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### Authors

Lu, Yandu

Zhang, Xu

Gu, Xinping

et al.



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# Engineering microalgae: transition from empirical design to programmable cells

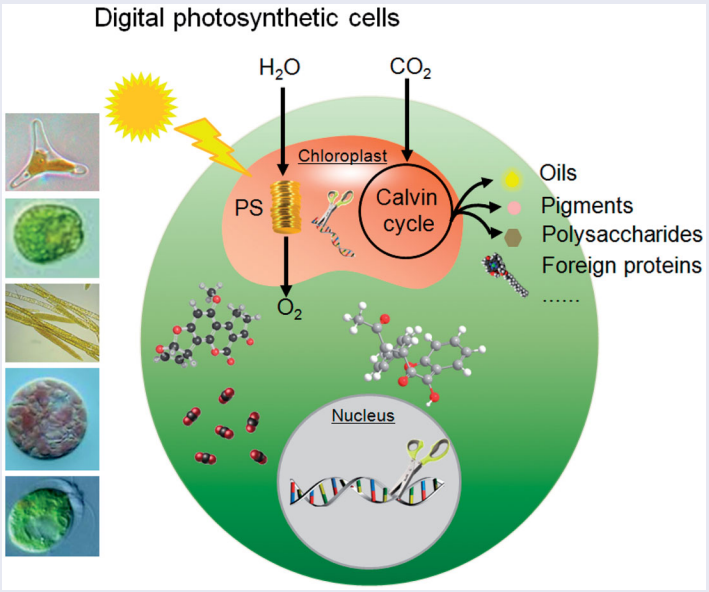
Yandu Lu[a,b], Xu Zhang[a], Xinping Gu[a], Hanzhi Lin[c] and Anastasios Melis[b]

[a]State Key Laboratory of Marine Resource Utilization in the South China Sea, College of Oceanology, Hainan University, Haikou, China; [b]Department of Plant and Microbial Biology, University of California, Berkeley, CA, USA; [c]Institute of Marine & Environmental Technology, Center for Environmental Science, University of Maryland, College Park, MD, USA

**ABSTRACT**

Domesticated microalgae hold great promise for the sustainable provision of various bioresources for human domestic and industrial consumption. Efforts to exploit their potential are far from being fully realized due to limitations in the know-how of microalgal engineering. The associated technologies are not as well developed as those for heterotrophic microbes, cyanobacteria, and plants. However, recent studies on microalgal metabolic engineering, genome editing, and synthetic biology have immensely helped to enhance transformation efficiencies and are bringing new insights into this field. Therefore, this article, summarizes recent developments in microalgal biotechnology and examines the prospects for generating specialty and commodity products through the processes of metabolic engineering and synthetic biology. After a brief examination of empirical engineering methods and vector design, this article focuses on quantitative transformation cassette design, elaborates on target editing methods and emerging digital design of algal cellular metabolism to arrive at high yields of valuable products. These advances have enabled a transition of manners in microalgal engineering from single-gene and enzyme-based metabolic engineering to systems-level precision engineering, from cells created with genetically modified (GM) tags to that without GM tags, and ultimately from proof of concept to tangible industrial applications. Finally, future trends are proposed in microalgal engineering, aiming to establish individualized transformation systems in newly identified species for strain-specific specialty and commodity products, while developing sophisticated universal toolkits in model algal species.

**GRAPHIC ABSTRACT**

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**CONTACT** Yandu Lu ydlu@hainanu.edu.cn College of Oceanology, Hainan University, Haikou 570228, China; Anastasios Melis melis@berkeley.edu Department of Plant and Microbial Biology, University of California, Berkeley, CA 94720-3102, USA
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## Introduction

Microalgae, with an estimate of 72,500 species [1], are of great ecological importance, as they contribute almost half of the global organic carbon fixation [2]. They provide a variety of natural products to support ecosystems (e.g. coral reef ecosystems [3]) by photosynthesis with efficiencies approximately three times greater than those achieved by land plants [4]. The microalgal lifecycle of fast cell division and continuous biomass accumulation highlights the advantages gained from their sustainable cultivation [5]. Accordingly, various attempts have been made to exploit microalgae for the production of commodity and specialty chemicals to meet human domestic, and industrial demands. To identify and improve species that naturally produce valuable compounds, mutation breeding and genetic modifications have been utilized for a long period, but are largely via empirical approaches [6].

Admittedly, extensive reviews cover the bioengineering of cyanobacteria [7], the genomic context underpinning microalgal diversity [8], and the ecology, evolution, and applications of microalgae [9,10]. Therefore, this article primarily focuses on eukaryotic microalgae with a brief comparative study between microalgae and cyanobacteria. Two primary problems are preventing microalgal biotechnology from further development and industrialization. One is that only a few model microalgal species can be routinely transformed and ready for downstream industrialization. The exploitation of all but a handful of algal species is thus severely impeded by a limitation in molecular tools for competent engineering. The other problem resides in the extremely low transformation efficiencies (even compared to those achieved with plants) that are prevailing when working with microalgae, clearly demonstrating a need for novel and improved transformation techniques.

Recent studies on microalgal metabolic engineering, genome editing, and synthetic biology are facilitating the improvement of transformation efficiencies and are bringing new insights into cellular metabolic processes [11]. There exists a demand to jointly consider these developments through a synthetic perspective of microalgal breeding. Therefore, after briefly reviewing empirical methods for vector design and engineering, specifically, this article focuses on (i) quantitative transformation cassette designs, (ii) elaborate target editing methods, (iii) digital design of algal cellular metabolism for the high yield of valuable products, and (iv) problems and countermeasures of industrial application. Drawing lessons from the broader history of the field and emerging advances, it is anticipated that a new era of rational design of digital microalgal cells is coming. This era would substantially benefit human society on food supply, energy consumption, and environmental sustainability.

## Relevance of microalgae as photosynthetic cell factories

Broadly speaking, strategies for engineering microalgal metabolic pathways can be divided into two categories: those based on endogenous pathways, and those derived from non-native pathways by involving heterologous genes.

### *Natural compounds*

Long-term adaptation to a wide range of ecotypes has engendered a diverse phenotype and genotype of microalgae, as well as helping algae evolve robust acclimation plasticity [8], enabling them to adapt to various niches and produce a vast array of compounds. Microalgae are highly efficient at sequestrating $CO_2$, accumulating biomass, and many secondary metabolites including pharmaceutically and nutritionally active compounds for human beings (or precursors for such compounds) (Table 1). It has therefore been suggested that genetically-tailored microalgae could serve as "platform strains" to convert $CO_2$ into diverse and useful compounds from metabolic intermediates [26]. However, current microalgal product yields are generally too low to meet the cost of commercial exploitation. Thus gene engineering approaches have been adopted in an attempt to overcome this drawback by modulating the activity of endogenous rate-limiting enzymes.

### *Heterologous compounds*

Beyond producing endogenous compounds, microalgae could potentially be recruited as cell factories to produce many different non-native compounds, ranging from small organic molecules to large recombinant proteins. Examples of the small molecules that could be produced using microalgae are provided in the report entitled "Top Value-Added Chemicals from Biomass" [30], which identified twelve platform chemicals, that is, small organic compounds that can be produced from sugars by microorganisms and subsequently converted into industrially relevant molecules.

The attractiveness of using microalgae as hosts to produce larger biomacromolecules (e.g. recombinant

