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REVIEW

Microalgae as platforms for production of recombinant proteins and valuable compounds: progress and prospects

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Abstract Over the last few years microalgae have gained increasing interest as a natural source of valuable compounds and as bioreactors for recombinant protein production. Natural high-value compounds including pigments, long-chain polyunsaturated fatty acids, and polysaccharides, which have a wide range of applications in the food, feed, cosmetics, and pharmaceutical industries, are currently produced with nontransgenic microalgae. However, transgenic microalgae can be used as bioreactors for the production of therapeutic and industrially relevant recombinant proteins. This technology shows great promise to simplify the production process and significantly decrease the production costs. To date, a variety of recombinant proteins have been produced experimentally from the nuclear or chloroplast genome of transgenic *Chlamydomonas reinhardtii*. These include monoclonal antibodies, vaccines, hormones, pharmaceutical proteins, and others. In this review, we outline recent progress in the production of recombinant proteins with transgenic microalgae as bioreactors, methods for genetic transformation of microalgae, and strategies for highly efficient expression of heterologous genes. In particular, we highlight the importance of maximizing the value of transgenic microalgae through producing recombinant proteins together with recovery of natural high-value compounds. Finally, we outline some important issues that need to be addressed before commercial-scale production of high-value recombinant proteins and compounds from transgenic microalgae can be realized.

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Introduction

Microalgae are a diverse group of photosynthetic microorganisms that can use sunlight to synthesize chemical energy carriers such as carbohydrates, lipids, and proteins [12]. Microalgae are typically unicellular and eukaryotic, although prokaryotic cyanobacteria are also frequently referred to as microalgae [53]. In the past decade, there has been a surge of interest in microalgal biotechnology for producing valuable molecules ranging from therapeutic proteins to biofuels [49, 65]. Numerous microalgal species can synthesize valuable compounds naturally, making them a potentially important source of chemical products that are applied in the feed, food, nutrition, cosmetics, and pharmaceutical industries. Moreover, some species of microalgae can be efficiently transformed, which makes it possible to enhance the productivity of natural compounds through metabolic engineering [10, 73]. For example, overexpression of the phytoene synthase gene from the green algae *Chlorella zofingiensis* in *Chlamydomonas reinhardtii* led to a twofold increase in the content of carotenoids violaxanthin and lutein [10]. Like bacteria, yeasts, higher plants, and other bioreactor systems, microalgae can also be engineered to express heterologous genes for the production of recombinant proteins that have important value in industrial and pharmaceutical applications. To date, some mammalian proteins, such as antibodies, hormones, and vaccines, have been expressed in microalgae at economically viable levels.

Table 1 Principal high-value compounds derived from some species of microalgae

Compounds for utilization	Microalgal species used for the production	References
β-Carotene and other carotenoids for health food, dietary supplements, cosmetics, and feed	*Dunaliella salina*, *Dunaliella bardawil*	[44, 61, 85]
Astaxanthin for health food, pharmaceuticals, and feed additives	*Haematococcus pluvialis*	[61]
Polysaccharides for dietary supplements	*Chlorella* sp.	[85]
Polysaccharides for dietary supplements, pharmaceuticals, cosmetics, and nutrition	*Chlorella* sp., *Porphyridium cruentum*	[61, 72]
Lipids and long-chain polyunsaturated fatty acids (PUFAs) for nutrition, pharmaceuticals, cosmetics, and others	*Phaeodactylum tricornutum*, *Isochrysis galbana*, *Odontella aurita*, *Crypthecodinium cohnii*, *Nitzschia laevis*	[50, 61, 85]
Extracts for cosmetics	*Nannochloropsis oculata*	[72]

Microalgae as new bioreactors have several distinct advantages compared with other bioreactor systems. First, microalgae commonly double their biomass within 24 h, and there is a relatively short period of time (generally a few weeks) from the generation of initial microalgal transformants to scaling up to volumes sufficient for large-scale production. Second, recombinant proteins can be expressed from nuclear, chloroplast, and mitochondrial genomes of some microalgal species. Unlike bacteria, eukaryotic microalgae possess complex post-translational modification pathways, and thus microalgae can produce secreted, glycosylated proteins [67, 82]. Third, microalgae can be grown either phototrophically or heterotrophically. Transgenic microalgae are especially suitable for growth in enclosed photobioreactors, in which the culture conditions such as light, temperature, nutrients, and mixing, can be well controlled. The culture of transgenic microalgae in photobioreactors can also prevent transgenes from escaping into the environment, which may potentially occur in higher plants by the means of pollen [33]. Fourth, some species of microalgae have been recognized as edible food sources and fall into the GRAS (Generally Regarded As Safe) category. Microalgae producing therapeutic proteins or other valuable compounds may potentially be sources for oral administration as a powder of lyophilized algae with little or no purification [49, 85]. These advantages make microalgae attractive systems for the production of recombinant proteins and other high-value compounds.

