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Review

Microalgal metabolic engineering strategies for the production of fuels and chemicals

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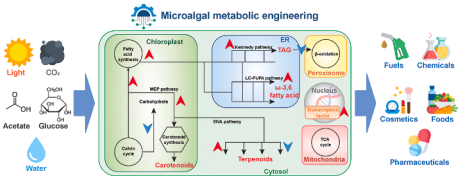
HIGHLIGHTS

- Microalgae metabolic engineering for lipid and isoprenoid production is reviewed.
- Comprehensive metabolic maps with metabolic engineering strategies are presented.
- Challenges and perspectives of microalgae metabolic engineering are discussed.

GRAPHICAL ABSTRACT



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ABSTRACT

Microalgae are promising sustainable resources because of their ability to convert CO_2 into biofuels and chemicals directly. However, the industrial production and economic feasibility of microalgal bioproducts are still limited. As such, metabolic engineering approaches have been undertaken to enhance the productivities of microalgal bioproducts. In the last decade, impressive advances in microalgae metabolic engineering have been made by developing genetic engineering tools and multi-omics analysis. This review presents comprehensive microalgal metabolic pathways and metabolic engineering strategies for producing lipids, long chain-polyunsaturated fatty acids, terpenoids, and carotenoids. Additionally, promising metabolic engineering approaches specific to target products are summarized. Finally, this review discusses current challenges and provides future perspectives for the effective production of chemicals and fuels via microalgal metabolic engineering.

1. Introduction

The high dependence on fossil fuels led to energy crises, and continuous CO_2 accumulation by petrochemical industries caused many environmental problems, such as global warming and climate change. Thus, research interest in sustainable and renewable energy sources continues to grow. In particular, autotrophic microalgae have been spotlighted because they can efficiently convert CO_2 to high-value products directly (Ahmad et al., 2011; Jeon et al., 2016). Microalgae can also be used for wastewater treatment because they consume

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inorganic nutrients such as nitrates and phosphates in wastewater and reduce biochemical oxygen demand and chemical oxygen demand (Arora et al., 2021; Bhatia et al., 2018). Likewise, microalgae are promising biomass that can solve environmental problems and produce value-added products simultaneously.

Microalgal cultures can be employed to produce biofuels and chemicals due to their fast growth rates and high lipid contents (Chisti, 2007). Considering scale-up and commercialization, microalgal bioprocess and biorefinery, consisting of cultivation, harvesting, recovery, purification, extraction, and conversion, have been studied (Khoo et al., 2019; Khoo et al., 2020; Yin et al., 2020). However, high process costs and low overall productivities of the microalgal bioprocess are undesirable for the industrial production of microalgal-bioproducts (Gifuni et al., 2019). Thus, researchers try to optimize cultivation and downstream process to reduce production costs. Besides the process development, the fundamental problems can be solved by increasing productivity via metabolic and genetic engineering (Brar et al., 2021).

However, sophisticated metabolic engineering is limited in microalgae. First of all, microalgal metabolic pathways are compartmentalized into several organelles with many gene homologs. In lipid synthesis, fatty acids are synthesized from the fixed CO_2 by photosynthesis and the Calvin cycle in the chloroplast and from organic carbon substrates such as glucose and acetate via glycolysis and central carbon metabolism. Generally, the synthesized fatty acids are stored in the form of triacylglycerols (TAGs) by sequential acylation of glycerol-3-phosphate (referred to as the Kennedy pathway) in the endoplasmic reticulum (ER) (Radakovits et al., 2010). Besides, isoprenoid is reported to be produced in two locations: the mevalonate (MVA) pathway in the cytosol and the 2-c-methyl-d-erythritol 4-phosphate/1-deoxy-d-xylulose 5-phosphate (MEP/DOXP) pathway in the chloroplast (Lauersen, 2019; Wichmann et al., 2020). However, as each microalgal species has a different organelle structure and metabolic pathways, it is necessary to accurately identify pathways of target products and their localization according to microalgae strains.

Furthermore, conventional genetic engineering tools, such as episomal plasmid expression and homologous recombination, are not generally applicable to microalgae. Thus, single-gene manipulation has been mainly conducted for microalgae metabolic engineering. However, because eukaryotic microalgae have several gene homologs and complicated metabolic pathways through many organelles, the regulation of a few genes poses limitations in eliciting an apparent phenotypic change (Radakovits et al., 2010). Although microalgal metabolic engineering studies are still in the early stages as compared to bacterial and yeast metabolic engineering, advanced metabolic engineering is being facilitated based on the recent development of genetic engineering tools and accumulating omics data (Brar et al., 2021). For example, as CRISPR/Cas9 has recently been applied to several microalgae, more systematic metabolic engineering is being enabled, constructing more stable transformants with improved lipid and carotenoid production (Ajjawi et al., 2017; Baek et al., 2018; Chang et al., 2020; Nguyen et al., 2020; Shin et al., 2019; Song et al., 2020).

In order to conduct efficient metabolic engineering in microalgae, it is necessary to comprehensively understand microalgal metabolic engineering studies that have been carried out so far. This review examines microalgal metabolic engineering strategies to increase the production of TAGs, long chain-poly unsaturated fatty acids (LC-PUFAs), terpenoids, and carotenoids. In addition, this review will present comprehensive metabolic maps, including the engineering strategies implemented in microalgae. This review will also conclude with a discussion of the challenges and future directions toward improving the production of biofuels and biochemicals.

2. Metabolic engineering strategies for biofuel production

Microalgae are a promising sustainable feedstock because they can accumulate large amounts of lipids from inorganic (CO_2) and organic carbon substrates (glucose and acetate). The carbon substrates are used for fatty acid synthesis in the chloroplast. The fatty acids can be stored in the form of TAGs in the chloroplast (prokaryotic pathway) and the ER (eukaryotic pathway). Numerous metabolic engineering studies for boosting the lipid synthesis pathway have been reported. Generally, the genes (*MCAT*, *DGAT*, *GPAT*, *LPAT*, *ME*, etc) involved in the fatty acid and TAG synthesis pathways, and increased production of NADPH, which is required for fatty acid synthesis, were overexpressed. Also, the genes (*AGPase*, *UDPase*, *lipase*, etc) involved in the competing pathway were down-regulated. Besides, transcription factor (TF) engineering was conducted to regulate the multi-genes involved in lipid synthesis simultaneously. Here, the reported metabolic engineering strategies to increase lipid production are categorized in Table 1, and comprehensive metabolic maps for the lipid synthesis are presented in Fig. 1.

2.1. Overexpressing the fatty acid synthesis pathway

Fatty acid synthesis begins with the conversion of CO_2 or glucose into pyruvate via subsequent metabolic reactions. The pyruvate dehydrogenase complex (PDC) converts pyruvate into acetyl-CoA, an important precursor for fatty acid production. Thus, several approaches to enhancing lipid production involved increasing the levels of pyruvate and acetyl-CoA in the chloroplast. The overexpression of pyruvate transporter-plastid type (*PTP*) in *Phaeodactylum tricornutum* increased the influx of pyruvate into plastids, leading to a 30% increase in the lipid content (Seo et al., 2020). As acetate can be utilized as a carbon source by microalgae, the overexpression of acetyl-CoA synthetase (*ACS*) might increase acetyl-CoA pools and thereby improve lipid production. Indeed, the overexpression of *Escherichia coli ACS* in *Schizochytrium* sp. resulted in an 11.3% increase in the lipid content (Yan et al., 2013), and the overexpression of endogenous chloroplastic *ACS* (*CrACS2*) in *Chlamydomonas reinhardtii* also led to a 2.4-fold increase in TAG content (Rengel et al., 2018). Acetyl-CoA carboxylase (ACC) and malonyl CoA-acyl carrier protein transacylase (MCAT) convert acetyl-CoA into malonyl-ACP, which is the first committed step in the fatty acid synthesis. The overexpression of endogenous *ACC2* (*PtACC2*) in *P. tricornutum* and plastid *MCAT* (*NoMCAT*) in *Nannochloropsis oceanica* led to 1.77-fold and 31% increases in neutral lipid content, respectively (Chen et al., 2017c; Li et al., 2018). Acyl-ACP, produced by the fatty acid synthesis cycle, is subsequently converted to fatty acids by a fatty acyl-ACP thioesterase (FAT). The overexpression of the endogenous *FAT* (*PtTE*) in *P. tricornutum* and the expression of *Dunaliella tertiolecta FAT* (*DtTE*) in *C. reinhardtii* successfully increased total lipid and TAG contents (Gong et al., 2011; Tan & Lee, 2017). Especially, the expression of myristic acid (C14-TE) or lauric acid biased thioesterase (C12-TE) enabled regulation of the fatty acid length in *P. tricornutum* (Radakovits et al., 2011). The expression of acyl-ACP reductase (AAR), which contributes to the bypass for producing fatty acids from acyl-ACP through fatty aldehyde, also increased the TAG contents by three-fold in *Cyanidioschyzon merolae* (Sumiya et al., 2015).

2.2. Overexpression of the TAG synthesis pathways

Fatty acids synthesized in the chloroplast are converted to acyl-CoA by long-chain acyl-CoA synthetase (LACS), and TAGs are produced by the conversion of acyl-CoA into glycerolipids. As the TAG synthesis occurs in the chloroplast and ER, the localization of transgene expression is an important consideration when optimizing the production of TAGs. In the TAG synthesis pathway, which is referred to as the Kennedy pathway, glycerol-3-phosphate dehydrogenase (GPDH), glycerol-3-phosphate acyltransferase (GPAT), lysophosphatidic acid acyltransferase (LPAT), phosphatidic acid phosphatase (PAP), and diacylglycerol acyltransferase (DGAT) enzymes contributes TAG production through successive acylation of a glycerol backbone derived from dihydroxyacetone phosphate (DHAP) (Fig. 1). Many studies attempted to increase TAG and lipid production by overexpressing the Kennedy pathway

Table 1
Summary of metabolic engineering studies to enhance lipid production in microalgae.