**Table 1.** Targeted compounds produced in microalgal cell factories.

| Target products | Human benefits | Species and contents |
|---|---|---|
| **Natural compounds** | | |
| Long-chained omega-3 PUFAs | Cardiovascular health | EPA: *Attheya septentrionalis* (4.58% DW), *Nannochloropsis oceanica* (23.52% TFA), *Nanofrustulum shiloi* (1.3% DW), *Phaeodactylum tricornutum* (3.4% DW), *Thalassiosira hispida* (4.63% DW); DHA: *Nitzschia laevis*(0.59% DW), *T. hispida* (0.61% DW), *A. septentrionalis* (0.60% DW); GLA: *Chlorella pyrenoidosa* (6.43% TFA) [12–14] |
| β-carotene | Antioxidants, improve cognitive function and skin and lung health, reduce macular degeneration and cancer risks | *Dunaliella salina* (10% DW), *Tetraselmis suecica* (8.6 mg/g oil), *Rhodomonas salina* (5.7 mg/g oil), *Thalassiosira pseudonana* (4.3 mg/g oil), *Nannochloropsis gaditana* (3.5 mg/g oil) [15,16] |
| Astaxanthin | Anti-inflammatory effects, antioxidants, anti-cancer, and cardiovascular health | *Haematococcus pluvialis* (5.79% DW), *Chromochloris zofingiensis* (0.22% DW) [17,18] |
| Fucoxanthin | Anti-obesity and anti-oxidant | *P. tricornutum* (38.3 mg/g oil), *T. pseudonana* (31.9 mg/g oil), *Isochrysis* (19.2 mg/g oil), *Pavlova lutheri* (14.8 mg/g oil), *Odontella aurita* (0.8% DW), *Cyclotella cryptic* (1.29% DW) [15,19] |
| Lutein | Prevents cataract, age-related macular degeneration, and cardiovascular diseases, and anti-oxidant and anti-cancer | *Scenedesmus obliquus* (4 mg/g), *R. salina* (8.7 mg/g oil), *Tetraselmis suecica* (5.6 mg/g oil) [15,20,21] |
| Violaxanthin | Prevent night blindness and anti-oxidant property | *Nannochloropsis gaditana* (14.3 mg/g), *N. oculata* (11.5 mg/g), *T. suecica* (7.8 mg/g) (oil:biomass) [15] |
| Zeaxanthin | Prevent night blindness, anti-oxidant property, and prevents liver fibrosis | *N. gaditana* (3.4 mg/g), *Porphyridium cruentum* (3.2 mg/g), *T. suecica* (2.0 mg/g) (oil:biomass) [15] |
| Phytosterols | Cholesterol reduction, anti-inflammatory activity, and even anti-cancer properties | *Isochrysis* (14.9 mg/g oil), *N. gaditana* (17.0 mg/g oil), *N. oculata* (6.1 mg/g oil), *P. lutheri* (97 mg/g oil), *P. tricornutum* (16.5 mg/g oil), *P. cruentum* (26.5 mg/g oil), *R. salina* (26 mg/g oil), *T. suecica* (10.9 mg/g oil), *T. pseudonana* (34 mg/g oil) [15,22] |
| Essential amino acids | Reduce symptoms of depression, boost mood and improve sleep | *C. pyrenoidosa* (21.76% DW) [14] |
| Triacylglycerol (TAG) | Biofuels | *Chlamydomonas reinhardtii* (20.5% DW), *P. tricornutum* (57.8% DW), *N. gaditana* (40–55% lipids of DW) [23–25] |
| **Foreign compounds** | | |
| Immuotoxins | Inhibit tumor growth | *C. reinhardtii* (0.2 − 0.4% TSPs) [26] |
| Vaccine antigens | Block transmission of infectious diseases such as malaria | *C. reinhardtii* (0.09% TSPs) [27,28] |
| Interferon | Cell defense signaling proteins in response to viral infections | *Synechocystis* PCC 6803 [29] |

DHA: docosahexaenoic acid; DW: dry cell weight; EPA: eicosapentaenoic acid; GLA: linolenic acid; TSPs: total soluble proteins; TFA: total fatty acids.

proteins) can be understood by comparing the properties with that of alternative biological production systems, such as bacteria, yeasts, mammals, insects, or plants (Table 2). These desirable qualities, together with the rising demand for recombinant proteins, have driven this pursuit to introduce druggability into transgenic microalgae [26]. Consequently, increasing numbers of recombinant proteins, including antibodies, immunotoxins, vaccine antigens, and mammary-associated serum amyloid, have been produced from metabolic intermediates in microalgae (Table 1). Yet, for all that, it still remains challenging to the sustainable production of foreign chemicals by introducing a *de novo* engineered pathway, necessitating the development of new approaches and advanced engineering strategies.

As putative cell factories, cyanobacteria offer distinct advantages but also usually have some drawbacks, when compared with microalgae. Among the advantages are the ease of transformation and the absence of epigenetic regulation or suppressor mutations to counter the effects of transformation. As a result, cyanobacteria have been successfully engineered to make a variety of heterologous fuels and useful chemicals [41–43]. A breakthrough has been achieved with the design of oligonucleotide fusion constructs (target genes are fused to the highly-expressed endogenous [44] or exogenous genes [45], as protein over-expression vectors that have been used in cyanobacteria to produce plant and human genes that are otherwise difficult to express [44–46]. The "fusion constructs" could

**Table 2.** Characteristics and advantages of representative expression systems.

| Expression systems | Cost | Growth Rate | Post-translational modification (PTMs) | Cultivation Systems |
|---|---|---|---|---|
| Bacteria | • Moderate | • Rapid | • Occur in a relatively low number of bacterial proteins in comparison with eukaryotic proteins [31]<br>• Incorrect folding and assembly [32] | • Heterotrophic cultivation<br>• Enclosed bioreactors |
| Cyano-bacteria | • Low | • Rapid | • No [33] | • Phototrophic or heterotrophic cultivation<br>• Enclosed bioreactors or open race ponds |
| Yeast | • Moderate | • Rapid | • Yes<br>• Unsuitable glycosylation [34] | • Heterotrophic cultivation<br>• Enclosed bioreactors |
| Mammals or insects | • High cost | • Slow | • Yes<br>• Human-like PTMs with some discrepancies [32] | • Heterotrophic cultivation<br>• Complex nutrient requirements<br>• Difficult to scale up<br>• Easily contaminated by animal pathogens [35] |
| Plants | • Less expensive | • Slow | • Yes [36]<br>• The glycosylation patterns often differ from those in mammals [37] | • Phototrophic cultivation<br>• Easy to release the genetically modified materials [38] |
| Microalgae | • Low | • Rapid | • Yes<br>• Species-specific PTMs<br>• The glycosylation patterns are more similar to humans than *E. coli* and yeasts [39,40] | • Phototrophic or heterotrophic cultivation<br>• Enclosed bioreactors or open race ponds |

facilitate the heterologous proteins being accumulated as dominant cyanobacterial proteins, accounting for 20-25% of the total cell proteins [44]. However, pertinent in this respect is that genes from eukaryotic organisms, for example, plants, animals, yeasts, and humans, are consistently expressed at low levels, in both microalgae and cyanobacteria, in spite of the use of strong promoters designed to confer "over-expression" of transgenes [44]. Compared to microalgae, another drawback of the cyanobacterial system is that despite intensive industrial cultivation of cyanobacteria *Spirulina (Arthrospira)* species, the productivity of most cyanobacteria is lower under mass culture and bright sunlight conditions [4]. There are reports of very-rapid-growth of unicellular cyanobacteria [47,48], however, they have not yet been tested on the industrial scale.

## Engineering vectors: transition from empirical to quantitative designs

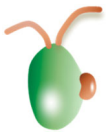
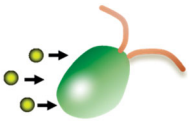
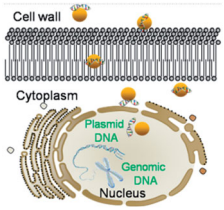
To deliver transgenes into microalgae, several methods have been developed, such as the glass-bead method, *Agrobacterium*-mediated transformation, electroporation, and particle bombardment. The comparison of these transformation methods and the characteristics of transformed microalgae have been listed in Table 3. They have been intensively reviewed elsewhere [75] and thus are not discussed in detail in this review. It should be noted that despite the pros and cons, electroporation-based transformation outweighs other methods in terms of the wide applicable range in microalgal species. Another promising and potentially species-independent method is nanoparticle-mediated DNA delivery, yet to be applied in microalgae. It was first developed by using bacterial magnetic particles (50–100 nm in diameter) in the 1990s [76] and was restricted due to difficulties in particle preparation [77] but recently refined in plants [73,74]. Moreover, despite many challenges, particularly for microalgae holding a tough cell wall, when combined with a droplet microfluidics platform, electroporation [78,79] and nanoparticles [80] are promising methods to convert the current "population transformation" of microalgae into high throughput "single-cell engineering" [81]. One of the necessary jobs that remain for practical application is to decrease the cost and simplify the manipulation of microfluidics.

Notwithstanding, the progress achieved in developing tools for delivering exogenous DNA, routine transformation is available only for a restricted number of microalgae [82]. Moreover, even in established species, obstacles pertaining to gene delivery efficiency, transgene stability, or heritability are preventing the transformation systems from practice. Therefore, it necessitates a methodological transition from the tools used to manipulate metabolism, relying on experience-dependent strategies and constrained in particular species or strains [83], to methods of quantitative and mathematic design.