In contrast to other expression hosts, genetic engineering of transgenic microalgae is still in its infancy. Here, we examine progress in the expression of recombinant proteins in microalgae, review the methods for microalgal transformation, and the constructs (promoters, marker, and reporter genes) used. The strategies employed for highly efficient expression of heterologous genes are then outlined. Finally, we discuss the potential of microalgae as production platforms for recombinant proteins and other valuable compounds.

Utilization of natural compounds from microalgae

Commercial applications of high-value compounds derived from microalgae are only a few decades old, although the use of microalgae as a food source or supplement has occurred for centuries [72]. Nowadays, the utilization of high-value compounds derived from microalgae as natural sources is restricted to only a few species of microalgae (Table 1). Microalgae not only contain a high content of proteins, carbohydrates, and lipids, but also produce metabolites such as pigments and many kinds of vitamins. These natural compounds have been widely used in the food, feed, cosmetics, and pharmaceutical industries (Table 1).

The freshwater green algae *Chlorella* and *Scenedesmus* are used in human food, animal feed, and aquaculture, partially because of their high protein content (50–60% of dry biomass) and nutritive value [61]. Microalgae can synthesize carbohydrates mainly in the form of starch, glucose, sugars, and polysaccharides. *Chlorella*-derived starch may substitute for starch-rich terrestrial plants in bioethanol production [4]. Effective polysaccharide fractions are found mainly in cyanobacteria, but also in green microalgae such as *Chlorella* or *Dunaliella*, and are used as dietary supplements or pharmaceuticals [69]. A few species of diatoms and dinoflagellata are a highly productive source of long-chain polyunsaturated fatty acids (LCPUFAs) that are nutritionally and therapeutically important as potential food additives or pharmaceuticals. Currently, profitable production of docosahexaenoic acid (DHA) has been achieved using *Crypthecodinium cohnii* as feedstock. Other LCPUFAs like eicosapentaenoic acid (EPA) from *Phaeodactylum*, *Nannochloropsis*, or *Nitzschia* have also demonstrated potential for industrial production [7, 64].

Microalgae not only contain chlorophyll as primary photosynthetic pigments, but also synthesize many other pigments that play important roles in improving the efficiency of light energy utilization and protecting microalgal cells from photodamage effects [85]. Carotenoids and phycobiliproteins seem to be the most important pigments from a commercial perspective. The carotenoid β-carotene is used as a vitamin A precursor and biological antioxidant in health foods and cosmetics. It is produced mainly by large-scale outdoor culture of the halophilic green microalgae *Dunaliella salina*, which accumulates β-carotene up to 14% of its dry weight [13]. *Haematococcus pluvialis* is capable of synthesizing 1.5–3% of natural astaxanthin by dry weight through a two-stage culture process, in which microalgal growth is optimized to optimal biomass concentration, followed by an astaxanthin-accumulating stage under conditions of intense light and limited nutrient [83]. Natural astaxanthin can be used for coloring muscles in fish and shows pharmaceutical efficacies, such as protecting against chemically induced cancers, increasing high-density lipoproteins, and enhancing the immune system [44]. It might be able to compete favorably with the synthetic astaxanthin when the costs of mass culture, harvesting, and extraction are significantly reduced. Other carotenoids, such as lutein, zeaxanthin, and canthaxanthin, are used for chicken skin coloration or as antioxidants for pharmaceutical purposes.

Although numerous types of valuable compounds have been found in microalgae, there are only a few high-value compounds commercially available today. The carotenoid β-carotene from *Dunaliella*, natural astaxanthin from *Haematococcus*, and DHA from *Crypthecodinium* are the three well-known compounds that are derived from microalgae and brought to market. It must be noted that these commercially utilized high-value compounds are almost all from nontransgenic microalgae. However, over the past 20 years, transgenic microalgae have proved to be excellent bioreactors for producing many other novel valuable compounds, especially recombinant therapeutic proteins.

Transgenic microalgae as bioreactors for recombinant protein production

Although no recombinant protein produced by transgenic microalgae is available on the market at present, transgenic microalgae offer increasing possibility to commercially produce therapeutic and industrially relevant recombinant proteins in a cost-effective manner. To date, more than 20 therapeutically important proteins have been successfully expressed in microalgae, mainly in *Chlamydomonas reinhardtii* [71]. The unicellular green algae *Chlamydomonas* is preferred for recombinant protein production owing to its many beneficial attributes, such as stable and relatively easy transformation of nuclear, chloroplast, and mitochondrial organelles, quick generation of positive transformants, alternative culture of phototrophic or heterotrophic growth, and the availability of various molecular toolkits. The expression levels of recombinant proteins are in the range of 0.16–5% of total soluble protein (TSP) (Table 2). The proteins expressed in chloroplasts generally have higher levels than those expressed in the nucleus. For a review of recombinant protein production in *Chlamydomonas* chloroplasts, see Mayfield et al. [49].

