coupled to H^+ transfer from stroma to lumen, resulting in a H^+ gradient across the thylakoid membrane, facilitating ATP synthesis by an ATP synthase (ATPase). A part of the energy and reducing equivalents produced in light reactions are subsequently consumed in the Calvin-Benson-Bassham (CBB) cycle, which converts CO_2 into glyceraldehyde 3-phosphate (GAP). GAP can follow different routes and can be converted into sugars (gluconeogenesis) or pyruvate and subsequently acetyl-CoA, which provides energy to the cells for lipid formation, maintenance, anabolism and growth (Subramanian et al., 2013). Because lipid synthesis usually occurs in the chloroplast, it is influenced by light conditions (Lehmuskero et al., 2018; Masojídek et al., 2013; Sato and Moriyama, 2018), carbon flux from GAP to acetyl-CoA and involvement of other cellular compartments. Recent studies have improved carbon flux towards lipid synthesis in microalgae either by improving photosynthetic capacity or by increasing the supply of reducing equivalents via genetic engineering. In this section we review relevant advances in both genetic strategies that lead to improved lipid contents.

2.5.2. Engineering photosynthetic performance for enhanced lipid production

Improving light reactions was attempted by overexpressing ferredoxin 1 (PETF) and 5 (FDX5) in *C. reinhardtii* (Huang et al., 2015). PETF is involved in electron transfer from PSI to $NADP^+$ through ferredoxin $NADP^+$ oxidoreductase (FNR) and FDX5 and it has been linked to reactive oxygen species (ROS) detoxification. Moreover, FDX5 was shown to physically interact with the fatty acid desaturases CrΔ4FAD and CrFAD6, likely donating electrons for the desaturation of fatty acids that stabilize monogalactosyldiacylglycerol (Yang et al., 2015). Separate overexpression of both proteins increased resistance to heat, salt stress, lowered intracellular levels of H_2O_2 and increased lipid contents by 13–56% and 50–250% under replete and deplete conditions, respectively. Furthermore, promising results were also obtained when overexpressing a ROS stress-response related enzyme superoxide dismutase (SOD1) in *Schizochytrium* sp. (Zhang et al., 2018). *SOD1* overexpression not only increased ROS detoxification but also enhanced TFA accumulation without affecting biomass yields. An 18% and 37% increase in SFA and PUFA contents were observed, respectively. Since PUFAs have antioxidant properties (Liu et al., 2013; Richard et al., 2008; Schmid-Siegert et al., 2016), higher levels of SOD1-mediated ROS scavenging may have caused a decreased depletion of PUFA pools via alleviation of lipid peroxidation, although further investigation is necessary.

Gargouri et al. (2017) observed that expression levels of PSI translation initiation factor *TAB2* in *C. reinhardtii* are markedly increased during early nutrient stress conditions. A *TAB2* knockout strain showed lower levels of PSI and significantly increased starch (>800%) and TAG (>100%) contents compared to WT strains under replete condition and mixotrophic cultivation. Additionally, *TAB2* knockouts showed lower $NADPH/NADP^+$ and ATP/NADPH ratios under replete and deplete conditions, respectively. These results suggest that carbon partitioning to carbohydrates and lipids is regulated by PSI activity and it is likely linked to ATP and NADPH generation in green algae. Furthermore, Koh et al. (2019) expressed the heterologous chlorophyllide an oxygenase gene from *C. reinhardtii* (*CrCAO*) in *N. salina* strains. *CrCAO* overexpressing strains showed significant contents of chlorophyll a (Chl a), chlorophyll b (Chl b), light-harvesting complex II (LHCII) antenna proteins and PSII reaction center protein D1, leading to a slight increase in photosynthetic performance under higher light conditions. Under lower light conditions an increased growth rate was observed leading to an overall increase in lipid productivity of ~42% compared to the wild type.

