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Genome engineering via gene editing technologies in microalgae

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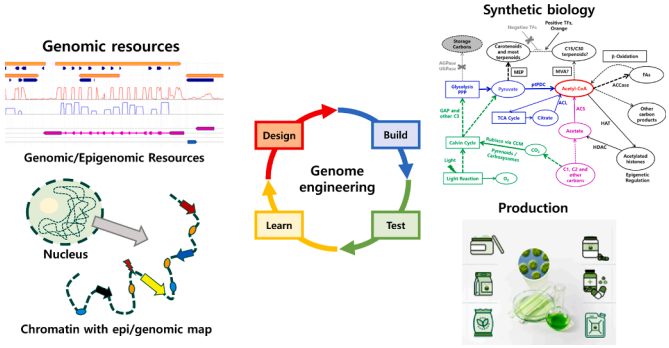
HIGHLIGHTS

- CRISPR-Cas has revolutionized gene editing in microalgae.
- Genome engineering using CRISPR-Cas results in genome deletion and replacement.
- *Epi*/genomic resources can be combined with CRISPR-Cas for improved engineering.
- Acetyl-CoA can be used as carbon cash for the metabolic hubs of carbon-based molecules.
- Synthetic biology of microalgae is proposed for terpenoid production.

GRAPHICAL ABSTRACT



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ABSTRACT

CRISPR-Cas has revolutionized genetic modification with its comparative simplicity and accuracy, and it can be used even at the genomic level. Microalgae are excellent feedstocks for biofuels and nutraceuticals because they contain high levels of fatty acids, carotenoids, and other metabolites; however, genome engineering for microalgae is not yet as developed as for other model organisms. Microalgal engineering at the genetic and metabolic levels is relatively well established, and a few genomic resources are available. Their genomic information was used for a "safe harbor" site for stable transgene expression in microalgae. This review proposes further genome engineering schemes including the construction of sgRNA libraries, pan-genomic and epigenomic resources, and mini-genomes, which can together be developed into synthetic biology for carbon-based engineering in microalgae. Acetyl-CoA is at the center of carbon metabolic pathways and is further reviewed for the production of molecules including terpenoids in microalgae.

1. Introduction

Modification of the genome has been achieved by random mutagenesis and selection of mutations; however, editing tools can now allow specific genomic mutations. Different methods of genome editing have been developed, including meganuclease, transcription activator-

Abbreviations: ACCase, acetyl-CoA carboxylase; ACL, ATP-citrate lyase; ACS, acetyl-CoA synthetase; CCM, carbon concentration mechanism; GAP, D-glyceraldehyde 3-phosphate; HAT, histone acetyltransferase; HDAC, histone deacetylase; TCA, tricarboxylic acid cycle; MEP, methyl-erythritol-4-phosphate pathway; MVA, mevalonic acid pathway; PPP, pentose phosphate pathway; ptPDC, plastidic pyruvate dehydrogenase complex.

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like effector nucleases (TALENs), zinc-finger nucleases (ZFNs), and clustered regularly interspaced short palindromic repeats/Cas9 (CRISPR-Cas9) (Gaj et al., 2013). For meganucleases, ZFNs, and TALENs, proteins must be designed to recognize specific genes. However, the CRISPR-Cas system is much simpler because it recognizes specific genes using designed guide RNAs (gRNAs) (Jeon et al., 2017; Knott & Doudna, 2018). CRISPR-Cas can now target genes at the genomic level, allowing genome engineering or "genome editing," although that term has been used interchangeably with gene editing. CRISPR-Cas systems have already been used for gene knockouts and knock-ins, multiplex gene targeting, and gene regulation in many animals, plants, and microalgae (Chen et al., 2019; Pickar-Oliver & Gersbach, 2019; Ryu et al., 2021).

Microalgae are sustainable and renewable feedstocks for biomolecules that can be used as essential nutrients and nutraceuticals (Borowitzka, 2013). Additionally, some species accumulate large quantities of energy-dense oils that can be converted into biofuels including biodiesel and aviation biofuels (Kumar et al 2023; Joshi & Mishra 2022; Park et al., 2019), and they are also being considered as sources of bioplastics (Madadi et al., 2021; Schmidtchen et al., 2022). To date, heterotrophic microbes have been the production platform of choice. However, microbial production requires expensive, large-scale fermenters, and the extraction of metabolites produced. However, microalgae are ideally suited for producing natural photosynthetic products because they natively host many biosynthetic pathways and produce essential precursor molecules using light and water (Sun et al., 2021).

Genomic resources are available for a few groups of microalgae, including *Chlamydomonas, Nannochloropsis*, and diatoms (Armbrust et al., 2004; Gong et al., 2020; Merchant et al., 2007), and that list is growing with advances in next-generation sequencing and ensuing cost reductions (Kselíková et al., 2022). It is now estimated that drafts and genomes are available for nearly 120 algal species (Kselíková et al., 2022). Together with multi-omics data and comparative genomics, these genomic resources enable the determination of target genes that can be mutagenized and characterized with CRISPR-Cas systems, as summarized in Table 1 and Table 2.

This review focuses on the latest developments in gene editing technologies, particularly using CRISPR-Cas in eukaryotic microalgae to achieve synthetic biology–based metabolic engineering. Many reviews of CRISPR-related genetic engineering in microalgae have been published (Brar et al., 2021; Jeon et al., 2017; Lee et al., 2023b; Sproles et al., 2021) however, this review provides genomic perspectives on CRISPR technologies with a focus on genome engineering and synthetic biology for the rational design of metabolic pathways that could improve the production of microalgal biofuels and metabolites, including carotenoids. Acetyl-CoA is at the centre of central carbon pathways, so we provide a detailed description of how it is produced and used as *carbon cash* in the biosynthesis of carbon-based molecules. In addition, we review new concepts in genome-level screening for safe harbor sites for transgene expression, mini-genomes, and proposals for the construction of sgRNA libraries.

2. Advances in gene editing technologies for microalgae

Gene editing technologies have revolutionized the reverse genetic field of biology and biotechnology by using sequence-specific nucleases such as ZFNs, TALENs, and CRISPR-associated protein 9 (Cas9) (Fig. 1). These nucleases enable the disruption of specific genes, and Cas9 and related Cas nucleases have gained popularity due to their simplicity, accuracy, and efficiency (Jain et al., 2021; Knott & Doudna, 2018). Initially referred to as genome editing (Komor et al., 2016), these reverse genetic techniques are often called *gene editing* due to the limited number of targeted genes (Jeon et al., 2017). However, it is time to consider actual genome editing as a tool for genome engineering to establish comprehensive genetic engineering and synthetic biology in industrial microalgae.

2.1. Microalgal gene editing using the CRISPR-Cas system

The first gene editing in microalgae was reported in *Chlamydomonas reinhardtii*, in which ZFNs were used to target the *COP3* gene by performing a proof-of-concept experiment (Sizova et al., 2013). Also, engineered TALENs were used in microalgae (Daboussi et al., 2014). However, due to the versatility and simplicity of the CRISPR-Cas system, ZFNs and TALENs are rarely used (Greiner et al., 2017; Kurita et al., 2020). Since the first genome editing using CRSPR-Cas9 was attempted in *Chlamydomonas* (Jiang et al., 2014), 10 different genera of eukaryotic microalgae have been studied, and their striking results are listed in Table 1 and Table 2.

2.1.1. Various Cas nucleases have been used for microalgae

To date, six different types of CRISPR-Cas systems (I–VI) have been described, and they are divided into two major classes (Class 1 and Class 2) (see detailed description in Jeon et al., 2017) (Jeon et al., 2017). In microalgae, DNA-targeting Class 2 CRISPR systems (types II and V) consist of large multi-domain effector proteins that use crRNA guides to target complementary DNA. After recognizing a protospacer adjacent motif (PAM) sequence near the target sequence, the Cas9 protein of the type II CRISPR-Cas system, which contains two nucleolytic domains, RuvC and HNH (Fig. 1) cleaves the target DNA strand and generates a double-stranded break (DSB) in the DNA.

Cas12a (also called Cpf1, a type V) was identified later and does not require *trans*-activating CRISPR RNA (tracrRNA) because it can cleave dsDNA as directed by crRNA. Cas12a contains no HNH domain, but its RuvC domain has been demonstrated to cleave both non-target and target DNA strands (Swarts & Jinek, 2018). In addition, Cas12a can autonomously process its crRNA (Swarts et al., 2017; Zetsche et al., 2017), which has been exploited to enable efficient multiple gene editing in microalgae (Naduthodi et al., 2019; Naduthodi et al., 2021; Serif et al., 2018). These results suggest the potential to expand the use of Cas12a into microalgae for efficient and high-throughput genome engineering. Greiner et al. (2017) compared the efficiency of Cas9 from *Streptococcus pyogenes* (SpCas9) and Cas9 from *Staphylococcus aureus* (SaCas9) in generating insertion/deletion (in/del) mutations in *C. reinhardtii* (Greiner et al., 2017). SaCas9 showed enhanced performance in *C. reinhardtii*, indicating the potential of evaluating its functional and toxic effects on various unexplored strains of Cas9 and Cas12a in microalgae.

Mutation of the catalytically active site of the Cas9 protein produces dead Cas9 (dCas9) that can enable CRISPR-customized gene regulation (Fig. 2). One method performs CRISPR-mediated knockdown using dCas9 linked to a transcriptional repressor to produce CRISPR interference (CRISPRi) (Pickar-Oliver & Gersbach, 2019). In *Chlamydomonas*, CRISPRi has been demonstrated to downregulate the phosphoenolpyruvate carboxylase gene involved in carbon partitioning (Kao & Ng, 2017). In *Chlorella sorokiniana,* multiple genes have been shown to be regulated by the CRISPRi system by using dCas9 and guanine-rich sgRNA (Lin et al., 2022). In *Nannochloropsis oceanica*, the CRISPRi system using dCas9 produced 85 % reduction in the transcript levels (Naduthodi et al., 2021). CRISPR activation (CRISPRa) uses the dCas9 protein with fused transcriptional activators such as VP64, p65, and RTA (Fig. 2). In *N. oceanica*, CRISPRa of the g1248 gene, a potential methyltransferase for DNA or mRNA, produced a 23 % increase in growth (Wei et al., 2022).

2.1.2. State-of-the-art precision base changes

Despite the ease of use and wide application of the conventional CRISPR-Cas system, concerns about the precision of this technology remain, and a number of unpredictable consequences, most of which results from DSB induced by conventional CRISPR editing, have been reported. However, CRISPR-Cas has been developed to perform precise

Table 1
Genome editing via CRRISPR-Cas in eukaryotic model microalgae.