### *Empirical designs*

Critical to the creation of transgenic microalgae is the ability to transform cells with specific DNA sequences using vector constructs. To ensure the proper transcription, marker genes and/or genes of interest are typically

**Table 3.** DNA delivery methods of modern breeding strategies for microalgae.

| Methods | Illustration | Pros | Cons | Examples |
|---|---|---|---|---|
| The glass bead method |  | More convenient and much less costly | Less efficient | *Chlamydomonas reinhardtii* [49], *Dunaliella salina* [50], and *Platymonas subcordiformis*) [51] |
| *Agrobacterium*-mediated transformation |  | No need to generate protoplasts<br>Holds great promise to transform large DNA segments (>100 kb) into cells | Low and unpredictable variation of efficiencies | Freshwater *C. reinhardtii* [52], *Haematococcus pluvialis* [53] and the marine *Symbiodinium* spp., [54], *Isochrysis* spp. [55], *Schizochytrium* sp. [56], *Nannochloropsis* sp. [57], and *Dunaliella bardawil* [58,59] |
| Biolistic transformation |  | Eliminates the need for production of plant protoplasts or infection by *Agrobacterium* | A rigid requirement to the diameters of the target cells<br>A tendency to generate rearranged and broken transgene copies | The green microalga *H. pluvialis* [60], the diatom *P. tricornutum* [61], *Thalassiosira pseudonana* [62], *Cyclotella cryptic* [63], *Navicula saprophila* [63], *Chaetoceros* sp [64], and *Cylindrotheca fusiformis* [65] |
| Electroporation |  | Simple, easily applicable, and very efficient | Needs a single-cell tissue homogenate | *C. reinhardtii* [66], *Scenedesmus obliquus* [67], *Monoraphidium neglectum* [68], *Nannochloropsis* [69], *P. tricornutum* [70], *Chlorella pyrenoidosa* [71], and *Dunaliella* [72] |
| Nanoparticle-mediated DNA delivery |  | Species-independent<br>Protects polynucleotides against nuclease degradation | Has not yet been applied in microalgae | Both model and crop plants, including both dicots and monocots [73,74] |

expressed in individual cassettes harboring a 5′ promoter and a 3′ terminator. Conventional protocols for selecting marker genes for microalgal transformation have been extensively reviewed [84]. Promoters are critical components that work in concert with other genetic elements (enhancers, silencers, transcription factors, and boundary elements/insulators) to direct the transcription of marker genes and other sequences. Promoter availability and selection thus profoundly influence the success of constructing robust genetic transformation systems. However, until now, few promoters are available for algal vectors, partially due to the shortcomings of the respective genetic toolkits (particularly the limited number of known regulatory elements).

Most attempts to engineer microalgae have been conducted empirically using a handful of repurposed tools. To achieve viable transformants, strong constitutive promoters from phylogenetically closely related algal species, viruses, or occasionally higher plants have been harnessed for transgene expression in microalgae. The viral promoters of CaMV35S (the cauliflower mosaic virus) and SV40 (Simian virus 40; an oncogenic simian polyomavirus) have been utilized for transient expression in some algal species, but heterologous promoter regions are usually inadequately recognized and regulated in microalgae [85] (Table 4). In this respect, there are publications describing the successful transformation of microalgae with results that, unfortunately, could not be reproduced in other laboratories [125]. For example, while the *Arabidopsis thaliana* U6 gene promoter was able to drive gene transcription in *Chlamydomonas reinhardtii* [85], vectors feature in either *Arabidopsis* or *Chlamydomonas* promoters were not successfully recognized in *Nannochloropsis* sp. [109].

As alternatives to exogenous promoters, vectors can be constructed using an orthologous promoter related to one that has been previously characterized. For instance, heat shock proteins [86] and tubulin [126] are highly expressed proteins in microalgae such as *Chlamydomonas* (Table 4). Their promoters are therefore regularly used to drive the constitutive nuclear expression of numerous genes in *Chlamydomonas*, and orthologous promoters have been used successfully in a number of other microalgae. However, microalgae tend to have numerous orthologs with quantitively unknown transcriptional and protein expression levels. In many cases, it is difficult to select suitable driving promoters for vector design. For example,

**Table 4.** Examples of genetic engineering in a variety of microalgae.

| Species | Promoters | Target genes | Highlights | References |
|---|---|---|---|---|
| **Nuclear transformation** | | | | |
| Pyrrophyta | | | | |
| *Symbiodinium* spp. | CaMV35S or NOS promoter | GFP | The first report on stable nuclear transformation of dinoflagellate, *Symbiodinium* by *Agrobacterium*-mediated transformation | [54] |
| Chlorophyta | | | | |
| *Chlamydomonas reinhardtii* | Endogenous promoter of *NIT* | *NIT* | The first report on stable nuclear transformation in microalgae; Glass bead-mediate transformation was used to recover a *NIT*-deficient strain with the wild type gene *NIT1* | [49] |
| *C. reinhardtii* | CaMV 35S promoter | β-glucuronidase (UIDA), green fluorescent protein (GFP) and hygromycin phosphotransferase (HPT) genes | The first *Agrobacterium*-mediated nuclear transformation in microalgae | [52] |
| *C. reinhardtii* | *CaMV35S* promoter, *Arabidopsis U6* gene promoter, or endogenous promoter of the gene encoding Photosystem I reaction center subunit II (*PSAD*) | *Cas9*, guide RNA, mutant GFP (mGFP), or Gaussia luciferase (Gluc) genes | First attempt to express Cas9 and single guide RNA genes in microalgae | [85] |
| *C. reinhardtii* | Endogenous *HSP70A*, *RBCS2*, or fused *HSP70A–RBCS2* promoter | The eubacterial resistance gene *AADA* | Systematically studied the driving efficiency of endogenous algal promoters | [86] |
| *C. reinhardtii* | *β-TUB* promoter | Arylsulfatase gene | First evidence to show the driving efficiency of *β-TUB* promoter | [87] |
| *C. reinhardtii* | Endogenous *PSAD* promoter or a hybrid *HSP70- RBCS2* promoter | *APHVII*" and yellow fluorescence protein (*YFP*) gene | Determined the relative contributions of GC content and codon usage to the efficiency of nuclear gene expression | [88] |
| *C. reinhardtii* | Endogenous *PSAD* or fused *HSP70/RBCS2* promoter | Codon optimized Paromomycin (PARO) gene and *Renilla reniformis* luciferase (LUC) gene | Compared the efficiency, stability and insertion sites of *Agrobacterium*- versus electroporation-mediated transformation | [89] |
| *C. reinhardtii* | Endogenous promoter of the gene encoding argininosuccinate lyase (*ARG7*) | *ARG7* | Employed a cell wall-less *C. reinhardtii* mutant devoid of *ARG7* as host | [90] |
| *C. reinhardtii* | Endogenous promoter of *ARG7* or the gene encoding nitrate reductase (*NIT*) | *NIT1* and *ARG7* | Used silicon carbide whiskers to mediate the transformation of *NIT or ARG7*-deficient strain with the wild type gene *NIT1* or *ARG7* | [91] |
| *C. reinhardtii* | Endogenous *RBCS2* promoter | *BLE* | Highlighted a potential role of introns as modulators of gene expression in microalgae | [92] |
| *C. reinhardtii* | Hybrid *HSP70/RBCS2* promoter | *BLE*, *mCerulean*, or *mCherry* | Developed a set of genetic tools that enable proteins targeting to distinct subcellular locations | [93] |
| *C. reinhardtii* | The *HSP70/RBCS2* promoter containing four copies of the first intron of *RBCS2* between the *HSP70A* or *RBCS2* promoter | *BLE-2A-GFP* and BLE-2A-XYLANASE 1 (XYN1) | Developed a nuclear multigene expression strategy using the foot-and-mouth-disease-virus 2A self-cleavage peptide (2A) | [94] |
| *C. reinhardtii* | Endogenous *PSAD* promoter | Ferredoxin-hydrogenase (*FD-HYD*) gene | Showed that codon usage and mRNA folding energy in the vicinity of translation initiation affect foreign gene expression | [95] |
| *C. reinhardtii* | Hybrid *HSP70/RBCS2* promoter | Codon adapted *LUC* human erythropoietin (*crEPO*) gene | RBCS2 introns alone had a positive effect on expression; The secretion of the LUC protein into the medium was achieved by using the export sequence of the *Chlamydomonas* ARS2 gene | [96] |

(*continued*)