2.5.3. Engineering NADPH generation for enhanced lipid production

Promising research has been directed towards increasing levels of reducing equivalents in order to boost FA synthesis in microalgae. Potential targets are malic enzymes (ME) which are $NAD(P)^+$-dependent oxidoreductases that perform the oxidative decarboxylation of malate to pyruvate (Cornish-Bowden, 2014). In plants and microalgae, different types of MEs have preference for either NAD^+, $NADP^+$ or both. In C4-plants, they are localized in the chloroplast and participate in carbon-concentrating mechanisms to increase efficiency of RuBisCO activity (Gerald and Carlos, 1992; Madhavan et al., 2002). On the other hand, cytosolic isoforms involved in redox homeostasis and energy metabolism supply reducing power to biosynthetic pathways and generate pyruvate for ATP production in the mitochondria (Drincovich et al., 2001). FA synthesis requires substantial NADPH supply and it was suggested to be the limiting factor for lipid synthesis in oleaginous organisms (Ratledge, 2014; Wynn et al., 1999). Talebi et al. (2014) have reported for the first time the expression of ME in the microalga *Dunaliella salina*. Successful simultaneous overexpression of the putative cytosolic $NADP^+$-dependent malic enzyme *DsME1* and acetyl CoA-carboxylase subunit D *DsAccD* was achieved in the chloroplast. Mutant strains showed a 12% and 23% increase in TFAs and neutral lipids, respectively, and the FA profile was substantially shifted towards saturated fatty acids at the expense of unsaturated ones. A putative mitochondrial ME was overexpressed in *P. tricornutum* by Xue et al. (2015). Overexpression of *PtME* resulted in 150% increase in TFA content reaching ~58% FA dcw^{-1} with a slight negative affect on growth rates. The authors also transferred the mitochondrial-localized *PtME* to *Chlorella pyrenoidosa* (Xue et al., 2016). They report a >300% increase in ME activity in cell extracts along with 220% increase in TFAs under replete conditions and 360% increase under deplete conditions, reaching 40.9% and 58.7% dcw^{-1} respectively. Zhu et al. (2018a) also investigated the cytosolic ME expression in *P. tricornutum*. *PtME1* overexpression led to a 52–81% increase in $NADP^+$-ME activity in cell extracts and 28.4–80.3% increase in intracellular NADPH levels, resulting in 150% increase in TFA content (57.8% dcw^{-1} during non-stressed conditions). The MUFA content was decreased from ~25 to ~20%, while the SFAs and PUFAs contents showed a slight increase from ~23 to ~25% and from 49 to 54%, respectively.

Another source of cellular reducing power is the oxidative pentose phosphate pathway (oxPPP) (Kruger and von Schaewen, 2003). The oxPPP provides metabolic intermediates such as ribose-5-phosphate and generates up to 12 NADPH molecules per molecule of G6P. However, the oxPPP involves an oxidative decarboxylation step leading to CO_2 production resulting in lower triose phosphate yields compared to other glycolytic pathways. While oxPPP lowers the lipid yield on substrate in heterotrophic processes, photoautotrophic cultivation may not be limited as CO_2 can be re-assimilated. Enzymes in the oxPPP are present not only in the cytosol but also in chloroplasts, indicating close spatial proximity of decarboxylation reactions and carbon fixation mechanisms (Kruger and von Schaewen, 2003). The plastidic isoforms of these enzymes are redox-regulated and have been proposed to supply NADPH in

Table 5
Genetic engineering of NADPH generation for enhanced lipid production.

Algal strain	Targeted gene (s)	Strategy	Effect on lipid synthesis	Comments	References
N. oceanica	nRca	OE	-	30% increased QY and growth > 30% increased lipid productivity	Wei et al. (2017b)
C. reinhardtii	PETC FDX5	OE	50–250% increased lipid content during deplete for both proteins	110–170% increased starch concomitantly	Huang et al. (2015)
Schizochytrium sp.	SOD1	OE	18% increased SFA, 37% increased PUFA	Lower levels of ROS	Zhang et al. (2018)
C. reinhardtii	TAB2	KO	>100% increased TAG during mixotrophic replete cultivation	>800% increased starch levels. Energy metabolism severely impaired, higher cell mortality under -N	Gargouri et al. (2017)
N. salina	CrCAO	HE	42% increased productivity due to increased growth under light-limited conditions		Koh et al. (2019)
D. salina	AccD ME	OE	12% increased TFA, 23% increased NL		Talebi et al. (2014)
P. tricornutum	ME		150% increased NL during replete, 66% increased NL during deplete		Xue et al. (2015)
C. pyrenoidosa	PtME	HE	220% increased TFA during replete, 360% during deplete		Xue et al. (2016)
P. tricornutum	ME	OE	150% increased TFA during replete	Decreased MUFA	Zhu et al. (2018a)
P. tricornutum	G6PD	OE	170% increased total lipids during replete phase, 100% during deplete		Xue et al. (2017)
C. pyrenoidosa	NoG6PD	HE	230–260% increased TFA during starvation phase. 209% increase in TAGs	119% increased intracellular NADPH	Xue et al. (2020)
C. pyrenoidosa	AtNADK3	HE	45–110% increased total lipids during growth		Fan et al. (2015)

