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Review

Landscape of microalgae omics and metabolic engineering research for strain improvement: An overview

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ABSTRACT

The unique metabolic capabilities and fast growth rates of microalgae render them promising candidates for various industrial applications, such as biofuel production, pharmaceuticals, nutraceuticals, and wastewater treatment. Metabolic engineering is a powerful approach used to enhance the sustainable production of high-value compounds in microalgae, improve their stress tolerance, growth characteristics and suitability for large-scale cultivation. This review provides a snapshot of the current state of knowledge on omics and metabolic engineering research to further enhance our understanding on microalgal metabolism and enable the development of optimized strains with improved productivity and functionality. More specifically, it focuses on the recent breakthroughs in microalgal omics, driven by advancements in genomics technologies, such as improved sequencing platforms and bioinformatics tools, that have enabled the functional characterization of key genes, identification of metabolic pathways, and elucidation of microalgae cell physiology.

Conventional and state-of-the-art genetic engineering approaches used in the last decades to manipulate the metabolic pathways of microalgae in a targeted manner, are highlighted in the scope of microalgal optimization. In this review, the different applications of genetic engineering and their impact on microalgae industry are also discussed. Integrating pan-omics data in future research is crucial for predicting novel functional interactions and identifying aspects of metabolic flux, towards enhancing algal strain-engineering techniques.

Keywords: genomics, transcriptomics, genetic engineering metabolism, microalgae applications, gene-editing, genetic transformation

1. INTRODUCTION

Microalgae, one of the most ancient living forms on earth, are microscopic mono or multicellular organisms that include the prokaryotic cyanobacteria, and the eukaryotic, green algae and diatoms (Mishra et al., 2019a; Mobin & Alam, 2017). Microalgae are autotrophic organisms having high photosynthetic efficiency, and no intrinsic sensibility to seasonality, due to the multiple coping mechanisms developed under different light regimes (Perin et al., 2019). They can recycle atmospheric carbon dioxide (CO_2) (Zhu et al., 2017) and combined with the use of non-agricultural land for biomass production can minimize the associated environmental and food-security impacts (Levasseur et al., 2020). The ability of microalgae to adapt to various abiotic stress factors, such as light, salinity, temperature, provides an interesting potential for the induction of high-value metabolites production (Gauthier et al., 2020; Little et al., 2021; Mishra et al., 2019; Rastogi et al., 2020; Ren et al., 2021; Suparmaniam et al., 2022).

There is a great diversity of microalgal species that provide energy-rich compounds for sustainable energy production, and other high value bioactive molecules of significant commercial interest, such as proteins, lipids and pigments used in pharmaceutical, nutraceutical, and cosmetics industries (Abu-Ghosh et al., 2021; Chung et al., 2017; Coulombier et al., 2021; De Luca et al., 2021; Martínez-Ruiz et al., 2022; Morais Junior et al., 2020). As such, a possible pipeline to optimize traits of high importance (i.e. yield, nutritional content) for large scale sustainable production may include i) species selection, ii) use of the trait plasticity/evolution through random DNA alteration or controlled DNA manipulation and iii) bioprocess optimization of the developed strain (Torres-Tiji et al., 2020).

Recent developments in genome sequencing, strain development, and genome editing technologies offer great opportunities for boosting microalgae productivity. Genomics, transcriptomics and the use of cutting-edge single-cell technology, as well as comparative data from different microalgae, would be beneficial in developing a comprehensive model of algae genomes (Shi et al., 2021). Algae Omics technologies, often known as "Algomics," provide insights into the evolutionary origins of algae and a comprehensive image of algae structure and system-function (Mishra et al., 2019; Ribeiro et al., 2017). In general, aspects of interest in microalgae research are focusing on omics approaches regarding biofuel and biofertilizer production, heavy metal bioremediation, pharmaceuticals, nutraceuticals, and cosmetics (Ahmad et al., 2022; Ranjbar & Malcata, 2022; Rumin et al., 2020).

The microalgae genomes database could enable the identification and distribution of gene families involved in high value molecules synthesis, among various selected microalgae models. However, only a limited number of whole genome sequences is available and is restricted to microalgae models (Brar et al., 2021; Fu et al., 2019), and therefore a significant and relevant mapping may not be possible. Gene characterization related to high value compounds synthesis using the -omics approach could facilitate the establishment of phyla-, genera-, or species-specific expression patterns, whereas genomics could be used as a quick guide in bioprospecting to discover compounds of interest. Further algal genome research will facilitate the access to functional and important genes, such as genes of industrial and biotechnological interest, for future applications (Abu-Ghosh et al., 2021).

Microalgae hold great potential for genetic engineering applications for enhancing productivity, accelerating growth rates, optimizing biofuel production, and generating valuable compounds. The omics technologies and the availability of numerous molecular tools and methods have significantly contributed to the advancement of algae biotechnology. Over the past five years, there has been a significant expansion in the genetic manipulation toolkit available for industrially important microalgal strains (Fajardo et al., 2020; Grama et al., 2022; Sproles et al., 2021). Numerous genetic transformation methods were initially created and improved using the green algae *Chlamydomonas reinhardtii*, and their use has subsequently been extended to other groups of algae, including red algae (rhodophytes), brown algae (phaeophytes), euglenoids, diatoms, and dinoflagellates. Recent efforts to enhance the quality and quantity of desired products in microalgae have concentrated on the development of targeted genome editing tools, such as the CRISPR/Cas9, TALEN, zinc-finger nuclease (ZFN) and RNA interference (RNAi) technologies, along with the development of high-throughput screening methodologies for fast and robust strain improvement procedures (Liang et al., 2019; Lin et al., 2019; Patel et al., 2019).

The current knowledge on the strategies used to induce and improve the bioproduction of high-added value metabolites in microalgae, including natural diversity, optimization of growth and productivity in a crosstalk with omics technologies and genetic engineering tools, towards application in various industrial sectors is presented in Figure 1. This review focuses on the omics combinatorial approach to high-throughput analysis of microalgae for trait improvement. It broadly encompasses genomics, transcriptomics, and genetic engineering tools applied to improve microalgal traits. It first covers a global presentation of genomics, transcriptomics and genetic engineering, to finally summarize and evaluate the application of such technologies in microalgae industry. This review aims to explore the different genetic engineering methods employed in algae and their potential applications in greater detail. The objective of this review is to provide insights into the recent landscape of omics approaches to unravel the intricate metabolic pathways and regulatory networks within microalgae and the use of genetic engineering tools in boosting microalgae strain improvement through targeted enhancement of desirable traits highlighting the challenges that need to be remediated. The different applications of genetic engineering and their impact on microalgae industry are also discussed. It also emphasizes and discusses the potential aspects of using genetic engineering for industrial production of bioactive compounds.