The first important human therapeutic protein expressed in *Chlamydomonas* was HSV8-lsc containing the entire IgA heavy chain protein fused to the variable region of the light chain [48]. This large single-chain (lsc) antibody assembled into a dimer in chloroplast and accumulated as a fully soluble protein with the ability to bind to herpes virus proteins. Moreover, a simplified version (HSV8-scFv) containing a single-chain fragment variable region was also expressed in *Chlamydomonas* chloroplasts in a soluble form [47]. More recently, a functional human IgG1 monoclonal antibody (mAb) was synthesized and properly assembled in *Chlamydomonas* chloroplasts. This antibody (83K7C) was able to bind its target and showed similar affinity to traditional mammalian-expressed 83K7C [84]. Transgenic microalgae are really a robust platform for human therapeutic protein production, which is evidenced by expressing seven proteins with or potentially with therapeutic value [63]. All recombinant proteins were soluble, and over 50% of the proteins were expressed at levels (2–3% TSP) sufficient for commercial production. These examples demonstrate the potential of *Chlamydomonas* chloroplasts in expressing and assembling complex human antibodies. Chloroplasts possess the modification apparatus for disulfide-bond formation, but are not able to perform other complex post-translational modifications, e.g., glycosylation, which is not essential for therapeutic function of human monoclonal antibodies in many cases [19, 84].

It is feasible to use *Chlamydomonas* for vaccine production. A fusion protein containing the foot and mouth disease virus (FMDV) VP1 and the cholera toxin B subunit (CTB) was expressed in *Chlamydomonas* chloroplast and accounted for up to 3% of TSP [75]. The CTBVP1 fusion protein also showed both GM1-ganglioside binding affinity and antigenicity for separate VP1 and CTB proteins. There are two examples demonstrating the potential of transgenic microalgae as a source for oral delivery of functional proteins. The transgenic *Chlamydomonas* expressing the D2 fibronectin binding domain of *Staphylococcus aureus* fused with the cholera toxin B subunit (CTB) was generated and then fed to mice for 5 weeks. Microalgae-based vaccination protected 80% of mice from *S. aureus* infection. The stability of algae-based vaccine could be maintained for more than 1.5 years at room temperature [20]. Another protein,

Table 2 Some pharmaceutically and industrially relevant proteins that could be produced in microalgae

Recombinant product and functions	Expressed in	Expression level achieved	References
HSV8-lsc and HSV8-scFv, a human IgA anti-herpes monoclonal, large single-chain (lsc) antibody, and a single-chain fragment variable (scFv) antibody	*C. reinhardtii* chloroplast	0.5% TSP in the chloroplast	[47, 48]
Foot and mouth disease virus (FMDV) VP1 fused to cholera toxin B subunit (CTB), facilitates the production of a new and safer FMDV mucosal vaccine	*C. reinhardtii* chloroplast	3% TSP	[75]
Human tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)	*C. reinhardtii* chloroplast	0.43–0.67% TSP	[88]
Human glutamic acid decarboxylase 65 (hGAD65), key autoantigen in type I diabetes; important marker for the prediction and diagnosis of type I diabetes	*C. reinhardtii* chloroplast	0.25–0.3% TSP	[86]
D2 fibronectin binding domain of *Staphylococcus aureus* fused with the cholera toxin B subunit (CTB); stable algae-based oral vaccine	*C. reinhardtii* chloroplast	0.7% TSP	[20]
Full-length IgG1 human monoclonal antibody (mAb), against anthrax protective antigen 83 (PA83); potentially blocks the effects of anthrax toxin	*C. reinhardtii* chloroplast	100 μg of purified protein per 1 g of dry algal biomass	[84]
High mobility group protein B1 (HMGB1); mediates a number of important functions involved in wound healing, has the potential to enhance the effectiveness of some anticancer therapies	*C. reinhardtii* chloroplast	2.5% TSP	[63]
Bovine mammary-associated serum amyloid (M-SAA); stimulates the production of mucin in the gut, acting in the prophylaxis of bacterial and viral infections	*C. reinhardtii* chloroplast	5% TSP	[46]
Hepatitis B surface antigen (HBsAg); low-cost HBsAg vaccine for prophylaxis of hepatitis B virus infection	*Dunaliella salina* nuclear	0.16–0.31% TSP	[27]
Flounder growth hormone; has a growth-enhancing effect on flounder fry	*Chlorella ellipsoidea* nuclear	420 μg of fGH protein per liter of culture	[36]

the flounder growth hormone (fGH), was expressed in *Chlorella ellipsoidea* at 400 μg per liter of culture. Flounder fry fed on the transgenic *Chlorella* experienced a 25% growth increase after 30 days of feeding [36]. These examples offer the possibility of utilizing transgenic algae as producer and carrier for oral delivery of therapeutic proteins although further studies are needed before they can be used for human populations.

Microalgal transformation

The utilization of transgenic microalgae as effective platforms for recombinant protein production depends on the establishment of stable transformation systems. Over the last 20 years, successful genetic transformation has been reported in ~22 species of microalgae, most of which are achieved by nuclear transformation. Table 3 outlines key features of some microalgal species that can be successfully transformed. Despite these advances, up to now routine transformation is achievable only for very few species including *C. reinhardtii*, *Volvox carteri*, some species of *Chlorella*, and the diatom *Phaeodactylum tricornutum*.

