Engineering microalgae through chloroplast transformation to produce high-value industrial products

Ayesha Siddiqui[1]
Zhengyi Wei[2]
Marko Boehm[3]
Niaz Ahmad[1*]

[1]Agricultural Biotechnology Division, National Institute for Biotechnology & Genetic Engineering (NIBGE), Faisalabad, Pakistan

[2]Institute of Agricultural Biotechnology, Jilin Academy of Agricultural Sciences, Changchun, Jilin Province, People's Republic of China

[3]Botanical Institute, Christian-Albrechts-University, Kiel, Germany

Abstract

The last few years have seen an ever-increasing interest in the exploitation of microalgae as an alternative platform to produce high-value products such as biofuels, industrial enzymes, therapeutic proteins, including antibodies, hormones, and vaccines. Due to some unique attractive features, engineering of the chloroplast genome provides a promising platform for the production of high-value targets because it allows manipulation of metabolic processes in ways that would be impossible, or at least prohibitively difficult through traditional approaches. Since its initial demonstration in 1988 in *Chlamydomonas reinhardtii*, genetic tools have been developed, which have made it possible to produce high-value molecules in different species. However, the commercial application of microalgae as production platform is hindered by many factors like poor biomass, low product yields, and costly downstream processing methodologies. In this review, we discuss the potential of microalgae to use as an alternative production platform for high-value targets using chloroplast transformation technology. Volume 00, Number 0, Pages 1–11, 2019

Keywords: *high-value targets, microalgae, chloroplast transformation*

1. Introduction

The world population is increasing at an exponential rate, which is expected to cross the 9 billion mark by 2050. As a result, the demand for several important industrial products is also increasing. The high demand and low product yields by conventional production systems are spurring their costs, which is a compelling factor to devise strategies for their low-cost production. Different platforms such as bacteria, yeast, plants, insect cells, and so on have been reported for the heterologous production of high-value targets with commercial importance [1, 2]. Photosynthetic organisms especially green plants and algae have emerged as the most affordable systems for large-scale production of high-value targets (Table 1). Microalgae are capable to grow either photoautotrophically or heterotrophically, depending on the type of available carbon source, making its cultivation potentially simple and cost effective.

Microalgae are an extremely diverse group of eukaryotic organisms, thriving in a variety of habitats including fresh water, saltwater, marine as well as unproductive terrestrial environments, high temperature, and UV/high light radiations [3]. Consequently, many microalgal species have evolved unique metabolic pathways whose products could be a source of commercially valuable products such as carotenoids, polyunsaturated fatty acids, proteins, phycobiliproteins, polysaccharides, and antioxidants [4, 5]. Up to 90% of algal biomass can be converted into food, feed, fuel, and high-value compounds [6]. Several properties including better growth rates (5–10 times faster than crop plants), high lipid content (up to 50% of their body mass), and mass culturing on less nutritive or even on wastewater make microalgae a suitable host for the production of high-value targets [3]. The concept of using microalgae as "cellular factories" provides a low cost, low tech, and

Abbreviations: *TSP, total soluble proteins; SDR, short dispersed repeats.*

*Address for correspondence: Niaz Ahmad, PhD, Agricultural Biotechnology Division, National Institute for Biotechnology & Genetic Engineering (NIBGE), Jhang Road, Faisalabad, Pakistan. Tel.: +92 0 41 920 1316x243; Fax: +92 0 41 920 1322; e-mail: niazbloch@yahoo.com; nahmad@nibge.org.

Received 17 July 2019; accepted 16 September 2019

DOI: 10.1002/bab.1823

Published online in Wiley Online Library (wileyonlinelibrary.com)

TABLE 1 *Comparison of different expression systems*

Parameter	*Microalgae*	*Plants*	*Bacteria*	*Yeast*	*Insect cells*	*Mammalian cells*
Capital cost	Medium	Low	Medium	Medium	High	Very High
Operating cost	Low	Low	Low	Medium	High	Very high
Scale up cost	Medium	Very low	High	High	Very high	Very high
Production scale	Short	Long	Short	Short	Medium	Long
Speed	Fast	Slow	Fast	Fast	Medium	Slow
Multigene engineering	Yes	Yes	Yes	No	No	No
Glycosylation	Yes, but absent in chloroplasts	Yes, but absent in chloroplasts	Absent	Incorrect	Yes	Yes
Contamination risk	Low	Low	High	Medium	High	High
Multimeric assembly	Yes	Yes	No	No	No	No
Protein folding	High	High	Low	Medium	High	High
Protein yield	High	Low-High	High	Medium-high	Medium	Medium
Safety		High	Low	Unknown	Medium	Low
Storage	Low	Low	Costly	Costly	Expensive	Very expensive
Distribution	Easy	Easy	Feasible	Feasible	Difficult	Difficult

Source: Refs. 2, 7.

sustainable approach to produce high-value compounds. Several microalgal species have been given the status of GRAS (generally recognized as safe) such as *Chlorella*, *Dunaliella*, and *Haematococcus* because of the absence of endotoxins, viruses, or pathogens [8].

Microalgae offer a low-cost production platform for different recombinant proteins, vaccines, antigens, and commercial enzymes. As unicellular organisms, microalgae devoid of differentiation, and for the most of their vegetative cycle, they remain haploid, which makes genetic manipulation of their genomes relatively easier compared to plants. All its three genomes—nuclear, mitochondrial, and plastidial—can be transformed. The green alga *Chlamydomonas reinhardtii* has emerged as a model microalgal platform for carrying out genetic research as well as a host to produce different recombinant proteins and metabolites. According to a recent report, over 100 recombinant proteins have been produced in algae, majority of which were expressed in chloroplast [9]. Many of them are shown to be active in laboratory trials.

Transformation of the chloroplast genome offers several advantages compared to nuclear transformation such as stable and uniform gene expression, site-specific integration of transgene(s) into chloroplast genome, and compartmentalization of the recombinant proteins inside the chloroplasts without effecting the rest of the cellular activities. Therefore, there is a considerable interest in exploiting the potential of chloroplast transformation for rapid and efficient production of recombinant proteins and commercially important compounds [9]. As the chloroplast transformation is considered more suitable compared with nuclear transformation, therefore, the focus of this review would be on the production of recombinant protein in the algal chloroplast. In this review, we give a brief overview of chloroplast transformation in microalgae and discuss the potential of microalgal chloroplast for the production of high-value targets at large scale. We also discuss different bottlenecks that currently hinder the commercial applications of this platform.