Target gene	Strain	Enzyme location	Metabolic engineering strategy	Carbon source	Result	Reference
Fatty acid synthesis						
PTP	*P. tricornutum*	Chloroplast	• Overexpression of the endogenous *PTP*	CO_2	• 21.9% and 30% increases in biomass and lipid contents	(Seo et al., 2020)
ACS	*C. reinhardtii*	Chloroplast	• Overexpression of endogenous chloroplastic *CrACS2*	CO_2 + acetate	• 2.4-fold increase in TAG content	(Rengel et al., 2018)
ACS	*Schizochytrium* sp. TIO1101	na	• Expression of *E. coli ACS*	Glucose	• 29.9% and 11.3% increases in biomass and lipid contents	(Yan et al., 2013)
ACC	*P.tricornutum*	Chloroplast	• Overexpression of endogenous *PtACC2*	CO_2	• 1.77-fold increase in neutral lipid content	(Li et al., 2018)
MCAT	*N. oceanica*	Chloroplast	• Overexpression of endogenous *NoMCAT*	CO_2	• 31% increase in neutral lipid content	(Chen et al., 2017c)
FAT	*C. reinhardtii*	Chloroplast	• Overexpression of heterologous *DtTE*	CO_2 + acetate	• 56% increase in total lipid content	(Tan & Lee, 2017)
FAT	*P. tricornutum*	na	• Overexpression of endogenous *PtTE*	CO_2	• 72% increase in shorter chain length fatty acids	(Gong et al., 2011)
FAT	*P. tricornutum*	na	• Overexpression of *C. campohora C14-TE* or *U. californica C12-TE*	CO_2	• 75–90% increase in shorter chain length fatty acids	(Radakovits et al., 2011)
AAR	*C. merolae*	Chloroplast	• Overexpression of cyanobacterial *AAR*	CO_2	• 3-fold increase in TAG content	(Sumiya et al., 2015)
TAG synthesis						
GPDH	*P. tricornutum*	na	• Overexpression of endogenous *GPDH*	CO_2	• 60% increase in lipid content with 20% decrease in cell concentration	(Yao et al., 2014)
GPAT	*P. tricornutum*	Chloroplast	• Overexpression of endogenous *AGPAT1*	CO_2	• 1.8-fold increase in TAG with an increase in PUFA (EPA and DHA)	(Balamurugan et al., 2017)
GPAT	*P. tricornutum*	Chloroplast	• Overexpression of endogenous *PtGPAT*	CO_2	• 2-fold increase in neutral lipid content (Fatty acid change)	(Niu et al., 2016)
GPAT	*P. tricornutum*	Chloroplast	• Overexpression of endogenous *GPAT2*	CO_2	• 2.9-fold increase in TAG content with the increase in C16:0, conferring hyposaline and chilling tolerance	(Wang et al., 2020)
LPAT	*N. oleoabundans*	Chloroplast	• Overexpression of endogenous *NeoLPAAT1*	CO_2	• 2.4- and 2.8-fold increases in total lipid and TAG productivities, respectively	(Chungjatupornchai et al., 2019)
GK	*F. solaris* JPCC DA0580	na	• Overexpression of endogenous *GK*	CO_2	• 12% increase in lipid productivity	(Muto et al., 2015)
DGAT	*N. oceanica*	Chloroplast ER (cER)	• Overexpression of endogenous *NoDGAT1A*	CO_2	• 2.4-fold and 47% increase in TAG content and yield without a cell growth defect	(Wei et al., 2017)
DGAT	*N. oceanica*	na	• Overexpression of endogenous type 2 *DGAT*	CO_2	• 69% and 129% increase in TAG content under N repletion and depletion conditions, respectively	(Li et al., 2016)
DGAT	*N. oleoabundans*	ER	• Overexpression of endogenous *NeoDGAT2*	CO_2	• 2.3-, 3.2-, and 4.3-fold increases in lipid content, TAG content, and TAG productivity, respectively	(Klaitong et al., 2017)
DGAT	*P. tricornutum*	na	• Overexpression of endogenous type 2 *DGAT*	CO_2	• 35% increase in neutral lipid content with a 76.2% increase in EPA	(Niu et al., 2013)
DGAT	*C. reinhardtii*	ER	• Overexpression of *Brassica napus DGAT2*	CO_2 + acetate	• 52%, 159%, and 166% increase in total lipid, FAME, and neutral lipid contents, respectively	(Ahmad et al., 2015)
DGAT	*S. obliquus*	ER	• Overexpression of *C. reinhardtii* type 2 *DGTT1*	CO_2	• 2-fold increase in lipid content under autotrophic conditions • 127.8% increase in lipid content in 40-L tubular type bioreactor under autotrophic conditions	(Chen et al., 2016)
DGAT	*P. tricornutum*	na	• Overexpression of endogenous *PtWS/DGAT*	CO_2	• 40% and 25% increases in the TAG content under N repletion and depletion conditions, respectively • 18.4% increase in the TAG productivity in the 10-L PBR	(Cui et al., 2018)
DGAT, OLEO	*P. tricornutum*	na	• Co-overexpression of *ScDGA1* and *AtOLEO3*	CO_2	• 3.6-fold increase in TAG content	(Zulu et al., 2017)
LPAT, DGAT	*N. oleoabundans*	Chloroplast (LPAT), ER (DGAT)	• Overexpression of endogenous *NeoLPAAT1* and *NeoDGAT2*	CO_2	• 1.6- and 2.1-fold increases in total lipid and TAG contents, respectively	(Chungjatupornchai & Fa-Aroonsawat, 2021)
GPAT, LPAT, DGAT	*N. oleoabundans*	ER	• Co-expression of *Acutodesmus obliquus GPAT*, *LPAT*, and *DGAT*	CO_2	• 4.5-fold increase in TAG productivity under N repletion conditions	(Munoz et al., 2019)
GPAT, LPAT	*P. tricornutum*	Chloroplast	• Co-overexpression of endogenous *GPAT1* and *LPAT1*	CO_2	• 2.3-fold increase in TAG content	(Wang et al., 2018)
GPAT, DGAT	*P. tricornutum*	na	• Co-overexpression of endogenous *GPAT* and *DGAT2*	CO_2	• 2.6-fold increase in total lipid content	(Zou et al., 2018)
GPDH, GPAT, LPAT,	*C. minutissima*	ER	• Co-overexpression of *GPDH*, *GPAT*, *LPAAT*, and *PAP* from	CO_2	• 2-fold increase in lipid content	(Hsieh et al., 2012)

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Table 1 (*continued*)

Target gene	Strain	Enzyme location	Metabolic engineering strategy	Carbon source	Result	Reference
PAP, DGAT			*S. cerevisiae* and *DGAT* from *Y. lipolytica*			
FAT, DGAT	*Coccomyxa* sp.	na	• Co-overexpression of endogenous *FAT* and *DGAT2*	CO_2	• 1.4-fold increase in lipid productivity	(Kasai et al., 2018)
ACC, GK, GPDH	*S. quadricauda*	na	• Co-expression of *S. cerevisiae* carboxylase *ACC*, *GK*, and *GPDH*	CO_2	• 1.45-fold increase in lipid content	(Gomma et al., 2015)
Blocking competing pathways						
AGPase	*Tetraselmis* sp.	Chloroplast	• CRISPR/Cas9 knock-out of *AGPase*	CO_2	• 3.1-fold increase in lipid content under nitrogen starvation	(Chang et al., 2020)
UGPase	*P. tricornutum*	Chloroplast	• TALEN knock-out of *UGPase*	CO_2	• 45-fold increase in TAG content	(Daboussi et al., 2014)
UGPase	*P. tricornutum*	Chloroplast	• Antisense knock-down of *UGPase*	CO_2	• 24.58% increase in lipid content with 61.93% decrease of chrysolaminarin	(Zhu et al., 2016)
PDK	*N. salina*	Mitochondria	• RNAi knock-down of *PDK*	CO_2	• 30.1% increase in total lipid content under high light conditions (Change of fatty acid profile)	(Ma et al., 2017)
PDK	*P. tricornutum*	na	• Antisense knock-down of *PDK*	CO_2	• 82% increase in neutral lipid content	(Ma et al., 2014)
CIS	*C. reinhardtii*	Mitochondria	• RNAi knock-down of *CIS*	CO_2 + acetate	• 169.5% increase in TAG content	(Deng et al., 2013)
PEPC	*C. reinhardtii*	na	• RNAi knock-down of *PEPC*	CO_2 + acetate	• 20% increase in TAG content	(Deng et al., 2014)
ACX	*C. reinhardtii*	Peroxisome	• Random insertional knock-out of *CrACX2*	CO_2 + acetate	• 20% increase in TAG content under nitrogen starvation conditions	(Kong et al., 2017)
TAGL	*P. tricornutum*	na	• Antisense Knock-down of *TAGL*	CO_2	• 2-fold increase in TAG content	(Barka et al., 2016)
ELT	*C. reinhardtii*	Chloroplast	• CRISPR/Cas9 Knock-out of *ELT*	CO_2 + acetate	• 27% increase in total lipid content	(Nguyen et al., 2020)
PL	*C. reinhardtii*	ER membrane	• CRISPR/Cas9 Knock-out of *PLA2*	CO_2 + acetate	• 37% and 111% increase in lipid content at the growth and N starvation phases, respectively	(Shin et al., 2019)
PL	*T. pseudonana*	na	• RNAi knock-down of lipase/ phospholipase/acyltransferase	CO_2	• 4.1-fold increase in lipid content under silicon starvation conditions	(Trentacoste et al., 2013)
Transcription factor engineering						
Dof	*C. reinhardtii*	Nucleus	• Overexpression of soybean *Dof* TF	CO_2 + acetate	• 2-fold increase in total lipids which are mainly fatty acids	(Ibanez-Salazar et al., 2014)
Dof	*C. ellipsoidea*	Nucleus	• Overexpression of heterologous *GmDof4*	CO_2	• 52.9% increase in lipid content with significant upregulation of *ACCase*	(Zhang et al., 2014)
Dof	*C. reinhardtii*	Nucleus	• Overexpression of endogenous *CrDof*	CO_2 + acetate	• 23.24% increase in total fatty acid content with the positive alteration of the expression level of fatty acid synthesis genes	(Jia et al., 2019)
ZnCys	*N. gaditana*	Nucleus	• CRISPR/Cas9 Knock-out of *ZnCys*	CO_2	• 2-fold increase in lipid content with a decrease of protein synthesis by ZnCys TF deletion	(Ajjawi et al., 2017)
PSR1	*C. reinhardtii*	Nucleus	• Overexpression of endogenous *PSR1*	CO_2 + acetate	• Enhanced lipid and TAG production under nutrient starvation conditions	(Ngan et al., 2015)
bZIP	*N. salina*	Nucleus	• Overexpression of endogenous *NsbZIP1*	CO_2	• 60% increase in FAME content under high salt conditions with the upregulation of *ACBP*, *KAS*, *LC-FACS*, and *LPAT*	(Kwon et al., 2018)
bZIP	*Chlorella* sp. HS2	Nucleus	• Overexpression of endogenous *HSbZIP1*	Glucose	• 113% increase in FAME yields under heterotrophic conditions with upregulation of *ACC1*, *KCS4*, and *KCS11*	(Lee et al., 2020)
bHLH	*N. salina*	Nucleus	• Overexpression of endogenous *NsbHLH2*	CO_2	• 49% increase in FAME productivity under N limited continuous culture conditions	(Kang et al., 2015) (Kang et al., 2019)
WRI1	*N. salina*	Nucleus	• Overexpression of heterologous AtWRI1	CO_2	• 64% increase in FAME yield under normal conditions with the upregulation of *PPDK*, *LPL*, *LPGAT1*, and *PDH* and the downregulation of *TAGL* and *DAGK*	(Kang et al., 2017)
bZIP	*N. oceanica*	Nucleus	• Overexpression of endogenous *NobZIP1*	CO_2	• 16.2-fold increase in extracellular lipid content through the weakened cell wall • ChIP and qRT-PCR revealed that the overexpression of *NobZIP1* upregulated *ACBP*, *KAS*, *LC-FACS*, *LPAT*, *CPS*, and *UDPase*.	(Li et al., 2019)
Enhancing reducing equivalents (NADPH)						
G6PDH	*P. tricornutum*	Chloroplast	• Overexpression of endogenous *G6PDH*	CO_2	• 15.76% increase in lipid content	(Wu et al., 2019)
G6PDH	*P. tricornutum*	Chloroplast	• Overexpression of endogenous *G6PDH* (*PtG6PD*)	CO_2	• 2.7-fold increase in lipid content with the fatty acid composition change	(Xue et al., 2017)
G6PDH	*C. pyrenoidosa*	na	• Overexpression of heterologous *NoG6PD*	CO_2	• 3.09-fold increase in neutral lipid content	(Xue et al., 2020)
ME	*P. tricornutum*	Cytoplasm	• Overexpression of endogenous *PtME*	CO_2	• 2.5-fold increase in lipid content	(Xue et al., 2015)
ME	*C. protothecoides*	na	• Overexpression of endogenous *ME*	CO_2	• 2.8-fold increase in total lipid content	(Yan et al., 2019)

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Table 1 (*continued*)

Target gene	Strain	Enzyme location	Metabolic engineering strategy	Carbon source	Result	Reference
ME	*C. reinhardtii* PTS42	Cytoplasm	• Overexpression of endogenous *ME2*	CO_2 + acetate	• 23.4% and 19.9% increase in fatty acid and lipid contents, respectively	(Kim et al., 2019)
ME	*C. pyrenoidosa*	na	• Overexpression of NADP-*PtME*	CO_2	• 4.6-fold increase in total lipid content under nitrogen deprivation conditions	(Xue et al., 2016)
ME	*T. obliquus*	na	• Overexpression of NADP-*PtME*	CO_2	• 2.4-fold increase in lipid content	(Xue et al., 2021)
ME	*N. salina*	Chloroplast	• Overexpression of endogenous *NsME1*	CO_2	• 53% increase in FAME yield by enhanced CO_2 fixation and reducing power	(Jeon et al., 2021)
ME, ACC	*D. salina*	Chloroplast	• Overexpression of *Brassica napus AccD* and *C. reinhardtii ME*	CO_2	• 12% increase in total lipid content	(Talebi et al., 2014)

na: data not available.
See Fig. 1 legend regarding abbreviations of metabolites and genes.

genes.