The commonly used methods for transforming microalgae are based on the fact that microalgal cells can endure temporary permeabilization of the cell membrane and invasion of DNA molecules while preserving viability. Microprojectile bombardment, also referred to as microparticle bombardment, gene gun transformation, or simply biolistics, is the most frequently used method (Table 4). This method uses DNA-coated gold or tungsten microprojectiles that are delivered into cells by a particle gun apparatus, and is applicable for almost any type of cell, regardless of the thickness or rigidity of cell wall. In addition, almost all reported successful transformations of diatoms use this method [21, 23, 24, 52]. Organelles such as chloroplasts and mitochondria have also been transformed in this way [3, 17, 41, 62]. Unlike nuclear genome transformation, in which foreign genes integrate randomly, microalgal chloroplasts support homologous recombination allowing targeted integration of transgenes [47, 75]. Microparticle bombardment is the preferred method when a transformation

Table 3 Representative species of microalgae that can be successfully transformed and their key features

Microalgal species	Taxa	Key features related to genetic transformation
Chlamydomonas reinhardtii	*Chlorophyta*	Unicellular; cell-wall-deficient mutants; achievement of stable transformation of nuclear, chloroplast, and mitochondrial; diverse methods of transformation; GC-bias for nuclear gene codons and AT-bias for chloroplast gene codons; extremely high GC content; available genome sequences and metabolic pathways
Chlorella sp.	*Chlorophyta*	Unicellular; small genome size; food supplement; fast growth rate; easy to culture; similar metabolic pathways to higher plants; often insensitive to certain antibiotics such as chloramphenicol and kanamycin
Dunaliella salina	*Chlorophyta*	Unicellular; resistant to high salinity; not easily contaminated by other organisms during mass culture; lack of a rigid polysaccharide cell wall; natural protoplast; not producing toxins and classified as a food source
Volvox carteri	*Chlorophyta*	Multicellular; closely related to unicellular *Chlamydomonas reinhardtii*; sequenced genome (approximately 138 Mb in size); composed of two cell types, somatic and reproductive; sexual development is initiated by a glycoprotein pheromone
Ostreococcus tauri	*Prasinophyta*	Unicellular; minimal cellular organization; lacking flagella; the smallest living eukaryote; compact and sequenced genome (12.56 Mb, one-tenth the size of *Chlamydomonas*)
Phaeodactylum tricornutum	*Bacillariophyta*	Multiple cell morphology; insensitive to most of the common antibiotics; well-established genetic systems; multiple marker genes available; diverse and advanced molecular toolkits; available genome sequences; routine transformation method; biased codon usage with a preference for G/C in the third position of the codon; GC content (48%) represents a typical value for eukaryotes
Cyanidioschyzon merolae	*Rhodophyta*	Unicellular; small genome size; available genome sequences; the cell contains a single nucleus, a single mitochondrion and a single chloroplast; unique chloroplast genome; resistant to extremely acid environment; simple life cycle; gene-targeting nuclear genome can be achieved by homologous recombination

method needs to be established for a previously untransformed species of microalgae.

Electroporation is commonly used for transforming special cells of microalgae, such as naked cells, protoplasts, cell-wall-reduced mutants, and other thin-walled cells. This method uses an electronic pulse to temporarily disturb the bilayer lipid membrane and allows DNA molecules to pass. Successful transformation has been achieved in *C. reinhardtii*, *Dunaliella salina*, *Chlorella vulgaris*, *Ostreococcus tauri*, and red algae *Cyanidioschyzon merolae*. The simplest method is to agitate cell-wall-deficient *C. reinhardtii* cells in the presence of DNA, glass beads, and polyethylene glycol (PEG) [37]. In cell-wall-free protoplasts of *Chlorella ellipsoidea*, agitation of protoplasts in the presence of PEG and DNA is sufficient to generate transformants [34]. However, in walled dinoflagellate cells, silicon carbide (SiC) whiskers are essential for transformation [80]. SiC-mediated microinjection has also been employed to transform *Chlamydomonas*. The limitations of this method are the availability and health hazard of SiC. Another method used for transformation of microalgae is *Agrobacterium tumefaciens*-mediated transformation that has so far been mainly used for transformation of plant cells [40]. Microalgal species that are successfully transformed and the transformation methods are listed in Table 4.

Selectable markers are generally required for efficient selection of microalgal transformants. Several dominant and recessive markers have been used in *Chlamydomonas*, the latter of which require auxotrophic mutants with mutations in the corresponding endogenous genes and the mutants can be rescued by functional complementation [85]. As one of the recessive markers, the *nit* gene encoding nitrate reductase has been used in *Chlamydomonas reinhardtii* [38], *Volvox carteri* [66], *Dunaliella viridis* [76], and *Chlorella sorokiniana* [15]. Recessive markers are only applicable for those species that are capable of generating defective mutants. The commonly used selectable markers are those dominant markers conferring resistance against antibiotics or herbicides (Table 5). Numerous dominant markers are proven effective in *Chlamydomonas*, which include *aadA* conferring resistance to spectinomycin and streptomycin [6], the *ble* gene to zeomycin and phleomycin [74], the *aphVIII* gene to paromomycin [70], and the mutated *als* gene to sulfonylurea herbicides [39]. The *ble* gene is also shown to confer resistance to zeomycin or phleomycin in *Volvox carteri* [29], *Phaeodactylum tricornutum* [1, 23], and *Cylindrotheca fusiformis* [24]. In addition, the *nptII* gene conferring resistance to aminoglycoside antibiotic G418 is used as a dominant marker in the diatoms *Phaeodactylum tricornutum* [89], *Navicula saprophila*, and *Cyclotella cryptica* [21], and the dinoflagellates *Amphidinium* sp. and *Symbiodinium microadriaticum* [80]. Table 5 lists some selectable marker genes frequently used in microalgal transformation, and it has been found that different