**Table 4.** Continued.

| Species | Promoters | Target genes | Highlights | References |
|---|---|---|---|---|
| *Chlorella pyrenoidosa* | Heterologous *UBI* promoter or endogenous promoters of heat shock protein70 (*HSP70*) or tubulin (*TUB*) gene | *EGFP* and *NPTII* | Electroporation mediated transformation | [97] |
| *C. pyrenoidosa* | Heterologous Ubiquitin (*UBI*) gene promoter | *EGFP* and the gene encoding neomycin phosphotransferase (*NPT II*) | Electroporation mediated transformation | [71] |
| *Dunaliella bardawil* | *CaMV 35S* promoter | GFP, *HPT*, and UIDA gene encoding β-glucuronidase (GUS) | *Agrobacterium*-mediated transformation | [58] |
| *Dunaliella salina* | *CaMV 35S* or *C. reinhardtii RBCS2* promoter | *BLE* and the gene encoding chloramphenicol acetyl transferase (*CAT*) | Electroporation mediated transformation | [72] |
| *Haematococcus pluvialis* | *CaMV 35S* promoter | *HPT* | *Agrobacterium*-mediated transformation | [53] |
| *H. pluvialis* | Endogenous *PDS* promoter | Phytoene desaturase (*PDS*) gene | Biolistic transformation | [60] |
| *Monoraphidium neglectum* | Endogenous light-harvesting protein (*CAB2*) promoter | The genes encoding hygromycin B phosphotransferase (*APHVII''*) and codon optimized mVenus variant (*mVenus*) | A pretreatment with lithium acetate and DTT increased electroporation efficiency | [68] |
| *Neochloris oleoabundans* | *C. reinhardtii HSP70A-RBCS2* hybrid promoter | *GFP* and the hygromycin B-resistant (*HYG3*) gene | Electroporation mediated transformation | [98] |
| *N. oleoabundans* | *CaMV35* promoter | Genes encoding glycerol 3-phosphate acyltransferase, lysophosphatidic acid acyltransferase, and diacylglycerol acyltransferase | Manipulate lipid biosynthesis, use the self-cleaving peptide F2A to simultaneously co-express three genes | [99] |
| *Scenedesmus obliquus* | *CaMV 35S* promoter | *CAT* and *GFP* | Electroporation mediated transformation | [67] |
| Bacillariophyta | | | | |
| *Chaetoceros* sp | *T. pseudonana FCP* or *NR* gene promoters | Nourseothricin resistance (NAT) gene and GFP | Biolistic transformation | [64] |
| *Cylindrotheca fusiformis* | Endogenous promoter of fructanase gene | *ble* and *N. pelliculosa* frustulin (FRUE) genes | Biolistic transformation | [65] |
| *P. tricornutum* | Endogenous fucoxanthin chlorophyll binding proteins (FCP) gene promoter | *Streptoaltoteichus hindustanus BLE* gene | Biolistic transformation | [61] |
| *P. tricornutum* | Endogenous *NR* or *FCPC* promoters | *CAT* and *EGFP* | Electroporation-mediated transformation; Inducible promoter | [70] |
| *P. tricornutum* | Endogenous FCPA promoter | *BLE*, *UIDA*, *GFP*, *NPTII*, and genes encoding nourseothricin acetyl transferase (*NAT*) and streptothricin acetyl transferase (*SAT-1*) | Codon usage has a significant effect on the efficient expression of reporter genes in *P. tricornutum* | [100] |
| *P. tricornutum* | Endogenous FCPA or FCPB promoters | *BLE*, *EGFP*, and *UIDA* | First attempt to establish electroporation-based transformation for diatoms | [101] |
| *P. tricornutum* | Endogenous FCPA or FCPB promoters | A basta resistance (BAR) and the gene encoding a dual-function diacylglycerol acyltransferase (*PtWS/DGAT*) | Revealed that PtWS/DGAT functions as either a wax ester synthase or a diacylglycerol acyltransferase, exhibiting a preference on saturated FA substrates | [102] |
| *P. tricornutum* | Endogenous FCPC promoter | Malic enzyme (*ME*) gene and *CAT* | An Omega leader sequence and "ACC" nucleotide motif were added before *PtME* to enhance its translation; Overexpression PtME boosted neutral lipid accumulation. | [23] |
| *P. tricornutum* | Endogenous histone H4 promoter | *GFP* and *BLE* | Targeted GFP to specific organelles by using mitochondrial transit peptide, nuclear signal peptide or chloroplast transit peptide | [103] |

(*continued*)

**Table 4.** Continued.

| Species | Promoters | Target genes | Highlights | References |
|---|---|---|---|---|
| *P. tricornutum* | *FCPA* promoter or promoter of the gene encoding ammonium transporter, purine permease, or actin-like 2 | *EGFP* and *BLE* | Characterized a serial of promoters, either constitutive or inducible under nitrogen starvation | [104] |
| *Thalassiosira pseudonana* | Endogenous *FCP* or nitrate reductase (NR) gene promoters | *BLE* and GFP | Biolistic transformation | [62] |
| Chrysophyta | | | | |
| *Isochrysis* spp. | CaMV35S promoter | *PDS* | *Agrobacterium*-mediated transformation | [55] |
| **Eustigmatophyta** | | | | |
| *Nannochloropsis* sp. | *CaMV 35S* promoter | UIDA | *Agrobacterium*-mediated transformation | [57] |
| *Nannochloropsis* sp. | Endogenous violaxanthin/chlorophyll a-binding protein (VCP2) gene promoter | BLE | Bidirectional promoter; Homologous recombination | [69] |
| *Nannochloropsis* sp. | Either endogenous or exogenous *β-TUB* promoter | *BLE* | Efficiency of PCR fragment-based transformation was higher than that based on plasmids | [105] |
| *Nannochloropsis gaditana* | Endogenous promoters of the genes encoding β-TUB, HSP70, and the ubiquitin extension protein (UEP) | *BLE* | Quantitative transforming cassette designs; The transformation efficiency of the chosen endogenous promoters approximately corresponds with their RNAseq quantification values; Tested the *P. tricornutum FCPB* promoter, but without success. | [106] |
| *N. gaditana* | Endogenous *UEP* promoter | *BLE* | Targeted gene knock out via homologous recombination; A palmitic acid elongase affects eicosapentaenoic acid (EPA) and plastidial monogalactosyldiacylglycerol levels | [107] |
| *Nannochloropsis oceanica* | *C. reinhardtii* α-tubulin promoter, 35S promoter, or endogenous *LDSP* promoter | *Streptomyces hygroscopicus APH7* gene | Electroporation-mediated transformation; Tested a serial of promoters | [108] |
| *N. ocenica* | Endogenous promoters of genes encoding VCP, V-type ATPase, HSP, and β-TUB | *BLE, HYG, CAS9*, guide RNA genes, hair-pin structure DNAs, or a serial of *DGAT*s | Select endogenous promoters based on the RNAseq quantification values | [109,110] |
| *N. ocenica* | Endogenous ribosomal subunits (*RIBI*) or *LDSP* promoter | *BLE*, *HYG*, *GFP*, *YFP*, the cyan fluorescent protein (CFP), and Luciferase (*LUX*) gene | *Ribi* promoter is a bidirectional promoter; Optimize 2A peptide ribosomal skipping efficiency; Engineered EPA pathway for enhanced long-chain polyunsaturated fatty acid production | |
| *N. oceanica* | Endogenous *DGTT5* or elongation factor (*EF*) promoter | *GFP, type-2 DGAT-encoding genes* (*DGTT1-DGTT6*), and Venus fluorescent protein (*VFP*) gene | Assessed six DGAT-encoding genes for TAG biosynthesis | [111] |
| *N. oceanica* | Lipid droplet surface protein *(LDSP) gene* promoter | Microsomal Δ12-desaturase gene and *APHVII* | Stress-inducible promoter; Overexpression of endogenous Δ12-desaturase improved deposition of unsaturated fatty acids in TAG | [113] |
| *N. oceanica* | *LDSP* promoter | *BLE, HYG, Aph7*, *VFP, GFP,* lysophosphatidic acid acyltransferase (*LPAT1–LPAT4*) genes | Functional investigated on four LPATs for triacylglycerol biosynthesis | [114] |
| *Nannochloropsis salina* | Endogenous *UEP* or *TUB* promoter | Endogenous basic helix-loop-helix (bHLH) transcription factors | Overexpression of *bHLH2* led to increased growth rate, nutrient uptake, and productivity of biomass and fatty acids | [115] |
| *N. salina* | *N. oceanica VCP2* promoter or endogenous *β-TUB* promoter | *GFP*, *BLE*, and hair-pin structure DNAs | Silencing of a pyruvate dehydrogenase kinase enhances TAG biosynthesis | [116] |

(*continued*)

**Table 4.** Continued.

| Species | Promoters | Target genes | Highlights | References |
|---|---|---|---|---|
| **Chloroplast transformation** | | | | |
| Chlorophyta | | | | |
| *C. reinhardtii* | Endogenous promoter of chloroplast *ATPB* gene encoding the subunit of CF1 complex of the chloroplast adenosine triphosphate (ATP) synthase | A DNA fragment harboring the *ATPB* | The first report on microalgal stable chloroplast transformation; Particle bombardment-mediated transformation<br>Employed mutants defective in *atpB* and incapable of photosynthesis as hosts | [117] |
| *C. reinhardtii* | The *16S/ATPA* promoter/UTR combination and endogenous *16S*, *RBCL*, *PSBD*, *ATPA* and *PSBA* promoter | Codon-optimized luciferase gene *LUXCT* | Heterologous protein expression was improved in the chloroplast of through promoter and 5′ untranslated region optimization | [118] |
| *C. reinhardtii* | Endogenous promoter of the gene encoding Core protein of photosystem I (*PSAA*) | *PSAA* or the gene encoding cytosine deaminase, endolysin, or hypothetical protein which is very toxic to *E. coli* | Biocontainment was built into the transgenes by replacing several tryptophan codons (UGG) with the UGA stop codon and using an orthogonal tryptophan tRNA to recognize these internal stop codons | [119] |
| *C. reinhardtii* | Variants of the library for the 5′-UTR of the genes encoding subunits of photosystem I (*PSAA*) and photosystem II (*PSBD*) | A codon-optimized luciferase reporter (*LUXAB*) gene | This study presents a synthetic biology approach to examine *in vivo* of designed variants of endogenous UTRs and quantitatively identify essential regions | [120] |
| *C. reinhardtii* | | | This study represents the first step toward *de novo* creation of chloroplast genomes | [121] |
| **Rhodophyta** | | | | |
| *Porphyridium* sp. | Native promoter of acetohydroxyacid synthase (*AHAS*) gene | A mutant *AHAS* gene | The first genetic transformation system for Rhodophytes; Particle bombardment-mediated transformation | [122] |
| **Eustigmatophyta** | | | | |
| *Nannochloropsis* sp. | Endogenous promoter of large subunit of RuBisCO (RBCL) gene | *GFP* or *BLE* | Electroporation-based chloroplast transformation | [123] |
| **Diatom** | | | | |
| *P. tricornutum* | Endogenous promoter of *RBCL* | *CAT* and E*GFP* | The first plastid gene expression system for diatoms | [124] |