2. Different Features of the Algal Chloroplast Genome

All plastids contain an inner envelope membrane bound protein–DNA complex that is termed as nucleoid. The plastid genome, also termed plastome, is composed of 10–20 copies

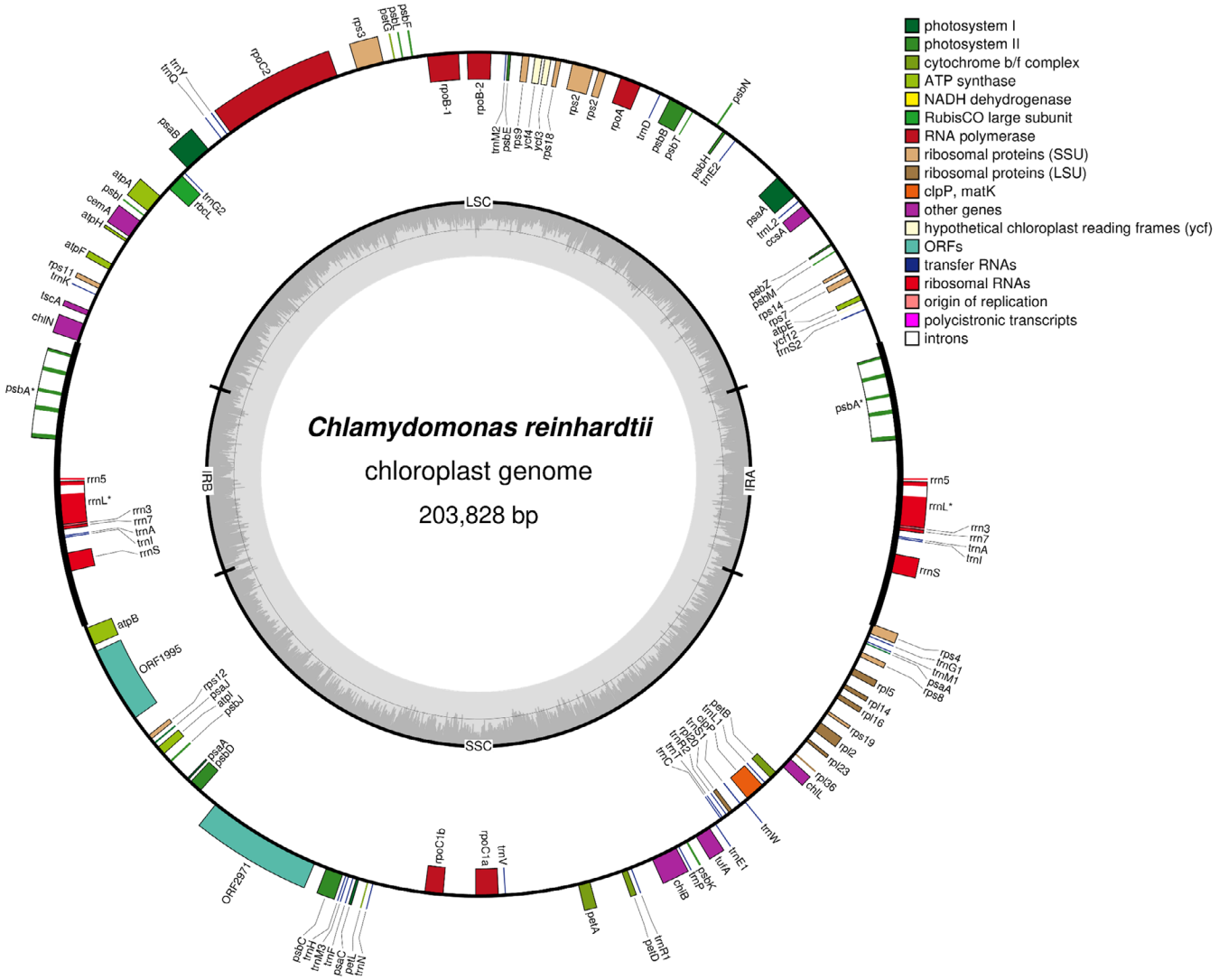

FIG. 1 *Map of the* Chlamydomonas reinhardtii *chloroplast genome (NC_005353.1) showing the organization of different plastid genes. Genes on the inside are transcribed clockwise, whereas genes on the outside are transcribed counterclockwise. The open reading frames of unknown function are shown by ycf plus designation number. The circle line inside the GC content graph (shown by gray color) marks the 50% threshold with positions of LSC, SSC, and IRs marked. Genes containing introns are marked by an asterisk (*). Genes coding for tRNA for an amino acid are indicated by its one-letter code of that amino acid. The map was constructed using a Web-based tool, OGDRAW v1.3.1 [10]. Abbreviations: LSC, large single copy; SSC, small single copy; IR, inverted repeat.*

of the DNA. It usually exists in a monomeric form, but may also form dimers, trimers, and sometimes tetramers. The size of algal chloroplast genomes varies between 100 and 200 kbp, with 100–120 protein-coding genes [11]. The largest variation in chloroplast genome size has been observed for green algae, ranging from 37.4 kbp (*Simulium jonesie*) to 269 kbp (*Dunaliella salina*) [12]. Such large variations in the genome size of land plant chloroplasts have not been observed. Their genome size remains fairly conserve between 140 ± 20 kbp, for example. The plastome size for *C. reinhardtii* is 203.8 kbp (Fig. 1), with 34.6% GC content [13]. The entire *C. reinhardtii* chloroplast genome contains 99 protein-coding genes including five genes coding for rRNA and 30 coding for tRNA, 17 coding for ribosomal proteins and five genes coding for the PEP core complex. The GC content and base pairing of the *C. reinhardtii* plastome is comparable to that of *Chlorella* (31.6%) and *Arabidopsis* (36.3%). The *C. reinhardtii* plastome contains two inverted repeat (IR) regions of 22,211 bp each in the outermost circle. These IR regions have been separated into unique regions with 80,873 and 78,100 bp in size (Fig. 1). The gene arrangement in *C. reinhardtii* is like that in land plants with the exception of the ribosomal RNA gene (23S rRNA) that is separated by an intronic region [14]. Figure 2 compares number of genes involved in different cellular functions in *C. reinhardtii* and tobacco (*Nicotiana tabacum*) chloroplast