Table 4 Microalgal species that can be transformed and methods used

Species	Transformation method	Genome	References
Green algae			
C. reinhardtii	Microprojectile bombardment	Nuclear	[16]
	Electroporation	Nuclear	[5]
	Glass bead	Nuclear	[37]
	Agrobacterium tumefaciens	Nuclear	[40]
	Microprojectile	Chloroplast	[3]
	Microprojectile	Mitochondrial	[62]
Dunaliella salina	Electroporation	Nuclear	[27]
Chlorella ellipsoidea	Polyethylene glycol	Nuclear	[34]
Chlorella sorokiniana	Microprojectile bombardment	Nuclear	[15]
Chlorella vulgaris	Electroporation	Nuclear	[8]
Chlorella kessleri	Microprojectile bombardment	Nuclear	[22]
Haematococcus pluvialis	Microprojectile bombardment	Nuclear	[73, 81]
Ostreococcus tauri	Electroporation	Nuclear	[11]
Gonium pectorale	Microprojectile bombardment	Nuclear	[43]
Volvox carteri	Microprojectile bombardment	Nuclear	[66]
Diatoms			
Cyclotella cryptica	Microprojectile bombardment	Nuclear	[21]
Navicula saprophila	Microprojectile bombardment	Nuclear	[21]
Phaeodactylum tricornutum	Microprojectile bombardment	Nuclear	[1]
Thalassiosira weissflogii	Microparticle bombardment	Nuclear	[23]
Cylindrotheca fusiformis	Microprojectile bombardment	Nuclear	[24]
Chaetoceros sp.	Microparticle bombardment	Nuclear	[52]
Dinoflagellates			
Amphidinium	Silicon carbide whiskers	Nuclear	[80]
Symbiodinium microadriaticum	Silicon carbide whiskers	Nuclear	[80]
Red algae			
Cyanidioschyzon merolae	Electroporation	Nucleus	[51]
Porphyridium spp.	Microprojectile bombardment	Chloroplast	[41]
Chlorarachniophytes			
Lotharella amoebiformis	Microparticle bombardment	Nuclear	[31, 32]
Euglenoids			
Euglena gracilis	Microprojectile bombardment	Chloroplast	[17]

species of microalgae show varying sensitivities to antibiotics resulting from these marker genes [23, 89].

Strategies for highly efficient expression of foreign genes

The economically efficient production of recombinant proteins using microalgae as bioreactors depends on high and consistent protein yields. There are many factors that influence the expression levels of recombinant proteins, including the choice of promoters, biased codon usage, integration of regulatory elements, gene silencing, and the action of proteases.

Promoters

The promoters determine transcriptional activities of transgenes. Although heterologous promoters have been proven appropriate for reporters or chimeric gene constructs, strong constitutive endogenous promoters are frequently employed for efficient expression of foreign genes. The promoters commonly used in microalgal transgenes are outlined in Table 6. The promoter of cauliflower mosaic virus 35S (CaMV35S) is the typical heterologous promoter used in dinoflagellates and chlorophyta [8, 34, 80]. In *Chlamydomonas*, the endogenous promoters of *RBCS2* (ribulose bisphosphate carboxylase, small subunit) and *PsaD* (abundant protein of photosystem I complex) can drive efficient

Table 5 Selectable marker and reporter genes commonly used in microalgal transformation

Marker or reporter	Coding protein or product	Gene source	References
AphVIII	Aminoglycoside 3' phosphotransferase, resistance to paromomycin, kanamycin, and neomycin	*Streptomyces rimosus*	[43, 70]
Ble	Bleomycin resistance protein, resistance to tallysomycin and related antibiotics	*Streptoalloteichus hindustanus*	[74]
Cat	Chloramphenicol acetyltransferase, resistance to chloramphenicol	Transposon Tn9	[79]
nptII	Neomycin phosphotransferase II, resistance to G418	*E.coli* transposon Tn5	[28]
aadA	Adenylyltransferase, resistance to spectinomycin	Eubacteria	[6]
Hpt	Hygromycin B phosphotransferase	*E. coli*	[80]
ChGfp	Modified green fluorescent protein, adapted to *Chlamydomonas* codon usage, reporter in *Chlamydomonas*	Synthetic	[26]
eGfp	Modified green fluorescent protein, adapted to human codon usage, reporter in *P. tricornutum*	Synthetic	[89]
Gus/uidA	*β*-Glucuronidase, reporter in *Amphidinium* and *Symbiodinium*	*E. coli*	[80]
Hup1	Hexose transporter, marker or reporter in *P. tricornutum* and *Cylindrotheca fusiformis*	*Chlorella kessleri*	[24, 89]
Luc	Luciferase, reporter in *P. tricornutum* and *Gonium pectorale*	*Hotaria parvula*	[23, 43]