*Nannochloropsis oceanica* strain IMET1 harbors nine orthologs for heat shock proteins and eight orthologs for tubulin. Each of these promoter regions was used separately to construct vectors and drive gene expression [128]. However, success was achieved with only 50% of the assembled vectors even though the same transformation protocol was used in all cases. Similarly in *N. oceanica* strain 1779, the constructs harboring a *C. reinhardtii* α-tubulin promoter or a native lipid droplet surface protein (LDSP) promoter were employed to drive the expression of the *Streptomyces hygroscopicus aph7* gene, conferring resistance to Hygromycin B. The latter achieves a more than 10-fold increase in transformation events compared with the former [108]. The limited transformation efficiency of microalgal expression systems using conventional strategies has resulted in poorly reproducible transformation protocols and has constrained the scope of reverse genetic tool development. Therefore, a more rational approach for promoter dissection and the design of biological engineering systems is needed.

## Quantitative methods for transforming cassette design

The dissection of regulatory elements is essential for the design of engineering systems, which can in turn facilitate an understanding of their natural counterparts [129] (Figure 1). However, the regulatory mechanisms remain unenlightened in microalgae, even with the model species *C. reinhardtii*. Therefore, characterized endogenous promoters have been the primary options when constructing customized vectors for specific microalgae. For example, a promoter for the gene encoding fucoxanthin-chlorophyll binding proteins (FCP) was isolated from diatoms, thoroughly tested, and widely used for the transformation of vector design (Table 4). It is noticeable that vectors incorporating this promoter exhibited stable and relatively high transformation frequencies in both biolistic and electroporation-induced transformations [102,130]. Additionally, several endogenous regulatory elements were characterized and incorporated into vectors for *Chlamydomonas* transformations using *Agrobacterium* [89], electroporation [89], silicon carbide whiskers [91], glass beads [49], and biolistic methods [117], achieving comparable efficiencies. Thus, vector assembly is a key determinant of transformation efficiency. The frontiers of this field have been advanced by the development of novel promoter engineering strategies which are generally classified as (a) random mutagenesis; (b) hybrid promoter design; (c) *de-novo* promoter synthesis [131].

In addition to promoters, incorporating regulatory elements such as introns and featured transcript sequences were also applied to improve the transformation efficiency and increase exogenous gene expression [95]. Synergistic effects were utilized to improve transgene expression by either incorporating different intron portfolios into vectors or including a consensus Kozak sequence in the 5′ UTRs [132] (Table 4). To allow proteins to target specific organelles, a leader-targeting sequence [23], comprising transit peptides [133], could be designed. These results highlight the key role of regulatory elements in the design of engineering systems and the necessity of dissecting the mechanisms and functions of different regulatory elements. Unfortunately, the diversity and potential of these regulatory mechanisms (or elements) in microalgae are largely unknown [125] as a result of the low-throughput methods for characterizing the molecular mechanisms.

The ongoing expansion of sequenced genomes of microalgae (60 already completed or in preparation) and several cyanobacteria, and the associated omics information facilitates quantitative dissection of the mechanisms underpinning the functionality of promoters and other regulatory elements in a wide range of microalgae [134]. Together with the development of sophisticated trapping systems [135] and the computational analysis of biological components, it is increasingly viable to rationally and systematically identify, characterize, and standardize promoters, untranslated regions, terminators, enhancers, silencers, codon preferences, and other yet-to-be-discovered elements [136]. This knowledge-driven strategy offers several potential advantages over traditional methods for establishing microalgal transformation systems. For instance, *in silico* prediction and investigation of regulatory elements considerably increases the likelihood of discovering active regulatory components. Additionally, system-level investigations on gene structure can help identify species-specific regulatory mechanisms and biological components, which facilitates the customized design of synthetic promoters, biological bricks, and circuits [137]. Finally, cross-species genome comparisons are helpful to unveil universal regulatory rules operating in different microalgae and thereby enable the design of universal (at the species or genus levels) transgene vectors.

We see notable progress in the dissection of algal genetic elements in such a knowledge-driven manner. A number of strong promoters have been discerned from different *Nannochloropsis* species [109] (Table 4). To enable multiple-gene expression, bidirectional

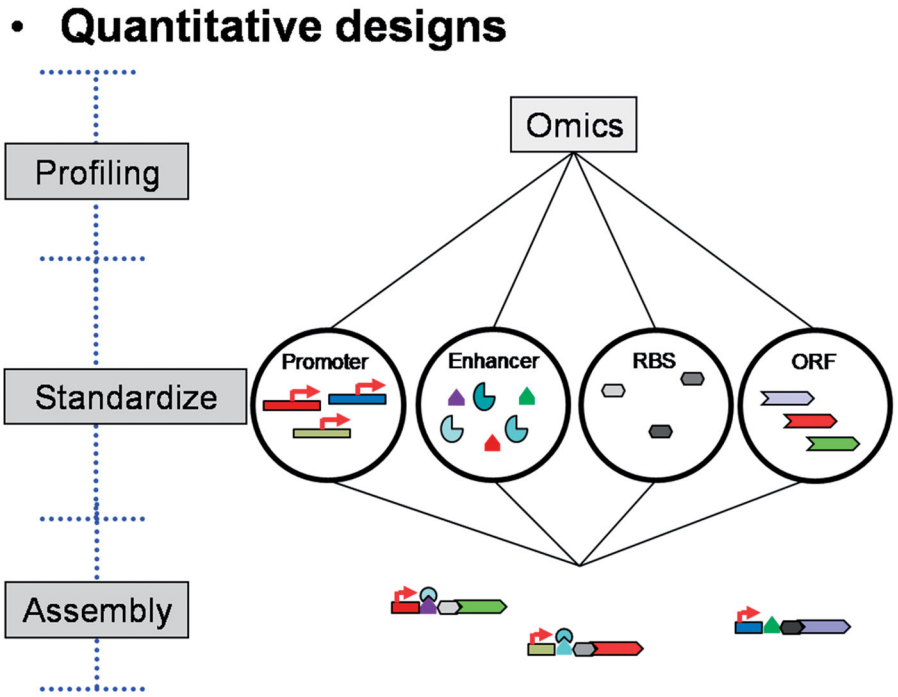



**Figure 1.** Approaches of quantitative design for the engineering vectors. Abbreviations: RBS, ribosomal binding site; ORF, open reading frame.

promoters [111] have also been isolated from this microalgal genus. Promoters, both constitutive and inducible under nitrogen starvation, were employed for customized transgene expression in the diatom *P. tricornutum* [104]. Quantitative profiling of such components can enable their use in BioBricks – DNA sequences conforming to a restriction enzyme assembly standard and encoding one or more functional units, that can be used as part of a scalable transgenic toolbox. The assembly and engineering of individual components with defined functions from libraries of such standardized interchangeable parts could enable the design and the development of generalizable engineering tools.

## Engineering strategies: a trend to editing methods

### *Gene overexpression*

Deliberate overexpression of genes of interest (GOIs) is widely used for functional analysis (Figure 2). Distinctive to the capacity for expressing multigene in a single operon in prokaryotic or prokaryotic-derived genomes (e.g. microalgal chloroplasts), and marker genes should be located in different expression cassettes in microalgae. Overexpression of multiple genes is a distinct advantage and requirement for trait optimization. Various strategies have been designed for multi-gene expression in microalgae (Figure 2(a)). For example, co-expressed genes can be cloned into separate vectors with different selective markers and then transformed into one strain sequentially [140]. Alternatively, recently developed strategies for multigene engineering, involve designing expression vectors using bidirectional promoters [111,139], isocaudamers [141], or using the self-cleaving peptide F2A (e.g. in *C. reinhardtii* [142], *Nannochloropsis* [111], or *Neochloris oleoabundans* [99]). Gene stacking overexpression systems have been established in some microalgae (e.g. *C. reiharditti*, *Nannochloropsis* sp., *Chlorella* sp., and *P. tricornutum*). This system is used to study specific metabolic pathways and to express intracellular [143] or secreted [144] recombinant proteins. These technologies simplify the transformation process, diminish the number of marker genes required for multi-step integration, and improve the dosage of target protein(s) after fusion with proper selectable markers.