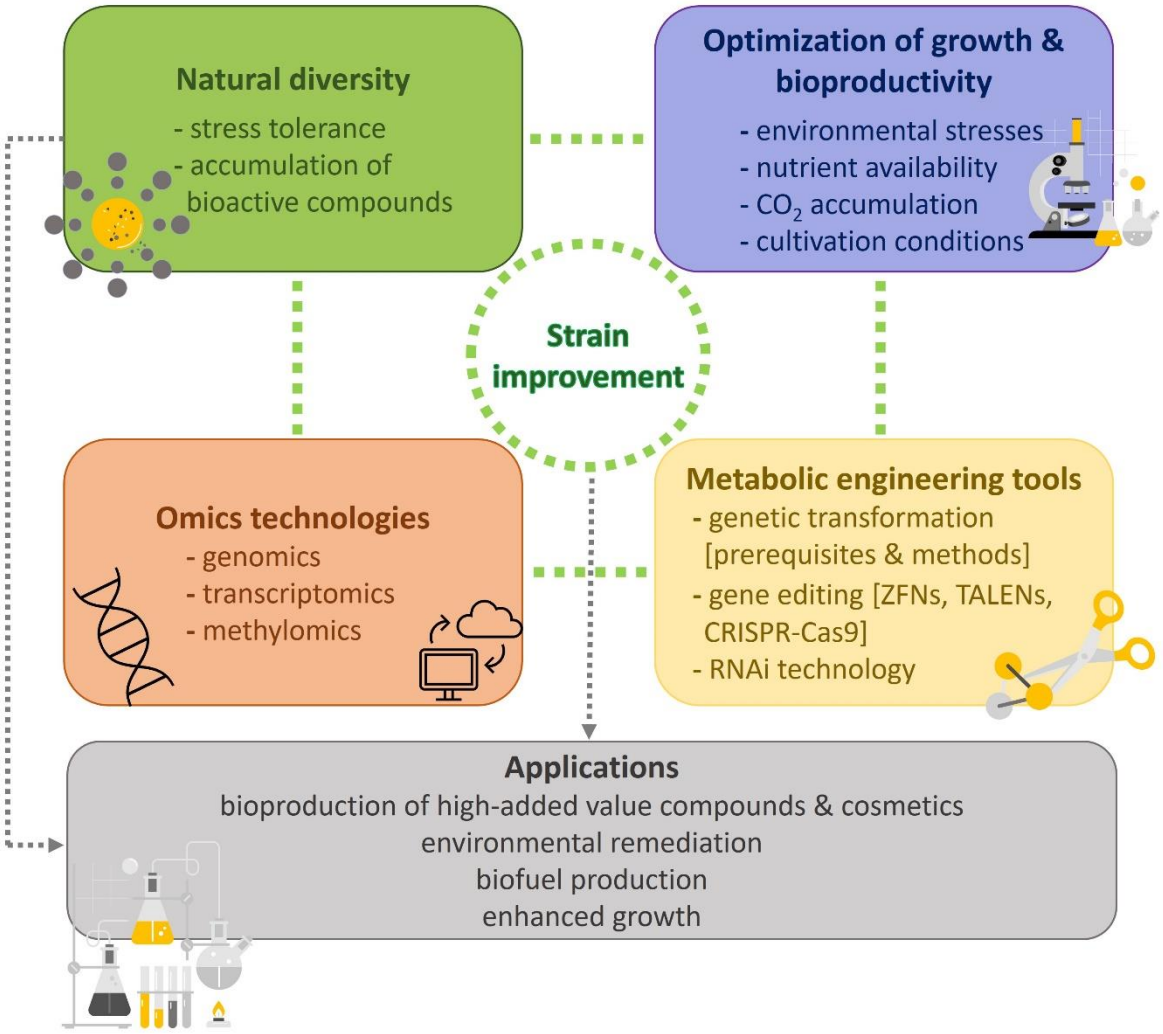

Figure 1. Graphical outline on the current knowledge of microalgae strain improvement using the omics resources and genetic engineering strategies. Natural diversity in microalgae and optimization techniques of growth and bioproductivity also play an important role in strain improvement towards enhancing stress tolerance and production of high added value compounds.

2. Genomics

Algae are among the most genetically complex organisms known. Genomic analysis is a powerful tool, providing crucial insights into the genetic complexity ofmicroalgae. Genome sequencing projects for different species of microalgae have been completed, allowing the discovery and annotation of genes involved in numerous biological processes. Genomic resources have been established for several groups of microalgae, notably Chlamydomonas, Nannochloropsis, and diatoms. This availability of genomic data has provided valuable insights into the genetic makeup and molecular mechanisms of these microalgae species (Armbrust et al., 2012; Gong et al., 2020; Merchant et al., 2010). Comparative genomics

studies have played a crucial role in investigating the evolutionary connections between different microalgal species, identifying expansions or contractions in gene families, and pinpointing conserved regions shared among diverse microalgal taxa. The genome of *Cyanidioschyzon merolae*, a red extremophile alga, was the first to be published in 2004 (Matsuzaki et al., 2004), and although *Chlamydomonas reinhardtii*, a single-celled flagellate freshwater green alga, is one of the most researched species (Mishra et al., 2019) its genome was published only later in 2007 (Merchant et al., 2010).

Currently, 191 algal genomes are publicly available, representing a diverse spectrum of algae taxa. Among these are 117 green algae (111 Chlorophyta and six Charophyta), 34 Heterokonts (diatoms), 12 Rhodophytes (red algae), ten Dinoflagellates, six Haptophytes, five Cryptophyceae, and a Glaucophyte (Shi et al., 2021) (Table 1). Green algae have the highest representation, accounting for approximately 58% of the sequenced algal genomes, followed by Heterokonts (18%), red algae (6%), the toxin producing Dinoflagellates (5%), and Haptophytes (5%). Together, these five groups represent 92% of the published algae genomes. Green algae are the most extensively studied, constituting 91% (174) of all published algal genomes. Among the sequenced genomes, the green alga *Ostreococcus tauri* (Prasinophyceae) is the smallest known free-living eukaryote, with a genome size of 12.56 Mb (Shi et al., 2021), whereas the biggest known the genome is that of the Dinoflagellate *Lingulodinium polyedrum* having a size of 185 Gbp (Casabianca et al., 2017). Regarding the organelle genomes, *Grateloupia taiwanensis* stands out with the highest gene richness, having 233 protein-coding genes (DePriest et al., 2013). In contrast, *Cyanidioschyzon merolae*, a red microalga, has the least gene-rich nuclear genome, with only 4,775 protein-coding genes (Nozaki et al., 2007). Interestingly, up to 20% of the nuclear genes in the green microalgae appear to have originated from the cyanobacterial endosymbiont through a process called endosymbiotic gene transfer (Dagan et al., 2013; Deusch et al., 2008) highlighting the significant impact of endosymbiosis on the evolution and genetic composition of microalgae.

Thus far, and in terms of completed genomes, only a few species have been fully sequenced without any gaps (Table 1). These include the relatively small nuclear genomes of the red alga *Cyanidioschyzon merolae* (16.5 Mbp) (Nozaki et al., 2007) and the Prasinophytes *Micromonas commoda* RCC299 (Worden et al., 2009) and *Ostreococcus lucimarinus* (Palenik et al., 2007). The number of published algal genome sequences continues to rise, driven by advancements in sequencing technologies, reduction in sequencing costs, and improved sequencing quality. The ALG-ALL-CODE project focused on sequencing over 120 genomes of microalgae, obtained from newly isolated and maintained stocks provided by culture collection centers, such as UTEX and NMCA (Ashworth & Ralph, 2018). According to the latest update of the project (March 2021), 22 subtropical microalgal species have been isolated and sequenced from the UAE and 107 additional species were obtained from international algal culture collections, including UTEX (www.utex.org) and the Bigelow National Center for Algae and Marine Microbiota (https://ncma.bigelow.org/). Notably, the 10KP Genome Sequencing project initiative, launched in 2017, aims at further expanding this dataset by sequencing the genomes of 1000 green algae (chlorophytes and streptophytes) and 3000 protists (photosynthetic and heterotrophic) (Sirohi et al., 2021), with, thus far, having sequenced and assembled over 300 green algae species.

The diversity of transposable elements (TEs), some of which form new classes, is another aspect revealed by algal whole genome sequencing (Tirichine & Bowler, 2011). Transposable elements are repetitive genetic sequences that play a vital role in the genome by being capable of relocating to different positions within the genome. This mobility can cause disruptions in the normal functioning of genes and induce alterations in the overall structure of the genome. The ratios of TEs in algae range from 5% to 40%, compared to 161 terrestrial plants, which normally contain approximately 50% of repeated sequences (Shi et al., 2021).

Table 1. Summary of the complete or nearly complete genomes of different microalgae species.

Species	Genome Size (Mb)	References
Auxenochlorella protothecoides	22,92	[Vogler et al., 2018; Xing et al., 2018]
Bathycoccus prasinos	15,07	[Moreau et. al., 2012]
Botryococcus braunii	164,2	[Browne et. al., 2017]
Chlamydomonas debaryana	120,36	[Yoshitomi et al., 2019]
Chlamydomonas reinhardtii	120,4	[Sithtisarn et al., 2017; Salguero et al., 2019]
Chlamydomonas sphaeroides	126	[Hirashima et. al. 2016]
Chlorella pyrenoidosa	56,99	[Run et. al., 2016]
Chlorella sorokiniana	58,53	[Dawson et al., 1997]
Chlorella variabilis	46,16	[Juneja et al., 2016; Sorigue et al., 2016]
Chlorella vulgaris	*39,08*	[Zuñiga et al., 2018; Mocsai et al., 2019]
Chloroidium sp.	54,31	[Nelson et al., 2019]
Chondrus crispus	104,98	[Collén et. al., 2013]
Chromochloris zofingiensis	58	[Liu et al., 2014]
Coccomyxa sp.	48,54	[Koechler et al., 2016]
Cyanidioschyzon merolae 10D	17	[Nozaki et. al., 2007]
Dunaliella salina	550	[Polle et. al., 2017]
Galdieria phlegrea DBV009	11,4	[Qiu et. al., 2013]
Galdieria sulphuraria	13,71	[Thangaraj et al., 2010]
Gonium pectorale	148,8	[Hanschen et. al., 2016]
Haematococcus pluvialis	669	[Luo et.al., 2019]
Helicosporidium sp.	13,2	[Pombert et. al., 2014]
Klebsormidium nitens	104,21	[Nagao et al., 2008]
Micromonas pusilla	21,96	[Kujawinski et al., 2017]
Monoraphidium neglectum	69,71	[Jaeger et al.,2017]
Nannochloropsis gaditana	29	[Fernandez-Acero et al., 2019; Patelou et al., 2020]
Nanochloropsis oceanica	29,3	[Guo et al., 2019]
Ostreococcus lucimarinus	13,2	[Krumholz et al., 2012]
Ostreococcus tauri	13,03	[Blanc-Mathieu et. al., 2014]
Parachlorella kessleri	64,9	[You et al., 2019]
Picochlorum sp.	13,5	[Foflonker et. al., 2015]
Porphyra umbilicalis	87,7	[Brawley et. al., 2017]
Porphyridium purpureum CCMP1328	19	[Bhattacharya et. al. 2013]
Pyropia yezoensis U-51	108	[Wang et. al., 2020]
Scenedesmus obliquus	107,72	[Wang et al., 2019]
Scenedesmus sp.	93,24	[Wang et al., 2019]
Tetraselmis striata	227,95	[Steadman et. al. 2019]
Volvox carteri f.Magariensis	137,68	[Schoch et. al., 2020]

Studies have shown that horizontal gene transfer (HGT) in green and red algae is associated with various functions such as carbohydrate metabolism, osmolyte management, sulfate scavenging, cell cycle control, energy shuttle, arsenic detoxification, and stress adaptation mechanisms, with many algae exhibiting relatively low levels of HGT gene percentages

except for a few specific species, such as Charophyta, Dinophyceae, Haptophyta and Stramenopiles (Heterokonts) (Foflonker et al., 2015; Hirooka et al., 2017). These genomic resources serve as invaluable tools for studying microalgae biology, identifying important genes and pathways, and facilitating genetic engineering and biotechnological applications in the field.