The overexpression of endogenous *GPDH* led to a 60% increase in the lipid content (Yao et al., 2014) in *P. tricornutum*. Glycerol kinase (*GK*) can contribute the TAG synthesis by catalyzing the conversion of glycerol to G3P. Indeed, the endogenous *GK* overexpressing *Fistulifera solaris* JPCC DA0580 showed a 12% increase in lipid productivity (Muto et al., 2015). The overexpression of plastidial GPATs (*AGPAT1* and *PtGPAT*) also resulted in a two-fold increase in TAG or neutral lipid contents in *P. tricornutum* (Balamurugan et al., 2017; Niu et al., 2016). The overexpression of *GPAT2* in *P. tricornutum* increased lipid production with alteration of the fatty acid composition (a significant increase in C16:0), conferring abiotic stress resistance to hyposaline and chilling. (Wang et al., 2020). The overexpression of the plastid *LPAT* (*NeoLPAAT1*) in *Neochloris oleoabundans* also increased the lipid and TAG productivities by 2.4- and 2.8-fold, respectively (Chungjatupornchai et al., 2019).

DGAT is the most studied enzyme to increase lipid production in microalgae because it is the last acylation step of TAG production and is considered a rate-limiting step. In microalgae, there are four types of DGAT; type-I (DGAT1), type-II (DGAT2), type-III (DGAT3), and wax ester synthase/acyl-coenzyme A (acyl-CoA):diacylglycerol acyltransferase (WS/DGAT). The endogenous *DGAT1* (*NoDGAT1A*) of *N. oceanica* was overexpressed in the chloroplast ER (cER), resulting in 2.4-fold and 47% increases in the TAG content and yield (Wei et al., 2017). The overexpression of the endogenous *DGAT2* in *N. oceanica* increased TAG contents by 69% and 129%, relative to wild type (WT), under nitrogen repletion and depletion conditions, respectively (Li et al., 2016). The overexpression of endogenous *DGAT2* in *N. oleoabundans* and *P. tricornutum* also successfully enhanced TAG and total lipid contents (Klaitong et al., 2017; Niu et al., 2013), and the heterologous expression of *Brassica napus DGAT2* in *C. reinhardtii* increased total lipid, fatty acid, and neutral lipid contents (Ahmad et al., 2015). The engineered *Scenedesmus obliquus* with the heterologous expression of *C. reinhardtii DGAT2* (*DGTT1*) showed a 127.8% increase in the lipid content in 40-L tubular type bioreactor under autotrophic conditions (Chen et al., 2016). The multifunctional wax ester synthase and Acyl-CoA:diacylglycerol acyltransferases (PtWS/DGAT) overexpressing *P. tricornutum* also showed a 40% increase in the TAG content (Cui et al., 2018).

The synthesized TAGs remain in the cytosol as lipid droplets (LDs). Thus, the TAG content was increased by 3.6-fold (Zulu et al., 2017) in *P. tricornutum* when the lipid droplet (LD) stabilizing oleosin protein 3 from *Arabidopsis thaliana* (*AtOLEO3*) was overexpressed with *S. cerevisiae* DGAT (*ScDGA1*).

As the genetic engineering tools for microalgae grow, promising results by co-overexpression of multiple genes involved in the TAG synthesis pathway have been reported (Chungjatupornchai & Fa-Aroonsawat, 2021; Munoz et al., 2019; Wang et al., 2018; Zou et al., 2018). Interestingly, Hsieh et al. 2012 expressed the five heterologous genes involved in the TAG pathway (*S. cerevisiae GPDH*, *GPAT*, *LPAAT*, *PAP*, and *Y. lipolytica DGAT*) in *Chlorella minutissima* simultaneously, leading to a two-fold increase in lipid content (Hsieh et al., 2012).

There were approaches to co-overexpress genes involved in the fatty acid and TAG synthesis. The overexpression of the endogenous *FAT* and *DGAT2* in *Coccomyxa* sp. resulted in a 1.4-fold increase in the lipid productivity (Kasai et al., 2018), and the expression of *S. cerevisiae ACC*, *GK*, and *GPDH* in *Scenedesmus quadricauda* also led to a 1.45-fold increase in lipid content (Gomma et al., 2015).

2.3. Blocking competing pathways

Blocking the competing metabolic pathways of the lipid synthesis is another effective strategy to improve lipid production in microalgae. As knockdown and knockout are relatively tricky as compared to the overexpression of transgenes in microalgae, the competing pathways have been rather less regulated. RNAi and antisense RNA methods were employed to downregulate the expression levels of targeted genes (*TAGL*, *CIS*, *PEPC*, *PDK*, etc) in microalgae. Recently, as genome-editing techniques with CRISPR/Cas9 are available in microalgae, targeted knockout and knock-in of *AGPase* or *UDPase* genes via genome editing are also facilitated to block competing metabolic pathways for lipid production (Jeon et al., 2017).

Carbohydrate synthesis could be competitive with the lipid synthesis pathway because microalgae store carbohydrates as an energy reserve instead of lipids. Green algae usually accumulate carbohydrates in the form of starch. After carbon dioxide fixation via the Calvin cycle, glyceraldehyde-3-phosphate (GAP) is converted to glucose-1-phosphate (G1P) through several metabolic steps. The first committed step for starch production in green microalgae is the conversion of G1P to AGP-glucose by ADP-glucose pyrophosphorylase (AGPase). In *Tetraselmis* sp., the deletion of *AGPase* by CRISPR/Cas9 improved the lipid content by a 3.1-fold (Chang et al., 2020). Unlike green algae, heterokont and diatom accumulate carbohydrates as chrysolaminarin. G1P is converted to UDP-glucose, the precursor of chrysolaminarin, by UDP-glucose pyrophosphorylase (*UGPase*). In diatom *P. tricornutum*, *UGPase* was knocked out by transcription activator-like effector nuclease (TALEN), resulting in a 45-fold increase in TAG content (Daboussi et al., 2014). The antisense knockdown of the *P. tricornutum UGPase* also led to a 25% increase in lipid content with a 62% decrease of chrysolaminarin (Zhu et al., 2016).

Acetyl-CoA is a key precursor in lipid synthesis as well as growth and energy metabolism in the TCA cycle. Thus, lipid production can be enhanced by increasing acetyl-CoA and by preventing other uses of acetyl-CoA. Pyruvate dehydrogenase kinase (PDK) deactivates the pyruvate dehydrogenase complex (PDC). In *N. salina* and *P. tricornutum*, downregulation of pyruvate dehydrogenase kinase (PDK) activated the PDC, increasing acetyl-CoA and lipid contents (Ma et al., 2017; Ma et al., 2014). Downregulation of citrate synthase (*CIS*) and phosphoenolpyruvate carboxylase (*PEPC*), involved in the consumption of acetyl-CoA during the TCA cycle, also increased the TAG and lipid contents by 1.7-fold and 1.2-fold in *C. reinhardtii*, respectively (Deng et al., 2013; Deng et al., 2014).

Another competing pathway of lipid production is β-oxidation in the peroxisome where the acyl-CoA, a precursor of TAG, is catabolized to