### *Random mutagenesis*

Overexpression platforms serve as foundations for the establishment of a comprehensive engineering system incorporating technologies of insertional mutagenesis, RNAi-mediated gene knockdown, and genome editing. Identifying mutants with an interesting phenotype is a classical genetic approach for probing life processes (Figure 2(b)). One way is to use transfer DNA (T-DNA), which is randomly integrated into genomes. It features

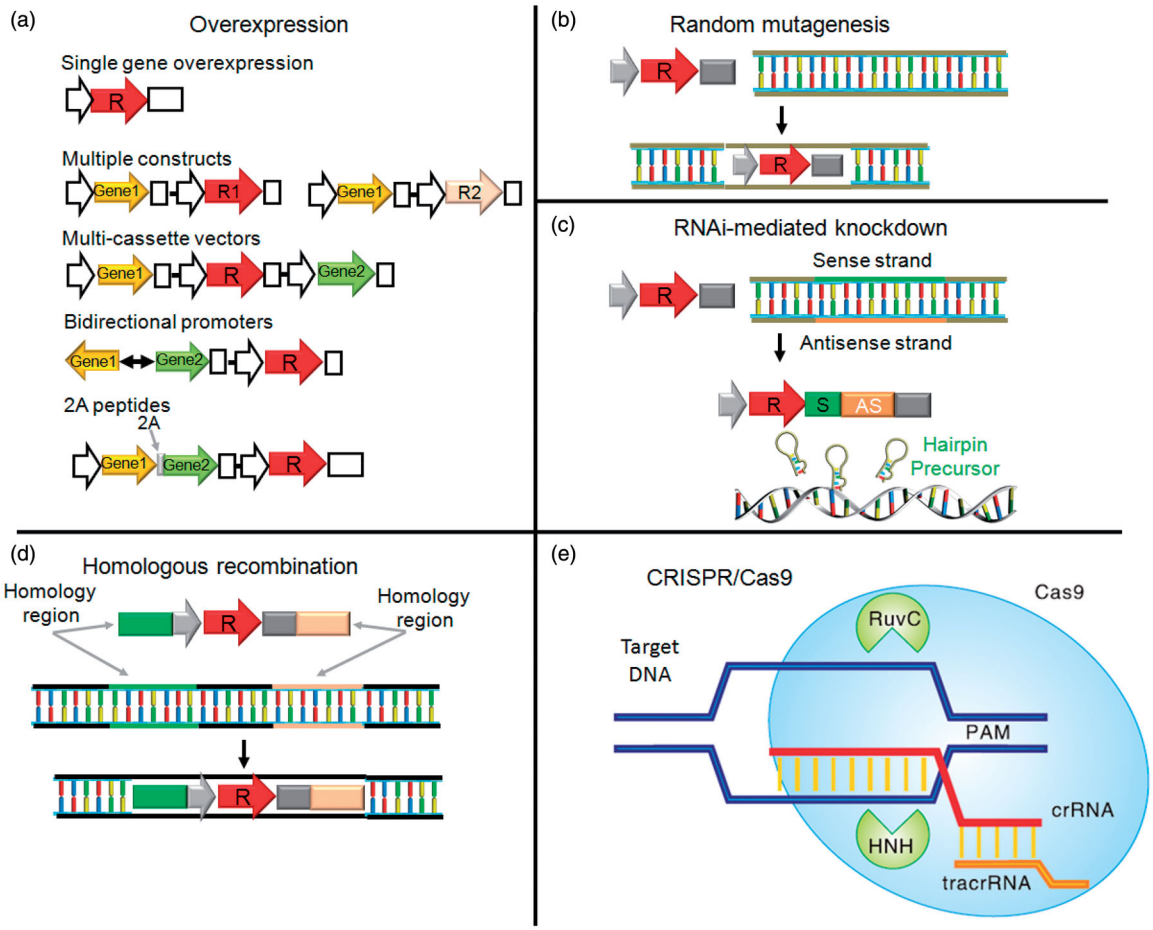



**Figure 2.** Engineering strategies for microalgae. (a) Overexpression strategies for a single gene or multiple genes. (b) Random DNA insertional mutagenesis. (c) RNAi-mediated gene knockdown. (d) Homologous recombination. (e) Targeted genome editing by CRISPR/Cas9 technology. Abbreviations: R, resistance marker; Gene, interested gene(s); S, sense strand; AS, antisense strand; RuvC, RuvC nuclease domain; PAM, protospacer adjacent motif; HNH, HNH nuclease domain; crRNA, CRISPR RNA; tracrRNA, trans-activating crRNA.

tag-like sequences that facilitate the identification of the affected gene(s) [145]. Therefore, gene inactivation by T-DNA mediated transposon insertion has been used in functional studies on diverse plant species that have in turn accelerated both basic research and applied plant biotechnology. However, T-DNA has not been demonstrated in algae thus far. Alternatively, by random insertion of transforming cassettes, whole-scale genome mutagenesis libraries have been generated only in a single microalga *C. reinhardtii* [146]. The mutant population has proved to be effective to dissect the function of key genes in photosynthesis [147] and TAG biosynthesis [148]. However, current methods for flanking sequence retrieval tend to be species-specific: the application of these methods is always constrained to a single species and can only determine insertion sites for that particular species, and even worse, at a low frequency [149]. A tagged mutant library was recently created for *C. reinhardtii* in a similar fashion to T-DNA that was successful in *A. thaliana* [148]. It was utilized to dissect genes involved in biological processes, such as photosynthesis [147] and $CO_2$ concentration [150,151]. The generality of this approach highlights its potential in investigating the function of non-lethal genes in many other microalgae. However, a drawback is that loss-of-function mutations caused by random disruptions are unlikely to probe the function of lethal genes. In addition, the random mutagenesis approach is of limited utility in studies targeting specific genes and in hypothesis-driven tests.

### *RNA interference (RNAi)-mediated gene knockdown*

RNAi-based gene silencing is a superior approach for the oriented knockdown of arbitrarily chosen genes (Figure 2(c)). Despite the apparent loss of core elements in many microalgal species [152], RNAi-mediated silencing appears to be widespread among algal lineages [128]. Although its biological machinery remains elusive

[153], sophisticated systems for small RNAs have been discovered in green, red, brown algae, diatoms, and dinoflagellates [154–158]. RNAi-mediated knockdown has been established for species including *C. reinhardtii* [159], *N. oceanica* [128], and *P. tricornutum* [160]. Nevertheless, the RNAi method has several general shortcomings such as unstable phenotype heritability and unpredictable activities of target genes [161].

### Targeted genome editing

To avoid unwanted phenotypes brought by random transforming cassette insertion (when using overexpression) or off-target knock-down (when using RNAi), it is desirable to develop tools for the precise modification of pre-selected sequences. Traditionally, homologous recombination is a routine practice for the genetic manipulation of prokaryotes. It was also developed in recombinogenic lower eukaryotes [162] (Figure 2(d)). However, this method delivers only limited success in most eukaryotic microalgae, where non-homologous recombination tends to occur in preference to the homologous one [163]. These problems have been circumvented by developing strategies based on engineered nucleases, which substantially facilitates targeted gene disruption and is revolutionizing many areas of science [164]. Although the zinc-finger nuclease (ZFN) and transcription activator-like effectors (TALEN) are excellent tools for targeted gene knockout, they have only been proven to be useful in *C. reinhardtii* [165] and *P. tricornutum* [166]. Clustered regularly interspaced palindromic sequences (CRISPR/Cas9) technology is more efficient and much easier in design than ZFN and TALEN technologies [167]. The straightforward design of CRISPR systems allow simultaneous disruption in multiple genes [168] and the creation of CRISPR mutant libraries [169] (Figure 2(e)). The list of applications of CRISPR has expanded to include knock-in [170] and CRISPR interference (CRISPRi), which involves inducing sequence-specific interference of a target gene's transcription [171]. By CRISPR/Cas9-derived activator systems, multiple endogenous (non)coding genes can be activated simultaneously [172]. The ability to upregulate any endogenous gene(s) provides unprecedented opportunities to look into and reconstruct cellular behavior. Therefore, an increasing number of microalgal species are engineered by CRISPR/Cas9 systems, such as *C. reinhardtii* [165], *Nannochloropsis* sp. [24], and *P. tricornutum* [173]. However, the CRISPR/Cas9-based technology in microalgae is still in its infancy, as evidenced by low efficiencies and the need for more intensive mutant screening than in plants and animals. To ensure efficiency and accuracy, standardized targeted genome editing protocols should be developed for microalgae on the basis of in-depth fundamental investigations, particularly on the effects of endogenous silence of introduced genetic materials [174].