3. Transcriptomics and methylomics

Transcriptomics is a powerful approach to unveil gene expression patterns across the entire transcriptome, allowing the investigation of an organism's capabilities and limitations related to various traits such as resistance, acclimatisation, and developmental processes (Page & Lawley, 2022). *De novo* assemblies (Blanc-Mathieu et al., 2014), microbiome investigation (Carney et al., 2014), and other genome sequencing applications (Teng et al., 2019), have become the gold standard method for transcriptome studies (Rismani-Yazdi et al., 2012), with more than 700 microalgae transcriptomes being available on algae database (www.algaedatabase.org/). The "Alganaut" is an interactive web resource available to the public, designed for the exploration of microalgae transcriptomes (Ashworth & Ralph, 2018). This resource combines a total of 1,375 samples obtained from 69 independent experimental trials, representing ten different microalgae species and four distinct clades. Additionally, the Marine Microbial Eukaryote Transcriptome Sequencing Project (MMETSP) (Keeling et al., 2014) has created 678 assembled, functionally annotated, and publicly available transcriptomes of 306 pelagic and endosymbiotic marine eukaryotic species representing more than 40 phyla.

The utilization of transcriptomic analyses can greatly accelerate advancements in harnessing algae for biofuel production. One example is the unicellular green algae species *Chlamydomonas moewusii*, which produce hydrogen under both light and dark anaerobic conditions (Sirohi et al., 2021). By conducting RNA sequencing (RNA seq) transcriptomic studies, over a specific time frame, valuable insights can be obtained regarding the expression patterns of genes involved in anaerobic fermentation, glycolysis, starch catabolism, and hydrogen evolution in dark anaerobic conditions. Such transcriptomic investigations serve as a foundation for characterizing and understanding the underlying genetic mechanisms and provide valuable information for potential genomic modifications aimed at enhancing specific production capabilities or improving stress tolerance in algae (Table 2). Knowledge on the genes and gene functions in microalgae species, such as the model organisms *Chlamydomonas* and *Nannochloropsis* spp., has assisted on the application of genetic engineering tools such as, gene editing and RNA interference (RNAi), given the available genetic resources and mutant collections (https://www.chlamylibrary.org/) of these species (further examples are presented in detail in section 4). Similarly, transcriptional engineering has also been used to overexpress the endogenous *bzip* transcription factors (TFs) mediated by the promoter of the heat-shock protein HSP20, increased the lipid content in the transformant lines compared to wild strains in *Nannochloropsis salina* (*NsbZIP1*) and N. oceanica (*NobZIP1*) (Kwon et al. 2018; Li et al. 2019).The processes underlying stress adaptation and metabolite production in microalgae at the epigenetic level are still unknown despite the crucial role in microalgae adaptation to the variable growth environments. Recent

studies have provided evidence of methylation events occurring in algae. The *C. reinhardtii* nuclear genome is methylated at low levels (5.4, 2.6 and 2.5% in CG, CHG and CHH context, respectively). Similarly, *Chlorella sp*. Demonstrates even lower levels of CG methylation, ranging from 4% to 5%. These findings shed light on the presence of methylation processes in algae genome (Ferrari et al., 2023). Microalgal genomes are shaped by epigenetic markers, which also control gene expression and may have an impact on how accessible the genome is to TFs or alter the expression of TFs. For instance, in *C. reinhardtii*, various epigenetic alterations altering TF activity have been discovered in relation to histone modifications (Bacova et al., 2020). At present, there is increasing interest in the microalgal epigenome, especially regarding the regulation of gene expression under variable conditions (Bacova et al., 2020; Ferrari et al., 2023). The genome of *C. reinhardtii* comprises of both ^{6}mA and ^{5}mC modified bases compared to those of other unicellular organisms (Hattman et al., 1978). After mapping the distribution of the ^{6}mA modification in more than 84% of genes of *C. reinhardtii*, the ^{6}mAs were found to cluster around the transcriptional start sites (TSSs) of active genes (Fu et al., 2015). Future studies are anticipated to unravel the role of epigenetics in microalgal biochemical pathways, which might be used to adjust cellular and physiological balance to boost the production of beneficial metabolites.

Table 2. Summary of the different conditions and abiotic stresses tested on various microalgae species in transcriptomics studies of the last decade from 2014-2023.

Conditions	Species	References
Growth conditions	*Chlorella sp.*	[Koh et al., 2023]
	A. protothecoides	[Steichen et al., 2022]
	Symbiodinium microadriaticum	[Aranda et al., 2016]
	Micromonas sp	[Baren et al., 2016]
Oil accumulation	*A. protothecoides*	[Gao et al., 2014]
	Ettlia oleoabundans	[Sturme et al., 2018]
	Lobosphaera bisecta	[Gao et al., 2020]
	Schizochytrium sp.	[Chang et al., 2021]
	S. obliqnus	[Silva et al., 2021], [Xi et al., 2021]
Different growth phases	*Chlorococcum sp*	[Lv et al., 2019]
	Messastrum gracile SE-MC4	[Afifudeen et al., 2021]
Day-night cycle	*Chlamydomonas sp.*	[Willamme et al., 2018]
Stresses		
Temperature stress	*A. protothecoides*	[Xing et al., 2018]
	Emiliana huxleyi	[Benner et al., 2013]
	Fragilariopsis cylindrus	[Jabre et al., 2021]
	Picochlorum sp.	[Barten et al., 2022
	Symbiodinium sp.	[Gierz et al., 2017]
	Chlorella sp.	[Poong et al., 2018]
	Schizochytrium sp.	[Hu et. Al., 2022]
	C. reinhardtii	[Légeret et al., 2016]

Phosphatase stress	*A. protothecoides*	[Park et al., 2018];
	M. pusilla	[Bachy et al., 2018]
Nitrogen deprivation	*B. braunii*	[Fang et al., 2015]
	C. reinhardtii	[Yang et al., 2022]
	C. vulgaris	[Nordin et al., 2020]
	C. sorokiniana (1230)	[Zhu et al., 2022]
	Neochloris oleoabundans	[Jaeger et al., 2018]
	M. neglectum	[Jaeger et al., 2017a]
Salinity	*D. salina*	[Polle et al., 2020],
	C. reinhardtii	[Wang et al., 2018]
	C. pyrenoidosa	[Sun et al., 2014]
	C. vulgaris	[Abdellaoui et al., 2019]
	N. oleoabundans	[Jaeger et al., 2018]
	P. kessleri	[You et al., 2019]
CO_2	*C. pyrenoidosa*	[Fan et al., 2016]
	C. sorokiniana	[Li et al., 2016]
	Coccomyxa subellipsoidea	[Peng et al., 2016]
	C. merolae	[Rademacher et al., 2016]
	E. huxleyi	[Wang et al., 2022]
	N. oceanica	[Wei et al., 2019]
	Thalassiosira pseudonana	[Clement et al., 2017]
	Dunaliella salina	[Huang et al., 2022]
Response to osmotic and oxidative stress	*D. salina*	[Chernobai et al., 2022]
	C. reinhardtii	[Choi et al., 2023]
Cold acclimation	*Galdieria sulphuraria*	[Rossoni et al., 2019]
Light intensity	*Chlorella sp.*	[Azaman et al., 2020]
	Monoraphidium sp.	[He et al., 2015]
	S. obliquus	[Breuer et al., 2013]
	Pavlova lutheri	[Carvalho & Malcata, 2005]
	N. gaditana	[Mitra et al., 2015]
UV radiation	*V. carteri f. Magariensis*	[Ekhtari et al., 2019]
	C. merolae	[Tardu et al., 2016]
Heavy metals	***Pb* – *C. reinhardti***	[Zheng et al., 2020; Guan et al., 2023]
	Cd – *C. sorokiniana*	[Ding et al., 2020]
	Cd – *Synechocystis sp*	[Tian et al., 2022]
	Cd – *D. salina*	[Zhu et al., 2019]
	Cd – *Chlamydomonas acidophila*	[Puente-s´anchez et al., 2018]
	Cd – *A. protothecoides*	[Lu et al., 2019]
	Cd – *C. reinhardtii*	[Simon et al., 2008]
	Cu – *Microcystis aeruginosa*	[Wang et al., 2020]
	Cu – *C. reinhardti*	[Beauvais-Flück et al., 2019]
	Co - *D. salina*	[Ling et. Al., 2021]
	Co – *B. braunii*	[Cheng et al., 2018]
	Hg – *Chromera velia*	[Sharaf et al., 2019]
	Hg – *C. Reinhardtii*	[Beauvais-Flück et al. 2017]