Table 6 Promoters frequently used for the expression of foreign genes

Promoter of gene and its product	Source	Host species of microalgae	References
RbcS2, rubisco small subuit 2	*Chlamydomonas*	*Chlamydomonas*	[2, 26]
Fcp, fucoxanthin chlorophyll-a or -c binding protein	*Phaeodactylum tricornutum*, *Thalassiosira pseudonana*	*P. tricornutum*, *Chaetoceros* sp.	[1, 23, 52, 89]
Hsp70, heat shock protein 70	*Chlamydomonas*	*Chlamydomonas*	[68]
35S, cauliflower mosaic virus 35S	Cauliflower mosaic virus	*Chlamydomonas*, *Amphidinium*, and *Symbiodinium*	[79, 80]
Nos, nopaline synthase	*Agrobacterium tumefaciens*	*Chlamydomonas*, *Amphidinium*, and *Symbiodinium*	[28]
NR, nitrate reductase	*Thalassiosira pseudonana*	*Chaetoceros* sp.	[52]
PsaD, photosystem I complex protein	*Chlamydomonas*	*Chlamydomonas*	[25]

gene expression [25, 26]. The fusion promoters of *HSP70A* (heat shock protein 70A)/*RBCS2*, and *HSP70A*/*β2TUB* (*β*2 tubulin) demonstrate improved expression of transgenes, in which *HSP70A* promoter may serve as a transcriptional activator when placed upstream of other promoters [68]. In diatoms, high-level expression of marker or reporter genes can be achieved under the control of an endogenous promoter of the *fcp* gene encoding the fucoxanthin-chlorophyll binding protein [23, 89]. Moreover, inducible promoters can provide rapid and tightly controlled expression of genes in transgenic microalgae. The promoter of the *NR* gene encoding nitrate reductase is such an inducible promoter. The *NR* promoter activity is suppressed by ammonium and induced when ammonium is replaced by nitrate [60], thus gene expression is switched off/on when *C. reinhardtii* cells are grown in the presence of ammonium/nitrate [56]. The regulated gene expression system will be attractive if a two-phase production approach is employed, in which normal culture conditions make the optimal growth of microalgal cells, followed by culturing cells under other conditions that drive high expression of transgenes to obtain the maximized protein yields.

Some introns or untranslated regions (UTRs) play an important role in improving the stability and efficiency of heterologous gene expression. In *Chlamydomonas*, insertion of endogenous *RbcS2* intron within the bacterial *Ble* gene (conferring resistance to zeomycin) can significantly increase the expression of the *Ble* gene [45]. The complete set of *Als* gene (encoding acetolactate synthase) introns are also essential to provide an adequate level of *Als* expression [39].

However, high-level expression of endogenous and exogenous genes requires only the flanking promoter and untranslated regions of *PsaD*, but not any introns [25]. Therefore, the effect of introns or UTRs on gene expression may depend on specific genes and expression hosts of microalgae.

Reporter genes

Reporter genes driven by the promoter of a particular gene are very useful for detecting and studying spatial or temporal expression of this gene. In *Chlamydomonas*, the endogenous *Ars* gene encoding periplasmic enzyme arylsulfatase, the heterologous *Gfp* encoding green fluorescent protein (GFP), and the *Luc* gene encoding luciferase are effective reporter genes [14, 26, 30]. The arylsulfatase activity can be used for quantification analysis or subcellular localization with chromogenic substrates 5-bromo-4-chloro-3-indolyl-sulfate (X-SO_4), *p*-nitrophenyl sulfate, or α-naphthylsulfate [14]. The synthetic *Gfp* adapted to the codon usage of host species provides a useful tool to visualize protein synthesis and localization in vivo in both green algae and diatoms [26, 52, 60, 89]. The expression of *Gfp* in *Cylindrotheca fusiformis* can be regulated by different nutrient conditions as a result of the use of an inducible *NR* promoter. In diatom *Cylindrotheca fusiformis* and multicellular green algae *Volvox carteri*, the *Hup1* gene (from *Chlorella kessleri*) encoding hexose/H^+ symporter has been used for monitoring gene expression or protein localization [24, 30]. The bacterial *Gus/uidA* gene encodes *β*-glucuronidase and can serve as a useful reporter gene in *Dunaliella salina* [78], *Chlorella* sp. [8, 22], *Phaeodactylum tricornutum* [89], and *Thalassiosira weissflogii* [23]. Notably, optimization of codon usage of reporter genes is necessary to achieve the optimal expression in certain species of microalgae [52, 89]. Together, these functional reporter genes (Table 5) play important roles in molecular dissection of microalgal biology and will allow an assessment of the biotechnological potential of transgenic microalgae.