Strain	Delivery of Cas nuclease/Cas type	Target gene (Abbreviation)	Resulting phenotype/related information	References
Chlamydomonas reinhardtii				
C. reinhardtii (CC-503)	Electroporation/plasmid/Cas9	*FKB12*	The first study of *Streptococcus pyogenes* cas9 (SpCas9) expression in *Chlamydomonas*. Expression of spCas9 protein using plasmid in *Chlamydomonas*. Transient expression of Cas9 and sgRNA genes suggest Cas9 toxicity.	(Jiang et al., 2014)
C. reinhardtii (CC-4349)	Electroporation/RNP/Cas9	*FTSY, ZEP*	Constitutively producing zeaxanthin (13-fold) with improved photosynthetic productivity (Pmax: ~2-fold/Chl basis at saturating irradiance), greater biomass accumulation under HL growth conditions.	(Baek et al., 2016)
C. reinhardtii (CC-124)	Electroporation/RNP (without or with Aph7)/Cas9	*MAA7, CpSRP43, ChlM*	Mutagenic efficiency was improved up to 100-fold compared to the first report of transgenic Cas9-induced mutagenesis. Findings of NHEJ-mediated knock-in events.	(Shin et al., 2016)
C. reinhardtii (CC-1883, CC-2931)	Electroporation/RNP (with ssODN(118nt, ~45nt homology arm)/Cas12a (formerly Cpf1)	*FKB12, CpFTSY, CpSRP43, PHT7*	The targeted knockout of genes using co-delivery of Cas12 a(Cpf1)-RNP with single-stranded DNA resulted in 0.5–16 % mutagenic efficiencies of cells.	(Ferenczi et al., 2017)
C. reinhardtii (CC-3403, CC-503, CC-125, SAG73.72)	Electroporation/plasmid, RNP (with selection marker)/Cas9	*COP½, COP3, COP4, COP5, PHOT, UVR8, VGCC, MAT3, aCRY, PSY1*	Optimized gene-editing protocols using Cas9 (plasmid vs RNP, homology arm: oligonucleotides vs PCR product vs circular plasmid, using SpCas9 vs SaCas9.	(Greiner et al., 2017)
C. reinhardtii (CC-503)	Electroporation/plasmid (or with 80nt ssDNA)/Cas9	*FKB12, ARG, ALS*	Cas9 containing an artificial intron with an inserted sgRNA gene was used for genome editing.	(Jiang & Weeks, 2017)
C. reinhardtii (CC-400)	Glass bead/plasmid/dCas9-KRAB (CRISPRi)	*RFP, PEPC1*	CRISPRi was first applied. Strains with downregulated CrPEPC1 showed increased lipid contents (highest: 28.5 % DCW; 74.4 % higher than WT) and productivity.	(Kao & Ng, 2017)
C. reinhardtii (CC-4349)	Electroporation/RNP (with *Aph7*)/Cas9	*PLA2*	The lipid productivity of *PLA2* knockout mutants increased by up to 64.25 % (to 80.92 g/L/d).	(Shin et al., 2019)
C. reinhardtii CW15	Electroporation/RNP (with *Aph7*and ~ 2 kb homology arm)/Cas9	*FTSY*	Transformation of synchronized cells and knock-in of specific foreign DNA at the desired genomic nuclear location by exploiting homologous recombination.	(Angstenberger et al., 2020)
C. reinhardtii (CC-503)	Electroporation/RNP (with or without *Aph7)*/Cas9	*CP26, CP29*	Photosystem II antenna complexes CP26 and CP29 were investigated. Absence of CP26 and CP29 impaired both photosynthetic efficiency and photoprotection: excitation energy transfer from the external antenna to the reaction center was reduced, and state transitions were completely impaired.	(Cazzaniga et al., 2020)
C. reinhardtii (CC-124, CC-400, UVM11)	Cell penetrating peptide/RNP/Cas9	*Maa7, FKB12*	Using cell-penetrating peptide(pVEC) transformation method.	(Kang et al., 2020)
C. reinhardtii (CC4349, CC124, CC503)	Electroporation/RNP (with *Aph7/ Gluc-Aph7 / Aph8-mVenus-Aph7* (6.4 kb)/Cas9	*FTSY*	CRISPR-Cas9-mediated knock-in was adopted to improve gene-editing efficiency and express the reporter genes (luciferase and mVenus) on the intended site.	(Kim et al., 2020)
C. reinhardtii (CC-125, CC-5415)	Electroporation, glass bead/RNP (with antibiotic-resistance cassette (0–50 bp homology arm))/Cas9	*IFT81, IFT43, MOT17, CDPK13, FAP70, CEP131*	Targeted insertional mutagenesis (TIM) utilizes delivery into the cell of a Cas9-RNP-mediated cell delivery with exogenous donor DNA. Mutation efficiencies ranged from 40 to 95 %.	(Picariello et al., 2020)
C. reinhardtii (CC-4349)	Electroporation/RNP (with *Aph7*)/Cas9	*ZEP, LCYE*	The double knockout mutants of *ZEP* and *LCYE* had a 60 % higher zeaxanthin yield (5.24 mg/L) than the single *ZEP* mutant.	(Song et al., 2020)
C. reinhardtii (CC-5415, CC-124)	Electroporation/RNP (with *Aph8* or ssODN, length of homology arms (30 to 70 nt) /Cas9	*PPX1, FTSY, WDTC1, ALS1*	Precise gene editing via HDR of *PPX1 (*as a selectable marker) to use for co-editing of *PPX1* and genes of interest such as *FTSY* or *WDTC1*.	(Akella et al., 2021)
C. reinhardtii (CC-1883)	Electroporation/RNP (without or with dsDNA (50 bp homology arm) or ssODN (<75nt homology arm)/Cas12a	*FKB12*	Studying single-strand template DNA repair (SSTR). SSTR at CRISPR/Cas12a-induced DNA DSBs relies precisely on NHEJ to maintain genomic stability in *Chlamydomonas.*	(Ferenczi et al., 2021)
C. reinhardtii (UVM4, N-UVM4)	Electroporation/RNP (with *Aph7* with 500 bp homology arm or a short multi-stop codon insert with 500 bp homology arm)/	*STA6, SPD1*	Making *SPD1* knock-out mutant (spermidine auxotrophy) for use as a novel selection marker.	(Freudenberg et al., 2022)

(*continued on next page*)

Table 1 (*continued*)

Strain	Delivery of Cas nuclease/Cas type	Target gene (Abbreviation)	Resulting phenotype/related information	References
	Cas9			
C. reinhardtii (CC-4349)	Electroporation/RNP/Cas9	*AGP, ZEP*	The oil productivity of the double knockout strains (*agp* and *zep)* in the one-step process was 1.81-fold that in the two-step process, but the zeaxanthin and lutein productivity remained the same.	(Song et al., 2022)
Phaeodactylum tricornutum				
P. tricornutum (CCMP2561)	Bombardment/plasmid/Cas9	*CpSRP54*	CRISPR/Cas9 technology can be used to generate stable targeted gene mutations in *P. tricornutum*.	(Nymark et al., 2016)
P. tricornutum (CCMP2561)	Bacterial conjugation/episomal plasmid/ Cas9	*Phatr3_J46193*	Cas9 delivery via bacterial conjugation can be applied for the delivery of episome to minimize unwanted changes in the genome.	(Russo et al., 2018)
P. tricornutum (CCMP2561)	Bombardment/plasmid (with NAT)/Cas9, Bombardment/RNP/Cas9	*UMPS, APT, Aureo1a*	Demonstration of the functionality of RNP delivery by using counter-selectable markers (*UMPS* and *APT*) and multiplexed genome editing by RNP complexes.	(Serif et al., 2018)
P. tricornutum (CCMP2561)	Bacterial conjugation/episomal plasmid/ Cas9, Bombardment/plasmid (without or with ShBle, 1 kb homology arm)/Cas9	*NR, GS-2, cGOGAT*	Both single- and two-gene knockout lines were generated with mutagenic efficiencies of 48 % and 25 %, respectively. HDR-mediated PtNR mutant was achieved.	(Moosburner et al., 2020)
P. tricornutum (CCMP2561)	Bombardment/plasmid/Cas9	*LHCFs*	Multiplexed knockout of LHCF gene family using single sgRNAs by exploiting the off-targeting of Cas9. Five of six LHCF genes were edited simultaneously.	(Sharma et al., 2021)
P. tricornutum (CCMP2561)	Bacterial conjugation/episomal plasmid/ Cas9	*ACSL1-ACSL5*	Generated knockout mutants with large fragment deletion using multiplexed CRISPR/Cas9.	(Hao et al., 2022)
P. tricornutum (CCAP1055/1)	Electroporation/plasmid (with NAT plasmid, 800 bp homology arm)/Cas9	*THIC, SSSP*	Investigation of the functions of the genes with associated TPP riboswitches. Through genome editing it is confirmed that *THIC* is essential for thiamine biosynthesis, and *SSSP* is necessary for thiamine uptake.	(Llavero-Pasquina et al., 2022)
P. tricornutum (CCMP2561)	Bacterial conjugation/plasmids/Cas9	*CryP*	Knockout lines had no change in cell morphology or growth but had increased fucoxanthin content (1.29-fold higher in *CryP* mutant than in WT) and FCP levels compared with the WT.	(Yang et al., 2022)
Thalassiosira pseudonana				
T. pseudonana (CCMP1335)	Bombardment/plasmid/Cas9	*Urease*	Demonstration of the feasibility of gene editing in *T. pseudonana*.	(Hopes et al., 2016)
T. pseudonana (CCMP1335)	Bombardment/Plasmid/Cas9	*Sin1*	The mutants exhibited reduced biosilica content with morphological aberration, compromising the strength and stiffness of their cell walls.	(Görlich et al., 2019)
T. pseudonana (CCMP1335)	Bombardment/Plasmid/Cas9 nickase (D10A) (dCas9)	*θ-type carbonic anhydrase*	Developed Cas9 nickase (D10A) system for genome editing of *T. pseudonana*.	(Nawaly et al., 2020)