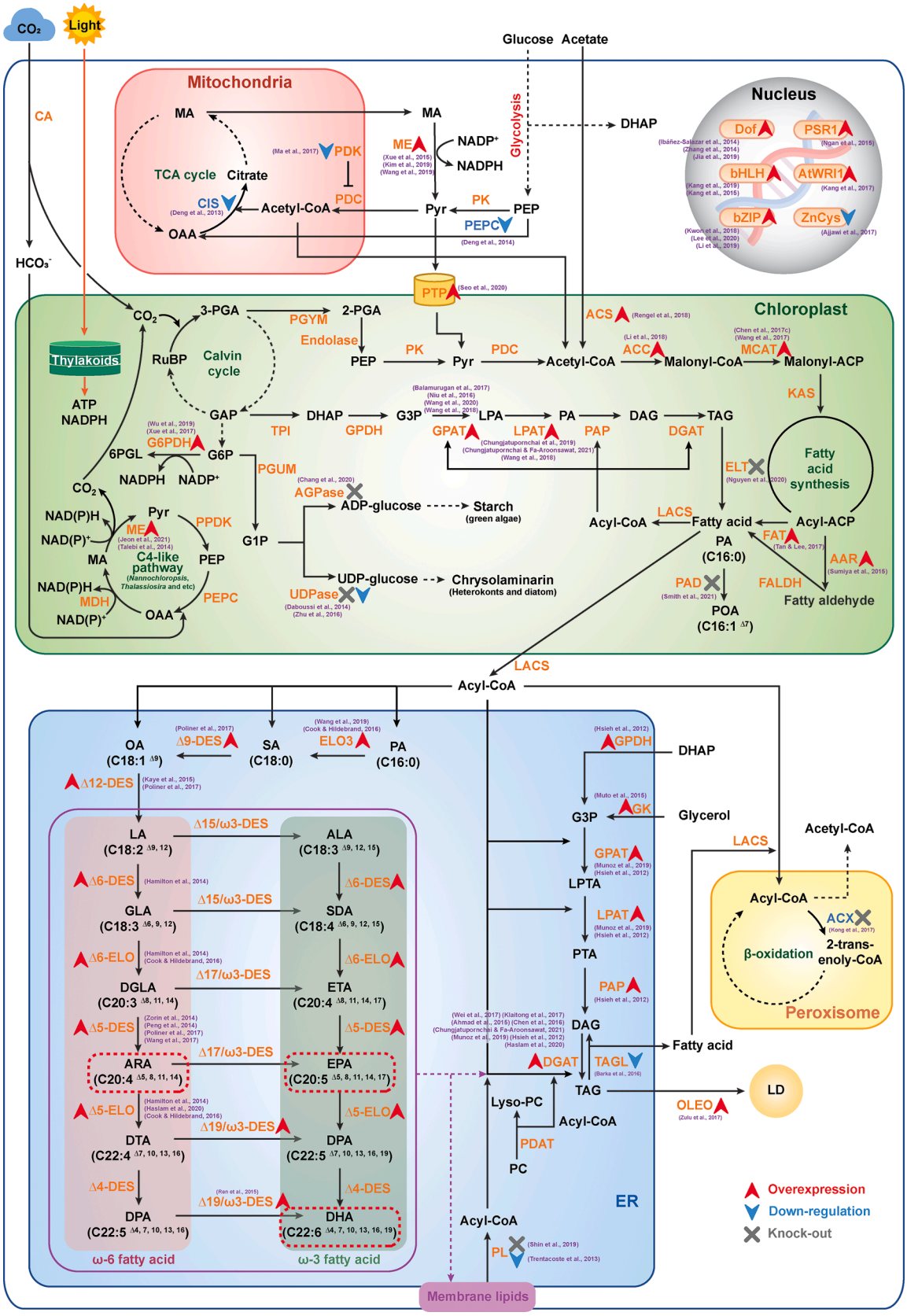

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Fig. 1. A schematic overview of metabolic engineering attempts for the enhanced production of lipids and LC-PUFAs. Dashed lines indicate simplified multi-reactions in a pathway. The red dashed box indicates the target products of LC-PUFAs. Black and orange letters represent metabolites and enzymes, respectively. Purple letters represent the references in which the genes were manipulated. The references in which the location of enzymes was not clearly identified were omitted in the map. The structure of the organelle may be slightly different depending on microalgae species. Abbreviations: 2-PGA, 2-phosphoglycerate; 3-PGA, 3-phosphoglycerate; 6PGL, 6-phosphogluconolactone; AAR, acyl-ACP reductase; ACC, acetyl-CoA carboxylase; ACS, acetyl-CoA synthetase; ACX, acyl-CoA oxidase dehydrogenase; AGPase, ADP-glucose pyrophosphorylase; ALA, α-linolenic acid; ARA, arachidonic acid; AtWRI1, Arabidopsis Wrinkled1; bHLH, basic helix-loop-helix; bZIP, basic leucine zipper; CA, carbonic anhydrase; CIS, citrate synthase; DAG, diacylglycerol; DES, desaturase; DGAT, diacylglycerol acyltransferase; DGLA, dihomo-γ-linolenic acid; DHA, docosahexaenoic acid; DHAP, dihydroxyacetone phosphate; DOF, DNA binding with one finger; DPA, docosapentaenoic acid; DTA, docosatetraenoic acid; ELO, elongase; ELT, esterase/lipase/thioesterase; EPA, eicosapentaenoic acid; ER, endoplasmic reticulum; ETA, eicosatetraenoic acid; FAT, fatty acyl-ACP thioesterase; FALDH, fatty aldehyde dehydrogenase; G1P, glucose-1-phosphate; G3P, glycerol-3-phosphate; G6P, glucose-6-phosphate; G6PDH, glucose-6-phosphate dehydrogenase; GAP, glyceraldehyde-3-phosphate; GK, glycerol kinase; GLA, γ-linolenic acid; GPAT, glycerol-3-phosphate acyltransferase; GPDH, glycerol-3-phosphate dehydrogenase; LA, linoleic acid; LACS, long-chain acyl-CoA synthetase; LD, lipid droplet; LPAT, lysophosphatidic acid acyltransferase; LPTA, lysophosphatidic acid; MA, malate; ME, malic enzyme; OA, oleic acid; OAA, oxaloacetate; OELO, oleosin; PA, palmitic acid; PAP, phosphatidic acid phosphatase; PEP, phosphoenolpyruvate; PEPC, Phosphoenolpyruvate carboxylase; PDC, Pyruvate dehydrogenase complex; PDK, pyruvate dehydrogenase kinase; PGUM, phosphoglucomutase; PGYM, phosphoglyceratemutase; PK, pyruvate kinase; POA, PSR1, phosphorus stress response; palmitoleic acid; PTA, phosphatidic acid; Pyr, pyruvate; RuBP, ribulose biphosphate; RuBisCO, ribulose-1,5-bisphosphate carboxylase/oxygenase; SA, stearic acid; SDA, stearidonic acid; TAG, triacylglycerol; TAGL, triacylglycerol lipase; TPI, triose phosphate isomerase; UGPase, UDP-glucose pyrophosphorylase; (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

acetyl-CoA. The metabolic reaction of acyl-CoA oxidase dehydrogenase (ACX), converting acyl-CoA to 2-*trans*-enoly-CoA, is the committed step of β-oxidation in the peroxisome. When the *C. reinhardtii* ACX (*CrACX2*) was knocked out, a 20% increase of the TAG content was shown under nitrogen starvation conditions (Kong et al., 2017).

Reducing the catabolism of lipids can also be an efficient tactic. TAG-lipase (TAGL) catalyzes the breakdown of TAGs, and the catabolized TAGs are finally converted to acetyl-CoA through β-oxidation. When *TAGL* was downregulated in *P. tricornutum*, there was a doubling of the TAG content (Barka et al., 2016). Nguyen et al. (2020) identified and deleted the esterase/lipase/thioesterase (*ELT*), leading to a 27% increase in total lipid content (Nguyen et al., 2020). Phospholipase (PL) contributes remodeling of membrane lipids for TAG synthesis. As such, the suppression of phospholipase A_2 (PLA2) in *C. reinhardtii* or lipase/phospholipase/acyltransferase in *Thalassiosira pseudonana* resulted in 2-fold or 4.1-fold increases in the lipid contents, respectively, under nutrient starvation conditions (Shin et al., 2019; Trentacoste et al., 2013).

2.4. Transcription factor engineering

Conventional metabolic engineering regulating individual genes is limited in making desirable phenotypes in microalgae. On the other hand, as one transcription factor (TF) regulates multiple genes, transcription factor engineering has attracted attention as an effective strategy.

It has been reported that DNA binding with one finger (Dof) type TF increased lipid production in soybean (Wang et al., 2007). As such, the expression of the soybean Dof-type TF into *C. reinhardtii* and *C. ellipsoidea* increased the lipid contents up to 2-fold and 52.9%, respectively, as compared to their parental strains (Ibanez-Salazar et al., 2014; Zhang et al., 2014). Jia et al. (2019) overexpressed endogenous Dof TF (*CrDof*) and observed a 23% increase of fatty acid contents in *C. reinhardtii*. They also found that the mRNA expression levels of genes involved in lipid metabolism were positively altered for lipid production (Jia et al., 2019).

In *Nannochloropsis gaditana*, the deletion of a *ZnCys* TF by CRISPR-Cas9 system improved carbon partitioning to lipid synthesis and thereby doubled lipid productivity (Ajjawi et al., 2017). Ngan et al. (2015) identified that the *C. reinhardtii* phosphorus stress response 1 (PSR1) TF triggered lipid accumulation and overexpressed the *PSR1* TF, resulting in an increase in lipid contents under nutrient starvation conditions (Ngan et al., 2015).

Transcription factor engineering was further developed with the binding site analysis. In *Nannochloropsis oceanica* IMET1, 78 TF-TFBS (transcription factor binding site) interaction pairs were identified, which consisted of 34 TFs, 30 TFBS motifs, and 2,368 regulatory connections (Hu et al., 2014). Through the analysis, it was suggested that a bZIP TF could regulate the expression of acyl-CoA-binding proteins (*ACBP*), 3-Ketoacyl-ACP synthase (*KAS*), long-chain acyl-CoA synthetases (*LC-FACS*), and *LPAT* which are involved in the lipid synthesis pathway (Hu et al., 2014). Indeed, the bZIP homolog was overexpressed in *N. salina*, leading to a 60% increase in lipid content with the upregulation of the four target genes (*ACBP, KAS, LCFACS, and LPAT*) (Kwon et al., 2018). The overexpression of the bZIP homolog in *Chlorella* sp. HS2 also increased FAME yields (113%) under heterotrophic conditions with the upregulation of *ACC* and two 3-ketoacyl-CoA synthases (*KCS*) (Lee et al., 2020). The overexpression of the endogenous bHLH2 (*NsbHLH2*) and the heterologous *Arabidopsis thaliana* Wrinkled1 (*AtWRI1*) also successfully enhanced lipid production in *N. salina* (Kang et al., 2015; Kang et al., 2017). Additionally, the potential NsbHLH2- and AtWRI1-regulated genes involved in lipid synthesis were identified by computational TFBS prediction. It was confirmed that the enhanced lipid production was attributed to the altered expression levels of the genes (Kang et al., 2019). Li et al. (2019) also overexpressed the endogenous bZIP1 (*NobZIP1*) in *N. oceanica*, and increased lipid production (Li et al., 2019). Interestingly, they experimentally identified the NobZIP1-regulated genes (*ACBP*, *KAS*, *LC-FACS*, *LPAT*, CPS (putative capsular polysaccharide synthesis gene), and *UDPase*) by a chromatin immunoprecipitation (ChIP) analysis, and confirmed that the upregulation of the NobZIP1-regulated genes enhanced the lipid content. In particular, the weakened cell wall by the upregulated *UDPase* induced lipid secretion, resulting in a 16.2-fold increase in the extracellular lipid content. These studies show that the multi-gene regulation by transcription factors can effectively enhance the production of lipids.

2.5. Enhancing the levels of NADPH

Fatty acid synthesis requires reducing equivalents such as NADPH, produced via the pentose phosphate pathway (PPP) and the transhydrogenase cycle. In the PPP, glucose-6-phosphate dehydrogenase (G6PDH) converts glucose-6-phosphate (G6P) to 6-phosphogluconolactone (6PGL), producing NADPH. Indeed, the overexpression of endogenous *G6PDH* in *P. tricornutum* and heterologous *N. oceanica G6PDH* (*NoG6PDH*) in *Chlorella pyrenoidosa* enhanced lipid production (Wu et al., 2019; Xue et al., 2017; Xue et al., 2020). In the transhydrogenase cycle, a malic enzyme (ME) also generates NADPH when converting malate to pyruvate. Thus, the overexpression of the endogenous *ME* in *P. tricornutum*, *C. protothecoides*, and *C. reinhardtii* resulted in 2.5-fold, 2.8-fold, and 23.4% increases in lipid contents, respectively (Kim et al., 2019; Xue et al., 2015; Yan et al., 2019), and the heterologous expression of *P. tricornutum* malic enzyme (*PtME*) also enhanced lipid production in *C. pyrenoidosa* and *Tetradesmus obliquus* (Xue et al., 2021; Xue et al., 2016). Jeon et al. (2021) found that the endogenous

chloroplastic NADP-dependent malic enzyme (NsME1) not only produced NAPDH, but also enhanced the C4-like carbon concentrating mechanism in *N. salina*. Thus, the overexpression of NADP-*NsME1* improved lipid production by the increased intracellular carbon and NADPH reducing power. (Jeon et al., 2021). Talebi et al. (2014) tried to supply NADPH with acetyl-CoA in *Dunaliella salina* by the overexpression of *Brassica napus ACC* subunit D (*AccD*) and *C. reinhardtii ME*, and observed a 15% increase in the lipid content (Talebi et al., 2014).

3. Metabolic engineering strategies for LC-PUFA

Microalgae produce long chain-polyunsaturated fatty acids (LC-PUFAs) which have many health benefits. Based on the length of their carbon chains and the number of conjugated double bonds in the position, LC-PUFAs are divided into two major groups: omega-3 (ω-3) and omega-6 (ω-6).

Eicosapentaenoic acid (EPA, C20:5) and docosahexaenoic acid (DHA, C22:6) in the ω-3 family of fatty acids and arachidonic acid (ARA, C20:4) in the ω-6 family of fatty acids were the main target LC-PUFAs of microalgae metabolic engineering. In particular, *Phaeodactylum*, *Schizochytrium*, and *Nannochloropsis,* which inherently accumulate high amounts of LC-PUFAs, were the primary strains used for metabolic engineering to increase LC-PUFA production further.