Codon usage

There are evident differences in the frequencies of codon usage between both genomes of different microalgal species, and between nuclear and chloroplast genomes of a certain species of microalgae [42]. For example, the chlorophytes *Chlamydomonas* (61%) and *Monoraphidium* sp. show high GC content (71%) when compared with other species of microalgae [35]. In diatoms, the GC contents of the nuclear genome of *Thalassiosira pseudonana* (47%) and *Phaeodactylum tricornutum* (48%) represent a typical value for eukaryotes, whereas GC contents of their plastid genomes are 31 and 33%, respectively [58]. Similar to the codon usage of these two diatoms, *Chlamydomonas* chloroplast also prefers A/T in the wobble position, whereas the nuclear genome prefers G/C [54]. Therefore, when a heterologous gene from certain organisms is expressed from the nuclear or chloroplast genome of microalgae, the translational efficiency and expression level may be influenced as a result of the difference in tRNA abundance. Optimization of the codon of heterologous genes can significantly improve protein expression in microalgae, which was exemplified by developing the *Gfp* reporter gene in *P. tricornutum*. Several adjustments of *Gfp* codons were made to achieve a high level of expression or increase the fluorescence level. The wild type and other forms of *Gfp* genes with codon usage different from that used by *P. tricornutum* were not functional; however, only *eGfp* employing codons similar to those preferred by *P. tricornutum* produced detectable fluorescence, and the eGFP level was estimated to be 0.3% of total proteins in transformed cells [89]. As in the cases of other expression systems, optimization of codons is one of the important aspects that should be taken into account for achieving the optimal expression levels.

Recombinant protein yields are also affected by regulatory mechanisms of cells and on the rates of protein synthesis and degradation, the latter of which is required by protease activities. The proteases are not only responsible for endogenous protein processing, but also appear to cleave heterologously expressed proteins [77, 84], which may lead to a lower overall yield of recombinant proteins. Several strategies are possible to minimize the protein degradation resulting from the action of proteases, including developing protease-deficient mutant strains, identification and modification of protease sites within target proteins that need to be produced in microalgae, and targeting nuclear-expressed proteins to the chloroplasts for storage [9, 59].

Although recombinant proteins can be successfully expressed in the microalgal nuclear, mitochondrial, and chloroplast genomes, commercially viable expression levels have only been achieved in the last of these. Transgene silencing is not reported in chloroplasts [87], but might be one of the possible causes for the low transgene expression levels attained in the nucleus. Although the molecular mechanisms for transgene silencing are not yet fully understood, position effects, nonconventional epigenetic effects, and an exceptionally compact chromatin structure have been proposed to possibly be associated with this process [55, 59]. It might be an effective strategy to select for mutants generated by artificial mutagenesis in which transgene silencing mechanisms are defective and thus mutants showing improved protein accumulation. Neupert et al. [55] have developed such a genetic screen and identified *Chlamydomonas* mutants that express introduced transgenes to high levels. However, it has been reported that the high-

est level of exogenous proteins expressed from nuclear transgenes is only 0.2% of TSP, which is still not comparable to that from chloroplasts. It appears that overcoming the effect of transgene silencing will be a major hurdle if really high levels of recombinant proteins can be expressed from microalgal nuclear genomes [71].

Maximizing the value of microalgae for cost-effective production

Transgenic microalgae show potential to produce high-value recombinant proteins as discussed above. Commercially viable production of recombinant proteins using microalgae needs to lower the overall costs to compete with other production systems. Increasing recombinant protein yields and maximizing the value of natural compounds derived from microalgae are thus two major strategies for cost reduction. An ideal production system may rely on the transgenic microalgae capable of producing high yields of recombinant proteins and also possessing the attributes to accumulate large amounts of valuable natural compounds. For transgenic microalgae, harvesting and purification of recombinant proteins from microalgae can be one of the major processes suitable for large-scale production. Once microalgal cells are disrupted, proteins, carbohydrates, fatty acids, pigments, and other compounds can be recovered with different methods and techniques. The accumulation of these biomolecules with maximal contents can be achieved by modulating growth conditions, whereas the most efficient recovery of valuable compounds may depend on the development of appropriate extraction process. Figure 1 illustrates such an approach, in which recombinant proteins are produced together with other valuable natural compounds and where industrial exhaust gases (e.g., flue gas emission from fuel-fired power plants) are used as a CO_2-rich source for microalgae cultivation [18, 90]. Many types of compounds may be simultaneously harvested. However, currently, almost no such microalgal species can fulfill these requirements. The establishment of stable transformation systems and highly efficient expression strategies for those species accumulating natural compounds might represent a major breakthrough for this purpose. With respect to this point, on the basis of the information currently available, several microalgal species including *Dunaliella salina*, *Haematococcus pluvialis*, *Chlorella* sp., *Phaeodactylum tricornutum,* and *Isochrysis galbana* should receive more attention as transgenic bioreactors in molecular farming.