Abbreviations: *ACSL*, long-chain acyl-CoA synthetases (LACS) isozymes; *AGP*, ADP-glucose pyrophosphorylase; *ALS*, acetolactate synthase; *APT*, adenine phosphoribosyl transferase; *ARG*, argininosuccinate lyase; *AUREO*, blue-light photoreceptor/transcription factor, aureochrome genes; *CDPK13*, calcium-dependent protein kinase 13; *CEP131*, centrosomal protein 131**;** *cGOGAT*, chloroplastic glutamate synthase; *ChlM*, Mg-protoporphyrin IX S-adenosyl methionine O-methyl transferase; *CP26, CP29*, photosystem II antenna complexes; *CpFTSY*, chloroplast signal recognition particle receptor protein; *CpSRP43*, chloroplast signal recognition particle 43; *CRY*, cryptochrome; *FAP*, flagella-associated proteins; *FKB12*, FK506-binding protein 12 (peptidyl-prolyl cis–trans isomerase); *GS-2*, glutamine synthetase II; *IFT*, Intraflagellar transport; *LCYE*, lycopene epsilon cyclase; *LHCF*, light-harvesting complexes family; *MAA7*, beta-subunit of tryptophan synthase; *MAT3*, strong homology to an animal retinoblastoma cancer gene involved in cell cycle control; *MOT17*, predicted protein(Identified by comparative genomics as being present only in organisms having motile (MOT) cilia); *NR*, nitrate reductase; *PEPC1*, phosphoenolpyruvate carboxylase isoform 1; *Phatr3_J46193*, *P. tricornutum* chr9: 533409–537647 locus; *PHT7*, phosphate transporter; *PLA2*, phospholipase A2; *PPX1*, protoporphyrinogen IX oxidase; *PSY1*, phytoene synthase-1; *RFP*, red fluorescent protein; *Sin1*, silicanin-1; *SPD*, spermidine synthase gene; SSSP, sodium-solute transporter (SSS) family; *STA6*, ADP-glucose pyrophosphorylase subunit; THIC, HMP-P synthase homologous; *UMPS*, uridine-monophosphate synthase; *VGCC*, voltage-gated calcium channel; *WDTC1*, WD and TetratriCopeptide repeats protein 1; *ZEP*, zeaxanthin epoxidase.

single-base editing of target sequences. Because dCas9 binds to the target without creating a DSB, it has been further developed for use as a *base editor* and *prime editor*.

To improve editing precision, several iterations of the technology been developed over the years. Base editors (BEs) enable the irreversible conversion of one base pair to another at a target genomic locus without the need for DNA DSBs, HDR, or donor DNA templates. BEs consist of the sgRNA-guided Cas9 nickase (nCas9) fused with a deaminase that causes C base to T/A base to G base conversions (Fig. 3A) (Komor et al., 2016). Compared with conventional genome editing systems that generate point mutations, BEs can proceed more efficiently and produce many fewer undesired in/del products or translocations (Kim et al., 2017; Komor et al., 2016). Cytosine and adenine base editing are now established as reliable methods to achieve precise genome editing in plant and animal research (Knott & Doudna, 2018; Mishra et al., 2020). Both cytosine and adenine BEs have achieved precise editing of point mutations in somatic cells, facilitating the development of gene therapy approaches (Knott & Doudna, 2018). Recently, dCas12a-assisted cytosine BE enabled base editing of the cyanobacterial genome by repressing uracil-DNA glycosylase (Lee et al., 2023a).

Table 2
Genome editing via CRRISPR-Cas in industrial model and non-model eukaryotic microalgae.

Strain	Delivery of Cas nuclease/Cas type	Target gene (Abbreviation)	Resulting phenotype/related information	References
Nannochloropsis				
N. oceanica IMET1	Electroporation/plasmid/Cas9	*NR*	Established a CRISPR/Cas9 genome-editing approach for the industrial oleaginous microalga.	(Wang et al., 2016)
N. gaditana (CCMP1894)	Electroporation/Cas9 editor line (with *Aph7*)/Cas9	18 genes of 20 transcription factors	Cas9 editor line. Mutants improved partitioning of total carbon to lipids from 20 % (WT) to 40–55 % (mutant) in nutrient-replete conditions.	(Ajjawi et al., 2017)
N. oceanica (CCMP1779)	Electroporation/episomal plasmid/ Cas9	*NR*	The use of CEN/ARS6 region from *Saccharomyces cerevisiae* as circular extrachromosomal DNA and, presentation of an episomal CRISPR/Cas9 system to generate marker-free non-transgenic gene disruption mutants.	(Poliner et al., 2018)
N. gaditana (CCMP1894)	Electroporation/Cas9 editor cell line/ Cas9	*Aco1, ZnCys 5′UTR, LHC-3X, LHC-3X/VCP-4X*	Combining Cas9 with Cre recombinase to generate a strain and demonstrated Cre-mediated marker recycling. Marker-free knockout in a gene encoding acyl-CoA oxidase (Aco1) and a marker-less 2 kb insertion in the 5′-UTR of the ZnCys gene resulted in a 2-fold increase in lipid productivity.	(Verruto et al., 2018)
N. salina (CCMP1776)	Electroporation/plasmid/Cas9	*CesA 1,4*	CesA1 knockout resulted in the reduction of chrysolaminarin and neutral lipid contents by 66.3 % and 37.1 %, respectively, and increased the soluble protein content by 1.8-fold.	(Jeong et al., 2020)
N. oceanica IMET1	Electroporation/RNP (with DNA, 1 kb homology), episomal plasmid with/ Cas12a, CRISPRi(dCas9-KRAB, dCas12-KRAB)	*NR, LPAT, tdTomato*	Generation of scar-less and marker-less mutants using a Cas12a system. Development of a Cas12a episomal plasmid for transgene-free system. Development of a CRISPRi system (Cas12a, 85 % reduction in the transcript levels using dCas9).	(Naduthodi et al., 2021)
N. salina (CCMP1776)	Electroporation/Cas9 editor cell line/ Cas9	'T1′ transcriptional hotspot	'T1′ transcriptional hotspot was identified from a mutant showing the best sfGFP expression but did not affect growth or total lipid content.	(Ryu et al., 2021)
N. gaditana (CCMP526)	Electroporation/Cas9 editor cell line (with *Sh ble* cassette/Cas9	*BGS, TGS*	Both mutants decreased soluble carbohydrates (~5-fold) following nitrogen starvation. *BGS* knock-out strain increased total fatty acid content (~25–40 %) under *N*-replete conditions.	(Vogler et al., 2021)
N. oceanica IMET1	Electroporation/episomal plasmid/ Cas9	*LER1, LER 2*	For minimal genomes, serial deletion of large genome fragments of ~ 214 kb from its 30.01 Mb nuclear genome resulted in a slightly higher growth rate and biomass productivity than the WT.	(Wang et al., 2021)
N. oceanica (CCMP1779)	Electroporation/episomal plasmid/ Cas9	*AUREO*	The deletion of three aureochrome genes led to a decrease in the blue light-specific induction of several fatty acid desaturase genes. EPA content in *NoAUREO2* and *NoAUREO4* mutants was also significantly reduced.	(Poliner et al., 2022)
N. oceanica IMET1	Electroporation/plasmid/CRISPRa (dCas9-VP64)	g1248	CRISPRa system (dCas9 protein-VP64) altered gene expression. The expression of the targeted gene was increased by ~ 2–6-fold at the transcriptional level.	(Wei et al., 2022)
Chlorella				
C. sorokiniana, C. vulgaris FSP-E	Electroporation/plasmid/Cas9	*Fad3*	*FAD3* knockout mutants increased the biomass and lipid content by 20 % and 46 %, respectively.	(Lin & Ng, 2020)
C. vulgaris UTEX395	Electroporation/plasmid, RNP/Cas9, Bombardment/RNP/Cas9	*NR, APT*	Genome editing in *C. vulgaris UTEX395* using the CRISPR-Cas9 system.	(Kim et al., 2021)
C. sorokiniana UTEX 1602	Electroporation/plasmid/dCas9-KRAB (CRISPRi), dCas9-VP64 (CRISPRa)	sgRNA (5′-AAGGGGGGGGGGGGGGGGG-3′)	Using the CRISPRi/a system, gene regulation via dCas9-VP64 (CRISPRa) increased the protein contents by up to 60 % (w/w), while that via dCas9-KRAB (CRISPRi) increased the protein content to 65 %, and lipid accumulated in the range of 150–250 mg/L (WT: 150 mg/L).	(Lin et al., 2022)
Others				
Coccomyxa sp. Strain KJ	Electroporation/RNP/Cas9	*FTSY*	Applicability of DNA-free genome editing in *Coccomyxa.*	(Yoshimitsu et al., 2018)
Euglena gracilis		*GSL2*		

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Table 2 (*continued*)

Strain	Delivery of Cas nuclease/Cas type	Target gene (Abbreviation)	Resulting phenotype/related information	References
	Electroporation/RNP (or with ssODN)/ Cas9		Transgene-free targeted mutagenesis in *E. gracilis* resulted in 70–90 % mutagenic efficacy, with cells exhibiting fewer but larger paramylon granules.	(Nomura et al., 2019)
Euglena gracilis	Microinjection/RNP/Cas9	*CrtP1*	Six out of 100 injected cells survived after microinjection of RNP complex, and the genome editing efficiency was 1.0 %.	(Chen et al., 2022b)
Euglena gracilis	Electroporation/RNP/Cas9	*BBS*	The generation of non-motile strains improved harvesting efficiency by up to 38 % higher compared with the WT.	(Ishikawa et al., 2022)
Picochlorum celeri	Electroporation/RNP (with NAT)/Cas9	*NR, CrtISO*	*CrtISO* mutants exhibited > 50 % decrease in lutein content compared to the WT.	(Krishnan et al., 2020)
Porphyridium purpureum CCMP 1328	Bombardment/RNP/Cas9	*CHS1*	Phycoerythrin contents of mutants were substantially increased to provide novel applicability for the CRISPR/ Cas9 RNP methods.	(Jeon et al., 2021)
Tetraselmis sp. KCTC12432BP	Bombardment/RNP/Cas9	*AGP*	Lipid content in two *AGP* mutants was significantly enhanced by 2.7- and 3.1-fold.	(Chang et al., 2020)
Volvox carteri strain EVE, 153–68-BN	Bombardment/plasmid/Cas9	*GlsA, RegA, InvA*	First to express Cas9 protein in *Volvox carteri* and use plasmid- mediated method. Observed mutants by morphological defects.	(Ortega-Escalante et al., 2019)

Abbreviations: *Aco1*, acyl-CoA oxidase; *AGP*, ADP-glucose pyrophosphorylase; *APT*, adenine phosphoribosyl transferase; *AUREO*, blue-light photoreceptor/transcription factor, aureochrome genes; *BBS*, bardet–biedl syndrome; *BGS*, beta-glucan synthase; *CesA*, cellulose synthase gene; *CHS1*, chlorophyll synthase; *cpFTSY*, Chloroplast signal recognition particle receptor protein; *CrtISO*, carotenoid isomerase; *crtP*: phytoene desaturase (PDS), *Fad3*, omega-3 fatty acid desaturase; g1248, potential methyltransferase responsible for DNA or mRNA methylation; *GlsA*, Gonidialess phenotype; *GSL2*, Glucan synthase-like 2; *InvA*, partial inversionless phenotype; *LER*, low expression region; *LHC*, light-harvesting complexes; *LPAT*, lysophosphatidyl acyltransferase; *NR*, Nitrate reductase; *RegA*, somatic regenerator; *tdTomato*, (constitutively fluorescent) orange fluorescent protein; *TGS*, transglycosylase; *ZnCys*, Tanscriptional regulator of lipid accumulation Zn(II) 2 Cys 6.