Microalgae LC-PUFAs are synthesized by the aerobic desaturation and elongation pathway in the ER (Monroig et al., 2013). The synthesis of ω-3 and ω-6 fatty acids begins with the sequential addition of double bonds to stearic acid (18:0, SA) by Δ9 desaturase (Δ9-DES) and Δ12 desaturases (Δ12-DES) to produce linoleic acid (18:2, LA). LA can be further desaturated by Δ15 desaturases (Δ15-DES), producing α-linoleic acid (C18:3, ALA). Then, Δ6 desaturases (Δ6-DES), Δ6 elongases (Δ6-ELO), and Δ5 desaturases (Δ5-DES) catalyze LA and ALA into ARA and EPA, respectively, through the sequential reactions (front-end desaturation and elongation). EPA can be further catalyzed by Δ5 elongases (Δ5-ELO) and Δ4 desaturases (Δ4-DES), thereby producing DHA. The reported metabolic engineering studies for LC-PUFAs are summarized in Table 2 and Fig. 1

3.1. Arachidonic acid

Enhancing ARA production using metabolic engineering was attempted in *N. oceanica* and *Lobosphaera incisa*. When the researchers overexpressed endogenous *Δ12-DES* (*NoD12*) in *N. oceanica*, ARA increased up to 50–75% of total fatty acids (Kaye et al., 2015). Zorin et al. (2014) complemented the *L. incisa* mutant strain containing the inactivated version of the Δ5-DES, and found that the ARA content was restored (25% of TFA) and even exceeded that of *L. incisa* WT (16% of TFA) (Zorin et al., 2014). Wang et al. (2017) overexpressed the endogenous *Δ5-DES* (*PtD5b*) with the endogenous *MCAT*, catalyzing the committed step of the fatty acid synthesis. The resulting strain showed a 2-fold increase in ARA contents as compared to the parental strain. This engineered strain also exhibited 3.42- and 2-fold increases in DHA and EPA contents (Wang et al., 2017).

3.2. Eicosapentaenoic acid

P. tricornutum and *Nannochloropsis* are mainly engineered for the production of EPA. The overexpression of *Δ5-DES* in *P. tricornutum* resulted in a 58% increase in EPA with a 65% increase in neutral lipid (Peng et al., 2014). Poliner et al. (2017) investigated the effects of Δ5-, Δ9-, and Δ12-DES on EPA production in *N. oceanica*. The overexpression of single *Δ5-FAD* or *Δ12-FAD* resulted in a 25% increase in EPA mol ratio, respectively. Although they co-overexpressed *Δ9/Δ12-DES* or *Δ5/Δ9/Δ12-DES*, the improvement of EPA was similar to the single overexpression lines (Poliner et al., 2018). Smith et al. (2021) identified and knocked out a plastidial palmitoyl-ACP D9-desaturase (*PAD*) which converts PA (C16:0) to palmitoleic acid (POA, C16:1) in *P. tricornutum*.

Table 2
Summary of metabolic engineering studies to enhance LC-PUFA production in microalgae.

Product	Strain	Target gene	Metabolic engineering strategy	Carbon source	Result	Reference
ARA	*N. oceanica*	*Δ12-DES*	• Overexpression of *NoD12*	CO_2	• 50–75% increase in ARA of total fatty acids at stationary phase under nitrogen starvation conditions	(Kaye et al., 2015)
ARA	*L. incisa*	*Δ5-DES*	• Complementation of P127 (inactive version of the Δ5-DES)	CO_2	• Restoration of ARA content (25% of TFA), which is even surpassed the WT ARA content (16% of TFA)	(Zorin et al., 2014)
EPA	*P. tricornutum*	*Δ5-DES*	• Overexpression of *PtD5b*	CO_2	• 58% and 65% increases in EPA and neutral lipid contents, respectively	(Peng et al., 2014)
EPA	*N. oceanica* CCMP1779	*Δ5-DES, Δ9-DES, Δ12-DES*	• Overexpression of endogenous *Δ5-DES*, *Δ9-DES*, and *Δ12-DES* with various combinations	CO_2	• 25% increase in EPA mol ratio	(Poliner et al., 2018)
EPA	*P. tricornutum*	*Plastid Δ9-DES*	• Knock-out of *PAD*	CO_2	• 1.32– to 1.42-fold increase in EPA content	(Smith et al., 2021)
DHA	*P. tricornutum*	*Δ5-ELO, Δ6-DES*	• Overexpression of heterologous *OtElo5* • Co-overexpression of heterologous *OtD6* and *OtElo5*	CO_2	• 8-fold increase in DHA content in TAG by the overexpression of *OtElo5* • A further increase in DHA content by the overexpression of *OtElo5* and *OtD6*	(Hamilton et al., 2014)
DHA	*Schizochytrium* sp.	*ω3-DES*	• Overexpression of *S. diclina* ω-3 *DES*	Glucose	• w-3/w-6 ratio was improved from 2.1 to 2.58, resulting in 49.23% DHA of total fatty acids	(Ren et al., 2015)
DHA	*P. tricornutum*	*DGAT, Δ5-ELO*	• Co-expression of endogenous *DGAT2B* and heterologous *OtElo5*	CO_2	• Significant increase in DHA levels	(Haslam et al., 2020)
DHA	*Schizochytrium* sp.	*ME, ELO3*	• Overexpression of *C. cohnii ME* and *M. alpina ELO3*	Glucose	• 1.39-fold increase in DHA content	(Wang et al., 2019)
DHA	*T. pseudonana*	*Δ6-ELO, Δ5-ELO, ELO3*	• Overexpression of endogenous *Δ6-ELO*, *Δ5-ELO*, and *ELO3*, respectively.	CO_2	• 2.3- to 4.3-fold increase in DHA content	(Cook & Hildebrand, 2015)
ARA, DHA, EPA	*P. tricornutum*	*MCAT, Δ5-DES*	• Co-overexpression of endogenous *MCAT* and *PtD5b*	CO_2	• 2-, 3.42-, and 2-fold increases in ARA (18.98 μg mg^{-1}), DHA (15 μg mg^{-1}), and EPA (85.35 μg mg^{-1}) contents	(Wang et al., 2017)

See Fig. 1 legend regarding abbreviations of metabolites and genes.

As oleic acid (OA, C18:1), stearic acid (SA, C18:0), and palmitic acid (PA, C16:0) are precursors for LC-PUFAs in the ER, the production of POA (C16:1) by PAD in the chloroplast might be a competitive metabolic reaction. Indeed, the deletion of *PAD* led to a 1.32 to 1.42-fold increase in the EPA content (Smith et al., 2021).

3.3. Docosahexaenoic acid

Metabolic engineering to increase DHA production was mainly conducted in *P. tricornutum* and *Schizochytrium* sp.. The expression of *Ostreococcus tauri Δ5-ELO* (*OtElo5*) led to an 8-fold increase in DHA content in TAG, and the co-expression of *OtElo5* and *O. tauri Δ6-DES*

Table 3
Summary of metabolic engineering studies for the production of terpenoids in microalgae.

Type of terpenoids (carbon number)	Product	Strain	Target gene (enzyme localization)	Metabolic engineering strategy	Carbon source	Result	Reference
Monoterpenes (C10)	Geraniol	*P. tricornutum*	• *CrGES* (cytosol)	• Episome-based expression of *CrGES* by the inducible alkaline phosphatase gene promoter (AP 1p) or the endogenous constitutive Phatr3_J492020p promoter.	CO_2	• 48.60 μg L^{-1} of geraniol production in 72 h by the inducible promoter (AP 1p) • 309 μg L^{-1} in 7 days by the constitutive promoter (Phatr3_J492020p)	(Fabris et al., 2020)
Sesquiterpenes (C15)	Patchoulol	*C. reinhardtii*	• *CrFPPS* (near the nucleus) • *E. coli IsPA* (cytosol) • *S. cerevisiae ERG20* (cytosol) • *PcPS* (cytosol)	• Overexpression of *C. reinhardtii FPPS*, *E. coli IsPA*, and *S. cerevisiae ERG20* to increase *FPP*, the precursor of Patchoulol • 3 times overexpression of *PcPS*	CO_2 + acetate	• The 3 times overexpression of *PcPS* was most effective. • 922 μg g^{-1} DCW of patchoulol production under mixotrophic conditions • 1.03 mg L^{-1} of sesquiterpenoids production under photoautotrophic conditions	(Lauersen et al., 2016)
Sesquiterpenes (C15)	(E)-α-bisabolene	*C. reinhardtii*	• *AgBs* (cytosol) • *SQS* (na) • *AGPase* (chloroplast) • *GGPPS* (chloroplast) • *PFT* (cytosol)	• 3 times overexpression of *AgBs* • amiRNA knock-down of *SQS*, *AGPase*, *GGPPS*, and *PFT*	CO_2 + acetate	• 3 times *AgBS* with *SQS* knock-down was most effective. • 11.0 mg L^{-1} of (E)-α-bisabolene under light/dark cycling and mixotrophic conditions	(Wichmann et al., 2018)
Sesquiterpenes (C15)	Squalene	*C. reinhardtii*	• *CrSQS* (ER membrane) • *CrSQE* (ER)	• Overexpression of endogenous *CrSQS* • amiRNA knock-down of endogenous *CrSQE*	CO_2 + acetate	• Only *CrSQE* knock-down strain produced 0.9–1.1 μg mg^{-1} DCW of squalene without any growth inhibition	(Kajikawa et al., 2015)
Diterpenes (C20)	Labdane diterpenes	*C. reinhardtii*	• *CcCLS* (chloroplast)	• Overexpression of *CcCLS* from *C. creticus*	CO_2 + acetate	• The production of four labdane-type diterpene compounds (ent-manoyl oxide, sclareol, labda-13-ene-8α,15-diol, and ent-13-epi-manoyl oxide) • Total labdane-type diterpene concentration of 1.172 ± 0.05 μg mg^{-1} DCW • Sclareol concentration 0.038 ± 0.001 μg mg^{-1} DCW	(Papaefthimiou et al., 2019)
Diterpenes (C20)	13R(+) manoyl oxide	*C. reinhardtii*	• *SpDXS* (chloroplast) • *TbTXS* (chloroplast) • *RcCBS* (chloroplast) • *ERG20(F96C)* (chloroplast) • *CfTPS* 2 and 3 (chloroplast) • *CfCYP76AH16* (chloroplast)	• Expression of *SpDXS* and *ERG20(F96C)* to increase GGPP • Expression of *RcCBS* and *TbTXS* for casbene and taxadiene production, respectively. • 3 times expression of *CfTPS2*, single expression of *CfTPS3*, and single expression of *ERG20 (F96C)* for *13R(+) Manloy oxide* production • Overexpression of *CfTPS2*, *CfTPS3*, and *CfCYP76AH16* for 9-OH 13R(+) manoyl oxide production	CO_2 + acetate	• Each engineered strain produced the target products (casbene, taxadiene, 13R(+) Manloy oxide, and 9-OH 13R(+) manoyl oxide. • The engineered strain (3 times expression of CfPS2, single expression of CfTPS3, and single expression of ERG20(F96C)) produced 40 mg L^{-1} of 13R(+) manoyl oxide under light/dark cycling (16:8 L:D) and CO_2 conditions.	(Lauersen et al., 2018)
Triterpenes (C30)	Lupenol, betulin	*P. tricornutum*	• *LjLUS* (cytosol) • *MtCYP716A12* (na) • *MtCPR* (na)	• Co-expression of *LjLUS*, *MtCYP716A12*, and *MtCPR*	CO_2	• 0.1 mg L^{-1} of lupeol in 2 days in the LjLUS expressing strain • Successful production of betulin with the engineered strain (*LjLUS*, *MtCYP716A12*, and *MtCPR*) in 550-L pilot-scale photobioreactor	(D'Adamo et al., 2019)

na: data not available.
See Fig. 2 legend regarding abbreviations of metabolites and genes.