Nanoparticles	***FeO*** *– O. Tauri* ***ZnO*** *– M. Commoda* ***α-Fe2O3*** *– C. vulgaris*	[Genevière et al., 2020] [Bibi et. Al., 2021]
Wastewater	*Desmodesmus sp*	[Wang et al., 2022]

3.1.Environmental Stresses

Transcriptomics have been used in microalgae research to mainly elucidate the mechanisms involved in environmental adaptation and acclimation (Rismani-Yazdi et al., 2012) (Table 2). Environmental stresses, such as nutrient deprivation, increased salinity, and temperature fluctuations, shifting light intensities and toxic compounds including heavy metals and organic toxins, that have a significant impact on microalgae growth and metabolism (Al-Ealayawi & Al-Dulaimy, 2023)and (Tirichine & Bowler, 2011). Nevertheless, different taxa have variable natural tolerance to such environmental stressors. The red algae *Galdieria sulphuraria* can thrive in a pH range of 0 to 4 and at temperatures as high as 56°C (Shi et al., 2021). The halophytic algae *Emiliania huxleyi* creates algal blooms from the equator to the subarctic, and under a wide range of climatic conditions. Whereas the green alga *D. salina* (Polle et al., 2017), *Picochlorum SENEW3* (Foflonker et al., 2015) and *Picochlorum renovo* (Dahlin et al., 2019) exhibit enhanced salt tolerance, under a range of 1,4 to 1,6 M NaCl.

Microalgae cultivation under different abiotic stress conditions may increase the synthesis of high-value metabolites, whilst in the past decades research has focused on the effects of variable conditions on metabolic pathways. Many research groups are focusing on microalgae adaptation in fuel gas settings, primarily comprising CO_2, Nox, and Sox (Cheng et al., 2016; Listmann et al., 2020; Schaum et al., 2017). The adaptation strategies of microalgae under nutrient stress conditions have also been evaluated unlocking their mechanisms for survival and growth in nutrient-limited environments (Helliwell et al., 2015; T. Li et al., 2016). Conventional lipid enhancement strategies for sustainable microalgal biodiesel production include nutrient stress and alterations in cultivation conditions (Singh et al., 2016; Zienkiewicz et al., 2016). One of the leading approaches in deciphering the mechanisms of algal lipid metabolism is the functional analysis of potential candidate genes governing lipid synthesis in algal cells through comparing global gene expression in nutrient-replete and nutrient-deprived cells of different microalgal species (Zienkiewicz et al., 2016). More specifically, nitrogen and silicon limitation along with CO_2 enrichment coupled with low temperature shock were shown to improve lipid content and stimulate lipid productivity in the diatom *Skeletonema costatum* (Gao et al., 2019; Xie et al., 2022).

Transcriptomics approach has also been used to assess and enhance the heat tolerance of *Dunaliella bardawil*, a halophilic green algae strain widely utilized for outdoor production of *β*-carotene showing that genes encoding for heat shock proteins (HSP) and antioxidant enzymes were up- regulated (Sirohi et al., 2021). Stress tolerance of microalgae to ultraviolet radiation (UVR) and high light intensity has also been investigated in the frame of inducing co-tolerance between abiotic and chemical stress factors, such as herbicides (Deblois et al., 2013; Korkaric et al., 2015), indicating that abiotic stress acclimation can provide evidence on the mechanistic effect of both stressors and the underlying co-tolerance mechanisms.

Moreover, research on salt tolerance mechanisms in microalgae has increased our understanding on osmolyte accumulation and physiological changes (Hu et al., 2020; Kato et al., 2022; X. Li et al., 2018; Meijer et al., 2017; Shetty et al., 2019; X. M. Sun et al., 2018). Transcriptomic analysis of the green microalga *C. sorokiniana* under high salt conditions, have unraveled the upregulation of genes involved in osmoregulation and ion transport processes (Azaman et al., 2020). Similarly, different microalgae strains exhibit distinctive iron uptake mechanisms under iron deficiency, such as diversity in proteins responsible for iron absorption and their regulation under variable iron levels or iron-starvation-induced proteins and iron-reductases through siderophore-mediated iron assimilation (reviewed by Rana & Prajapati, 2021). Recent genomics analysis of the diatom *T. pseudonana* under iron deficiency has revealed the presence of multiple genes related to iron assimilation and transport, indicating a robust adaptive response to iron scarcity (Al-Ealayawi & Al-Dulaimy, 2023).

Microalgal species play a significant role in environmental pollution monitoring and bioremediation through the phytoremediation of both hazardous metals and nanometals (Abdel-Ghany et al., 2015). Microalgae play an important role in environmental remediation by absorbing, sequestering, and altering toxic metals and nanometals. Their participation in phytoremediation and monitoring renders them valuable tools for tackling the consequences of metal contamination in aquatic environments (Touliabah et al., 2022) The tolerance of microalgae to organic pollutants (Cho et al., 2016; H. Li et al., 2021; X. Wang et al., 2019), high concentrations of heavy metals, and under wastewater conditions (Okurowska et al., 2021; L. Wang et al., 2018; Yu et al., 2020) has been examined. Research on algae transcriptome in response to different pollutant stresses is a commonly employed approach to uncover molecular changes and assess biological risks accurately and sensitively (Duan et al., 2019; L. Wang et al., 2018; Q. Zhu et al., 2021) that can potentially enhance the bioremediation ability of microalgae.

4. Genetic Engineering

The advancement of algae biotechnology greatly benefits from the valuable and comprehensive data repositories provided by omics technologies. The field of microalgae biotechnology is gaining increasing commercial significance across a wide range of industries including food, pharmaceuticals, nutraceuticals, cosmetics, animal feed, energy, and environmental applications, such as water and soil remediation (Singh et al., 2021; Vanhoudt et al., 2018) as the genetic modification of microalgae expands the biotechnological applications. In this context, genetic engineering plays a key role in molecular farming, also known as gene pharming, that involves the production of enzymes, antibodies, immunotoxins, bioactive peptides, and hormones (Hempel et al., 2011; Mayfield, 2013; Rasala & Mayfield, 2011; Rosales-Mendoza, 2016; E. Specht et al., 2010; E. A. Specht & Stephen, 2014; Yan et al., 2016; Yusibov et al., 2016).

Thus far, the process of modifying the alga genome has traditionally relied on known genetic engineering tools (Table 3) followed by selecting desired mutations. However, with the advent of genome editing tools, or gene editing techniques given their focus on the modification of specific targeted genes in microalgae (Jeon et al., 2017), it is possible to achieve precise and specific genomic mutations (Table 3). These editing tools enable the

targeting and modification of specific regions in the microalgae genome, utilizing sequence-specific nucleases, such as zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and CRISPR-associated protein 9 (Cas9). Among these nucleases, Cas9 and related Cas proteins are preferred in microalgae genetic engineering due to their simplicity, precision, and efficiency (Fayyaz et al., 2020; Jeong et al., 2023). Furthermore, chloroplast transformation techniques have been successfully developed and applied to *C. reinhardtii* and various other microalgal species. In these approaches, antibiotic resistance genes, herbicide resistance genes, and metabolic marker genes have been implemented (Doron et al., 2016; Esland et al., 2018).

Table 3. Transformation methods and gene mutants used for different algae species and strains for genetic engineering from 1997-2022.