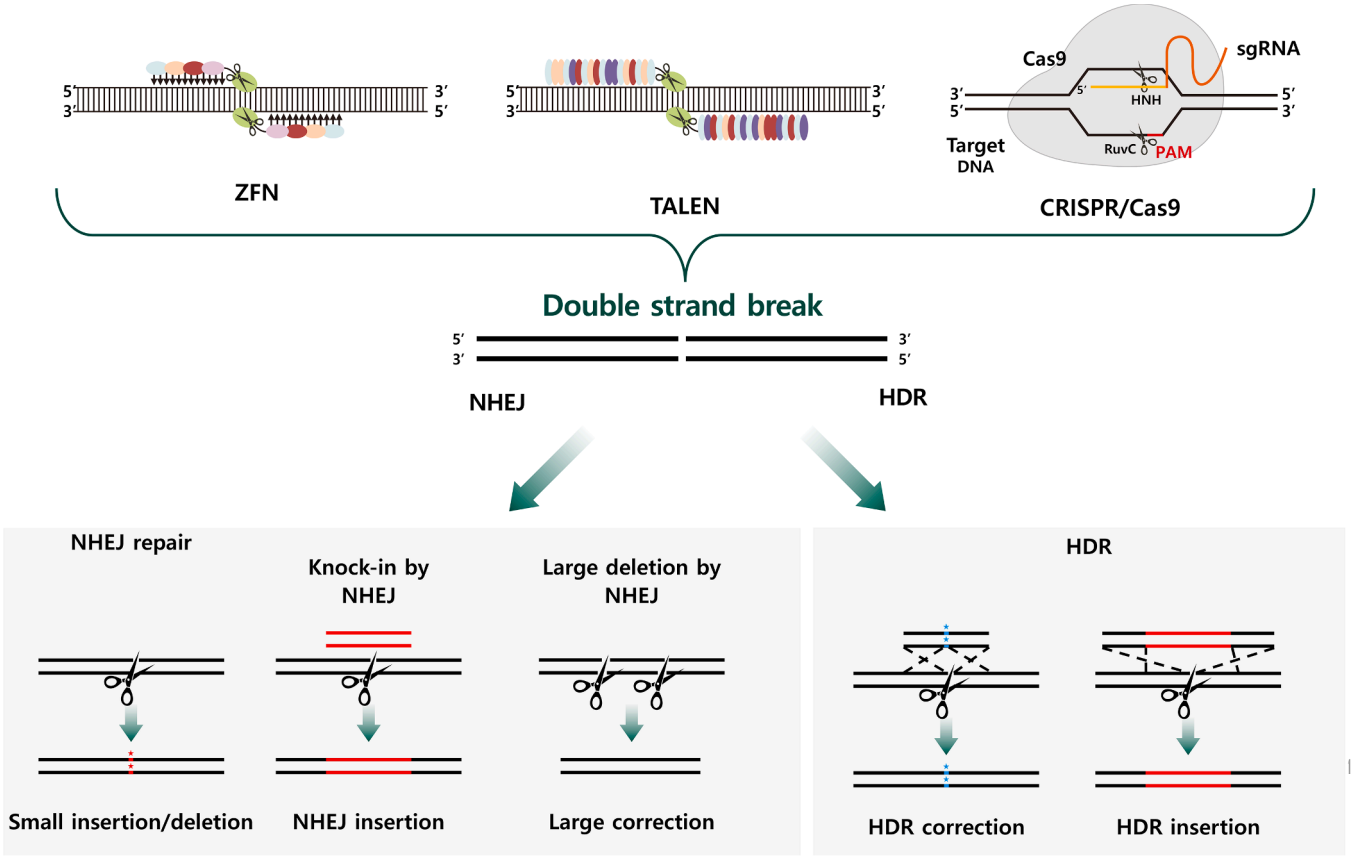

Fig. 1. Genome editing using engineered nucleases, ZFN, TALEN, CRISPR/Cas9, and repair mechanisms. All nucleases break down the target DNA to create a double-strain breakages (DSBs), that lead to genome editing via the non-homologous end joining (NHEJ) and homology-directed repair (HDR) pathway. Small insertions/deletions can be produced via an error-prone DNA repair NHEJ. If the donor DNA (red) is provided, knock-in events can be generated via NHEJ or HDR. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

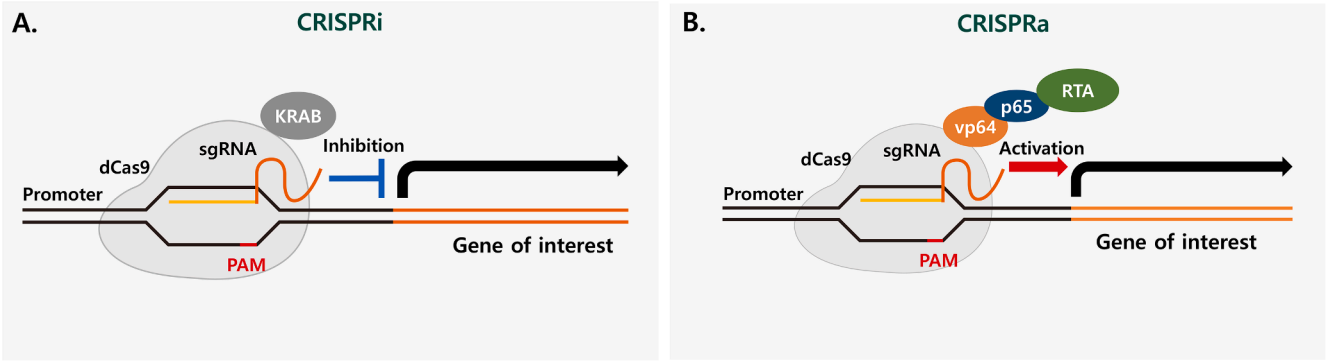

Fig. 2. Schematic diagram of the CRISPR-Cas9 system. The gRNA is complementary to the target sequence, and the Cas9 protein (light grey) (A). CRISPRi: CRISPR interference consists of the dCas9 protein (light grey) and the fused transcriptional repressor, such as KRAB (dark grey). (B). CRISPRa: CRISPR activation consists of the dCas9 protein (light grey) and the fused transcriptional activator (e.g., VP64 (orange), p65 (blue), RTA (green), etc.). The dCas9 protein binds to the targets without making generating DBS. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

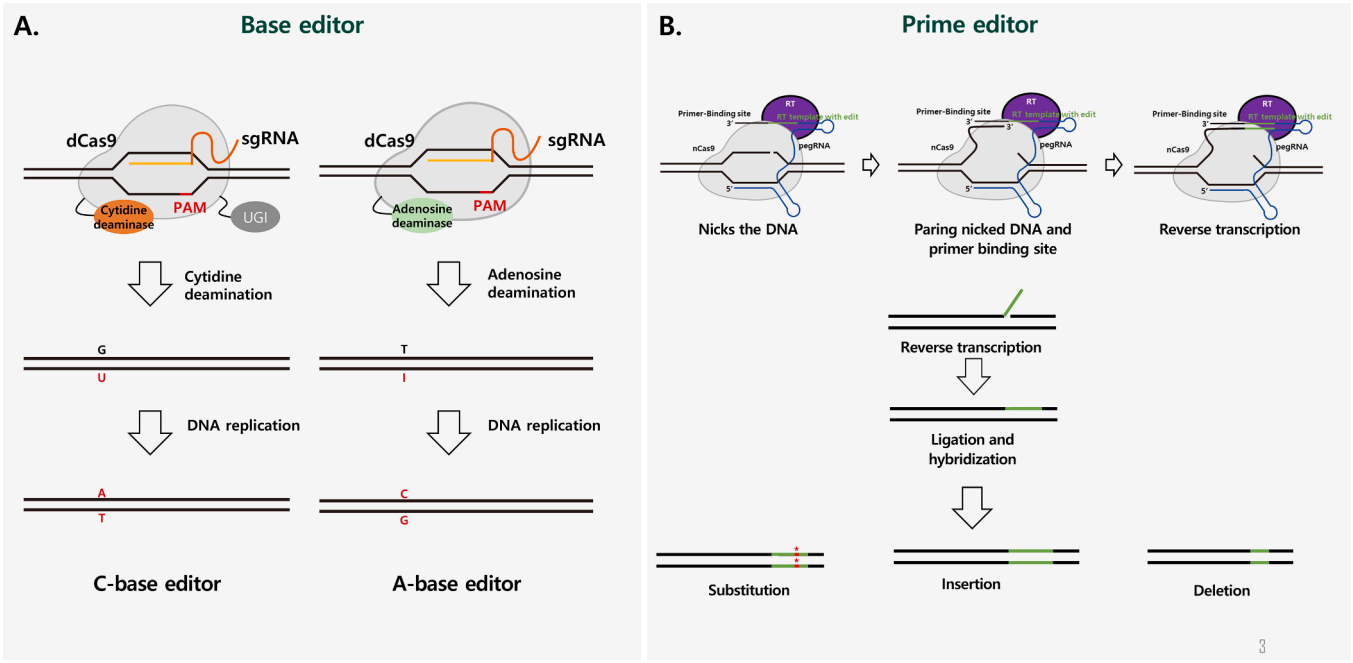

Fig. 3. Schematic diagram of the CRISPR-Cas9 system. The gRNA is complementary to the target sequence and the Cas9 protein (light grey) introduces a double-strand break. (A) Base editor: Cytosine base (C-base editor) or adenine base editor (A-base editor) is engineered via the fusion of dCas9 and a cytidine (orange) or adenine deaminase enzyme (green). It mediates the direct conversion of cytidine to uridine or adenine to inosine. Following DNA repair or replication, the original C-G base pair is replaced with a *T*-A base pair and vice versa. (B) Prime editing contains two components, a Cas9 nickase fused to a modified reverse-transcriptase (RT) (purple) and a multifunctional prime editing guide RNA (pegRNA) (blue line). RT uses a 3′-hydroxyl group to initiate the reverse transcription of pegRNA. As a result, the mismatched heteroduplex DNA undergoes a repair process that can create base substitution, insertion, or deletion in the target DNA. RT transcribed DNA (green). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Despite rapid progress in optimizing gene editing tools, base editing can address only a subset of possible base conversions within a relatively narrow window; it cannot handle larger genomic manipulations. Therefore, prime editing (PE), originally defined as a simple search-and-replace editing tool, has emerged. PE uses a fusion protein composed of dCas9 fused to an engineered reverse transcriptase and a PE-gRNA for targeted insertions, deletions, and base-to-base conversions without DSBs or donor DNA templates (Fig. 3B) (Anzalone et al., 2019). PE has been used in some animal models of genetic diseases (Jang et al., 2022). Unfortunately, BE and PE systems have not yet been realized in eukaryotic microalgae, but they can broaden the potential range of genome engineering.