(*OtD6*) further improved DHA production (Hamilton et al., 2014). Ren et al. (2015) expressed *Saprolegnia diclina* ω-3 desaturase which converts ω-6 to ω-3 in *Schizochytrium* sp. and observed that ω-3/ω-6 ratio was improved from 2.1 to 2.58, resulting in 49.23% of DHA content in total fatty acids (Ren et al., 2015). The individual overexpression of three elongases (*Δ6-ELO*, *Δ5-ELO*, and *ELO3*) in *T. pseudonana* also led to a 2.3 to 4.3-fold increase in DHA content (Cook & Hildebrand, 2015).

As LC-PUFAs can be acylated into glycerolipids for TAG synthesis, the improvement of TAG production could positively affect DHA production and storage. Haslam et al. (2020) overexpressed the endogenous type 2 *DGAT* (*DGAT2B*) with *O. tauri Δ5-ELO* (*OtElo5*), resulting in a significant increase in DHA-containing TAGs (Haslam et al., 2020). Wang et al. (2019) overexpressed the *Crypthecodinium cohnii ME* and the *Mortierella alpina ELO3* in *Schizochytrium* sp. to provide NADPH to LC-PUFA synthesis. The engineered *Schizochytrium* sp. by the co-overexpression of *C. cohnii ME* and the *M. alpina ELO3* showed a 1.39-fold increase in the DHA content (Wang et al., 2019).

4. Metabolic engineering strategies for terpenoids

Isoprenoids, including terpenoids and carotenoids, are widely used for pigments, perfumes, cosmetics, and pharmaceuticals. As chlorophyll and carotenoid pigments, which are molecules derived from isoprenoid metabolism, are necessary for the photosynthesis of microalgae, a carbon flux should be directed to the isoprenoid pathway in microalgae.

There are two pathways for producing isoprenoids: 2-c-methyl-d-erythritol 4-phosphate/1-deoxy-d-xylulose 5-phosphate (MEP/DOXP) pathway, referred to as the MEP pathway, and the mevalonate (MVA) pathway. In microalgae, the MEP and MVA pathways are located in the chloroplast and cytosol, respectively. Generally, green algae have only the MEP pathway, and diatoms have both MVA and MEP pathways. Both pathways produce 5-carbon precursors, isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) from which various isoprenoid products begin. Prenyl transferases catalyze the serial condensation of IPP and DMAPP to make higher-carbon terpenoids. According to the number of carbons, terpenoids are classified as follows: hemiterpenes (C5), monoterpenes (C10), sesquiterpenes (C15), diterpenes (C20), triterpenes (C30), and tetraterpenes (C40). In the MEP and MVA pathways, geranyl pyrophosphate (GPP, C10), farnesyl pyrophosphate (FPP, C15), and geranylgeranyl pyrophosphate (GGPP, C20) are important precursors for the production of various terpenoids. The reported metabolic engineering studies for terpenoid production in microalgae are summarized in Table 3 and Fig. 2.

4.1. Monoterpenoids

Fabris et al. (2020) produced heterologous monoterpenoid geraniol, a component of perfumes and insect repellents, in diatom *P. tricornutum* (Fabris et al., 2020). They found that *P. tricornutum* did not make geraniol, although they have a GPP (C10) pool which is a precursor of geraniol. Thus, they expressed *Catharanthus roseus* geraniol synthase (CrGES), which is verified as the most effective enzyme for geraniol production in *S. cerevisiae*, into the *P. tricornutum* cytosol (Jiang et al., 2017). Interestingly, they used an episome vector for the extrachromosomal expression of *CrGES* under the control of inducible alkaline phosphatase gene promoter (AP1p) and the endogenous constitutive promoter (Phatr3_J492020p), resulting in 48.60 μg L^{-1} and 309 μg L^{-1} of geraniol production, respectively. For the first time, they reported not only the heterologous production of monoterpenoids but also the extrachromosomal expression using a plasmid in *P. tricornutum*. This plasmid-based expression can be a useful tool for future metabolic engineering in *P. tricornutum*.

4.2. Sesquiterpenoids

Metabolic engineering of microalgae for the production of sesquiterpenoids was attempted with *C. reinhardtii*. Generally, FPP is produced through the MVA pathway in the cytosol and serves as an essential precursor for sesquiterpenoids. Although green algae *C. reinhardtii* has only the MEP pathway in the chloroplast, the endogenous FPP synthase (FPPS) was identified near the nucleus (Lauersen et al., 2016). However, it is still unclear how the IPP and DMAPP in the chloroplast are transferred and converted to FPP.

Lauersen et al. (2016) produced the sesquiterpenoid patchoulol as a marker of sesquiterpenoid production capacity by introducing the *Pogostemon cabin* patchoulol synthase (PcPS) into the *C. reinhardtii* cytosol (Lauersen et al., 2016). They tried to increase the FPP pool by expressing *Chlamydomonas FPPS*, *E. coli FPPS* (*ispA*), and *S. cerevisiae FPPS* (*ERG20*). However, the expression of the *FFPSs* did not induce a significant increase in patchoulol production, indicating that the FPP pool was not limited. Instead, as insufficient enzyme expression titers could prevent the desired phenotype, they overexpressed *PcPS* three times, resulting in 922 μg g^{-1} dry cell weight (DCW) in TAP medium.

Wichmann et al. (2018) produced (E)-α-bisabolene, the sesquiterpene biodiesel precursor, in the *C. reinhardtii* cytosol (Wichmann et al., 2018). They conducted three times overexpression of *Abies grandis* bisabolene synthase (*AgBS*), which converts FPP to (E)-α-bisabolene. Furthermore, they attempted to downregulate competing pathways via amiRNA knockdown. First, the ADP-glucose pyrophosphorylase small subunit (AGPP), a key enzyme for starch synthesis in *C. reinhardtii*, was downregulated to increase the flux towards the MEP pathway. As the chloroplast GGPP synthesis, which can be the competing pathway for the production of (E)-α-bisabolene in the cytosol, was also knocked down. They also downregulated the protein farnesyltransferase (*PFT*) and squalene synthase (*SQS*) which convert FPP to protein farnesyl tails and squalene, respectively, to prevent the flux from FPP to other metabolites. As the knockdown of SQS was most effective for the production of (E)-α-bisabolene, they produced the engineered strain by combining the three times sequential *AgBS* expression and the amiRNA knockdown of *SQS*. The engineered strain produced 10.3 ± 0.7 mg g^{-1} DCW of (E)-α-bisabolene which is a 15-fold increase as compared to the single expression of AgBs. They further increased (E)-α-bisabolene titer (11.0 mg L^{-1}) under light/dark cycle conditions, because FPP pools could increase to make ubiquinone (UQ) in the mitochondria for respiration during dark cultivation.

Squalene, a versatile sesquiterpenoid, can be produced from FPP by SQS, and converted to 2,3-oxidosqualene by squalene epoxidase (SQE) in the ER (Kajikawa et al., 2015). Although Kajikawa et al. (2015) attempted to overexpress the endogenous squalene synthase (*CrSQS*) and downregulate squalene epoxidase (*CrSQE*) to increase squalene production, the CrSQE-knockdown mutant only accumulated 0.9–1.1 μg mg^{-1} DCW of squalene.

4.3. Diterpenoids

GGPP (C20), produced through the MEP pathway in the chloroplast, is a pivotal precursor for diterpenes. Thus, the reported diterpenoid production studies conducted the metabolic engineering in the chloroplast.

Papaefthimiou et al. (2019) produced labdane-type diterpenes by overexpressing *Cistus creticus* copal-8-ol diphosphate synthase (*CcCLS*) (Papaefthimiou et al., 2019). They integrated *CcCLS* into the chloroplast genome to express the CcCLS in the chloroplast. The CcCLS converted GGPP to copal-8-ol-diphosphate, and copal-8-ol-diphosphate is cyclized to the four labdane-type diterpene compounds (ent-manoyl oxide, sclareol, labda-13-ene-8α,15-diol, and ent-13-epi-manoyl oxide). The total diterpenoid concentration of the CcCLS overexpressing strain was increased to 1.172 ± 0.05 μg mg^{-1} DCW. Particularly, sclareol which is an important precursor for pharmaceutical and cosmetics was produced at about 0.038 ± 0.001 μg mg^{-1} DCW.

Lauersen et al. (2018) reported metabolic engineering strategies to make various diterpenes such as taxadiene, casbene, and 13R(+) manoyl