OE, overexpression; HE, heterologous expression; KO, knock-out.

absence of photosynthesis (Hauschild and von Schaewen, 2003). The rate-limiting step of the oxPPP is the conversion of G6P to 6-phosphogluconolactone coupled to the reduction of $NADP^+$, which is catalyzed by G6P dehydrogenase (G6PD). Xue et al. (2017) characterized G6PD in *P. tricornutum*. Overexpression of the plastidic PtG6PD resulted in a 170% increase in TFA levels (55.7% dcw^{-1}) without affecting growth of mutant strains. The SFA and MUFA contents increased while the PUFA content decreased. Moreover, metabolomic analyses revealed elevated levels of glycolytic and TCA cycle intermediates such as pyruvate and acetyl-CoA, which were increased by 300% and 710%, respectively. Additionally, 3-phosphoglycerate was elevated by 570%, suggesting a possible increase of FA synthesis. Furthermore, the heterologous expression of *NoG6PD* from *N. oceanica* in *C. pyrenoidosa* (Xue et al., 2020) increased NADPH and TAG levels by 119% and 209% during both growth and stationary phases. In this study, the TFA content was increased by 230–260% under nitrogen deplete conditions, with an increase in MUFA and PUFA contents at the expense of SFAs. Carbohydrates and protein levels were drastically reduced in the engineered strains without negatively impacting the growth rate.

Fan et al. (2015) observed 40–80% increased NADPH levels in *C. pyrenoidosa* strains expressing an NAD kinase enzyme from *A. thaliana* (AtNADK3). This resulted in a 45–110% increase in TFA content under heterotrophic and mixotrophic cultivation conditions.

Alternatively, increasing cellular reducing power towards microalgal lipid biosynthesis can be achieved by expressing, for instance, transhydrogenase enzymes. This has been attempted and reported in *E. coli* (He et al., 2014). Soluble transhydrogenase transfers electrons from NADPH to NAD^+ and membrane-bound isoforms transfer electrons from NADH to $NADP^+$ driven by a proton gradient to overcome high NADPH/NADH ratios in prokaryotes and in eukaryotic mitochondria (Sauer et al., 2004). Overexpression of the membrane-bound isoform or knockdown/knockout of soluble transhydrogenase might increase NADPH/NADH ratios and FA synthesis in microalgae. Another enzyme involved in generating cellular reducing power is the $NADP^+$-dependent isocitrate dehydrogenase (IDH) which has been reported to be the main source for NADPH required for fatty acid synthesis in mammalian adipocytes (Koh et al., 2004; Lee et al., 2002; Shechter et al., 2003). Cytosolic and chloroplastic IDH are present in microalgae but have thus far not been given attention as targets for increasing NADPH production rates (Martínez-Rivas and Vega, 1994) (Table 5).

2.6. Central carbon metabolism

The central carbon metabolism plays a fundamental role in directing carbon fluxes to different metabolic pathways, and it regulates *inter alia* acetyl-CoA and G3P availability (Subramanian et al., 2013). For instance, G3P can function as substrate for TAG biosynthesis, but it can also be reconverted to DHAP and enter glycolysis or gluconeogenesis (Han et al., 2016). In this context, Muto et al. (2015) attempted to alter glycerol availability by overexpression of an endogenous glycerol kinase (GK) gene in the oleaginous diatom *Fistulifera solaris*. Although only 12% increase in lipid yield was observed, externally supplied glycerol was utilized 40% more efficiently in overexpression strains resulting in a slight increase in biomass productivity.