2.1.3. Improvement of Cas9 expression and selection in gene modification

When the first CRISPR-Cas9-based genome editing was reported in *C. reinhardtii* (Jiang et al., 2014), there were issues with the low efficiency of plasmid-based expression and the cytotoxicity of Cas9. Later, the same laboratory implemented a hybrid version of Cas9 to improve the efficiency of plasmid-based Cas9 activity in *C. reinhardtii* (Jiang & Weeks, 2017). As an alternative delivery method, Cas9 RNP (ribonucleoprotein) generated from recombinant Cas9 using *in vitro*–transcribed, target-specific sgRNAs to *C. reinhardtii* has substantially resolved the issues with plasmid-based Cas9 genome editing (Baek et al., 2016; Shin et al., 2016) (Fig. 4). However, no cytotoxic effects of plasmid-driven expression of Cas9 have been observed in *Nannochloropsis, Phaeodactylum tricornutum*, or *Thalassiosira pseudonana*, so efficient and targeted genome editing in those hosts is fairly

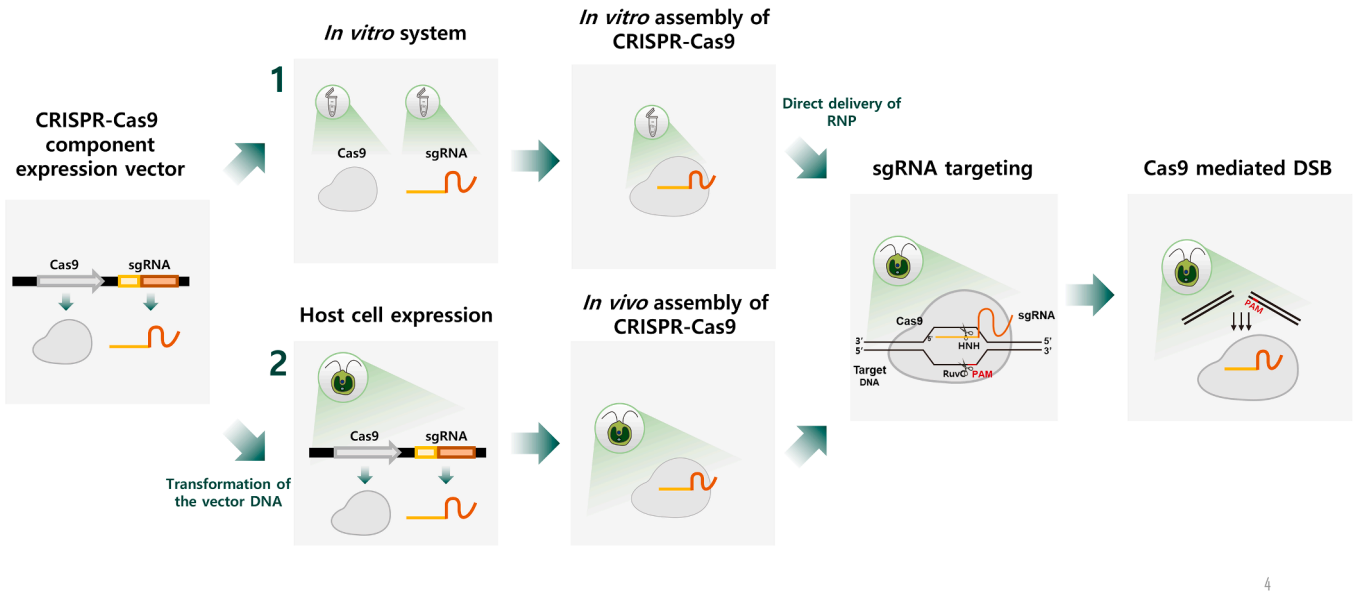

Fig. 4. Schematic diagram of the major delivery methods of CRISPR Cas9 complex into microalgae. (A) Ribonucleoprotein (RNP), which consists of Cas9 and a single guide RNA (sgRNA) (B) Plasmid vector with an embedded Cas9-coding gene and a guide RNA (sgRNA)-coding gene.

straightforward (Ajjawi et al., 2017; Hopes et al., 2016; Nymark et al., 2016).

Direct delivery of the CRISPR-Cas9 system as an RNP complex has advantages over the use of a vector-driven Cas9 expression system for several reasons: it prevents random insertional mutagenesis, is less likely to produce off-target cleavages, and can avoid GMO (genetically modified organism) conflicts (Sproles et al., 2021). Cas9 RNP has also been applied together with a cell-penetrating peptide, pVEC (LLIILRRRIRKQAHAHSK), in *Chlamydomonas* (Kang et al., 2020). However, the efficiency of gene editing via Cas9 RNP is still low (<10 %). In addition, the selection of genome-edited cells based on phenotypic changes, such as visual changes, is still low, and the process is labour and time intensive (Baek et al., 2016; Chang et al., 2020; Shin et al., 2016).

The use of endogenous selectable markers is one strategy to overcome those challenges. To date, endogenous selective markers such as nitrate reductase, adenine phosphoribosyl transferase, peptidylprolyl isomerase, tryptophan synthase beta subunit, and orotidine 5′-phosphate decarboxylase, protoporphyrinogen IX oxidase, spermidine synthase have been used for gene editing in microalgae (Akella et al., 2021; Freudenberg et al., 2022; Jiang & Weeks, 2017; Serif et al., 2018; Shin et al., 2016; Wang et al., 2016). Therefore, co-targeting a gene-of-interest with one endogenous selective marker could significantly improve the number of intended gene modifications in microalgae without transgene integration. However, pre-selection using antibiotic resistance has also been used for gene editing (Greiner et al., 2017; Kim et al., 2020; Picariello et al., 2020; Shin et al., 2016) and improved to achieve up to 90 % selection efficiency. Furthermore, reporter genes, including luciferase or mVenus, have been precisely knock-in in the desired site of gene, which provides an easy selection of mutants (Freudenberg et al., 2022; Kim et al., 2020).

2.1.4. *Multiplex genome editing of microalgae using CRISPR-Cas technology*

By generating multiple gRNAs and Cas proteins *in vivo*, researchers can build layered genetic circuits that regulate metabolic pathways by simultaneously editing, activating, and downregulating multiple target genes (McCarty et al., 2020). The expression of multiple gRNAs can be achieved by cloning multiple gRNAs separated by ribozymes such as hammerhead (HH) or hepatitis delta virus (Naduthodi et al., 2021; Poliner et al., 2018; Tang et al., 2019). Ribozymes are a family of naturally occurring RNA motifs that catalyse a self-transesterification reaction by cleaving their own RNAs (Zetsche et al., 2017). However, in *N. oceanica*, the removal of the HH ribozyme from the plasmid for self-processing of crRNA by the Cas12a enzyme was attempted, and multiplexed using an additional spacer to the crRNA array to target multiple regions in a single transformation (Naduthodi et al., 2021).

Alternatively, individual gRNAs can be transcribed *in vitro* and supplied directly to microalgae. Multiple gRNAs can be used to simultaneously generate DSBs at different target loci in the genome (Serif et al., 2018). Recently, to optimize and improve the efficiency of genome editing, physical delivery methods for the Cas9 RNP, including electroporation, microinjection, biolistic and microfluidic techniques, and synthetic carriers such as lipid nanoparticles and polymers, nanogels, inorganic nanoparticles, and cell-derived vesicles, have been developed (Chen et al., 2022b; Kang et al., 2020; Zhang et al., 2021). Multiplex gRNAs have also been used to delete large fragments of chromosomes in *Nannochloropsis* (Wang et al., 2021). In addition, paralogous genes can be targeted simultaneously by using one or two sgRNAs located in the homologous sequences, which has been verified for antennal *LHCF* genes in diatoms (Sharma et al., 2021). In this study, multiple genes within the same family were knocked out by exploiting off-targeting of Cas9 outside the seed sequences. Six light-harvesting complex genes belonging to the *LHCF* gene family were targeted, and five *LHCF* genes were edited. Multiplexing gRNAs should be considered for genome engineering, particularly when targeting multiple genes in a metabolic pathway.

2.2. *Induction of homology-directed repair in microalgae*

CRISPR-mediated gene editing in microalgae has mostly been limited to creating gene knockout mutants, rather than inserting protein-encoding transgenes. Therefore, it has yet to be used as a tool for the overexpression of transgenes or the production of high-value recombinant proteins in microalgae (Sproles et al., 2021). Compared with nonhomologous end joining (NHEJ), which uses two cellular DNA repair pathways (Fig. 1) to re-join the ends of DSBs (causing in/del mutations in the target genes), HDR is a more precise process in which two similar sequences interact and exchange information (Gaj et al., 2013). Thus, HDR is essential for inserting foreign DNA into specific sequences in the genome.

Complete HDR usually requires a repair template containing 500–2,000 bp of homologous DNA in the left and right arms. However,

the efficiency of HDR is species-dependent, and it rarely occurs in *Chlamydomonas* (Sproles et al., 2021). On the other hand, microhomology-mediated end joining –based editing using microhomologous sequence arms (5–30 bp) and the CRISPR-Cas9 system has been used via DNA integration in *Chlamydomonas* (Ferenczi et al., 2021; Picariello et al., 2020; Sizova et al., 2021). To facilitate HDR and achieve scar-free DNA repair at the target site, linear DNA fragments with short gene-specific homology arms could be the best donors for efficient CRISPR-Cas-mediated gene targeting in *Chlamydomonas* (Sizova et al., 2021).