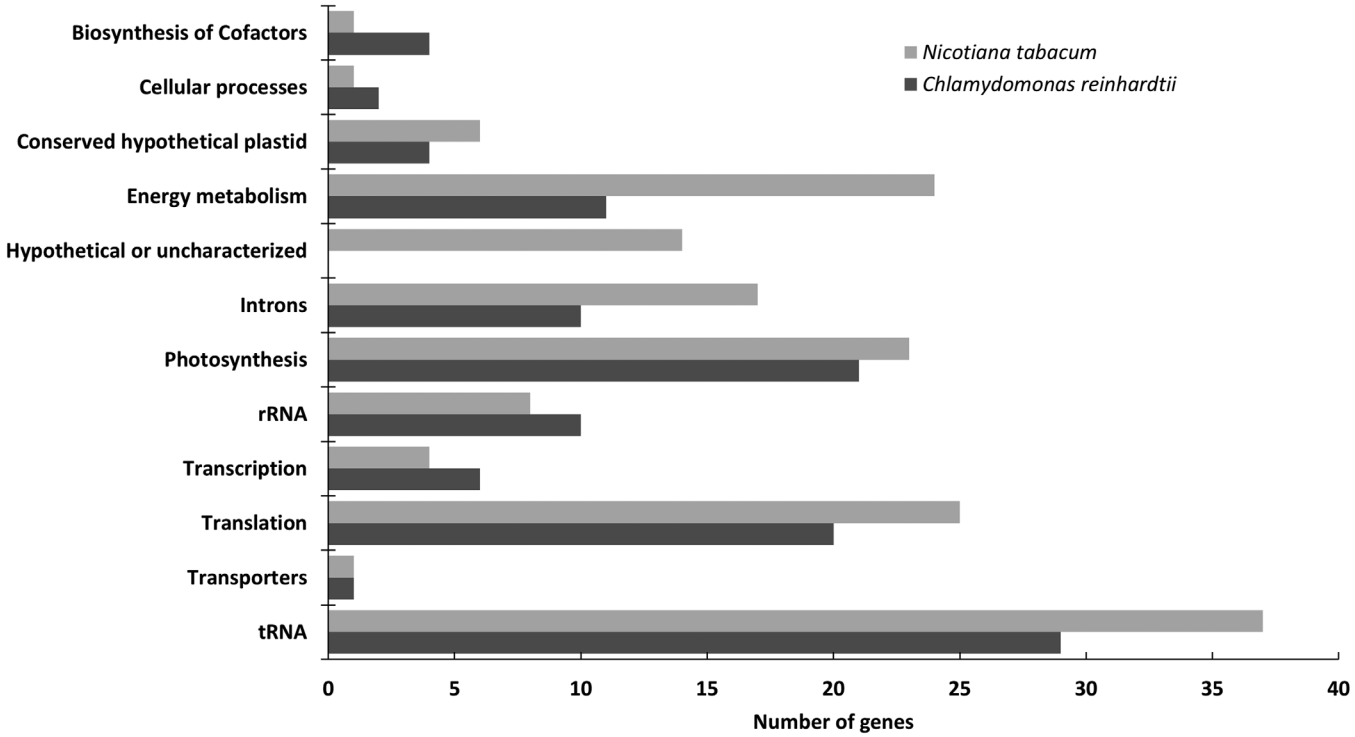

FIG. 2 *Comparison of gene distribution of* Chlamydomonas reinhardtii *and tobacco (*Nicotiana tabacum*) plastid genomes for different cellular functions.*

genomes. The *psb*A gene is interrupted by four intronic regions [15]. Interestingly, the plastid genome of *C. reinhardtii* has several characteristic features that are not found in land plants: (i) the presence of an unusual gene *tsc*A gene that encodes an RNA molecule involved in the trans-splicing of the *psa*A transcript, (ii) the presence of *tuf*A gene encoding the transcription elongation factor Ef-Tu, (iii) the *rpo*C1 gene in split form, (iv) two large ORFs (ORF1995 and ORF2971) of unknown but essential functions, and (v) the loss of *ndh* genes [16]. More than 20% of its genome are repetitive sequences known as short dispersed repeats (SDRs). Despite similarity in their function, the SDRs vary in size among different species [16]. Such SDR variations may be due to evolutionary changes in the genome. The addition of transposable elements or the loss of genes as a result of mutations might have played a role in the process [17]. Table 2 gives a summary of different features of *C. reinhardtii* chloroplast genome.

TABLE 2 ***Summary of different features of the* C. reinhardtii *chloroplast genome***

Feature	*Values*
Total sequence length	203,828 bp
Total CDS bases	77,235 bp
Average CDS length	1,119 bp
Total RNA bases	11,499 bp
Total number of protein genes	99
Total repeat bases	2,353 bp
Average repeat length	98 bp
Average intergenic distance	1,055 bp
Overall GC content	34.36%
Overall AT content	65.64%
Annotation	Curated

Source: https://rocaplab.ocean.washington.edu/tools/cpbase/run/?genome_id=1698&view=genome

3. Transformation of the Chloroplast Genome: An Overview

Genetic engineering of the algal chloroplast holds a great promise for the development of a variety of different products, for example, recombinant proteins, vaccine antigens, industrial enzymes, and biofuels [18]. There are many advantages of expressing transgenes in chloroplasts. For example, transgene(s)

can be integrated at a specific location on the chloroplast genome through homologous recombination, avoiding the disruption of the reading frames of any local genes. Genes in the chloroplast genome are organized in the form of groups, called operons often transcribed from the same promoter. Chloroplasts have the necessary machinery for cutting and processing the polycistrons into individual readable transcripts, which provides the opportunity to express multiple genes. This feature is particularly attractive for engineering metabolic pathways in which often expression of multiple genes is required [19–21]. The absence of gene silencing mechanisms in the chloroplasts and their non-Mendelian inheritance ensure uniform gene expression. Chloroplasts have the capacity to express foreign genes at extraordinary levels [22, 23]. Moreover, as a cellular compartment, chloroplasts can act as a safe subcellular compartment for the accumulation of foreign protein(s), without disturbing the biology of the rest of the cell [24]. Overall, transplastomic technology represents an important and feasible approach for the production of high-value targets at large scale [19].

Although chloroplast transformation has been reported in several higher plant species too [25–30], the transformation of algal chloroplast offers certain advantages over higher plants. For example, higher plants contain several thousand copies of the chloroplast genome in each cell, transformation of each of those is quite challenging [31]. Contrary to higher plants, most of the algal species contain only one chloroplast per cell. The process of transforming the higher plant chloroplast genome is, therefore, quite lengthy and complicated. It takes several months to develop stable homoplasmic plant lines compared to microalgae, which can be transformed and harvested in just weeks. The open field cultivation of transplastomic crops expressing foreign genes run the risk of contaminating food crops. The microalgal platform offers tight control over its cultivation by their growth in fermenters or photobioreactors, without the fear of releasing transgenes mixed into the food chain.

The fundamental difference between plastid and nuclear transformation is in the construction of the vector carrying the transgene of interest. Plastid transformation vector consists of two flanking elements homologous to the host plastid DNA ranging from 1 to 2 kbp in size. The transgene expression cassette and the selection cassettes are introduced in between these regions. It is possible to construct host specific vectors that can be used as a universal chloroplast vector. However, low transformation efficiency and reduced expression levels have been reported for this approach [32]. After construction and confirmation of the gene orientation, the vectors are then delivered to the explants. Figure 3 outlines the vector construction scheme and the transformation procedure for microalgae chloroplast.