Isotopic labelling studies in higher plants have shown that increased levels of intracellular G3P correlate with carbon partitioning of acetyl-CoA to TAG (Vigeolas and Geigenberger, 2004). These findings support the idea that G3P levels may be limiting for TAG synthesis only under conditions of increased acyl-CoA availability. It was also found that overexpression of G3P dehydrogenase (G3PDH) in *P. tricornutum* led to a 580% increase in intracellular glycerol levels and improvement of lipid content by 60% during the stationary phase (Yao et al., 2014). The growth rates of mutant strains were lower compared to the wild type and the neutral lipid content was elevated by 90% with an increase in MUFA content at the expense of PUFAs. These findings have shown that G3P is a limiting substrate for glycerolipid synthesis in *P. tricornutum* under nutrient stress conditions thereby emphasizing the importance of G3PDH as a link between glycolysis and glycerol metabolism.

Furthermore, the connection between glycolytic flux and lipid production was investigated in the marine diatom *Thalassiosira pseudonana* (Abbriano et al., 2018). Overexpression of the glycolytic regulator 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK2/F2BP) enhanced the flux through the glycolytic pathway, resulting in increased production of neutral lipids by 116% and proteins by 35% at the expense of storage carbohydrates under both stressed and non-stressed conditions. Engineered strains showed a delayed progression through the cell cycle (G1 to the S phase), resulting in an overall decrease in growth rate. Hypothetically, this may be the effect of a decreased availability of glucose for the PPP and consequently less synthesis of C5 sugars required for nucleotide synthesis. Although diatoms are complex study organisms due to the presence of glycolytic pathways in the cytosol,

Table 6
Genetic engineering of central carbon metabolism for enhanced lipid production.

Algal strain	Targeted gene (s)	Strategy	Effect on lipid synthesis	Comments	References
F. solaris	GK	OE	12% increase in lipid yield	Glycerol usage up 40% in mixotrophic cultivation. No analysis done during stationary phase	Muto et al. (2015)
P. tricornutum	G3PDH	OE	60% increased total lipids, 90% increased neutral lipids during stationary phase	20% reduced cell concentration	Yao et al. (2014)
T. pseudonana	PFK2/F2BP		116% increased TAG under growth and starvation conditions	Cell cycle progression slowed, lower growth rate.	Abbriano et al. (2018)

OE, overexpression.

mitochondria and plastid, the results from Abbriano and co-workers highlight the potential of redirecting the carbon flux by modifying glycolytic pathways (Table 6).

2.7. Other approaches

2.7.1. Lipid catabolism

In higher eukaryotes, TAG is stored in multi-functional cytosolic lipid droplets (LDs) (Huang, 1992; Olzmann and Carvalho, 2019; Thiam and Beller, 2017; Zhang and Liu, 2017). Although TAG assembly and LD formation have been reported for some microalgae to occur also in the chloroplast, the subject is still under debate (Balamurugan et al., 2017; Eugeni Piller et al., 2011; Fan et al., 2011; Moriyama et al., 2018). While the mechanism involved in LD formation remains poorly understood, it has been reported that LDs bud from the ER membrane towards the cytosol with the aid of specialized proteins (Jacquier et al., 2013; Morris and Olzmann, 2019). LDs are composed of a hydrophobic core, consisting mostly of TAG and enclosed by a phospholipid monolayer with a broad range of proteins. These proteins can be involved in RNA binding, translation (Zhang and Liu, 2017), membrane trafficking (Olzmann and Carvalho, 2019), signal integration, lipogenesis and lipolysis (Kong et al., 2018). Moreover, several components involved in these processes have been identified in *Chlamydomonas reinhardtii* such as CXC-domain containing regulatory protein, phosphatidylethanolamine-binding delayed in TAG hydrolysis-1 (DTH1) (Lee et al., 2020), two lipases and two enzymes involved in FA β-oxidation (Li-Beisson et al., 2021). However, the most well-characterized function is that of oleosins and perilipins families which prevent LD coalescence.

The Stramenopile lineage-specific LD protein StLDP was identified in *P. tricornutum* as a major class of LD coat proteins. Recently, Yoneda et al. (2018) have reported that *StLDP* overexpression in *P. tricornutum* leads to an increased number of LDs and ~25% higher neutral lipid content during late stage of nutrient starvation. The authors hypothesized that StLDP sequesters LDs during LD formation and prevents LD coalescence during nitrogen starvation. A high number of lipid droplets implies a high surface to volume ratio of individual LDs, thereby increasing the available area for lipogenic and lipolytic coat proteins such as DGAT2 and lipases, resulting i.a. in increased remobilization capacity of stored TAGs (Thiam and Beller, 2017).