### From nucleus to organelle engineering

Nuclei, chloroplasts, and mitochondria are three membrane-bounded organelles in the microalgal cell. Although most of the preceding examples involve nuclear transformation, the nuclear expression has the drawback of sensitivity to epigenetic effects and random insertion, which can cause variable transgene expression. In contrast, chloroplast engineering shows several key advantages, such as absence of epigenetic gene silencing, targeted localization of transgenes in the genomes, and relatively high expression levels [175]. Microalgal chloroplast genomes (e.g. in *Nannochloroposis* sp.) possess approximately 120 genes involved in photosynthesis and the gene expression system [176]. In principle, all of these genes could be precisely manipulated. Furthermore, a number of nuclear gene products (such as those involved in photosynthesis, oil metabolism, or plant hormone synthesis) are targeted to plastids [177,178]. For example, in *Arabidopsis*, more than 2000 nuclear-encoded proteins are predicted to localize in chloroplasts and contribute to cellular properties critical for the growth or the production of essential cellular compounds [179]. This further enlarges the category of biological traits which could be optimized by plastome engineering.

Although chloroplast transformation was initially developed in the green microalga *C. reinhardtii* [117], plastome genetic engineering was more thoroughly developed in and applied to plant species. The use of chloroplast engineering to produce pharmaceuticals in crop plants presents several fundamental challenges [180]. Problems include slow plant growth [4], especially in cases when a high yield and a recombinant product amount is required. This problem is further accentuated by the seasonal growth of crops, as opposed to the year-round cultivation of microalgae. Moreover, transgene containment [181] is always more difficult to cope with in plants than that in photobioreactor-enclosed photosynthetic microalgae. Microalgal platforms avoid many undesirable issues since these systems can be cultivated under tightly controlled and contained conditions in closed photobioreactors. In addition, the difference in codon usage in microalgae from that of plants helps avoid contamination of the human food chain with heterologous proteins due to cross-pollination [182]. Biocontainment can be improved further by codon

reassignment of the transgenes in the chloroplast [119]. Taken together, barriers encountered in the use of plant-based systems to produce recombinant protein or other heterologous products would make them less attractive than photosynthetic microorganisms, for example, microalgae and cyanobacteria [29]. More than 100 foreign or native proteins have thus far been produced in algal chloroplasts, including 40 different therapeutic proteins produced in *C. reinhardtii* chloroplasts [183]. Table 4 shows some case studies of chloroplast transformation. Further examples are given in recent reviews [183].

However, despite some notable advances, microalgal plastid transformation is only available for a relatively small number of species [82]. Chloroplast transformation has mainly relied on bombardment with gold microparticles laced with the transforming DNA, which is intractable for most microalgae. The smallest readily available gold particles have a diameter of around 0.6 μm which exceeds the dimensions of most microalgal chloroplasts [123]. An alternative method is via polyethylene glycol (PEG)-mediated protoplast transformation [184]. However, an intrinsic drawback of this method is the requirement of cell wall removal prior to transformation. Protoplast preparation is technically demanding and effectively impossible for many microalgal species with complex cell walls [185]. A simple and straightforward electroporation-based method was recently devised for transforming *Nannochloropsis* chloroplasts [123]. However, expression levels of transgenes in microalgal chloroplasts remain low compared to plant plastid and cyanobacterial expression systems. To bring plastome engineering into full play, it is critical to establish protocols that enable efficient and reliable expression for individual transgenes or entire pathways. It is thus indispensable to identify several components, such as constitutive promoters for robust expression [118], controllable expression systems for spatially or temporally inducible expression, and optimized genetic elements for multigene expression [84,120]. Synthetic biology principles have been applied to plastome engineering, allowing *ex vivo* assembly, modification and duplication of the entire *Chlamydomonas* chloroplast genome [186]. This proof of concept experiment demonstrates that *de novo* synthesis of algal chloroplast genomes is now possible.

### *From genetically modified (GM) to "non-GM" engineering*

GM crops have been commercially used for decades. Available impact studies of pest-resistant crops show that these technologies are beneficial to farmers and consumers, producing large aggregate welfare gains as well as positive effects on the environment and human health [187,188]. Nonetheless, there has been substantial debate regarding the merits and ethics of GM and non-GM breeding technologies. Widespread public reservations have led to a complex system of regulations and in most countries, the commercial use and cultivation of GM organisms (GMOs) is heavily regulated [189]. For example, the EU arguably has the strictest regulations in the world for the use of GMOs in both food and feed. Specifically, organisms obtained by techniques of genome editing (that cannot be distinguished from a conventionally-bred variety or a naturally-occurring variant; without GM tags) are regarded as GMOs and subject to the same restriction as other transgenic organisms [189]. GM microalgae are created by inserting DNA-containing marker genes and additional GOIs into the host's genome. A critical concern regarding the GM approach is the risk of promoting antibiotic resistance. To address this concern, techniques for generating transgenic algae should not rely on antibiotic resistance markers. Thus, successful *C. reinhardtii* chloroplast transformation and strain selection protocol with zero false-positives was achieved upon reconstitution of a functional *rbcL* gene recovery of Rubisco catalytic activity, coupled with the heterologous expression of the *Saccharomyces cerevisiae* alcohol dehydrogenase *ADH1* gene in the algal chloroplast [190]. This recovery of function, however, requires that photosynthesis-lethal microalgal mutants, which act as the transgene recipient strain, can be propagated heterotrophically. Alternatively, a recombinase has been applied to remove marker genes in engineered cyanobacteria [191]. Extrachromosomal vectors, or episomes, offer another tool for "non-GM" breeding in microalgae [192]. Combined with genome editing techniques, engineered cells of diatoms [193] and *N. oceanica* [194] maintained the desired traits while episomes (harboring the marker gene) were lost. It highlights the potential to create a "non-GM" microalga by the development of tools derived from episomes with specific and universal algal hosts [195]. This highly efficient genetic system will accelerate knowledge transfer from fundamental research to tangible applications.

## History of microalgal engineering approaches: a starting point for future development

Genetic manipulation has been accomplished in a wide spectrum of microalgae. In this section, we will revisit viable ways to generate useful products by engineered microalgae.

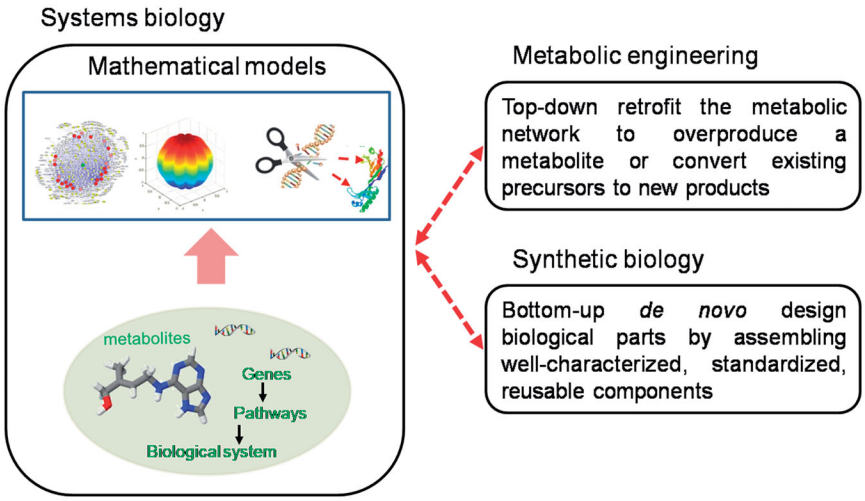



**Figure 3.** Comparison between metabolic engineering and system biology. Adapted from [6].

### *Conventional gene engineering – technologies based on single-enzyme encoding genes*

Metabolic engineering is a purposeful modification of metabolic pathways for customized or improved production of useful compounds [196] (Figure 3). Historically, metabolic engineering efforts in microalgae have largely centered on modifying a single gene at a time, typically one encoding a rate-limiting enzyme. Biosynthetic pathways are retrofitted to preferentially flux endogenous carbon toward desirable-end chemicals [23], but not to byproducts [197]. Strategies adopted for this purpose include yield improvement [102,198], substrate range expansion [138], novel chemicals synthesis [26], and optimization of cellular robustness [199,200]. Subsequent developments in molecular and chemical biology led to the design and reconstruction of complete metabolic networks and even whole organisms rather than just individual reactions. It is from these studies that the phrase "cell factories" emerged. High-throughput omics techniques make it possible to rapidly link genotypes and phenotypes and to accelerate the engineering processes [201]. These revolutionary techniques and mindsets have transformed metabolic engineering into a new field comprising genome-scale engineering, which incorporates computational biology, versatile approaches for interrogating and understanding cellular metabolism, and multiplex optimizations to reach diverse phenotypes [202].