As cellular compartments that are separated from the rest of the cell by typically two membranes, for example, the outer and inner envelope membrane, chloroplasts provide an excellent containment for toxic recombinant proteins. They lack the mechanism or machinery to export foreign proteins. This feature allows the use of plastids as a potential site for heterologous protein expression that would be toxic when expressed in the cytosol (via nucleus). The transformation of microalgae chloroplast (*C. reinhardtii*) was reported in 1988 by using particle bombardment [33]. This transformation method used tungsten particles to penetrate agar-plated *Chlamydomonas* cells. Apart from tungsten particles, gold nanocarriers coated with vector have also been successfully applied in *C. reinhardtii* chloroplast transformation. Once inside the chloroplast, insertion of the foreign gene occurs through the resident homologous recombination process (Fig. 3). Early devices used for the biolistic transformation used gunpowder for the bombardment process [34]. Nowadays, gene guns are powered by helium, as it is a better propellant and offers higher transformation rates [35]. Apart from the biolistic method, several other chloroplast transformation techniques have been introduced that involve chloroplast targeting by using transit peptide sequences [36] and agitation of cells with glass beads (reviewed in Ref. [35]). During the biolistic method, cells are kept in dark for some period after particle bombardment and are then transferred to selection plates. The selective agar plates contain usually an antibiotic that allows for selection of transformants based on the inserted selectable marker. Only cells that carry the antibiotic resistant gene can survive on the selective plates. The most commonly used selectable markers for *C. reinhardtii* are the bacterial *aadA* and *aphA6* genes that confer spectinomycin and amikacin resistance, respectively [35]. Initially, few copies of the plastome are transformed, which are then allowed to multiply in the presence of selection to achieve homoplasmy. Once the homoplasmy is achieved, transformants can be stably maintained even without selection. Finally, the putatively transformed cells are subjected to molecular screening (PCR analysis, southern blotting), biochemical analyses (immunoblotting, mass spectrometry, activity assays), or microscopy (when transgenes are tagged with a reporter gene like GFP) to confirm the presence and expression of the transgene. Once a stable transplastomic line is obtained, the marker gene(s) can be excised as they are no longer required. Various systems have been developed for the excision of selective markers, for example, the integrase (Int) phage recombinase system, Cre/loxP, or via direct repeats for intrinsic homologous recombination [37].

4. Various Products from Transplastomic Algae

Several recombinant proteins, therapeutics, commercial enzymes, and metabolites have been produced in microalgae by transforming the chloroplast genome. Predominantly, these have been produced in *C. reinhardtii* because of the availability of transformation protocols for this alga. The first attempt to produce commercially high-value compounds was the expression of a human large single-chain antibody in the chloroplast of the green algae, *C. reinhardtii* [38]. A patented chloroplast

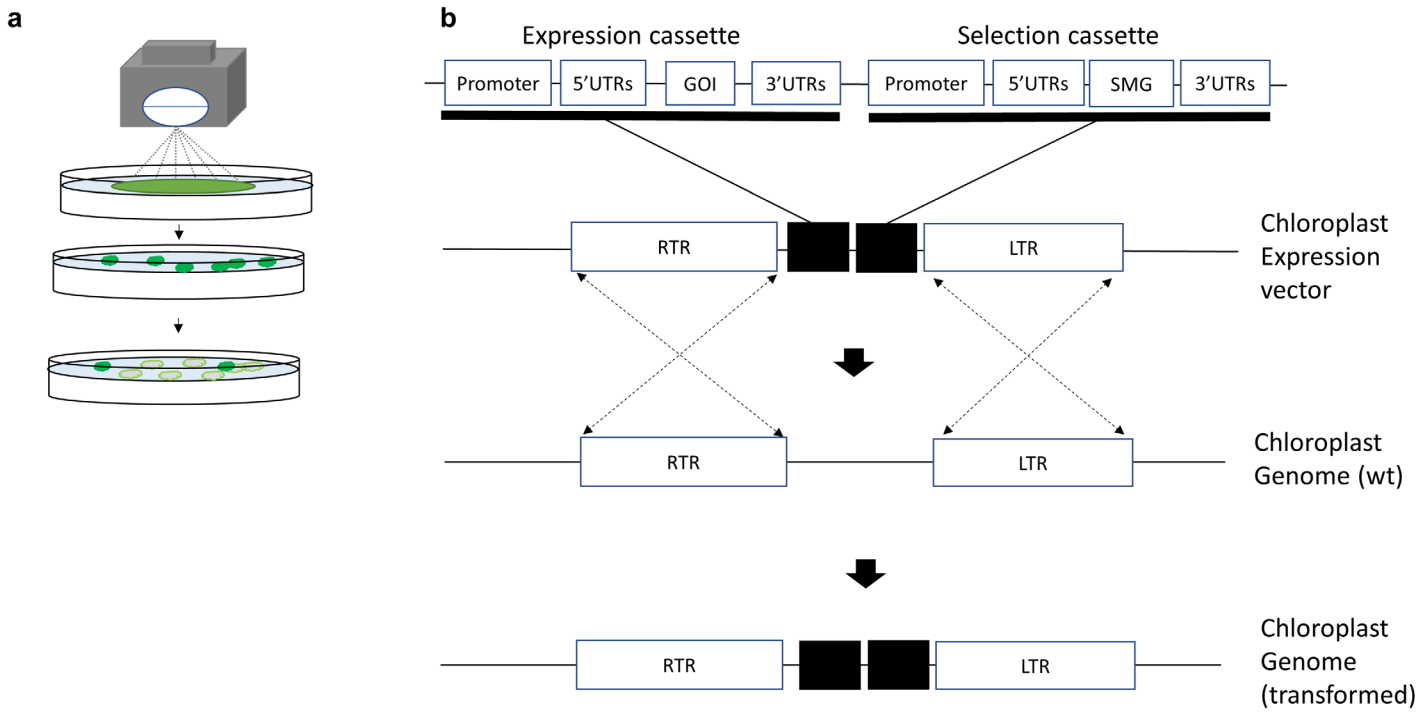

FIG. 3 *Schematic representation of transgene integration into a chloroplast genome. (a) Steps for recovering transplastomic algal cells. (b) Schematic map of a chloroplast expression vector. Abbreviations: UTRs, untranslated region; GOI, gene of interest; SMG, selectable marker gene; RTR, right targeting region; LTR, left targeting region.*

transformation technology using particle bombardment for *C. reinhardtii* describes the robust expression of a mammalian gene mammary-associated serum amyloid protein (M-SAA). The M-SAA is usually present in the colostrum of mammals that helps fight bacterial and viral infections in newborns. When produced in larger quantities using an algal expression system, this protein could be administered orally to animals lacking this protein in their colostrum. Therefore, large-scale production of this protein would be industrially important. The patent describes the methodology to achieve high-level expression of M-SSA (~10% total soluble proteins [TSP]) in *C. reinhardtii* chloroplast [23].