Patatin-like phospholipase domain-containing protein 3 (PNPLA3) is a LD membrane protein identified in animal cells which has a controversial biological function (Kienesberger et al., 2009). An amino acid substitution in mammalian PNPLA3 causes fatty liver disease (Kienesberger et al., 2009) and expression of the gene encoding catalytically inactive PNPLA3^{I148M} in mice causes increased hepatic TAG content (Li et al., 2012). On the other hand, in yeast, human PNPLA3 drives TAG hydrolysis (Pingitore et al., 2014) while murine PNPLA3 exhibits LPAT activity *in vitro* (Kumari et al., 2012). Wang et al., 2015 identified and characterized a PNPLA3 ortholog in *P. tricornutum*. *PtPNPLA3*-overexpressing strains showed a 55% increase in neutral lipid content and a 26% increase in PUFA content (mainly C20:4), during stationary phase. The protein shows homology to the catalytic domain of a cytosolic phospholipase A2 which hydrolyzes the sn-2-acyl ester bonds of C20:4 FAs from phospholipids. Accordingly, PtPNPLA3 may have phospholipase activity and thereby increase the availability of C20:4 FAs for CoA esterification and incorporation into TAG. Similar results were observed when expressing the heterologous human *HsPNPLA3^{I148M}* in *P. tricornutum*. Results showed a 64% increase in TAG content and 52.5% increased TFA dcw^{-1} during the transition to the stationary phase (Wang et al., 2018c). Whereas PtPNPLA3 was proposed to exhibit phospholipase activity, which increases FA availability for TAG synthesis, phospholipid diacylglycerol acyl transferases (PDATs) catalyze the direct transfer of an acyl group from lipid donors like membrane phospholipids to DAG (Dahlqvist et al., 2000; Yoon et al., 2012). Heterologous expression of *PDAT* from *S. cerevisiae* led to 22% and 32% increase in TFA and TAG content in *C. reinhardtii* during growth and early stationary phases when targeted to the chloroplast (Zhu et al., 2018b). A substantial increase in PUFAs was observed whereas MGDG content was lower in the mutant strain. Presumably, the transfer of acyl groups from PUFA-rich MGDGs to TAGs may enhance PUFA synthesis in order to replenish photosynthetically active compounds in the thylakoid membrane MGDGs. Although photosynthetic performance and growth rates were lower, final biomass densities were similar to the WT. *PDAT* overexpression seems to be a suitable approach to change FA distribution and lipid profiles in microalgae cells, though compromising the chloroplast integrity.

2.7.2. Others

The protein UBC2 is involved in Lys63-linked polyubiquitination in *C. reinhardtii* (Fei et al., 2017). This type of polyubiquitination is a regulatory mechanism unlike the Lys-48-linked polyubiquitination, which is a key step in proteolysis (Volk et al., 2005). In *C. reinhardtii* it has been proposed that UBC2 functions in DNA damage tolerance. However, Fei et al. (2017) have found that in *UBC2* knockdown mutants the neutral lipid content of cells was 13.5–35.2% lower than in wild type. *UBC2* overexpression strains showed a 100% increase in neutral lipids with no impact on growth rate. These findings suggest that UBC2 is involved in other cellular processes than DNA damage response in *C. reinhardtii*. Accordingly, similar observations were reported for a homologous protein UEV1A in *A. thaliana* (Wen et al., 2014).

Lastly, several studies have proposed that fatty acid content could be increased by shunting carbon precursors from starch by blocking or reducing starch biosynthesis in the cells. Starchless mutants of *Scenedesmus obliquus*, *Chlorella pyrenoidosa* and *Chlamydomonas reinhardtii* generated using ultraviolet radiation as a mutagen showed increased FA content (de Jaeger et al., 2014; Li et al., 2010; Ramazanov et al., 2006). The starchless mutant of *S. obliquus* showed an increase in TFA productivity of 41% after nitrogen depletion, reaching 49.4% of TAGs (% DW), without a significantly decreased biomass productivity (de Jaeger et al., 2014). Starchless mutant of *C. pyrenoidosa* showed an increase of PUFA content by 20.4% and decrease of saturated FA by 18% compared to WT strains. In addition, TFAs were increased from 25 to 38% (% DW) under nitrogen limitation and mutants presented higher growth rates and productivities compared to WT cells during nitrogen replete conditions (Ramazanov and Ramazanov, 2006). Moreover, the inactivation of an ADP-glucose pyrophosphorylase via UV mutagenesis in *C. reinhardtii* generated a starchless mutant with a 10-fold increase in TAG content and a TFA content of 47%, up from 13% for theWT strain.