In *Nannochloropsis,* homologous recombination (HR)-based genome editing has not been problematic. Cas-RNP-mediated HDR for genome editing in *N. oceanica* has been achieved by enhancing the native homologous recombination system through targeted DSB induction with the Cas9 protein to facilitate HDR (Naduthodi et al., 2019). Subsequently, Cas12a RNPs and HDR generated scarless and marker-less mutants of *N. oceanica* (Naduthodi et al., 2021). Transgene-free RNP-based genome editing has been demonstrated in *Euglena* (Nomura et al., 2019). In this study, the glucan synthase-like 2 gene (*EgGSL2*) was targeted with ssODNs containing 50 nt downstream and upstream of the *EgGSL2* cleavage site as homology arms and a 42 nt knock-in DNA fragment containing restriction enzyme sites. The editing efficiency rate was approximately 70–90 % based on morphology and amplicon sequencing, which is significantly higher than the rates for previously reported DNA-free targeted mutagenesis using Cas9 RNPs (~1%) or Cpf-1 RNPs with ssODN (~10 %) in *C. reinhardtii* (Ferenczi et al., 2017; Jeon et al., 2017).

3. Genomic perspectives of gene editing in microalgae

Gene editing technologies have been established in some microalgae by employing sequence-specific nucleases including ZFNs, TALENs, and Cas nucleases as summarized in Fig. 1. These reverse genetic techniques enable metabolic and genetic engineering as well as genetic and biological studies by manipulating a few genes in microalgae (Jeon et al., 2017; Park et al., 2019). Genomic resources are also available for a few microalgal species including *Chlamydomonas* and *Nannochloropsis* (Gong et al., 2020; Merchant et al., 2007), which can be employed for genome engineering. There have been attempts to utilize genomic resources for genetic engineering by combining CRISPR techniques, particularly in *Nannochloropsis*. The industrial model microalgae *Nannochloropsis* spp. have been used in genome engineering experiments because of their solid genome resources, which have enabled large chromosomal deletions with Cas9 (Gong et al., 2020; Wang et al., 2021). In addition, efforts to screen transcriptional hot spots for the Cas9-guided integration of a lipid metabolic transgene have been made to improve expression and function in a process known as safe harboring of transgenes (Ryu et al., 2021). These genome level applications of gene editing technology are further discussed below.

3.1. Safe harboring of transgene expression

Transgene expression is essential for successful genetic engineering, which requires stable and often strong expression. However, microalgae have evolved efficient silencing mechanisms against foreign DNA such as transposons and viruses, and those mechanisms are also effective against transgenes (Jeong et al., 2002). In addition, typical microalgal transformation relies on the random integration of transgenes, which leads to position effects. The transcriptional status of transgene expression depends on its position in the genome, making it difficult to achieve consistent or repeatable transformation using random integration (Doron et al., 2016).

3.1.1. Position effects

Position effects are a phenomenon by which the expression of a gene is influenced by its location in the genome. They were initially reported in the fruit fly *Drosophila* as the peculiar silencing of the euchromatic gene *brown* when it was translocated near the suppressive telomeric heterochromatin, later termed *position-effect variegation*. Such position effects are mediated by epigenetic regulation in human cells (Timms et al., 2016), which also applies to transgene expression in microalgae (Jeong et al., 2002). To prevent position effects and achieve strong and stable expression of transgenes, several approaches have been explored, including strong promoters (Dong et al., 2017) and the suppression/mutation of transgene silencing factors (Jeong et al., 2002). Boundary elements (or insulators) have also been used to achieve stable expression by "insulating" transgenes from the effects of surrounding chromatin structures such as matrix/scaffold-associated regions, hypersensitive site 4, and their combinations (Lu et al., 2020). In addition, the episomal expression has been used to prevent position effects in diatoms and *Nannochloropsis* (Liu et al., 2022; Poliner et al., 2018), even though heterologous centromere/autonomous replication sequence (CEN/ARS) can make transgenes less stable in the long run. However, those procedures are time-consuming and require complicated cloning, so better transgenic strategies are needed.

One solution for position effects would be the integration of transgenes at favourable sites, rather than randomly in the genome. Transgenes are randomly integrated into the genome mostly through the DNA DSB repair mechanism (NHEJ or HR), which joins linearized transgenes to the broken ends (Srivastava & Raghavan, 2015). In *Chlamydomonas*, DSB repair is thought to initiate transgene silencing by establishing epigenetic marks at the broken ends, including histone phosphorylation and methylation (Jeong et al., 2002). Together with position effects, the epigenetic transgene silencing mechanism results in weak and unstable transgene expression. One proposed way to avoid the problems of random integration is to integrate transgenes at specific genomic locations by providing an initial DSB with the aforementioned nucleases for transgene integration. Initially, this concept was successfully demonstrated by cleaving one of the hotspots, or safe harbor sites, with the TALEN nickase, followed by the integration of reporters via HR, which improved expression and transformation efficiency in animal cells (Wu et al., 2014). This strategy is called *safe harboring* transgenes for high and stable expression.

3.1.2. Examples of safe harboring

Safe harboring of transgenes is critical for both biotechnical and biomedical purposes. It is particularly important for biomedical applications, in which therapeutic or immunogenic transgenes are integrated into the genomes of animal or human cells. In such cases, additional safety measures are needed, including appropriate distances from known oncogenes and regulatory genes (Aznauryan et al., 2022). For biotechnological applications, stable transgene expression is also pursued, and safe harboring has been reported in *Nannochloropsis*. An initial screen for transcriptional hotspots produced a few candidates, and the integration of a lipid metabolic gene (fatty acid desaturase, FAD12) at the best hotspot using Cas9 led to higher expression of FAD12 and the production of more unsaturated fatty acids, including the highly valued eicosapentaenoic acid (Ryu et al., 2021). Interestingly, another safe harbor site in *Nannochloropsis*, an rDNA repeat in the nucleolus, was found after screening for strong expression of a promoter-less GFP reporter (Südfeld et al., 2022). Given that rRNA genes are transcribed by RNA polymerase I, the transcription of the reporter might have been enhanced by active transcriptional status near the integration site, although unforeseen issues could develop due to its heterologous nature. Taken together, the evidence shows that random integration of transgenes is unreliable because it produces unstable and unsafe transgene expression, so safe harboring should be used for future advances in genetic engineering and synthetic biology.

3.2. Proposal for CRISPR libraries for microalgae

Genomic resources for sgRNA libraries are unfortunately absent for

microalgae; however, similar libraries can be manually constructed. CRISPR-Cas9 has gained popularity as an excellent gene editing tool, and it has great potential for genetic screening via its mutagenic capabilities. However, its use in screening mutants at the genomic level is limited by its requirement for PAMs, which are necessary for Cas9 and similar Cas nucleases to precisely recognize the target site (Luo et al., 2016) and critical for precision modification and minimizing off-targeting, but they are not essential for genome-wide mutagenic screens. In genetic screening, priority is given to simplicity and convenience in library construction, even at the cost of precision, because follow-up confirmation with precise Cas9 is simple and straightforward. Therefore, a few alternatives can be considered, including PAM-less Cas9, which has a reduced requirement for PAMs (Tang, 2020). Similar screens at the genomic level have been developed for other regulatory schemes, including RNAi (Wang et al., 2008) and artificial microRNA (Xie et al., 2021), which are relatively simple; however, those RNA silencing mechanisms carry inherent issues of off-targeting, silent mutations, and low specificity (Mohr et al., 2010).

CRISPR libraries for higher eukaryotes, including human and other animals, are well established (Wang et al., 2022), and indexed libraries are readily available commercially. Some commercial sgRNA libraries are available and already packaged into viral vectors, such as lentiviral or adeno-associated viral vectors (Wang et al., 2018). Indexed libraries are also available for plants, and the identification and characterization of receptor-like kinases in rice have been successfully demonstrated (Chen et al., 2022a). Indexed sgRNAs can be designed using software such as CRISPR Library Designer or the Variant Library Annotation Tool, which is followed by synthesis and cloning into appropriate vectors (Barbon et al., 2022). Animal virus vectors are very efficient in delivering DNA, allowing relatively simple isolation and characterization of the mutations responsible for the phenotype of interest (Wang et al., 2018), as summarized in Fig. 5.

Unfortunately, such advanced resources are not available for microalgae, so CRISPR libraries must be manually constructed. To reduce the cost and time required to construct an indexed sgRNA library, it is proposed to isolate cDNAs enriched in the 5′ untranslated region (UTR), coding sequence (CDS), and 3′ UTR that can then be cloned into appropriate vectors for microalgal transformation (Fig. 5). Enrichment of the 5′ UTRs can be achieved using transcriptional inhibitors, including alpha-amanitin, cordycepin, and actinomycin D (Gao et al., 2018). The 3′ UTRs can be enriched by reverse transcriptase (RT) inhibitors or short RT reactions from oligomeric Ts (oligoTs) starting from the polyA tail (Suslov & Steindler, 2005). Because those bits of mRNA might lack the PAM sequence, it is critical to use PAM-less Cas9 (Tang, 2020). The combination of enriched RNA fragments and PAM-less Cas9 is expected to produce transformants, some of which will carry mutant phenotypes for selection. Mutant selection can be achieved by visual criteria such as colony size for growth, pale green for photosynthetic or antennal function, other colors for pigments, and auxotrophy (Jiang et al., 2014; Kim et al., 2021; Naduthodi et al., 2021; Shin et al., 2016). Once mutants have been selected, they should be verified by sequencing the whole genome, particularly when using PAM-less Cas9.

On the other hand, if a family of proteins or a metabolic pathway has only a few genes, multiple sgRNAs can be delivered together for mutagenesis. Endogenous CRISPR-Cas9 systems in bacterial cells are intended to target multiple sites by storing multiple gRNAs in the CRISPR array interspaced by a repeat, as indicated in the name CRISPR (Jeon et al., 2017). However, in heterologous systems, individual gRNAs should be provided in the form of sgRNAs, and a few sgRNAs can be delivered by separating them with a ribozyme such as HH (Poliner et al., 2018).

Another issue to be considered when designing CRISPR screens in microalgae is their ploidy. Many microalgae are haploid or haplontic, at least during their vegetative growth, including green algae, *Nannochloropsis*, and *Guillardia* (Hirakawa & Ishida, 2014), though some diatoms are known to be diploid (Armbrust et al., 2004). A haploid genome allows faster screening for otherwise recessive mutations. Higher organisms are diploids that require homozygotes to reveal recessive phenotypes or conversion to haploids before screening (Yilmaz et al., 2018). On the other hand, haploids do not allow the knockout of essential genes, which should be considered when making a gene list for mutagenesis. In this case, CRISPR knockdown approaches such as CRISPRi or the attenuation of gene expression by targeting UTRs could be considered (Song et al., 2017).