Another study conducted to test the heterologous expression of a bifunctional enzyme (diterpene synthase) with a molecular weight of 91 kDa in the chloroplast of *C. reinhardtii* demonstrated that the protein could accumulate to as much as 3.7% TSP [39]. This study concluded that algal biosystems could serve as a platform for a higher and stable expression system of relatively large proteins. Another study in which a plant cytochrome P450 encoding gene from *Sorghum bicolor* (CYP79A1) was expressed in the chloroplast of *C. reinhardtii* showed an increased production of diterpenoids (900 ng/mL of the culture) [36]. Wannathong et al. [40] used glass beads for the transformation of algal chloroplast and reported that recombinant human growth hormone could be stably expressed up to 25 μg/mL. In another study, Castellanos-Huerta et al. [41] performed biolistic transformation of *C. reinhardtii* chloroplast to express avian influenza virus (AIV) protein. The results of this study indicated stable expression of the recombinant AIV coat proteins, and ELISA results revealed the antigenic potency of the recombinant protein. The protein interaction revealed hemagglutination upon interaction with different monoclonal and polyclonal antibodies. This study demonstrated the potential of transplastomic algae in poultry industry for the production of recombinant proteins in bulk quantities. Dejtisakdi and Miller [42] overexpressed a cyanobacterial enzyme fructose-1, 6-bisphosphate in the chloroplast of *C. reinhardtii*. The overexpression of this enzyme in higher plants is known to increase biomass. Overexpression of this enzyme in the *C. reinhardtii* chloroplast resulted in an increase of up to 1.4-folds TSP as compared to the wild-type while negatively impacting the algal biomass production [42]. In a recent study, Faè et al. [43] performed a comparative analysis for the production of a bacterial endoglucanase in two expression systems (tobacco and *C. reinhardtii*). The expression levels in tobacco chloroplasts reached 8%–10% of total protein, whereas only 0.003% of TSP could be observed for *C. reinhardtii*. Although the microalgal platform did provide lower protein yields compared to tobacco chloroplasts, however, the protein was stably expressed. Recently, Yang and coworkers [44] expressed bacterial proteins in *C. reinhardtii* chloroplasts as a fusion of the foreign protein to highly expressing regulatory elements. For the bacterial Cel6A protein, that was fused to the downstream box of TetC (fragment from the tetanus toxin) promoter and terminator, protein accumulation of about 0.3% TSP could

TABLE 3 *Summary of different industrial products produced in microalgal chloroplasts*

Microalgal host	*Target gene*	*Transformation method*	*Outcome*	*Reference*
C. reinhardtii	VHH against Botulinum neurotoxin (BoNT/A)	Biolistic	5% of total soluble protein (TSP)	[45]
C. reinhardtii	AppA phytase gene from *E. coli*	Biolistic	10 units of phytase/gram	[46]
C. reinhardtii	*bkt1* gene from *H. pluvialis*	Chloroplast targeting	New ketocarotenoid detected namely, 4-keto-lutein/ketozeaxanthin	[47]
P. tricornutum	Human IgG against hepatitis B surface antigen (HBsAg)	Biolistic	8.7% of TSP	[48]
C. reinhardtii	Synthetic gene NCQ coding for a bioactive peptide (chimeric protein)	Biolistic	0.16%–2.4% of TSP	[49]
C. reinhardtii	E7GGG gene of human papilloma virus for vaccine development	Glass beads	0.12% of TSP	[50]
N. salina	AtWRI1 transcription factor gene from *Arabidopsis* for biofuel enhancement	Biolistic	Biofuel contents increased up to 64% as compared to wild-type	[51]
C. reinhardtii	VP28 expression against white spot syndrome virus (WSSV)	Biolistic	>20% of TSP	[52]
C. reinhardtii	CD22 (B-cell surface antigen) fused to PE40 endotoxin A of *Pseudomonas aeruginosa*	Biolistic	Immunotoxin accumulated successfully in the eukaryotic platform	[53]
C. reinhardtii	VP1 as mucosal vaccine against foot and mouth disease (FMD)	Biolistic	3% of TSP	[54]
C. reinhardtii	E2 gene expressing classical swine fever virus structural protein as vaccine	Biolistic	1.5%–2% of TSP	[55]
C. reinhardtii	CD22 (B-cell surface antigen) fused to gelonin endotoxin gene from Gelonium multiform	Biolistic	0.1%–0.3% of TSP	[24]
C. reinhardtii	*Staphylococcus aureus* binding domain D2 fused to cholera toxin B subunit (CTB)	Biolistic	0.7% of TSP and showed successful immunization of mice against *S. aureus*	[56]
C. reinhardtii	VEGF and HMGB1 genes expressed in algal plastids	Biolistic	VEGF levels showed up to 3% of TSP and HMGB1 up to 2.5% of TSP	[57]

be obtained. In another study performed by Carrizalez-López et al. [58], three peptides that have a role in reducing blood pressure (antihypertensive activity) were expressed in *C. reinhardtii* chloroplast under the control of the *rbc*L promoter. The results showed that the protein content was 34.4 ng of the algal biomass. When administered to laboratory rats that suffered from spontaneous hypertension, blood pressure was lowered after 6 h. Together these results demonstrate that algal chloroplasts could be an excellent platform to produce important pharmaceuticals. A recent study by Perozeni et al. [59] showcases the potential of genetic engineering and chloroplast targeting in *C. reinhardtii*. Typically, the β-ketolase enzyme is encoded in the nuclear genome of *C. reinhardtii* and expressed at an almost negligible level, so that it is even considered a pseudogene. The enzyme is usually actively involved in the conversion of β-carotene to the zeaxanthin and astaxanthin, which

are well-known antioxidants. Interestingly, by redesigning and optimizing the codons of this gene, by adding intronic regions from the host's own Rubisco small subunit II, and by adding the chloroplast transit peptide sequence from the subunit D of photosystem I, the enzyme was expressed and localized to the chloroplast. The transformed cells displayed a reddish-brown color and produced ketocarotenoid at up to 4.5 mg/L/day. This production was found to be in the approximate range of carotenoids produced by *H. pluvialis*, a natural high accumulator of carotenoids. See Table 3 for the list of salient commercially important compounds produced recombinantly in the algal chloroplast.

5. Extending Chloroplast Transformation to Other Algal Species

Research and development in plastid biotechnology are still a challenge for most of the microalgae species. With over 70,000 species, microalgae represent a vast unexplored potential resource. Several algal species have evolved exclusive metabolic pathways that lead to the production of commercially valued compounds [21]. Therefore, microalgae can be considered as biochemical factories that produce a range of valuable compounds of commercial importance [24, 60]. To date only a few of them have been exploited to produce recombinant proteins in their chloroplasts [7, 61]. Several attempts have been made to extend chloroplast transformation to other algal species. In this section, we discuss few examples that report successful transformation of the chloroplast genome in different microalgae other than *C. reinhardtii*.