Species/Strain	Transformation method	Gene mutants	References
C. reinhardtii	Biolistic, Glass bead agitation/ Electroporation/ *Agrobacterium*-mediated	Disruption of the photoreceptor genes *COP1/2*, *COP3* (encoding channelrhodopsin 1 [ChR1]), *COP4* (encoding ChR2), *COP5*, *PHOT*, *UVR8*, *VGCC*, *MAT3*, and *aCRY* and creation of the *chr1 chr2* and *uvr8 phot* double mutants; generation of an IR transgene targeting the *EXO* gene (protein ID 407657); insertion of *Paromomycin* (*Paro*) gene and a reporter gene (*Renilla reniformis* luciferase (Luc)	[Greiner et al., 2017; E. J. Kim & Cerutti, 2009; Mini et al., 2018]
C. reinhardtii (CW15, CC-503, CC4349, CC124, CC503, CC-4349, CC-5415, CC-124, UVM4, N-UVM4, CC-4349)	Electroporation/RNP/Cas9	Mutants of *PPX1* and *FTSY* gene ; knock-out of *cpftsy* gene; knock-out mutants of *cp29* and *cp26* gene; targeted knockout of *CrSPD1*; knock-in of the gene *CrFTSY* using a combination of ribonucleoprotein (RNP) complex and DNA fragment (antibiotics resistance gene); knockout mutant of the zeaxanthin epoxidase gene (*ZEP*), double deletion knock-out mutants (dZAs), for the *ZEP* (Zeaxanthin epoxidase) and *AGP* (ADP glucose pyrophosphorylase) genes	[Akella et al., 2021; Angstenberger et al., 2020; Cazzaniga et al., 2020; Freudenberg et al., 2022; J. H. Kim et al., 2020; Song et al., 2020, 2022]
C. reinhardtii (CC-124, CC-400, UVM11)	Cell penetrating peptide/RNP/Cas9	Disruption of the genes *Maa7* and *FKB12* through a ribonucleoprotein delivery system mediated by the cell penetrating peptide pVEC (LLIILRRRIRKQAHAHSK)	[Kang et al., 2020]
C. reinhardtii (CC-125, CC-5415)	Electroporation/ Glass bead/RNP/Cas9	Mutants of *IFT81* (first gDNA), *IFT43*, *MOT17*, *CDPK13*, *FAP70*, *IFT81* (second gRNA), and *CEP131*, through TIM	[Picariello et al., 2020]
C. reinhardtii (CC-1883)	Electroporation/RNP/Cas12a	Loss-of-function mutation of *FK506-binding protein 12* (*FKB12*) locus	[Ferenczi et al., 2021]
Phaeodactylum tricornutum (CCMP2561)	Bacterial conjugation/Episomal plasmid/ Cas9, Bombardment/Plasmid /Cas9	Mutants of *NR* gene	[Moosburner et al., 2020]
P. tricornutum	Bombardment/Plasmid/Cas9	Mutants of light-harvesting complex	[Sharma et al.,

(CCMP2561)		(*LHC*) genes belonging to the LHCF gene family	2021]
P. tricornutum (CCMP2561)	Bacterial conjugation/episomal plasmid/ Cas9	knock-out of cryptochrome CryP (*CryP* gene)	[Yang et al., 2022]
P. tricornutum	Biolistic/ Electroporation/ Bacterial conjugation	Knock-out mutants of *PtUMPS* (*Phatr3_J11740*) and *PtAureo1a* (*Phatr3_J8113*) genes; single base pair mutation of the Myb transcription factor (XM_002181623; named Pt_Myb1R_SHAQKYF5)	[Serif et al., 2018; Sharma et al., 2018]
P. tricornutum (CCAP1055/1)	Electroporation/plasmid/ Cas9	knock-out mutants of *THIC*, *SSSP*, *SSUA/THI5-like* (encoding NMT1 domains) and *FOLR* genes	[Llavero-Pasquina et. Al., 2022]
T.pseudonana	Biolistic/ Bacterial conjugation	Insertion of the CEN6-ARSH4-HIS3 fragment	[Karas et al., 2015]
T. pseudonana (CCMP1335)	Bombardment/Plasmid/Cas9	Indels mutations of the *TpθCA3* THAPSDRAFT_bd1765)	[Nawaly et al., 2020]
Nannochloropsis salina (CCMP1776)	Electroporation/plasmid/Cas9	Knockout and knockdown mutants of cellulose synthase gene (*CesA*)	[Jeong et al., 2020)
N. oceanica IMET1	Electroporation/RNP/ Cas12a, CRISPR	Down-regulation of *NR* (nitrate reductase) gene	[Naduthodi et al., 2021]
N. salina (CCMP1776)	Electroporation/Cas9	Knock-in of the *FAD12* gene, encoding Δ12-fatty acid desaturase	[Ryu et al., 2021]
N. gaditana (CCMP526)	Electroporation/Cas9	Knock-out of beta-glucan synthase (*BGS*) gene and transglycosylase (*TGS*) gene	[Vogler et al., 2021]
N. oceanica IMET1	Electroporation/episomal plasmid/Cas9	Telomere-deletion mutants and large genome fragment deletion	[Wang et. Al., 2021)
N. oceanica (CCMP1779)	Electroporation/episomal plasmid/Cas9	Deletion mutants of the *NoAUREO2* or *NoAUREO4* genes	[Poliner et al., 2018]
N. gaditana	Electroporation	knock-out mutants of a homolog of fungal Zn(ii)2Cys6-encoding genes	[Ajjawi et al., 2017]
N. limnetica	Electroporation	Insertion of bleomycin-resistant gene (*sh ble*) or hygromycin B-resistant gene (*hptII*)	[Y. Chen & Hu, 2019]
N. oceanica	Electroporation	Insertion of *β-tubulin* promoter, *sh ble* gene, and *fcpA* terminator; random mutation of genomic DNA by insertion of a Transposome complex Tn5; overexpression of EPA biosynthesis genes	[Li et al., 2014; Osorio et al., 2019; Poliner et al., 2018]
N. oculata	Electroporation	Insertion of *β-tubulin* promoter, *sh ble* gene, and *fcpA* terminator	[F. Li et al., 2014]
N. oceanica IMET1	Electroporation/plasmid/CRISPRa	Over-expression of gene *g1248*	[Wei et al., 2022]
C. pyrenoidosa	Electroporation	Insertion of *NptII* gene and *eGFP*	[Run et al., 2016]
C. sorokiniana	Biolistic	Insertion of the nitrate reductase (*NR*) gene into NR-deficient *C. sorokiniana* mutants	[Dawson et al., 1997]
C. vulgaris	Electroporation/ Glass bead agitation/ *Agrobacterium*-mediated,	Insertion of GFP*His6 or *aph7* gene	[Muñoz et al., 2018]
C. sorokiniana, C. Vulgaris FSP-E	Electroporation/Plasmid/Cas9	Deletion mutant (fatty acid desaturase-*fad3* gene)	[Lin & Ng, 2020]
C. vulgaris UTEX395	Electroporation/Plasmid, RNP/Cas9, Bombardment/RNP/Cas9	Null mutation of edited genes of nitrate reductase *(NR*) and adenine Phosphoribosyltransferase (*APT*)	[Kim et al., 2021]
C. sorokiniana UTEX 1602	Electroporation/Plasmid/Cas9/CRISPRi/ CRISPRa	Mutants of phosphoenolpyruvate carboxylase (*PEPC*) and glutamine	[Lin et al., 2022]

Other species	Transformation method	Gene mutants	References
		synthetase (*GS*) genes	
Euglena gracilis	Microinjection/RNP/Cas9	Deletion mutants of the partial *EgGSL2* and *crtP1* gene	[Chen et al., 2022]
E. gracilis	Electroporation/RNP/Cas9	Deletion mutants of the *Bardet–Biedl syndrome* (*BBS*) genes	[Ishikawa et al., 2022]
Picochlorum celeri	Electroporation/RNP/Cas9	Loss-of-function mutants of nitrate reductase and carotenoid isomerase genes	[Krishnan et al., 2020]
P. purpureum CCMP 1328	Bombardment/RNP/Cas9	Chlorophyll synthase loss-of-function mutants (*Δchs1*)	[Jeon et al., 2021]
Tetraselmis sp. KCTC12432BP	Bombardment/RNP/Cas9	Development of *AGP* gene (ADP-glucose pyrophosphorylase) loss-of-function mutants	[Chang et al., 2020]
B. braunii	Electroporation	Insertion of the *AphVIII* gene	[Berrios et al., 2016]
C. zofingiensis	Biolistic	Insertion of *CHYb* (β-carotene hydroxylase) and *BKT* (β-carotene ketolase) genes	[Liu, et al., 2014]
Coccomyxa sp.	Biolistic/ Electroporation	Overexpression of acyl-(acyl-carrier-protein) thioesterase gene, and a type-2 diacylglycerol acyltransferase gene; knock-out mutants of the *FTSY* gene	[Kasai et al., 2018; Yoshimitsu et al., 2018]
C. subellipsoidea	Electroporation	Insertion of the blue fluorescent protein (*BFP*) gene and *hptII* gene coding for hygromycin B phosphotransferase	[Kania et al., 2020]
C. merolae	PEG-mediated	double knock-in of HA-tagged cyclin 1 (*CYC1/CML219C*) and FLAG-tagged cyclin-dependent kinase A (*CDKA/CME119C*); Insertion of the antisense strand of the *catalase* gene; insertion of the acyl-ACP reductase gene of *Synechocystis sp*. PCC 6803 (sll0209)	[Fujiwara et al., 2017; Ohnuma et al., 2009; Sumiya et al., 2015]
D. salina	Electroporation/ Biolistic/ Glass beads agitation/*Agrobacterium*-mediated	Insertion of nitrate reductase (*NR*) gene and bar gene; insertion of GFP as a reporter gene and, kanamycin and hygromycin resistant genes	[Li et al., 2007; Srinivasan & Gothandam, 2016]
Fistulifera solaris	Biolistic	Overexpression of the endogenous glycerol kinase (*GK*) gene	[Muto et al., 2015]
Gonium pectorale	Biolistic	Insertion of *aphVIII* gene and luciferase gene	[Lerche & Hallmann, 2009]
H. pluvialis	Biolistic	Mutation and up-regulation of the *pds* gene; insertion of *tub/aadA*, *tub/hyr* and *tub/ble*	[Steinbrenner & Sandmann, 2006; Yuan et al., 2019]
M. neglectum	Electroporation	Insertion of *aphVII"* gene	[Jaeger et al., 2017b]
O. tauri	Electroporation/ PEG-based	Insertion of the aminoglycoside 30-phosphotransferase type I (*APHI*) coding gene of the bacterial transposon Tn903 for G418 resistance and the firefly luciferase CDS and expression assay by using four native *O. tauri* promoter sequences for histone 2A (*pOLK2*),	[Sanchez et al., 2019; van Ooijen et al., 2012]