4. Genomic engineering with gene editing techniques

Genome engineering involves genomic modifications for metabolic, biological, medical, and biotechnological improvements, rather than simple metabolic or genetic engineering. Such genomic modification is not easy and has thus been delayed by technical difficulties. However,

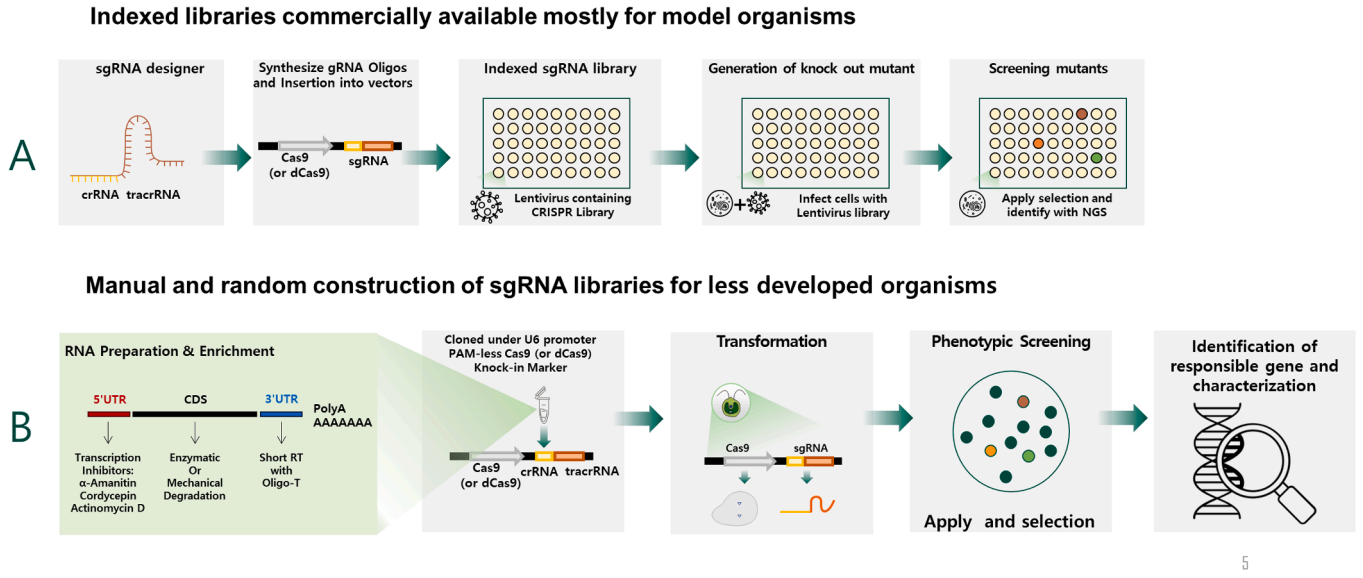

Fig. 5. Construction of sgRNA libraries. (A) Indexed libraries commercially available mostly for model organisms and (B) manual and random construction of sgRNA libraries for less developed organisms.

genomic resources have been established in many organisms to enable the precise design of engineering plans that can be synergistically improved by CRISPR-Cas9. Genomic resources have also been developed into pan-genomes for related strains of species or related organisms (Huang et al., 2022). Epigenetic modifications are critical to the genetic regulation of stress and metabolic responses, which should be understood at the genomic level, termed *epigenomics* (Michael & Thomä, 2021). In addition, large-scale modifications of the genome are being pursued by using CRISPR-Cas9 for precise deletions, which are not possible with classic genetic approaches (Gong et al., 2020). Large-scale genomic deletions could be a stepping stone to the construction of mini-genomes that could be used as chassis strains for further genetic and metabolic engineering. Those aspects of genome engineering are summarized next.

4.1. Pan-genomes for algal species

Genomic resources are required for genome engineering, and they are in fact available for most model organisms at different levels. However, pan-genomes are needed to enable comparative genomics of closely related strains and species, more accurate determination of gene functions, and legitimate synthetic designs for metabolic and genetic engineering (Huang et al., 2022). The efforts required to compile such resources have already been made for model microalgal organisms such as *Chlamydomonas* and *Nannochloropsis* (Gong et al., 2020; Merchant et al., 2007).

4.2. Epigenomic resources

Epigenetics is the non-genetic information carried by a genome that can be heritable to daughter cells or future generations, such as DNA modifications and histones (Kouzarides, 2007). Such epigenetic modifications play crucial roles in gene expression and biological regulation, including metabolic and stress responses, and should be understood at the genomic level as epigenomics (Michael & Thomä, 2021). Epigenomics can be incorporated into genomic resources together with transcriptomic data (Hu et al., 2022). Individual epigenetic modifications have already been reviewed, so this review focuses on overall epigenomic features that affect metabolic pathways and stress responses.

Epigenetic modifications of DNA and histones can influence not only metabolic pathways, including lipid metabolism, but also responses to stresses such as heat (Kim, 2021). Lipid metabolism and heat stress are selected for bio-mitigation of global warming because lipid-derived biofuels can improve the carbon balance, and heat tolerance is expected to make plants and microalgae resistant to warming trends and thereby also improve the carbon balance (Joshi, & Mishra 2022; Kim, 2021). Global DNA methylation changes can affect lipid metabolism in microalgae (Mikulski and Santos-Aberturas, 2022; Steadman et al., 2020). Similar effects on lipid metabolism are found in animals, in which histone methylation affects the expression of transcription factors involved in lipid metabolism and fatty acid desaturation (Lee et al., 2019; Steadman et al., 2020). This finding highlights the health benefits of epigenetic regulation, in addition to environmental bio-mitigation.

Stress responses are also affected by epigenetic modifications. For example, heat tolerance is affected by thermopriming in plants, as revealed by a genome-wide survey of epigenetic changes (Kim, 2021). Thermopriming is expected to enhance heat tolerance in plants, which will make plants better prepared for the forthcoming global warming (Liu et al., 2021). Similar thermotolerance is also found in microalgae, mainly involving shorter fatty acids (Ye et al., 2022), suggesting that microalgae use epigenetic regulation to manage their stress responses and lipid metabolism.

Epigenetic markers can be overlaid on the genomic map with transcriptomic profiles, which will aid comprehensive and rationally designed genetic and metabolic engineering (Hu et al., 2022). Furthermore, small RNAs such as siRNAs and miRNAs contribute to post-transcriptional epigenetic regulation. Interestingly the small RNAs are associated with DNA methylation, known as RNA-dependent DNA methylation (Singh et al., 2022), and histone modifications (Eimer et al., 2018). Taken together, the evidence suggests that it is time to build comprehensive epigenomic/genomic maps to enable the rational design of genetic and epigenetic engineering.

4.3. Mini-genomes and genome duplication

Genomes can be reduced or duplicated, depending on the genomic flexibility of an organism, and efforts to both minimize and double the genomes in microalgae are being made for genome engineering. Seemingly contradictory, the two processes are useful for different engineering purposes. Efforts are also being devoted to building mini-genomes in some model organisms. For small genomes, minimal sets of genes can be synthesized and transferred to DNA-free bacterial cells. For example, 473 genes in ~ 530 kb DNA can be implanted into a shell of *Mycoplasma*, resulting in a self-replicating bacterial cell (Breuer et al., 2019). The genomes of eukaryotes are generally too big to synthesize. Some groups have attempted to delete seemingly non-essential genes because higher eukaryotes in particular contain a massive number of repetitive and seemingly non-functional sequences, including transposons (Wessler, 2006). Microalgal genomes are generally compact, ranging from 30 to 100 Mb for *Chlamydomonas*, *Nannochloropsis*, *Chlorella*, and diatoms (Gong et al., 2020; Merchant et al., 2007). Even so, a recent report demonstrated that large fragments could be deleted from microalgae. Jian Xu's group reported that several hundred kilobases of DNA containing apparently non-essential genes could be deleted without having a significant effect on the growth of *Nannochloropsis* (Wang et al., 2021). Such non-essential genes might be necessary for survival in natural ecosystems; however, the conditions required to improve productivity in cultivation conditions can be provided artificially.

On the other hand, genome sizes can be increased to achieve improved yields and stress tolerance. Plants have evolved or been domesticated to improve in quantitative and qualitative ways that are associated with polyploidization (Zhang et al., 2019). Like plants, microalgae are flexible in their genome dosage, and diploids can be induced in *Chlamydomonas* by chemical treatment with colcemid. Interestingly, the artificial diploids showed improved biomass production and stress tolerance (Kwak et al., 2017). Such genomic flexibility is important for genome engineering purposes. The decision to increase or decrease the size of a genome would depend on the purpose of an individual project. One interesting idea would be the duplication of a mini-genome to achieve the benefits of both processes, but that idea still requires experimental verification.

5. Future engineering schemes toward microalgal synthetic biology

Previous sections described genomic and epigenomic resources that are expected to be essential tools for the rational design of genetic and metabolic engineering that will improve the production of target molecules, termed synthetic biology (Andrianantoandro et al., 2006). One of the most important metabolites to be considered for such engineering strategies is acetyl-CoA, a metabolic hub for the central carbon pathways (Lu et al., 2019), as schematized in Fig. 6. The backbone of most target molecules is composed of organic carbons, and acetyl-CoA is the carbon cash used in many biosynthetic pathways, including fatty acids and carotenoids. Acetyl-CoA is also the hub for inputs of carbon from degradation pathways such as the beta-oxidation of fatty acids, glycolysis, and the tricarboxylic acid (TCA) cycle and other mixotrophic supplies of C1 and C2 chemicals, including acetate. Such metabolic aspects of acetyl-CoA have been extensively reviewed in model organisms, such as bacteria and yeast (Xing & Poirier, 2012), and synthetic