Platymonas (Tetraselmis) subcordiformis is a marine unicellular green alga, which due to its high nutrient content is widely used as feed in aquaculture. In addition to its usage as feed, it has been reported to produce H_2 [62], making it a potential source for H_2 production for commercial use. Therefore, its genetic manipulation holds great promise for industrial applications. Cui et al. [63] reported successful chloroplast transformation in *P. subcordiformis* by expressing GFP in its chloroplast using Basta as selectable marker due to insensitivity of this alga to spectinomycin, streptomycin, or kanamycin. It is noteworthy that Basta has been failed as selectable marker for recovering tobacco transplastomic plants due to its lethality. The establishment of chloroplast transformation technology in this algal species may offer new opportunities for industrial applications.

Nannochloropsis is a genus of algae that includes different and important species for commercial applications to produce foreign proteins. These algal species have also been genetically engineered to produce various biofuels. They are relatively easy to culture and large-scale cultivation is conducted by various companies and institutes [64, 65]. Recently, Gan et al. [66] used electroporation to successfully transform the chloroplast genome of an oleaginous marine microalga *Nannochloropsis oceanica*, where an introduced GFP-tagged transgene could be detected by fluorescent microscopy as well as by laser confocal scanning microscopy. One notable observation of this study however was that the transformation efficiency remained quite low, which must be improved to make it commercially feasible. Nevertheless, the study demonstrated successful genetic manipulation of the *Nannochloropsis oceanica* chloroplast genome to use them as cellular factories for the production of commercially important compounds.

Haematococcus pluvialis is another algal species of high commercial potential. It is the main source of natural astaxanthin, a high-value secondary carotenoid with antioxidant properties. However, its accumulation takes place only under stressful conditions such as nutrient starvation, exposure to high salt concentrations or high light. Genetic manipulation of *H. pluvialis* chloroplast holds great potential for industrial applications in human health, for example, for treating chronic inflammatory diseases, cancer, metabolic disorders, and for preventing neurodegeneration processes [67]. Galarza et al. [68] showed that *H. pluvialis* chloroplasts could be transformed by particle bombardment. They overexpressed an endogenous enzyme phytoene desaturase under the light-inducible *psb*A promoter. The transformed cells could accumulate up to ~67% more carotenoid astaxanthin than the wild-type cells under high light intensities.

These examples are good indications that plastome engineering of microalgae is a promising approach for the large-scale production of important industrial products in the future, so that increasing energy and fuel demands might be met. Table 4 enlists different algal species in which chloroplast transformation has been established.

6. Conclusion and Perspectives

Algae are being used as a production platform for a range of products because of their comparatively easy and relatively cost-effective cultivation. Even though plant chloroplasts also provide an excellent platform for the efficient production of foreign proteins at quite high levels (~70% TSP) [22], a major concern with plants is the possibility of transgene outcrossing to other species [31, 69]. In contrast, microalgae are relatively easy to handle compared to transgenic plants, as they are typically maintained in liquid cultures or on petri-dishes and containment is much more easily ensured [8, 35, 70]. Products from GRAS-designated species can easily be used for topical applications by directly using the algal lysates that contain the therapeutic protein(s). Moreover, therapeutics produced in this way could also be modified further to yield certain vaccines and drugs that could be administered to patients orally without the risk of harmful effects linked to their dosage form [7].

Until recently, progress in the field of genetically engineering microalgae has been quite slow. Approaches that had been used effectively for the transformation of other systems like plants were ineffective for microalgae, primarily because of the extensive evolutionary distance between the organisms [19]. Therefore, new transformation techniques are being developed to extend chloroplast transformation to other algal species. The

TABLE 4 *List of algal species in which chloroplast transformation has been reported (only first report is included)*

Algal species	*Gene of interest*	*Trait*	*Reference*
Platymonas subcordiformis	*bar*	Phosphinothricin acetyltransferase	[63]
Porphyridium spp.	*AHAS*	Acetohydroxyacid synthase	[71]
Nannochloropsis oceanica	*gfp, BLE*	Green florescent protein, Zeocin resistance	[66]
Euglena gracils	*aadA*	Resistance to spectionomycin and streptomycin	[72]
Haematococcus pluvialis	*aadA*	Resistance to spectionomycin and streptomycin	[73]
Dunaliella tertiolecta	*Xylanase, α-galactosidase, phytase, phosphate anhydrolase, and β-mannanase*	Resistance to erythromycin endo-1,4-β-xylanase β-mannosidases α-D-Galactoside galactohydrolases Phytases, cysteine proteases	[74]
Amphidinium carterae	*ChI*	Resistance to chloramphenicol	[75]
Phaeodactylum tricornutum	*CAT*	Resistance to chloramphenicol	[76]

green alga *C. reinhardtii*—a long-standing model organism for molecular biology and chloroplast genetics of microalgae—has contributed significantly to establish new ways for the development of stable chloroplast transformation in other algal species

A major issue of expressing transgenes in chloroplasts as shown in Table 1 could be the absence of glycosylation, the enzymatic attachment of polysaccharide units to the proteins. Glycosylation is often considered necessary for the protein functioning and stability. However, some reports show that when glycoproteins were expressed in higher plant chloroplasts, for example, xylanases and Type I Interferon α2b; they were found active and stable like native proteins [77–80]. More work is needed though to rule out the effect of absence of glycosylation on protein stability of chloroplast-made proteins.

Overall, the microalgal chloroplast has emerged as an alternative platform to produce high-value targets at large scale; however, the successful demonstration for commercial application of this platform is hindered by low biomass production, poor gene expression levels, and costly downstream processing methodologies. The improved understanding of gene expression mechanisms due to research on *C. reinhardtii* has resulted in improving the genetic transformation methods of algal chloroplasts as well as expression levels of recombinant proteins. It is imperative to reduce the costs for productive recovery, which currently account for 60% of the total production cost [3]. Therefore, efforts should be made to genetically modify strains for efficient product recovery to reduce product recovery costs for example by the excretion of product to the medium, induction of auto flocculation, or autolysis. More work will be needed to address the issues like biomass production, development of strains with reduced antenna size for better light penetration especially under mass cultures, and designing efficient harvesting strategies for making microalgae a competitive production platform for pharmaceutical, industrial, and nutritional applications.

7. Acknowledgements

The authors thank Higher Education Commission (HEC) of Pakistan for funding work in the author's laboratory (NA). We sincerely apologize to all colleagues whose work could not be discussed here due to space constraints.

The authors declare that they have no conflict of interest.