### *Transcriptional engineering – a control-knob-gene-based approach*

Mixed success has been achieved in microalgae using the aforementioned conventional genetic engineering strategies. In some cases, the abundance of the desired metabolite remained unaltered. Transcriptional engineering provides an alternative strategy where a control-knob gene can be modified to simultaneously modulate multiple steps of a metabolic pathway [203]. Initial attempts at trait improvement of microalgae by this strategy involved expressing plant transcription factors (TFs) and resulted in enhanced production of target chemicals [204]. This success prompted a pursuit of genome-wide identification of microalgal TFs [205], which were subsequently utilized to optimize the desired properties [24,202].

### *Synthetic biology – a systems-level precise engineering*

The advance of metabolic engineering along with the increasing blending of DNA technology gave genesis to the field of synthetic biology (Figure 3). A genetic toggle switch prototype was first devised in bacteria by metabolic engineers [206], elaborated by synthetic biologists [207], and consummated in mice [208]. Unlike metabolic engineering, synthetic biology may use unnatural molecules to create artificial biological systems [6]. While this approach has been successfully implemented in bacteria, yeasts, higher plants, and animals by characterizing and collecting reusable and standard biological parts [209,210], microalgal synthetic biology remains in its infancy. Attempts have been undertaken in only a handful of species. Their successes have been modest because of the underdevelopment of genetic tools and the limited knowledge of gene regulation mechanisms in microalgae, as discussed precedingly [211]. However, advances in several species (e.g. *Nannochloropsis* sp., *C. reinhardtii*, and *P.*

*tricornutum*) are now allowing an expansion to the realm of genome-wide reprogramming beyond conventional individual gene manipulation [212]. Together with the ongoing expansion of microalgal omics datasets, it is possible to use these species as chassis for synthetic biology. A versatile and modular vector toolkit has been generated for *C. reinhardtii*, which features a standardized collection of reporters, selectable markers, and targeting peptides that can be assembled and repurposed [213]. Broadly speaking, this toolkit generation highlights the inaugural application of synthetic biology in microalgal engineering and enables the development of digital photosynthetic cells.

## Future perspectives

### *Digital photosynthetic cells*

Principles and methods of synthetic biology from well-established systems are increasingly introduced into microalgae. However, the development of microalgae as true "programmable" entities is hampered not only by the accessibility of well-characterized and repurposed genetic elements but also by a lack of high-throughput methodology to identify, standardize, and rationalize basic parts and modules, not to mention systems-level circuitry. This is true even for *C. reinhardtii*, of which, among all microalgal species, the transcriptional regulation and cellular metabolism are best understood. The systematic knowledge of its regulatory elements and potential BioBricks remains elusive. Despite these challenges, the development of standardized and scalable methods for module assembly (e.g. Gibson assembly and Golden Gate cloning), engineering (i.e. transformation), colony handling (e.g. colony picking robots), and high-throughput strain screening (e.g. microfluidics) will give microalgal researchers unprecedented access to "digital photosynthetic cells" in which each gene, pathway, and the biological process can be quantitatively designed and modulated.

### *From proof of concept to tangible industrial application*

Enhancement in the microalgal biofuel R&D occurred in the period between 2006 and 2011 because the global petroleum price skyrocketed [214]. However, with the 2014 collapse in the price of fossil fuels, the renewable biofuels field is at a crossroads and its further development becomes foggy. End-products with an interest of the algal community now mainly focus on primary and secondary metabolites, such as proteins, essential oils, specialty chemicals, and biopharmaceuticals. These comprise sizable markets, albeit not as large as that offered by the transportation fuels field. Moreover, the diversity of natural secondary metabolite in microalgae is far from being fully exploited, suggesting the promising development of future R&D and commercialization. Meanwhile, it may be necessary to fill the gaps between laboratory R&D and tangible industrial production by engineering microalgae with crucial and economically relevant pathways of value-added compounds, such as wax [102], astaxanthin [215], and non-native isoprenoids (e.g. β-phellandrene; lupeol, bisabolene, patchoulol, geranyllinalool, and 13R(+) manoyl oxide) [216,217]. Plant secondary metabolites are a valuable reservoir of drugs and numerous of these compounds, such as ginsenosides and artemisinin, are among the most promising and commercially important biopharmaceuticals that can be generated in microalgae and cyanobacteria. These pathways and the associated key genes could be characterized using microalgal systems which resemble those of plants, but possess unique advantages as mentioned above. Moreover, unlike yeast or bacterial systems, microalgae are promising hosts to produce these metabolites in scalable culturing systems. The development of market-ready drug-producing systems using industrial microalgal strains is just beginning [193,216–218].

To realize the economic viability of commercial microalgal products, there is a need to inform on several major areas, including: (i) For newly identified species with industrial potential, the challenge would be to deliver exogenous DNA into the cells. (ii) Universally applied sophisticated engineering toolkits are essential in efforts to produce versatile biochemicals and biomaterials for agricultural, biomedical, and industrial ends. (iii) The development of genetic circuits, gene switches, systems-level circuitry, synthetic devices, synthetic genomes, genome editing technologies, and practices for microbiome engineering [219] will benefit fundamental studies in microalgal synthetic biology. (iv) Genome-wide activation/knock down/knock out of multiple endogenous genes and simultaneous overexpression of multiple exogenous genes involved in target pathways or networks would greatly facilitate the creation of "super" microalgal strains as customized cell factories. (v) Although the application of glycosylation mechanisms is largely untapped in microalgae [220,221], "humanization" of microalgae glycans via glycoengineering would greatly facilitate the development of active and safe biopharmaceuticals production with proper folding and addition of immunogenic glycans [39].

The sustainability of the target chemical productivity in engineered algal cells has been assessed in bench-scale (10 L) [102] and in pilot-scale photobioreactors

(PBRs; 550 L) [216] in controllable laboratory conditions or in mimicked outdoor environments [24]. The scalability and stability of introduced traits in GM microalgae were also probed by culturing cells at different PBR configurations and volumes [222]. Although engineered phenotypes appeared to be stable and robust across these tested scales (from 800 mL to 100 L) and PBR configurations (i.e. vertical column, vertical flat-plate, and open raceway pond), reports on life-cycle assessments of bioenergy or other value-added chemicals using engineered microalgae are not yet available.

A barrier to the commercial-scale algal biofuel production is the relatively high capital and operating costs of PBRs, combined with the need to deliver fuel products at very low cost, typically less than $1 per kg. Coupling biofuel production with environmental applications, or the co-generation of high-value products, could achieve mutual benefits in renewable energy production and mitigation of greenhouse gas emission [223] or removal of pollutants from wastewater [224]. Moreover, extensive R&D is essential for studies on lowering the cost of downstream processing, such as industrial-scale cultivation, contamination control, cell harvesting and disruption, and extraction of target biomolecules. There is no doubt that engineering technology, in particular, simultaneously targeting/activation/overexpression/knockout of multiple genes, would play critical roles in achieving the objective. For example, truncated light-harvesting antennae of the photosystems can be generated to improve sunlight penetration and utilization, resulting in greater photosynthetic productivity of cultures with a high cell density under bright sunlight conditions [225]. Versatile contaminant control strategies have also been developed for open pond cultivation by modifying nutrition mode [226], expressing herbicide resistance genes [227], or genome-based chemical biology [228]. Target biomolecules could be extracted using cost-effective cell disruption methods by expressing algicides [229] or upon bypassing the disruption step employing an inducible green recovery strategy [230]. Overall, sound opportunities exist for the development of market-ready multiple-product systems by employing microalgae as digital photosynthetic cells.

## Conclusions

We have reviewed the trend of microalgal engineering in the era of synthetic biology. In this article, the bioengineering of cyanobacteria, genomes, and evolution, and applications of microalgae were not at the center of our attention, but the bottleneck and the development of the engineering techniques of eukaryotic microalgae were considered. As mentioned, microalgal metabolic engineering, genome editing, and synthetic biology are increasingly intervening in microalgal biotechnology due to their numerous advantages over mutagenesis strategies using chemical and physical mutagens. Microalgal engineering technologies rapidly transfer from empirical methods to quantitative design, as that target genome editing and synthetic biology play more and more important roles in breeding new algae and the digital design of algal cellular metabolism. The current trend explicitly demonstrates that alongside technological progress in transforming techniques, algal engineering develops mostly toward customized transformation systems, systems-level precision engineering, and cells created without GM tags. All these aspects are going to be integrated with big data analysis of the increasing omics dataset. Despite positive horizons, the costs, the development of scale-up configurations, as well as the public concerns about GMOs, are dominant challenges.

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## ORCID

Yandu Lu http://orcid.org/0000-0002-0136-2252

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