		histone 3 (*pOLK3*), ubiquitin (*pOLK4*) and thioredoxin (*pOLK5*); Genomic transformation protocol Iinsertion of hygromycin	
P. kessleri	Biolistic/ *Agrobacterium*-mediated	phosphotransferase (*hpt*) gene and a *GUS* gene under the CaMV promoter	[Rathod et al., 2013]
S. obliquus	Electroporation	Insertion of CAT gene	[Guo et al., 2013]
S. microadriaticum	Silicon carbide whiskers	Insertion of neomycin phosphotransferase gene (*nptII*) and hygromycin B phosphotransferase gene (*hpt*)	[Ten Lohuis & Miller, 1998]
V. carteri f. magariensis	Biolistic	Indel mutants of *glsA*, *regA* and i*nvA* genes	[Ortega-Escalante et. Al., 2019]

The first report on gene editing in microalgae was in *C. reinhardtii*, using zinc finger nucleases (ZFNs) to target the COP3 gene as a proof-of-concept experiment (Sizova et al., 2021). Furthermore, engineered transcription activator-like effector nucleases (TALENs) have also been employed in microalgae (Dubois et al., 2014). Nevertheless, the CRISPR-Cas system has become the preferred choice for genome editing in microalgae due to its versatility and simplicity compared to the ZFNs and TALENs (Greiner et al., 2017; Kurita et al., 2020). Since the initial efforts to use CRISPR-Cas9 in Chlamydomonas, further research studies have incorporated ten different genera of eukaryotic microalgae, leading to the development of new strains with improved productivity, nutrient content, and stress tolerance (Ryool Jeong et al., 2023).

Another powerful method is the use of RNA interference (RNAi) to selectively inhibit the expression of specific genes in organisms. Microalgae can undergo gene silencing or knockdown through the introduction or expression of artificial microRNAs or by using RNA interference (RNAi) techniques (Cerutti et al., 2011; Kim & Cerutti, 2009; Molnar et al., 2009). In algae, RNAi has been employed to suppress the activity of target genes by introducing small RNA molecules that can specifically recognize and degrade the mRNA transcripts of those genes. Antisense technology and RNAi have been successfully applied in the model microalga *C. reinhardtii* as reported in various studies (Kim & Cerutti, 2009; Moellering & Benning, 2010; Molnar et al., 2009). Characterization of the microRNA (miRNA) system and its potential for gene regulation has been performed in several microalgae species including *C. reinhardtii*, the red alga *Porphyra yezoensis*, and the diatoms *T. pseudonana* and *Phaeodactylum tricornutum* (Huang et al., 2011; Molnar et al., 2009; Norden-krichmar et al., 2011; Zhao et al., 2007). A successful application of RNAi in algae is the suppression of genes involved in lipid degradation, resulting in increased lipid accumulation within cells, which is important for sustainable biofuel production from algae (Aratboni et al., 2019).

4.1. DNA Elements Required for Transformation

4.1.1. Selectable marker genes and Reporter genes

Various endogenous selective markers have been used in gene editing in microalgae along with the co-targeted gene of interest, such as the nitrate reductase, adenine phosphoribosyl transferase, peptidylprolyl isomerase, tryptophan synthase beta subunit, orotidine 5'-phosphate decarboxylase, protoporphyrinogen IX oxidase, and spermidine synthase (Freudenberg et al., 2022; Jiang & Weeks, 2017; Serif et al., 2018). These markers have been employed to facilitate gene modifications without the need for transgene integration, thereby improving the efficiency of the intended gene modifications in microalgae. In addition to the endogenous selective markers, antibiotic and herbicide resistance-based pre-selection methods have also been used and have been refined to achieve high selection efficiency up to 90% (Greiner et al., 2017; J. H. Kim et al., 2020; Picariello et al., 2020). These markers are derived from bacteria and, when introduced into microalgae, confer resistance to specific antibiotics or herbicides (León-Bañares et al., 2004).

Moreover, reporter genes, such as luciferase or mVenus have been successfully inserted at desired gene sites, enabling easy selection of mutant strains (Freudenberg et al., 2022; J. H. Kim et al., 2020). Reporter genes typically encode enzymes or fluorescent proteins that generate a visible or detectable signal, allowing the monitoring of the activity and expression of the gene of interest. Among the widely used reporter genes are the green fluorescent protein (GFP) and its variants, such as blue (BFP), cyan (CFP), orange (OFP), red (RFP), and yellow (YFP) (Day & Davidson, 2009; Rasala et al., 2013). Reporter genes play a crucial role in genetic studies and biotechnological applications, by enabling the visualization and quantification of gene expression in real time and protein localization.

4.1.2. Regulatory sequences

Regulatory sequences play a crucial role in gene expression as they control the time and site of gene expression, especially for both selectable marker and reporter genes (Sproles et al., 2021). In microalgae, is often necessary to use endogenous regulatory sequences to ensure gene expression, as heterologous regulatory sequences may not be recognized or may result in minimal or no expression (Potvin & Zhang, 2010) As such, endogenous regulatory sequences are often replaced with different ones to modify gene expression patterns or increase expression rates. This can involve replacing the promoter region or using larger "regulatory regions" or "promoter regions" when the exact position of essential DNA elements is not known. Constitutive regulatory sequences, such as the small chain of ribulose bisphosphate carboxylase (RbcS2), heat shock protein 70A (HSP70A), or β-tubulin, are commonly used when continuous expression is desired, such as for selectable marker genes (Potvin & Zhang, 2010; Strenkert et al., 2013). In contrast, inducible regulatory sequences are useful when controlling gene expression and turning it on or off at specific times or under specific conditions. These inducible regulatory sequences can be designed to respond to specific chemicals or light, allowing precise control over the gene expression.

In *C. reinhardtii*, more than 60 working regulatory sequences (promoters, UTRs, terminators, tags, reporters, antibiotic resistance genes, and introns) have been identified and used for gene expression (Crozet et al., 2018). Some of the most implemented regulatory

sequences are small chain of ribulose bisphosphate carboxylase /oxygenase small subunit (RbcS2) (Stevens et al., 1996), β-tubulin (Blankenship & Kindle, 1992; Hallmann & Sumper, 1996), photosystem I complex proteins (psaD) (Fischer & Rochaix, 2001), cytochrome c6 (CYC6) (Quinn et al., 2003), nopaline synthase (NOS) (Díaz-Santos et al., 2013), and NAD(P)H nitrate reductase (nia1) (Llamas et al., 2002). For chloroplast transformation, regulatory sequences from genes, such as chlL, petD, rbcL, atpA, atpB, and rrn16 have been found to be suitable (Kim et al., 2015).

4.1.3. UTRs and Introns

The 5' and 3' untranslated regions (UTRs) of mRNA can impact the gene expression in microalgae. More specifically, the 3' UTRs affect the mRNA stability and polyadenylation. In *C. reinhardtii*, an effective polyadenylation signal such as UGUAA in the 3' UTR of genes can support expression (Bell et al., 2016) In the case of 3' UTRs, specific UTRs from genes such as RBCS2 or PSAD have been preferred in studies (Fischer & Rochaix, 2001; Lumbreras et al., 1998). On the other hand, the 5' UTRs contain introns and regulatory elements that could influence the transcriptional regulation (Specht et al., 2010). These regulatory elements within the intron sequences can affect gene expression by interacting with transcription factors (TF) or other regulatory proteins. The exact position and function of regulatory elements within the 5' UTRs may not always be known; therefore, larger promoter regions provide a better chance in capturing these important elements (Scranton et al., 2016).