8. 8. References

[1] Mahmoud, K. (2007) Res. J. Cell Mol. Biol. 1, 9–22.
[2] Ahmad, N., and Mukhtar, Z. (2013) Gen. Ther. Mol. Biol. 15, 14–29.
[3] Gomaa, M., Al-Haj, L., and Abed, R. (2016) J. Appl. Microbiol. 121, 919–931.
[4] Shahid, A., Khan, F., Ahmad, N., Farooq, M., Mehmood, M. A., (2019) In Microalgal Carbohydrates and Proteins: Synthesis, Extraction, Applications, and Challenges (Alam, M. A., ed.), Springer, New York.
[5] Abomohra, A. E.-F., and Elshobary, M., (2019) Biodiesel, Bioethanol, and Biobutanol Production from Microalgae. pp. 293–321, Springer, New York.
[6] Leu, S., and Boussiba, S. (2014) Indust. Biotechnol. 10, 169–183.

[7] Yan, N., Fan, C., Chen, Y., and Hu, Z. (2016) Int. J. Mol. Sci. 17, 962.
[8] Specht, E., Miyake-Stoner, S., and Mayfield, S. (2010) Biotechnol. Lett. 32, 1373–1383.
[9] Dyo, Y. M., and Purton, S. (2018) Microbiology.164, 113–121.
[10] Greiner, S., Lehwark, P., and Bock, R. (2019) Nucleic Acids Res. 47, W59–W64.
[11] Olejniczak, S. A., Łojewska, E., Kowalczyk, T., and Sakowicz, T. (2016) Planta 244, 517–527.
[12] Smith, D. R., Lee, R. W., Cushman, J. C., Magnuson, J. K., Tran, D., and Polle, J. E. (2010) BMC Plant Biol. 10, 83.
[13] Maul, J. E., Lilly, J. W., Cui, L., Miller, W., Harris, E. H., and Stern, D. B. (2002) Plant Cell 14, 2659–2679.
[14] Rochaix, J., and Malnoe, P. (1978) Cell 15, 661–670.
[15] Erickson, J. M., Rahire, M., and Rochaix, J. D. (1984) EMBO J. 3, 2753–2762.
[16] Grossman, A. R., Harris, E. E., Hauser, C., Lefebvre, P. A., Martinez, D., Rokhsar, D., Shrager, J., Silflow, C. D., Stern, D., and Vallon, O. (2003) Eukaryot. Cell 2, 1137–1150.
[17] Turmel, M., and Lemieux, C., (2018) Evolution of the Plastid Genome in Green Algae. pp. 157–193. Elsevier, Amsterdam, Netherlands.
[18] Ng, I. S., Tan, S. I., Kao, P. H., Chang, Y. K., and Chang, J. S. (2017) Biotechnol. J. 12, 1600644.
[19] Bock, R. (2014) Curr. Opin. Biotechnol. 26, 7–13.
[20] Doron, L., Segal, N. A., and Shapira, M. (2016) Front. Plant Sci. 7, 505.
[21] Rasala, B. A., and Mayfield, S. P. (2015) Photosynth. Res. 123, 227–239.
[22] Oey, M., Lohse, M., Kreikemeyer, B., and Bock, R. (2009) Plant J. 57, 436–445.
[23] Mayfield, S. P., (2010) Robust Expression of a Bioactive Mammalian Protein in *Chlamydomonas* Chloroplast. Google Patents.
[24] Tran, M., Henry, R. E., Siefker, D., Van, C., Newkirk, G., Kim, J., Bui, J., and Mayfield, S. P. (2013) Biotechnol. Bioeng. 110, 2826–2835.
[25] Sikdar, S., Serino, G., Chaudhuri, S., and Maliga, P. (1998) Plant Cell Rep. 18, 20–24.
[26] Skarjinskaia, M., Svab, Z., and Maliga, P. (2003) Trans. Res. 12, 115–122.
[27] Svab, Z., Hajdukiewicz, P., and Maliga, P. (1990) Proc. Natl. Acad. Sci. USA 87, 8526–8530.
[28] Svab, Z., and Maliga, P. (1993) Proc. Natl. Acad. Sci. USA 90, 913–917.
[29] Sidorov, V. A., Kasten, D., Pang, S. Z., Hajdukiewicz, P. T., Staub, J. M., and Nehra, N. S. (1999) Plant J. 19, 209–216.
[30] Ruf, S., Forner, J., Hasse, C., Kroop, X., Seeger, S., Schollbach, L., Schadach, A., and Bock, R. (2019) Nat. Plants 5, 282–289.
[31] Ahmad, N., and Mukhtar, Z. (2017) Genomics 109, 494–505.
[32] Ruhlman, T., Verma, D., Samson, N., and Daniell, H. (2010) Plant Physiol. 152, 2088–2104.
[33] Boynton, J. E., Gillham, N. W., Harris, E. H., Hosler, J. P., Johnson, A. M., Jones, A. R., Randolph-Anderson, B. L., Robertson, D., Klein, T. M., Shark, K. B., and Sanford, J. C. (1988) Science 240, 1534–1538.
[34] Sanford, J. C. (1988) Trends Biotechnol. 6, 299–302.
[35] Purton, S., (2007) Tools and Techniques for Chloroplast Transformation of *Chlamydomonas*. pp. 34–45, Springer, New York.
[36] Gangl, D., Zedler, J. A., Włodarczyk, A., Jensen, P. E., Purton, S., and Robinson, C. (2015) Phytochemistry 110, 22–28.
[37] Day, R. N., and Davidson, M. W. (2009) Chem. Soc. Rev. 38, 2887–2921.
[38] Mayfield, S. P., Franklin, S. E., and Lerner, R. A. (2003) Proc. Natl. Acad. Sci. USA 100, 438–442.
[39] Zedler, J. A., Gangl, D., Hamberger, B., Purton, S., and Robinson, C. (2015) J. Appl. Phycol. 27, 2271–2277.
[40] Wannathong, T., Waterhouse, J. C., Young, R. E., Economou, C. K., and Purton, S. (2016) Appl. Microbiol. Biotechnol. 100, 5467–5477.
[41] Castellanos-Huerta, I., Bañuelos-Hernández, B., Téllez, G., Rosales-Mendoza, S., Brieba, L. G., Esquivel-Ramos, E., Beltrán-López, J. I., and Velazquez, G., Fernandez-Siurob, I. (2016) Avian Dis. 60, 784–791.
[42] Dejtisakdi, W., Miller, S. M. (2016) Algal Res. 14, 116–126.
[43] Faè, M., Accossato, S., Cella, R., Fontana, F., Goldschmidt-Clermont, M., Leelavathi, S., Reddy, V. S., and Longoni, P. (2017) Appl. Microbiol. Biotechnol. 101, 4085–4092.