Concluding remarks and future prospects

Microalgae can be a consistent source of large numbers of natural compounds with high value, including pigments,

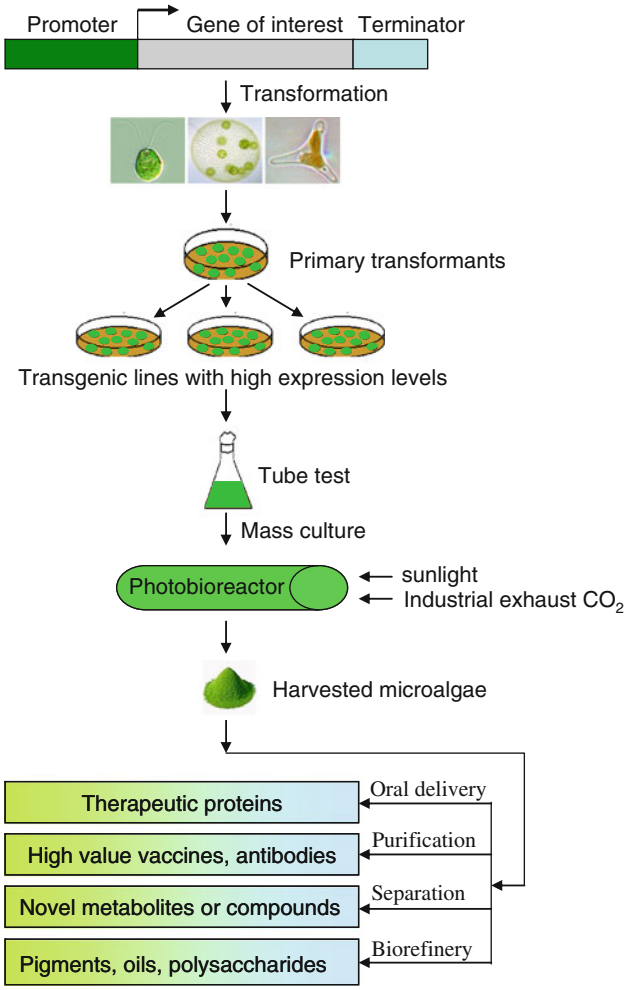

Fig. 1 Stages of development for the large-scale production of recombinant protein and valuable compounds in microalgae

PUFAs, carbohydrates, and others, which have a wide range of applications in the feed, food, nutrition, cosmetics, and pharmaceutical industries [57]. Microalgae as bioreactors have several advantages over bacteria, yeast, plants, and other systems for recombinant protein production, including low cost, safety, alternative culture methods, and rapid scalability. However, major advances achieved in pharmaceutical protein production with transgenic microalgae are from unicellular green algae *Chlamydomonas*, indicating that microalgae are not a well-studied group from a biotechnological perspective. The major obstacles for microalgal protein expression systems are the lack of standard procedures for genetic transformation of commercially important species of microalgae, limited availability of molecular toolkits for genetic engineering of microalgae, and relatively low expression levels of recombinant proteins resulting from several factors [77]. However, the cost of recombinant antibody production in microalgae is estimated to be US $0.002 per gram, which is much lower than

that in mammalian cell culture (US $150 per gram) and transgenic plants (US $0.05 per gram) [48]. The attribute of accumulating high-value compounds makes microalgae particularly attractive for recombinant protein production. In the near future some microalgal species are expected to emerge as bioreactors for efficiently producing pharmaceutical proteins, coupled with recovery of valuable natural products. Although in recent years considerable progress has been made in regards to utilizing microalgae, especially *Chlamydomonas*, as bioreactors for the production of recombinant proteins, there are many untouched research areas that are important before commercial production of microalgae-based recombinant proteins can be realized. We outline below some of these issues, which need to be addressed over the coming years:

1. The yields of recombinant proteins produced by transgenic microalgae were reported only at lab-scale. How do cultivation conditions, including nutrients, temperature, CO_2 concentration, light regime, and agitation, affect protein yields when large-scale cultivation of engineered microalgae is performed?
2. Unlike transgenic plants and animals, application of antibiotics is generally required to prevent microalgal transformants from losing the exogenous genes and this presents a potential health hazard. It remains unknown whether the genes conferring resistance to antibiotics in transgenic microalgae can be removed by homology-based excision, a cotransformation-segregation approach, or other strategies that are successfully used in higher plants.
3. If certain transgenic microalgal species are capable of expressing high levels of recombinant proteins and accumulating high-value natural compounds, it remains unknown whether the synthesis of recombinant proteins interferes with metabolic pathways of natural product accumulation.
4. The maintenance of acquired transformation phenotype during long-term culture is important for commercial production of recombinant proteins. Is there any correlation between different transformation methods and the stability of microalgal transformants?
5. If a cheap exogenous carbon source is available, heterotrophic and mixotrophic cultivation could be more economically viable when compared with light-dependent autotrophic production systems. However, many of the currently used promoters for heterologous gene expression are related to photosynthesis, such as *RBCS2* in green algae and *fcpA*/*B* in diatoms. Development of useful promoters that are not regulated by light is essential before large-scale heterotrophic culture of transgenic microalgae is used for the production of recombinant proteins.

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