Spliceosomal introns are a common feature in eukaryotic genomes, including microalgae, and hold important roles in gene expression regulation. While the number of introns per gene can vary greatly, it has been observed that the majority of microalgal genes contain at least one intron (Baier et al., 2018). Introns harbor various regulatory elements such as enhancers or silencers, transcription factor binding sites, and alternative splicing signals, which can influence the transcriptional activity of genes and affect the abundance, processing, stability, and translation efficiency of the resulting mRNA isoforms. Therefore, introns can be crucial for achieving an adequate expression of a gene of interest in microalgae. Insertion of introns from the target species at unnatural positions within heterologous or homologous cDNAs is an alternative strategy for enhancing gene expression in microalgae (Baier et al., 2018; Eichler-Stahlberg et al., 2009; Hallmann et al., 1999; Lumbreras et al., 1998). The insertion of the intron of the ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit 2 (rbcS2i1) throughout codon-optimized coding sequences (CDS) has been shown to generate optimized algal transgenes which have been expressed reliably in *C. reinhardtii* (Baier et al., 2018). Also, Baier and colleagues (2020) have identified several native and non-native introns in the green microalga *C. reinhardtii* with the ability to elicit strong Intron-Mediated Enhancement (IME) and were broadly applicable in different expression constructs.

4.1.4. De novo DNA synthesis

De novo DNA synthesis involves the chemical synthesis of DNA in the laboratory without the need for DNA or RNA template. This process allows for the development of custom DNA sequences based on electronically available or designed sequences. Until recently, the upper limit for the length of chemically synthesized single-stranded DNA was

around 200-300 nucleotides (Johnson & Widlanski, 2003; Palluk et al., 2018) But in 2021, first synthesis of synthesis of long (>10 kb) DNA was reported (Hoose et al., 2023) In microalgae, large-scale oligonucleotide synthesis was applied for the creation of libraries of variant 5′ UTRs of two *C. reinhardtii* genes, *psbD* and *psaA*, with the aim of identifying key regulators of chloroplast gene expression and developing tools for increased recombinant protein accumulation in algal plastids (Specht & Mayfield, 2013). In addition to endogenous regulatory sequences, synthetic regulatory sequences have also been developed that can drive robust gene expression in the nucleus of microalgae (Scranton et al., 2016). These synthetic regulatory sequences offer flexibility and control in gene expression for various biotechnological applications. Furthermore, DNA synthesis technology has enabled the synthesis of metabolic ORFeomes of two cyanobacteria strains of *Prochlorococcus marinus* (MED4, NATL1A) and *Chlamydomonas*, allowing for carrying out high throughput experiments with high levels of success rate (Fu et al., 2019). DNA synthesis is an indispensable tool of genetic engineering, allowing for the development of custom-designed DNA sequences (DNA molecules of desired composition and complexity) (Hoose et al., 2023) but its future application is highly affected by the cost and affordability (Patwari et al., 2023).

4.1.5. Vector construction

Vectors used for transformation of microalgae often incorporate elements from both eukaryotic microalgae and bacterial vectors. The bacterial vector backbone, such as pBluescript, allows for a vector construction in *Escherichia coli* strains like DH5α. The minimal requirements for propagation in *E. coli* include an origin of replication (e.g., pUC ori), an antibiotic resistance gene (e.g., AmpR), and a multiple cloning site for easy cloning (Rosales-Mendoza, 2016). One approach to simplify vector construction is the use of the λ-phage based gateway system, which utilizes recombination sequences for the transfer of DNA fragments between vectors *in vitro* (Hartley et al., 2000; Oey et al., 2014). Another strategy is the cassette multiplication technique, which enables the production of vectors with multiple gene copies, potentially leading to increased gene dosage and expression rates (Hallmann & Wodniok, 2006). Additionally, a modular cloning toolkit based on Golden Gate cloning with standard syntax has been developed for *C. reinhardtii*, containing various genetic parts for maximum modularity (Crozet et al., 2018). Transformation vectors have been designed to direct engineered gene products to different subcellular compartments, such as the chloroplast (Beth et al., 2014). Additionally, vectors that facilitate efficient secretion of recombinant proteins have been developed, which simplifies the purification process., for *C. reinhardtii* (Molino et al. 2018) and *P. tricornutum* (Vanier et al., 2017).

4.2.Transformation Methods

In parallel with selecting the most appropriate DNA elements required for transformation, various techniques have been developed to facilitate the uptake of foreign DNA into the cell and subsequently into the nucleus, chloroplast, or mitochondria of the algae, for ensuring higher yields of the targeted metabolite (lipids, antioxidants, high-value bioactive compounds, and proteins). Nevertheless, in contrast to the plant systems, the

transformation of microalgae continues to face challenges in achieving high efficiency, with the exception of Chlamydomonas (Kumar et al., 2020).

The primary challenge in accomplishing successful microalgae transformation is overcoming the cell wall to allow the entry of DNA, and as such, most protocols include a physical method to penetrate the cell wall (Ortiz-Matamoros et al., 2018). Integration of exogenous genes into the genome typically occurs at random sites through a process called recombination, which does not depend on homology or specific DNA sequences, albeit regions of microhomology may play a role. Technical limitations in the generation of transgenic lines in microalgae

The most commonly used transformation techniques employed in algal genetic engineering are presented below and examples can also be found in Table 3.

Electroporation involves the use of high-intensity electric pulses to create temporary pores in the cell membrane allowing foreign DNA to enter the cell walls and membrane's phospholipid bilayer (Rathod et al., 2017). This technique has been used successfully to transform many algae species, including *C. reinhardtii*, . *salina*, *Cyanidioschyzon merolae*, *Nannochloropsis sp.*, *C. vulgaris*, *Chlorella ellipsoidea*, and *Chlorella saccharophila* (Z. Chen & Lee, 2019; Garoma & Shackelford, 2014; Ladygin, 2003). In some cases, enzymatic pretreatment is required to disrupt the cell wall; however, this method has been successful only in some cases given the limited available information regarding the diverse composition of cell walls in different microalgae species (Ortiz-Matamoros et al., 2017). Nevertheless, this method is particularly effective for nuclear gene transformation in microalgae compared to other systems (Fajardo et al., 2020).

Microinjection method has been used to directly inject foreign DNA into the nucleus of the algal cell using a fine-tipped glass needle. This technique is highly precise and can be used to introduce DNA into specific cells within a population (Qin et al., 2012); yet it is labor-intensive and time-consuming.

Microparticle bombardment (biolistics) is a versatile method that enables transformation of various cell types regardless of their cell wall thickness or rigidity, as well as plastid transformation. This method was successfully used in microalgae to transform chloroplast genome and in studies for the production of different compounds (Gangl et al. 2015). This technique has been successfully applied in various microalgae species. These include *C. reinhardtii*, *V. carteri*, *Gonium*, *Pandorina morum*, *Eudorina elegans*, *H. pluvialis*, *Euglena gracilis*, *Porphyridium sp.*, *'Chlorella' kessleri*, *C. sorokiniana* and *D. salina* (Lapidot et al., 2002; Siddiqui et al., 2020). Diatoms such as *P. tricornutum*, *Cylindrotheca fusiformis*, *Navicula saprophila*, and *Cyclotella cryptica* can also undergo transformation through particle bombardment (Velmurugan & Deka, 2018). Nevertheless, the transformation efficiency is relatively low (Ng et al. 2017).

Agitation with glass beads is a popular transformation method due to its simplicity and reproducibility, especially for species that lack rigid cell wall, such as *D. salina* (Feng et al., 2009), however low transformation efficiencies and reduced cell recovery are some of the drawbacks (Ortiz-Matamoros et al., 2018). This method has also been applied successfully to *C. reinhardtti* (Wannathong et al., 2015) and *Symbiodinium* spp (Ortiz-Matamoros et al., 2015). The protocol involves an enzymatic pre-treatment for the degradation of the

eukaryotic cell wall and vigorous agitation of algal cells/DNA suspension with glass beads and the membrane fusion agent polyethylene glycol (PEG) (Ortiz-Matamoros et al., 2018).

Similar to higher plants, microalgae have also been successfully transformed using *Agrobacterium tumefaciens*. *Agrobacterium* possesses a tumor-inducing (Ti) plasmid that integrates into the genome of infected cells. *Agrobacterium*-mediated transformation has been used in microalgae species, such as *Chlamydomonas reinhardtii*, *H. pluvialis*, *D. bardawil*, *Parachlorella kessleri*, *Schizochytrium sp* and *Symbiodinium spp* (Anila et al., 2011; R. Cheng et al., 2012; Kathiresan et al., 2009; Kathiresan & Sarada, 2009; Kumar et al., 2004; Ortiz-Matamoros et al., 2015; Rathod et al., 2013).