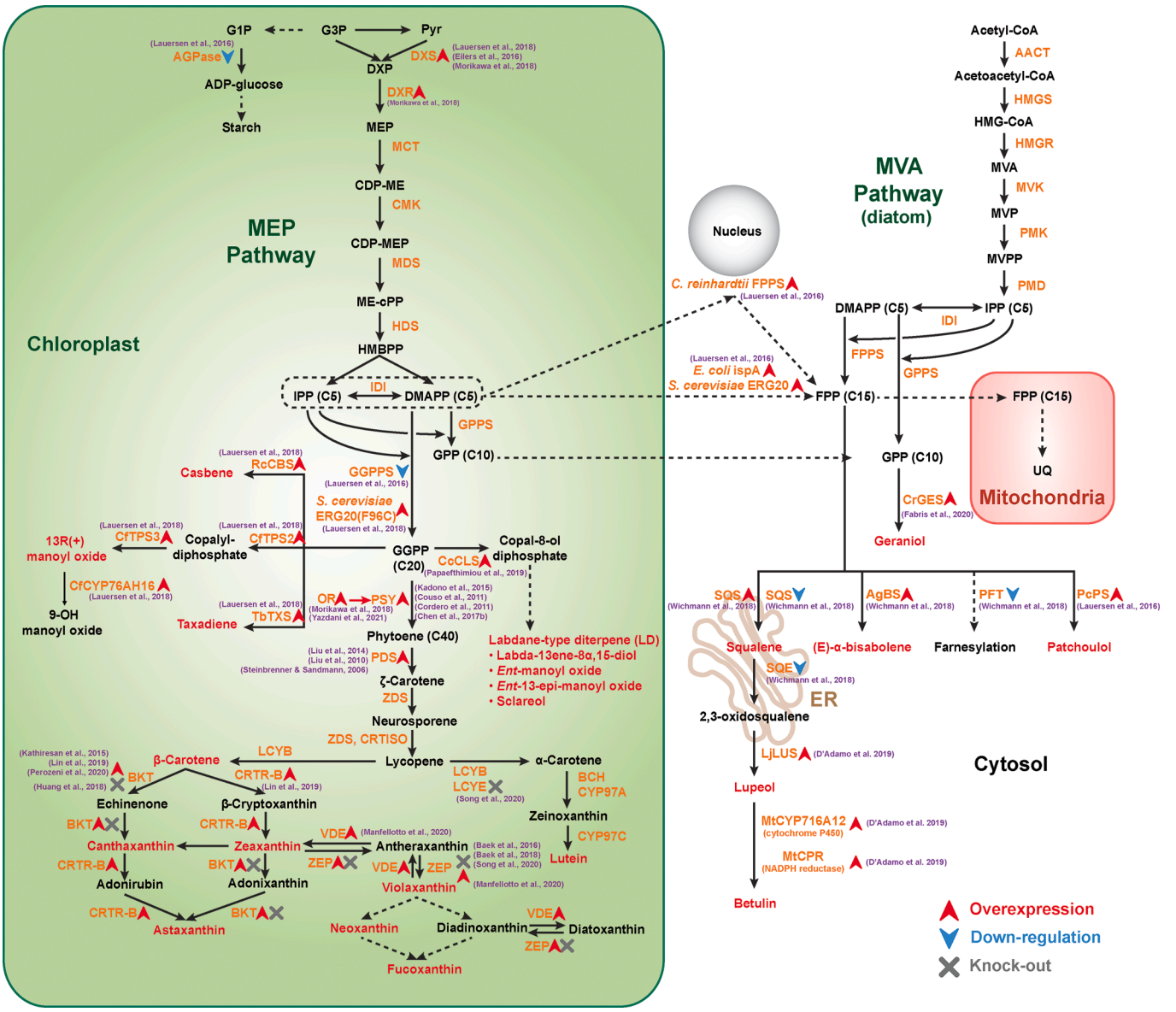

Fig. 2. A schematic overview of metabolic engineering attempts for the enhanced production of terpenoids and carotenoids. Dashed lines indicate simplified multi-reactions in a pathway. Black, orange, and red letters represent metabolites, enzymes, and target products, respectively. Purple letters represent the references in which the genes were manipulated. The structure of the organelle may be slightly different depending on microalgae species.Abbreviations: AACT, acetyl-CoA C-acetyltransferase; AgBS, *Abies grandis* bisabolene synthase; AGPase, ADP-glucose pyrophosphorylase; BCH, β-carotene hydrolase; BKT, β-carotene ketolase; CcCLS, *Cistus creticus* copal-8-ol diphosphate synthase; CDP-ME, 4-(cytidine 5′-diphospho)-2-C-methyl-D-erythritol; CDP-MEP, CDP-ME 2-phosphate; CfTPS, *Coleus forskohlii* diterpene synthase; CMK, 4-(cytidine 5′-diphospho)-2-C-methyl-D-erythritol kinase; CrGES, *Catharanthus roseus* geraniol synthase; CRTISO, carotene isomerase; CRTR-B, β-carotene hydroxylase; CYP97A, cytochrome P450 carotene β-hydroxylase; CYP97C, cytochrome P450 carotene ε-hydroxylase; DMAPP, dimethylallyl pyrophosphate; DXP, 1-deoxy-d-xylulose 5-phosphate; DXR, 1-deoxy-D-xylulose 5-phosphate reductase; ER, endoplasmic reticulum; FPP, farnesyl pyrophosphate; FPPS, farnesyl pyrophosphate synthase; G1P, glucose-1-phosphate; G3P, glycerol-3-phosphate; GGPP, geranylgeranyl pyrophosphate; GGPPS, geranylgeranyl pyrophosphate synthase; GPP, geranyl pyrophosphate; GPPS, geranyl pyrophosphate synthase; HDS, 1-hydroxy-2-methyl-2-butenyl 4-diphosphate synthase; HMBPP, 1-hydroxy-2-methyl-2-butenyl 4-diphosphate; HMG-CoA, 3-hydroxy-3-methylglutaryl CoA; HMGR, hydroxymethylglutaryl-CoA reductase; HMGS, hydroxymethylglutaryl-CoA synthase; IDI, isopentenyl-diphosphate delta-isomerase; IPP, isopentenyl pyrophosphate; LCYB, lycopene β-cyclase; LCYE, lycopene ε-cyclase; LjLUS, *Lotus japonicus* lupeol synthase; MCT, MEP cytidylyltransferase; MDS, 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase; MEP, 2-c-methyl-d-erythritol 4-phosphate; ME-cPP, 2-C-methyl-D-erythritol 2,4-cyclodiphosphate; Mito, mitochondria; MtCPR, *M. truncatula* P450-NADPH reductase; MtCYP716A12, *M. truncatula* CYP716A12; MVA, mevalonate; MVK, mevalonate kinase; MVP, 5-phosphomevalonate; MVPP, 5-diphosphomevalonate; OR, orange protein; PcPS, *Pogostemon cablin* patchoulolsynthase; PDS, phytoene desaturase; PFT, protein farnesyltransferase; PMD, diphosphomevalonate decarboxylase; PMK, phosphomevalonate kinase; PSY, phytoene synthase; PtDXS, *Phaeodactylum tricornutum* 1-deoxy-D-xylulose 5-phosphate synthase; Pyr, pyruvate; RcCBS, *Ricinus communis* casbene synthase; SpDXS, *Salvia pomifera* 1-deoxy-D-xylulose 5-phosphate synthase; SQS, squalene synthase; SQE, squalene epoxidase; TbTXS, *Taxus brevifolia* taxadiene synthases; VDE, violaxanthin de-epoxidase; UQ, ubiquinone; ZDS, ζ-carotene desaturase; ZEP, zeaxanthin epoxidase. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

oxide in *C. reinhardtii* (Lauersen et al., 2018). As these diterpenoids are also produced from GGPP in the chloroplast, chloroplast targeting peptide was used to express heterologous genes into the chloroplast. In order to increase fluxes towards GGPP, they introduced *Salvia pomifera* 1-deoxy-D-xylulose 5-phosphate synthase (*SpDXS*), which is the first committed step of the MEP pathway, and *S. cerevisiae* GGPPS *ERG20 (F96C)*. They then expressed *Ricinus communis* casbene synthase (*RcCBS*) and *Taxus brevifolia* taxadiene synthases (*TbTXS*) to produce casbene and taxadiene, respectively. *Coleus forskohlii* diterpene synthase 2 and 3 (*CfTPS2* and *CfTPS3*) were also expressed to produce 13R(+) manoyl oxide. Among the various combinations of the gene expression, the engineered strain with three times overexpression of the *CfTPS2* and a single time expression of the *CfTPS3* and *ERG20(F96C)* showed the highest yield of 13*R*(+) manoyl oxide (40 ± 8 mg L^{-1} and 1120 ± 220 fg $cell^{-1}$). Additionally, they produced 9-OH manoyl oxide through hydroxy-functionalization of 13*R*(+) manoyl oxide by introducing *C. forskohlii* cytochrome P450 monooxygenase (CfCYP76AH16) into the engineered *C. reinhardtti* (2X CfTPS2 and 1X CfTPS3). This indicated that the first controlled functionalization of a non-native metabolite in any green algae by metabolic engineering.

4.4. Triterpenoids

D'Adamo et al. (2019) aimed to make the triterpene betulin, which is

Table 4
Summary of metabolic engineering studies for the production of carotenoids in microalgae.

Target gene	Product	Strain	Metabolic engineering strategy	Carbon source	Result	Reference
DXS, PSY	Fucoxanthin	*P. tricornutum*	• Overexpression of endogenous *DXS* or *PSY*	CO_2	• The DXS and PSY transformants produced 24.2 mg g^{-1} and 18.4 mg g^{-1} DCW fucoxanthin, resulting in 2- and 1.8-fold increases, respectively.	(Eilers et al., 2015)
PSY	Fucoxanthin	*P. tricornutum*	• Overexpression of endogenous *PSY*	CO_2	• 1.45-fold increase in fucoxanthin content	(Kadono et al., 2015)
PSY	violaxanthin, lutein, β-carotene, and neoxanthin	*C. reinhardtii*	• Overexpression of *D. salina PSY*	CO_2 + acetate	• 2-, 2.6-, 1.25-, and 1.8-fold increases in violaxanthin, lutein, β-carotene, and neoxanthin contents, respectively	(Couso et al., 2011)
PSY	violaxanthin and lutein	*C. reinhardtii*	• Overexpression of *C. zofingiensis PSY*	CO_2 + acetate	• 2.0- and 2.2-fold increases in violaxanthin and lutein contents, respectively	(Cordero et al., 2011)
PSY	β-Carotene	*Scenedesmus* sp. *CPC2*	• Overexpression of synthesized *PSY* based on highly consensus regions of three *PSYs* from *C. reinhardtii*, *D. salina*, and *M. zofingiensis*	CO_2	• 31.8 mg g^{-1} DCW of β-carotene, resulting in a 3-fold increase	(Chen et al., 2017b)
DXS, DXR, OR	Lutein and β-carotene	*C. reinhardtii*	• Overexpression of each of *DXS*, *DXR*, and *OR* (DnaJ-like chaperone)	CO_2 + acetate	• 1.5- and 1.3-fold increases in the lutein and β-carotene titers, respectively, by overexpressing only *OR*	(Morikawa et al., 2018)
OR	α-carotene, lutein, β-carotene, and violaxanthin	*C. reinhardtii*	• Overexpression of endogenous OR ($CrOR^{WT}$) and the mutated OR ($CrOR^{His}$) containing a single histidine substitution	CO_2 + acetate	• 1.9-, 2-, 2.1-, and 2.1-fold increases in α-carotene, lutein, β-carotene, and violaxanthin contents by overexpressing $CrOR^{WT}$ • 4-, 3.1-, 3.2-, and 3.6-fold increases in α-carotene, lutein, β-carotene, and violaxanthin contents by overexpressing $CrOR^{His}$	(Yazdani et al., 2021)
PDS	Astaxanthin	*C. zofingiensis*	• Overexpression of *PDS-L516F*	CO_2	• 32.1% and 54.1% increases in total carotenoids and astaxanthin contents, respectively, with norflurazon-resistance	(Liu et al., 2014) (Liu et al., 2010)
PDS	Astaxanthin	*H. pluvialis*	• Overexpression of *PDS-L504R*	CO_2 + acetate	• 32.6% increase in astaxanthin content with norflurazon-resistance	(Steinbrenner & Sandmann, 2006)
BKT	Astaxanthin	*H. pluvialis*	• Overexpression of endogenous *BKT*	CO_2 + acetate	• 2- to 3-fold increase in total carotenoids and astaxanthin contents	(Kathiresan et al., 2015)
BKT, CRTR-B	Astaxanthin and canthaxanthin	*D. viridis*	• Overexpression of *H. pluvialis CRTR-B* and *BKT* from the chloroplast genome	CO_2	• 77.5 ± 7.7 μg g^{-1} DCW of astaxanthin and 50.1 ± 0.8 μg g^{-1} DCW canthaxanthin by high light conditions	(Lin et al., 2019)
BKT	Astaxanthin	*C. reinhardtii*	• Overexpression of the modified endogenous *CrBKT* (removing the 116 amino acid C-terminal extension, which is absent from all homologs in BKTs of other organisms)	CO_2 + acetate	• The overexpression of the engineered *CrBKT* in *UVM4* produced 2.6–3.1 mg astaxanthin L^{-1} day^{-1} and 3.7–4.3 mg ketocarotenoid L^{-1} day^{-1} under mixotrophic conditions	(Perozeni et al., 2020)
BKT	Zeaxanthin, β-carotene, and lutein	*C. zofingiensis*	• Generation of a dysfunctional CZ-bkt1 carotenoid ketolase by chemical mutagenesis	CO_2 + glucose	• 36.79, 34.64, and 33.97 mg L^{-1} of zeaxanthin, β-carotene, and lutein, respectively, under high light and nitrogen deficiency conditions with glucose feeding	(Huang et al., 2018)
ZEP	Zeaxanthin	*C. reinhardtii*	• Knock-out of *ZEP* by CRISPR-Cas9 RNP method	CO_2	• 56-fold increase in zeaxanthin content and productivity, respectively	(Baek et al., 2016) (Baek et al., 2018)
ZEP, LCYE	Zeaxanthin	*C. reinhardtii*	• Knock-out of *ZEP* and *LCYE* by CRISPR-Cas9 RNP method	CO_2 + acetate	• 60% higher zeaxanthin yield (5.24 mg L^{-1}) than that of the ZEP single deletion strain	(Song et al., 2020)
VDE, VDR, ZEP	Fucoxanthin	*P. tricornutum*	• Overexpression of endogenous *VDE*, *VDR*, and *ZEP3*	CO_2	• 4-fold increase in fucoxanthin content	(Manfellotto et al., 2020)

See Fig. 2 legend regarding abbreviations of metabolites and genes.

a precursor to pharmaceutical treatments used against cancers and HIV, in *P. tricornutum* (D'Adamo et al., 2019). They expressed a heterologous *Lotus japonicus* lupeol synthase (LjLUS), converting 2,3-oxidosqualene to lupeol, into the *P. tricornutum* cytosol. Subsequently, the *Medicago truncatula* P450-NADPH reductase (*MtCPR*) and *M. truncatula* cytochrome P450 (*MtCYP716A12*) were expressed in the *Lj*LUS-expressing *P. tricornutum* for the oxidation of lupeol to betulin. In terms of MtCPR, they removed the first 71 amino acids, which are part of the transmembrane domain, to avoid unwanted membrane insertion or association, and fused it with MtCYP716A12 (MtCYP716A12-λ-D72CPR). As CYP716A12 family enzymes generally produce partial C28 oxidation products, the expression of MtCYP716A12 could catalyze the oxidation of lupeol into betulin or betulin aldehyde. Consequently, they confirmed betulin production from the engineered strain with the expression of *Lj*LUS and MtCYP716A12-λ-D72CPR.