[44] Richter, L. V., Yang, H., Yazdani, M., Hanson, M. R., and Ahner, B. A. (2018) Biotechnol. Biofuels 11, 133.
[45] Barrera, D. J., Rosenberg, J. N., Chiu, J. G., Chang, Y.-N., Debatis, M., Ngoi, S.-M., Chang, J. T., Shoemaker, C. B., Oyler, G. A., and Mayfield, S. P. (2015) Plant Biotechnol. J. 13, 117–124.
[46] Yoon, S.-M., Kim, S. Y., Li, K. F., Yoon, B. H., Choe, S., and Kuo, M. M.-C. (2011) Appl. Microbiol. Biotechnol. 91, 553–563.
[47] León, R., Couso, I., and Fernández, E. (2007) J. Biotechnol. 130, 143–152.
[48] Hempel, F., Lau, J., Klingl, A., and Maier, U. G. (2011) . PLoS One 6, e28424.
[49] Campos-Quevedo, N., Rosales-Mendoza, S., Paz-Maldonado, L. M. T., Martínez-Salgado, L., Guevara-Arauza, J. C., and Soria-Guerra, R. E. (2013) Plant Cell Tissue Organ Cult. 113, 217–225.
[50] Demurtas, O. C., Massa, S., Ferrante, P., Venuti, A., Franconi, R., and Giuliano, G. (2013) PLoS One 8, e61473.
[51] Kang, N. K., Kim, E. K., Kim, Y. U., Lee, B., Jeong, W.-J., Jeong, B.-r., and Chang, Y. K. (2017) Biofuels 10, 231.
[52] Surzycki, R., Greenham, K., Kitayama, K., Dibal, F., Wagner, R., Rochaix, J. D., Ajam, T., and Surzycki, S. (2009) Biologicals 37, 133–138.
[53] Tran, M., Van, C., Barrera, D. J., Pettersson, P. L., Peinado, C. D., Bui, J., and Mayfield, S. P. (2013) Proc. Natl. Acad. Sci. USA 110, E15-E22.
[54] Sun, M., Qian, K., Su, N., Chang, H., and Liu, J., Shen, G. (2003) Biotechnol. Lett. 25, 1087–1092.
[55] He, D. M., Qian, K. X., Shen, G. F., Zhang, Z. F., Li, Y. N., Su, Z. L., and Shao, H. B. (2007) Colloids Surf. B Biointerfaces 55, 26–30.
[56] Dreesen, I. A., Charpin-El Hamri, G., and Fussenegger, M. (2010) J. Biotechnol. 145, 273–280.
[57] Rasala, B. A., Muto, M., Lee, P. A., Jager, M., Cardoso, R. M. F., Behnke, C. A., Kirk, P., Hokanson, C. A., Crea, R., Mendez, M., and Mayfield, S. P. (2010) Plant Biotechnol. J. 8, 719–733.
[58] Carrizalez-López, C., González-Ortega, O., Ochoa-Méndez, C. E., Galván-Moreno, F. U., Rosales-Mendoza, S., Monreal-Escalante, E., Bañuelos-Hernández, B., Paz-Maldonado, L. M. T., Castro-Moreno, P., and Ibarra-Barajas, M. (2018) J. Appl. Phycol. 30, 1701–1709.
[59] Perozeni, F., Cazzaniga, S., Baier, T., Zanoni, F., Zoccatelli, G., Lauersen, K. J., Wobbe, L., and Ballottari, M. (2019) bioRxiv, 535989.
[60] Moody, J. W., McGinty, C. M., and Quinn, J. C. (2014) Proc. Natl. Acad. Sci. USA 111, 8691–8696.
[61] Ahmad, N., Mehmood, M. A., and Malik, S. (2019) Protein Pept. Lett. 616, 34–45.
[62] Guo, Z., Chen, Z., Zhang, W., Yu, X., and Jin, M. (2008) Biotechnol. Lett. 30, 877–883.
[63] Cui, Y., Qin, S., and Jiang, P. (2014) PLoS One 9, e98607.
[64] Wei, L., Wang, Q., Xin, Y., Lu, Y., and Xu, J. (2017) Algal Res. 27, 366–375.
[65] Zienkiewicz, K., Zienkiewicz, A., Poliner, E., Du, Z.-Y., Vollheyde, K., Herrfurth, C., Marmon, S., Farré, E. M., Feussner, I., and Benning, C. (2017) Biotechnol. Biofuels 10, 8.
[66] Gan, Q., Jiang, J., Han, X., Wang, S., and Lu, Y. (2018) Plant Sci. 9, 439
[67] Han, D., Li, Y., and Hu, Q. (2013) Algae 28, 131–147.
[68] Galarza, J. I., Gimpel, J. A., Rojas, V., Arredondo-Vega, B. O., and Henríquez, V. (2018) Algal Res. 31, 291–297.
[69] Chen, Q., and Davis, K. R. (2016) F1000Research 5.
[70] Taunt, H. N., Stoffels, L., and Purton, S. (2018) Bioengineered 9, 48–54.
[71] Lapidot, M., Raveh, D., Sivan, A., Arad, S. M., and Shapira, M. (2002) Plant Physiol. 129, 7–12.
[72] Doetsch, N. A., Favreau, M. R., Kuscuoglu, N., Thompson, M. D., and Hallick, R. B. (2001) Curr. Genet. 39, 49–60.
[73] Gutierrez, C. L., Gimpel, J., Escobar, C., Marshall, S. H., and Henríquez, V. (2012) J. Phycol. 48, 976–983.
[74] Georgianna, D. R., Hannon, M. J., Marcuschi, M., Wu, S., Botsch, K., Lewis, A. J., Hyun, J., Mendez, M., and Mayfield, S. P. (2013) Algal Res. 2, 2–9.

[75] Nimmo, I. C., Barbrook, A. C., Lassadi, I., Chen, J. E., Geisler, K., Smith, A. G., Aranda, M., Purton, S., Waller, R. F., and Nisbet, R. E. R. (2019) eLife 8, e45292.
[76] Xie, W.-H., Zhu, C.-C., Zhang, N.-S., Li, D.-W., Yang, W.-D., Liu, J.-S., Sathishkumar, R., and Li, H.-Y. (2014) Marine Biotechnol. 16, 538–546.
[77] Kim, T., and Langridge, W. H. (2004) Plant Cell Rep.22, 382–387.
[78] Leelavathi, S., Gupta, N., Maiti, S., Ghosh, A., Reddy, V. S. (2003) Mol. Breed. 11, 59–67.
[79] Huang, Z., Santi, L., LePore, K., Kilbourne, J., Arntzen, C. J., and Mason, H. S. (2006) Vaccine 24, 2506–2513.
[80] Arlen, P. A., Falconer, R., Cherukumilli, S., Cole, A., Cole, A. M., Oishi, K. K., and Daniell, H. (2007) Plant Biotechnol. J. 5, 511–525.