Table 7
Other approaches for enhanced lipid production.

Algal strain	Targeted gene (s)	Strategy	Effect on lipid synthesis	Comments	References
P. tricornutum	StLDP	OE	25% increased TAG during late starvation	No expression of transgene during starvation phase	Yoneda et al. (2018)
P. tricornutum	PNPLA3	OE	55% increased TAG	26% increase in PUFA	Wang et al. (2015)
P. tricornutum	HsPNPLA3	HE	52% increased TFA, 64% increased TAG during late log/ early stationary	Increased PUFA, decreased MUFA levels	Wang et al. (2018c)
C. reinhardtii	ScPDAT	HE	22% increased TFA, 32% increased TAG during growth	Delayed growth, substantial increase in PUFAs, lower MGDG	Zhu et al. (2018b)
C. reinhardtii	UBC2	OE	~100% higher TAG during stationary phase	No impact of OE on growth. KD strains 13–35% lower in TAG	Fei et al. (2017)

OE, overexpression; HE, heterologous expression.

However, this resulted in 30% decrease in growth compared to the WT strain under autotrophic conditions (Li et al., 2010). Conversely, this approach did not lead to significant increase in lipid accumulation or change in fatty acid profile in lipids in the starchless mutant of *Chlorella sorokiniana* compared to WT (Vonlanthen et al., 2015) (Table 7).

3. Conclusion and future perspectives

The ability of microalgae to grow photo-autotrophically on non-arable land makes them a suitable platform for green chemical production. However, the high cost of biomass production has hampered the commercialization of various microalgal products including third generation biofuels (Ruiz et al., 2016). Genetic engineering of microalgae has been seen as a solution to this bottleneck. This review summarizes the various advancements in genetic engineering of microalgae with improved lipid productivities. Most of the discussed engineering strategies involve modifications of a single metabolic pathway with the aim to either channel carbon towards lipid synthesis or to improve carbon capture efficiency. Even though several approaches have improved lipid accumulation in transgenic strains, productivities remain too low for economically feasible production of biofuels from microalgae (Ajjawi et al., 2017; Remmers et al., 2018).To further improve lipid productivities, engineering strategies will have to simultaneously improve the photosynthesis reactions and channel the carbon flux towards lipids without limiting the growth in host species. Proposing detailed approaches to improve the microalgal productivities is outside the scope of this review. Nevertheless, a recent review has presented various synthetic biology approaches that could potentially improve the productivities of microalgae (Naduthodi et al., 2021).

Evidently, substantial advancements have been achieved in the field of microalgal biotechnology for sustainable production of third generation biofuels and green chemicals. Bioprospecting of more suitable host species and/or metabolic engineering of genetically accessible microalgal strains could accomplish the aim of commercially feasible lipid production. Future research may need to parallelly focus on microalgae that are the most promising to industrially produce high-value lipids, as these are most likely to reach economic feasibility on the short term. The heterokont genus *Nannochloropsis* has already received considerable attention and it is a promising candidate for EPA production. More recently, the heterokont *Schizochytrium*, and the haptophyte *Tisochrysis* are emerging as potential lipid production platforms due to high growth rates, as well as high natural EPA and DHA contents, respectively (Barten et al., 2020). However, the light harvesting and carbon capturing systems of fast-growing species need to be investigated to allow for the development of genetic engineering strategies aiming to increase biomass and lipid productivities.

Authors contribution

CFM, CS and MISN wrote the manuscript. RAW, MJB, RHW and SD supervised the project and edited the manuscript. All authors contributed to the work, discussed the results, read and approved the final version of this manuscript.

Declaration of interest

The authors declare that they have no conflict of interest.

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