Interestingly, transformation methods, such as electroporation, microinjection, biolistics and microfluidic techniques, used as physical delivery techniques coupled with the use of lipid nanoparticles, polymers, nanogels, inorganic nanoparticles, and cell-derived vesicles as synthetic carriers have been, thus far, successfully employed for enhancing the efficiency of genome editing using the Cas9 ribonucleoprotein (RNP) (Kang et al., 2020; Lu et al., 2021).

As interest grows in developing transgenic microalgae for biotechnological applications and research, overcoming issues related to transgene efficiency, integration, or stability are common is a major challenge. Selecting the most appropriate transformation strategy is crucial for ensuring a successful transformation using approaches specifically tailored to the diverse cellular characteristics of microalga. The main challenge lies in permeabilizing the cell walls and membranes to introduce DNA without causing significant damage that could harm or kill the cells. Other important factors for enhancing the transformation efficiency include the optimization of the transformation conditions, the type of genetic material to be transferred and that the cells can resume normal cell division after genetic modification.

5. Applications of Genetic Engineering and omics tools in Microalgae

The field of microalgae biotechnology is gaining increasing commercial significance across a wide range of industries including food, pharmaceuticals, nutraceuticals, cosmetics, animal feed, energy and can also be employed in environmental applications, such as water and soil remediation (Singh et al., 2021; Vanhoudt et al., 2018). Whilst applications of genetically engineered microalgae have also been found in other scientific fields, such as in neuroscience, where the light-dependent ion channels of microalgae allow for the manipulation of the neuronal activity (Ishizuka et al., 2006). The genetic modification of microalgae offers expanded possibilities for biotechnological applications in various contexts through enabling the production of compounds and introduction of traits that are not naturally present in algae. Various studies refer to molecular farming, molecular pharming, biopharming, or gene pharming, that involves the production of enzymes, antibodies, immunotoxins, bioactive peptides, hormones, and vaccines (Dyo & Purton, 2018; Rasala & Mayfield, 2015; Taunt et al., 2018).

5.1. Enhanced productivity of high-added value products and improved growth rates

Genetic engineering techniques can be employed to enhance the productivity of algae by manipulating their metabolic pathways. One approach is to overexpress specific enzymes that

play a crucial role in lipid biosynthesis (Mulgund, 2022). By increasing the expression of these key enzymes, the lipid content of algae can be augmented. This is particularly advantageous in the production of biofuels given that lipids are a valuable raw material. Through genetic modifications, algae can be engineered to have higher lipid content, and thus be more efficient and economically viable for biofuel production. Ultimately, the enhanced lipid content contributes to the development of sustainable and renewable energy sources (Ng et al., 2017).

Through genetic engineering, the bioproduction of pharmaceutical and nutraceutical compounds in algae can be optimized rendering them a more viable and sustainable source for the development of novel therapies (Garduño-González et al., 2022; Qiao et al., 2020). Overexpressing key enzymes in biosynthetic pathways can enhance the algae's capacity towards higher yields of the desired compounds, involving manipulation of genes for precursor synthesis, enzyme regulation, and other pertinent metabolic processes (Garduño-González et al., 2022; Qiao et al., 2020).

Genetic engineering techniques offer a means to enhance the growth rates of algae by manipulating their photosynthetic machinery. Additionally, the overall productivity and growth rates of algae can be significantly enhanced by optimizing the photosynthetic machinery through overexpressing essential genes involved in photosynthesis (Hitchcock et al., 2022).

5.2. Bioremediation

Algae have shown great potential for environmental remediation based on their ability to remove pollutants, such as heavy metals and organic compounds from the environment. Genetic engineering techniques can be employed to enhance this capability further. By overexpressing key enzymes involved in the degradation of pollutants, algae can be genetically modified to increase their efficiency in bioremediation processes. These enzymes play a crucial role in the breakdown and metabolism of specific pollutants, facilitating their removal or transformation into less harmful forms. Through genetic engineering, the expression of these enzymes can be upregulated, enabling the algae to effectively target and degrade pollutants in the environment (Martínez-Ruiz et al., 2022; Zeraatkar et al., 2016). This approach offers a promising avenue for the development of sustainable and eco-friendly strategies to address pollution challenges.

6. Impact of Genetic Engineering and omics data on Microalgae Industry

The integration of omics data and metabolic engineering allows researchers to design and implement precision modifications in microalgae strains for specific biotechnological applications, ranging from biofuel production and pharmaceuticals to environmental remediation. Overall, genetic engineering of algae involves the manipulation of the genetic material to achieve specific characteristics With notable implications for the algae industry, offering both advantages and potential drawbacks. While it can increase productivity, enhance stress tolerance, and improve the quality of algae products, it necessitates a careful consideration of potential risks and ethical implications More specifically, one of the positive effects is the improvement on growth rate and overall productivity resulting in higher

biomass yields, biofuels, or other high-added value microalgae derived products. It is also used effectively to optimize the composition of lipids, proteins, or other compounds with commercial or industrial value. Additionally, the development of resilient microalgae strains may not only contribute to the effective cultivation in adverse conditions but also in producing stress-induced high-added value products. Negative aspects of genetic engineering include the release of transgenic strains in the environment that could affect the natural diversity and possibly disrupt the balance of ecosystems, the production of algae-derived products that may not be suitable for human consumption. Therefore, there is a need for careful consideration of the benefits and risks of genetic engineering of algae, and for the development of regulations and guidelines that ensure its safe and sustainable use. Comprehensive risk assessments, ethical frameworks, and robust regulatory mechanisms are essential to guide the responsible deployment of genetically modified microalga.

The CRISPR/Cas technology is a potent genetic engineering tool with numerous potential applications in microalgae. It offers the opportunity to introduce specific genetic modifications that can enhance algae's productivity, improve biofuel yields, increase disease resistance, and promote carbon sequestration. However, there are still obstacles and limitations that must be addressed, including the development of efficient delivery methods, the potential for off-target effects, and safety concerns. Through ongoing research and development, CRISPR/Cas has the capacity to revolutionize algae biotechnology, offering sustainable solutions to a range of environmental challenges. It is essential to carefully weigh the benefits and risks associated with genetic engineering of algae to ensure its safe and sustainable use.

Despite the significant advancements in the field, the current knowledge on the metabolism of industrially relevant microalgae strains remains limited. A substantial portion of genes lack significant sequence similarity to known entities, making it challenging to assign them specific metabolic functions (Vieler et al., 2012). Additionally, uncertainties persist regarding protein localization, compartmentalization of metabolite pools, and trafficking of metabolites between organelles, further complicating the development of comprehensive metabolic models. These limitations must be addressed given that a deeper understanding of microalgae and their metabolism is crucial for guiding the design of strain engineering approaches. In the absence of high-quality metabolic models, genetic engineering strategies are often constrained to educated guesses based on knowledge obtained from other organisms.

The advancement of genetic tools and omic technologies has significantly accelerated research in a select few microalgae strains, leading to the accumulation of a large database of transcriptomic responses to various stimuli (Südfeld et al., 2021; Zienkiewicz et al., 2016). To enable metabolic engineering, it is crucial to have an advanced toolbox for genetic manipulation. However, developing genome editing methods specifically tailored for microalgae species is a time-consuming process that requires careful attention. Currently, the genetic toolbox for industrially relevant microalgae is relatively limited compared to other microbial systems, but efforts are underway to expand and enhance these tools.

7. CONCLUSIONS

Microalgal omics hold considerable promise due to the ongoing breakthroughs in genomics technologies, such as better sequencing platforms, bioinformatics tools, and data processing techniques. Transcriptional and metabolic engineering strategies have played vital roles in advancing microalgae research. These approaches have facilitated the functional characterization of key genes and regulators, the identification of metabolic pathways, and the elucidation of microalgae cell physiology (Bajhaiya et al., 2017). Moreover, the use of knowledge on epigenetic and metabolic cellular regulating processes may facilitate the confirmation of regulatory protein functions and their impact on diverse metabolic pathways and will contribute to the development of robust microalgal strains (Bacova et al., 2020). The rapid advancement of omics technologies in the field of microalgae have laid a strong foundation for genetic engineering. Extensive research conducted over several decades, especially in the model algae such as Chlamydomonas, has elucidated gene expression patterns, biochemical processes, and metabolic pathways in microalgae (Jeong et al., 2023). Overall, the review provides a comprehensive overview of the current state of microalgae research, emphasizing the synergistic integration of omics and metabolic engineering approaches to drive advancements in strain improvement for various practical and sustainable applications. Future research is required with a biology systems approach by integrating the pan-omics data of microalgae research to enable us to predict novel functional interactions between molecular mediators at multiple levels and identify points of metabolic flux for improving algal strain-engineering techniques.

Declaration of Competing Interests

The authors declare no conflict of interest.

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Declaration of interests

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.

Highlights:

- Coverage of the latest advancements in the microalgae omics technologies.
- The positive impact of omics and metabolic engineering on microalga is discussed.
- Limitations and risks of genetic engineering application are presented.