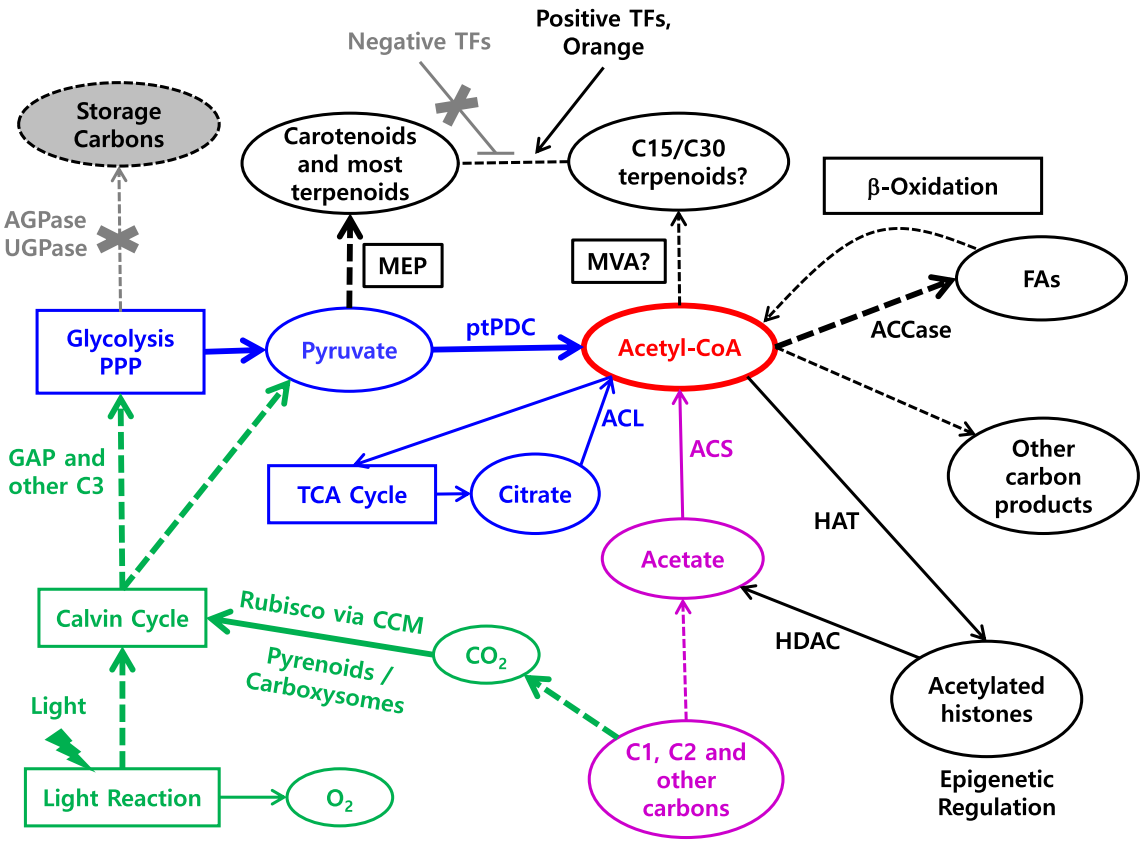

Fig. 6. Schematic illustration of the acetyl-CoA metabolic hub that supplies and sinks carbons in microalgae, including pathways (boxed) and key metabolites (circled). The enzymes are also included as possible targets of engineering. Direct or single-step reactions are presented as solid arrows, and multiple-step reactions are represented as dotted arrows. Photoautotrophic pathways are green and the central carbon pathways are shown in blue, and heterotrophic or mixotrophic carbon supplies are in purple. The main carbon fluxes in thicker lines can be considered for engineering strategies. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

biological engineering for acetyl-CoA supply is well established for production of value-added products and bioactive chemicals, including fatty acids, polyketides, isoprenoids, and lipstatin (Jatain et al 2022; Krivoruchko et al., 2015). While, synthetic biology in microalgae is not as advanced, some studies have reported complex genetic engineering to rationally design the chain length of fatty acids (Wang et al., 2021). Acetyl-CoA is also the source of two carbons for the acetylation of proteins and histones in the nucleus, contributing to epigenetic regulation (Xing & Poirier, 2012). It is time to use the genomic and epigenomic resources available to establish the rational design of metabolic pathways and thereby improve the production of target molecules in microalgae.

5.1. Acetyl-CoA

Many microalgae can grow in both photoautotrophic and heterotopic cultivation, in which carbons are supplied from CO_2 and organic carbons such as acetate or glucose, respectively, contributing to the acetyl-CoA pool in both cases. Acetyl-CoA is supplied mainly by degradative pathways, including glycolysis and the beta-oxidation of fatty acids, and can also be converted from citrate through the TCA cycle (Xing & Poirier, 2012). As photosynthetic organisms, microalgae and plants also have access to acetyl-CoA through the Calvin cycle, by which C3 intermediates, including glyceraldehyde 3-phosphate, are tightly linked to the plastidic glycolysis that leads to the production of pyruvate and acetyl-CoA (Li-Beisson et al., 2019).

For engineering schemes, it should be noted that acetyl-CoA is not permeable to the membrane and lacks appropriate transporters (Xing & Poirier, 2012). Instead, precursors such as pyruvate, citrate, and acetate are mobilized to appropriate compartments in the cell and then converted to acetyl-CoA by the pyruvate dehydrogenases ATP: citrate lyase (ACL) (Tan & Lee, 2016) and acetyl-CoA synthase (ACS) (Rengel et al., 2018). Therefore, compartmental distribution of acetyl-CoA and its precursors should be assessed according to the locations of the target molecules.

Carbons from acetyl-CoA are used for the biosynthesis of many organic molecules, including fatty acids, terpenoids, and smaller molecules such as amino acids (Xing & Poirier, 2012). Fatty acids are biosynthesized from acetyl-CoA; they are initially catalysed by acetyl-CoA carboxylase (ACCase) in the chloroplast and continue until C16 or C18 fatty acids are produced ACCase catalyses the rate limiting steps of fatty acid biosynthesis; however, overexpression of ACCase produced mixed results in increasing lipid content in microalgae. On the other hand, ACL (Tan & Lee, 2016) and ACS (Rengel et al., 2018) are key enzymes known to contribute carbon to fatty acid synthesis by increasing acetyl-CoA input, and that should be considered before planning experiments.

5.2. Terpenoids in microalgae

Terpenoids, including C40 carotenoids, are other lipids biosynthesized from carbons taken from acetyl-CoA or pyruvate. Terpenoid biosynthesis mainly uses the methyl-d-erythritol 4-phosphate (MEP) pathway, which starts from pyruvate in the plastid of microalgae (Banerjee & Sharkey, 2014). The mevalonate (MVA) pathway is the cytoplasmic alternative pathway for terpenoid biosynthesis, and it is conserved in many organisms (Davies et al., 2015; Zhang et al., 2019). However, the MVA pathway is known to be lost in green algae and *Nannochloropsis* (Davies et al., 2015), though diatoms might have

retained it (Athanasakoglou & Kampranis, 2019). Interestingly, heterologous expression of the MVA operon has been shown to increase terpenoid production in bacteria (Zurbriggen et al., 2012) and plants (Majer et al., 2017), which could also be applied to microalgae.

5.3. C40 carotenoids with biological and biotechnological importance

Many microalgal species have central terpenoid metabolic pathways that supply enough precursors to synthesize carotenoids and therefore have sufficient capacity to store them. Carotenoids are classified as carotenes and xanthophylls, linear or cyclic, and have oxygenated derivatives at both ends. Among all carotenoids, β-carotene, astaxanthin, lutein, zeaxanthin, and fucoxanthin are actively studied because of their health-promoting properties (Kang et al., 2022). Recently, genetic manipulation has been used to increase carotenoid production in microalgae, and metabolic engineering for carotenoid production in microalgae has been reviewed (Kang et al., 2022; Patel et al., 2022). Advances in efficient gene editing tools have made target-specific gene editing in microalgal carotenoid production possible. For example, in *Chlamydomonas,* Baek et al. (2016) generated zeaxanthin epoxidase (ZEP) knockout mutants using the Cas9 RNP system. This mutant had a 56-fold higher zeaxanthin content and 47-fold productivity compare to the wild type without a reduction in the lutein level. Using this *zep* mutant (Baek et al., 2016). Song et al. (2020) further targeted the lycopene epsilon cyclase Cas9 RNP system to eliminate the a-branch of xanthophyll biosynthesis in the ZEP mutant. The double knockout mutant had a 60 % higher zeaxanthin yield (5.24 mg/L) and content (7.28 mg/g) than the parental *zep* mutant (Song et al., 2020). *C. reinhardtii,* which does not produce astaxanthin, produced astaxanthin upon overexpression of the β-ketolase gene (Perozeni et al., 2020). The introduction of heterologous or endogenous genes (*PSY, PDS*, bacterial *CrtB*, and *CrtI*) increased the carbon flux, which increased the carotenoid content. To enhance the carotenoid content, the genes mentioned above can be introduced into the safe harbor regions of the genome, as described in section 3.2, which will prevent random insertion from a negative effect on growth.

Positive regulators of carotenoid production have been identified and characterized in microalgae, including the Orange (OR) DnaJ-type chaperone and MYB/Nin-like transcription factors (Yazdani et al., 2021). The *OR* genes encode DnaJ cysteine-rich zinc finger domains, and the interaction between PSY and OR proteins might enhance the stability and activity of PSY. Negative regulators of carotenoid biosynthesis exist in plants. For instance, in tomatoes, SGR knockout mutants show increased lycopene and β-carotene content (Li et al., 2018). In microalgae, negative regulators have not yet been reported, but negative regulators could be identified by CRISPR library screening and then applied to enhance carotenoid production.

5.4. Epigenetic roles of acetyl-CoA in microalgae

Acetyl-CoA is also involved in the epigenetic regulation of gene expression by providing acetate to histones and other proteins, i.e., histone/protein acetylation (Mews et al., 2017). Histone acetylation is catalyzed by histone acetyltransferases (HATs), and deacetylation occurs through histone deacetylases (HDACs), which release acetate that can be converted to acetyl-CoA by ACS (Mews et al., 2017). Epigenetic modifications regulate the expression of metabolic genes that are directly and indirectly involved in lipid biosynthesis, cancer therapy, and even aging. For example, the overexpression of Gcn5-related *N*-acetyltransferase (*GNAT19*), a HAT, increased the starch content in *Chlamydomonas* (Li et al., 2020). On the other hand, HDAC inhibitors affect stress responses that lead to cancer therapy or aging in animals (Li et al., 2020). Overall, acetyl-CoA is involved in the epigenetic regulation of metabolic pathways via histone acetylation/deacetylation, in addition to its biochemical function as a carbon donor and sink (Poliner et al., 2018; Xing & Poirier, 2012).

6. Conclusion

CRISPR-Cas system has advanced gene editing technologies in both academic and industrial fields, and some basic techniques have been successfully demonstrated in microalgae. Some applications have been reported at the genomic levels, including safe harboring and large chromosomal deletions. However, advanced techniques, such as sgRNA libraries and epigenomic resources, should be developed for microalgae. This review proposed ideas for developing genome engineering involving sgRNA libraries and genome manipulation in microalgae. In addition, synthetic biology has yet to be truly executed in microalgae, for which rational metabolic design for the key metabolite acetyl-CoA can be employed for the production of terpenoids.

CRediT authorship contribution statement

Byeong-ryool Jeong: Conceptualization, Writing – original draft. **Junhwan Jang:** Writing – original draft. **EonSeon Jin:** Conceptualization, Funding acquisition, Writing – review & editing, Writing – original draft.

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.

Data availability

No data was used for the research described in the article.

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