5. Carotenoids

Photosynthetic microalgae produce carotenoids, or tetraterpenoid (C40) pigments, in the chloroplast. Carotenoids act as light-harvesting antennas for photosynthesis and scavenge reactive oxygen species (ROS) that would otherwise cause photooxidative damage. (Kato & Hasunuma, 2021; Saini et al., 2020).

As carotenoids are isoprenoid products, they are produced through the MEP and carotenoid pathways in the chloroplast. The first and rate-limiting step of carotenogenesis is the condensation of GGPP to phytoene (C40) by phytoene synthase (PSY). Then, phytoene desaturase (PDS) converts phytoene to ζ-carotene, and subsequently, the ζ -carotene is converted to lycopene which is the branch point for α-carotene and β-carotene. The pathway which begins with β-carotene eventually produces zeaxanthin, astaxanthin, violaxanthin, and fucoxanthin, and the α-carotene pathway generates lutein. In this chapter, the studies of metabolic engineering for the production of carotenoids are summarized according to metabolic genes which were manipulated (Table 4). In addition, the metabolic pathways for carotenoids are also presented in Fig. 2.

5.1. Phytoene synthase, phytoene desaturase, and orange protein (enhancing from the MEP pathway to lycopene)

As the upstream pathway of lycopene is the same regardless of carotenoid pigments, several attempts have been made to increase inherent pigments by overexpressing major enzymes from the MEP pathway to the lycopene synthesis pathway. In order to increase fucoxanthin in *P. tricornutum*, Eilers et al. (2015) overexpressed endogenous DXS, which is the committed step of the MEP pathway, and PSY, which converts GGPP into phytoene. The DXS and PSY overexpressing *P. tricornutum* increased fucoxanthin by 2- and 1.8-fold increased, respectively (Eilers et al., 2015). Kadono et al. (2015) also overexpressed an endogenous *PSY* in *P. tricornutum*, resulting in a 1.45-fold increase in fucoxanthin, relative to WT (Kadono et al., 2015). In *C. reinhardtii*, the overexpression of *D. salina* or *Chlorella zofingiensis PSY* also increased inherent carotenoids such as violaxanthin, lutein, β-carotene, and neoxanthin (Cordero et al., 2011; Couso et al., 2011). Chen et al. (2017) overexpressed the synthesized *PSY* based on the high consensus regions of three PSYs from *C. reinhardtii*, *D. salina*, and *Mariella zofingiensis* and produced 31.8 mg g^{-1} DCW of β-carotene, a 3-fold higher than that of WT. (Chen et al., 2017b) Morikawa et al. (2018) overexpressed the endogenous DnaJ-like chaperone, orange protein (*OR*), which supports PSY, and observed 1.5- and 1.3-fold increases of the lutein and β-carotene titers, respectively, in *C. reinhardtii* (Morikawa et al., 2018). The overexpression of the mutated *OR* (*CrOR*^{His}) containing a single histidine substitution also enhanced overall carotenoid production in *C. reinhardtii* (Yazdani et al., 2021). Likewise, the overexpression of *PSY* positively affected the production of overall carotenoids.

Phytoene desaturase (PDS), which converts phytoene to ζ-carotene, could also affect carotenoid production. The mutated PDS of *Chlorella zofingiensis* (PDS-L516F) and *Haematococcus pluvialis* (PDS-L504R) showed higher efficiency in converting phytoene to ζ -carotene with resistance to norflurazon. Thus, the overexpression of the modified *PDS* showed enhanced astaxanthin production with norflurazon resistance in *C. zofingiensis* and *H. pluvialis* (Liu et al., 2014; Liu et al., 2010; Steinbrenner & Sandmann, 2006). As these modified PDSs can be used not only to enhance carotenoids but also as a selection marker, they could play an important role in metabolic engineering for the production of carotenoids

5.2. β-carotene ketolase and β-carotene hydroxylase (enhancing the β-carotene pathway)

A β-carotene ketolase (BKT) and a β-carotene hydroxylase (CRTR-B) are key enzymes for the production of β-carotene-derived carotenoids such as astaxanthin and canthaxanthin. The overexpression of the endogenous *BKT* in *H. pluvialis* resulted in a 2- to 3-fold increase in total carotenoids and astaxanthin contents with an 8- to 10-fold increase in the intermediates such as echinenone and canthaxanthin (Kathiresan et al., 2015). The overexpression of *H. pluvialis BKT and CRTR-B* in *Dunaliella viridis*, which do not produce astaxanthin inherently, showed 77.5 ± 7.7 and 50.1 ± 0.8 μg g^{-1} DCW of astaxanthin and canthaxanthin, respectively, under high light conditions (Lin et al., 2019). Perozeni et al. (2020) identified the endogenous BKT (CrBKT) in *C. reinhardtii* and modified the CrBKT by removing the 116 amino acid C-terminal extension, which is absent from all homologs in BKTs of other organisms. The overexpression of the modified *CrBKT* increased the productivity of astaxanthin (2.6–3.1 mg astaxanthin L^{-1} day^{-1}) and ketocarotenoid (3.7–4.3 mg ketocarotenoid L^{-1} day^{-1}) under mixotrophic conditions (Perozeni et al., 2020). On the other hand, the dysfunctional BKT in *C. zofingiensis* induced accumulation of zeaxanthin, β-carotene, and lutein up to 36.79, 34.64, and 33.97 mg L^{-1} instead of astaxanthin production because the flux might not go to astaxanthin (Huang et al., 2018).

5.3. Zeaxanthin epoxidase and violaxanthin de-epoxidase

Zeaxanthin epoxidase (ZEP) and violaxanthin de-epoxidase (VDE) regulate the violaxanthin cycle and eventually affect the production of zeaxanthin and fucoxanthin. The deletion of *ZEP* gene via CRISPR-Cas9 RNP-mediated mutagenesis in *C. reinhardtii* led to a 56-fold increase in zeaxanthin content as compared to WT (Baek et al., 2018). The double deletion of the *ZEP* and *CpFTSY* enhanced growth and zeaxanthin content with the reduction of antenna size (Baek et al., 2016). The deletion of *ZEP* and lycopene ε-cyclase (*LCYE*) also enhanced zeaxanthin production by reducing the flux towards α-carotene in *C. reinhardtii* (Song et al., 2020). The co-overexpression of endogenous *VDE*, VDE-related (*VDR*), and *ZEP* in *P. tricornutum* showed 4-fold higher fucoxanthin content (Manfellotto et al., 2020).

6. Challenges and future perspectives

As microalgae can convert CO_2 into chemicals and fuels directly, they are considered promising sustainable feedstocks. However, it is still questionable if a microalgal bioprocess can be economically feasible. Thus, there are many efforts to solve the economic issues by improving strains via metabolic engineering.

In order to increase lipid production, a variety of genes involved in the fatty acid synthesis and Kennedy (TAG) pathway were overexpressed, and the carbohydrate synthesis pathways were down-regulated. Multi-gene regulation by transcription factor engineering and the enhancement of reducing power for fatty acid synthesis also increased lipid production. ω-3 and ω-6 LC-PUFAs were increased by regulating various fatty acid elongases and desaturases. In terms of terpenoids and

carotenoids, a number of endogenous or heterologous genes have been up-or down-regulated to increase titers of inherent products or produce non-native products. Likewise, the metabolic engineering of microalgae has made lots of progress in the last decade. Based on the aforementioned metabolic engineering strategies, it would be enabled to find more efficient combinations of gene regulation to improve the productivity of the target products or to produce more diverse bioproducts in microalgae.

However, microalgal genetic and metabolic engineering still have many challenges. To date, microalgal genetic engineering has mainly relied on random integration, because episomal plasmid expression and homologous recombination are unavailable in most microalgae. Thus, unexpected and inconsistent phenotypes were issues according to the integrated location of transgenes. In addition, unstable expression and transgene silencing have hampered the development of microalgae metabolic engineering (Baier et al., 2018; Wu-Scharf et al., 2000). Recently, as various microalgae genetic engineering tools such as CRISPR/RNP or intron-mediated gene expression are innovated, more sophisticated and stable transformants will be produced (Baek et al., 2016; Baier et al., 2018; Shin et al., 2016).

Moreover, microalgae have complicated metabolic pathways that are compartmentalized through multiple organelles, and the structure of intracellular organelles and metabolic pathways are even different depending on the microalgae species (Radakovits et al., 2010; Wichmann et al., 2020). For example, in heterokont species such as *Nannochloropsis*, the ER is located in the chloroplast via endosymbiosis. Therefore, it is important to use an appropriate signal peptide to express a target protein at the desired site (Koh et al., 2019). As such, it is necessary to construct accurate metabolic models considering the organelles of target microalgae. Metabolic engineering advances would be enabled in microalgae if the location and function of targeted metabolic pathways can be accurately understood and controlled.

For the commercial production of microalgal chemicals and fuels, cultivation strategies should also be developed in parallel with metabolic engineering. In general, value-added products of microalgae are induced under stress conditions, and the required stress conditions are different depending on target products (e.g., lipids under nutrient starvation conditions and carotenoids under high light conditions) (Chen et al., 2017a; Paliwal et al., 2017). Thus, the development of the cultivation method specific for a targeted product should be optimized according to engineered strains. Additionally, as microalgae can use inorganic materials in wastewater, integration of wastewater treatment with microalgal cultivation and metabolic engineering will be eco-friendly and economical (Bhatia et al., 2021). Taken together, the commercial production of fuels and chemicals would be accelerated with microalgal metabolic engineering.

7. Conclusion

Over the few decades, scientists have continued to innovate new microalgae metabolic engineering strategies. Recently, as more sophisticated metabolic engineering strategies have been facilitated by developing genetic engineering tools for microalgae, a systematic metabolic engineering strategy needs to be designed. As microalgae metabolic pathways are compartmentalized in different organelles, an accurate understanding of metabolism and elaborated manipulation is required. This work comprehensively reviewed metabolic engineering strategies for the production of lipids, LC-PUFAs, terpenoids, and carotenoids. Through the insightful combination of the strategies, efficient metabolic engineering using microalgal hosts will become a reality.

CRediT authorship contribution statement

Nam Kyu Kang: Conceptualization, Writing – original draft, Visualization. **Kwangryul Baek:** Writing – review & editing. **Hyun Gi Koh:** Writing – review & editing. **Christine Anne Atkinson:** Writing – review & editing. **Donald R. Ort:** Writing – review & editing, Supervision. **Yong-Su Jin:** Conceptualization, Writing – review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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