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High-value Recombinant Protein Production in Microalgae

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Abstract
Increasing interest in recombinant protein technologies for human and animal health applications has spotlighted microalgae as a platform with the potential to meet a large impending demand. Here we describe an algae protein expression system and compare the advantages and disadvantages to other platforms currently operating on a commercial level. High-value recombinant proteins that have been produced in microalgae are presented, and strategies for developing production strains with improved commercial properties are discussed.

Keywords algae; therapeutics; recombinant protein; biotechnology; genetic engineering; transformation

27.1 INTRODUCTION

Microalgae are an ideal platform for large-scale production of high-value products because they are fast-growing solar-powered biofactories with minimal nutrient requirements. In addition, many species are generally regarded as safe (GRAS) for human consumption, and several are already commercially farmed for various bioproducts relevant to human or animal health (Pulz & Gross, 2004). Recombinant proteins such as protein vaccines, therapeutic antibodies, and industrial enzymes can also be produced in microalgae where low-cost production can greatly impact applicability. Furthermore, pending bioavailability, certain parenterally administered therapeutic proteins could be delivered in an edible format, greatly reducing the cost of these therapeutics.

Currently, mammalian cell cultures such as Chinese hamster ovary (CHO) cells dominate commercial production for complex eukaryotic therapeutic proteins (e.g., monoclonal antibodies), while bacterial systems are widely used for producing more simple proteins, such as proinsulin. Together, these two platforms represent 55% and 29% of a $100 billion/year recombinant protein market (Walsh, 2010). These platforms dominate the market because mammalian cells have the appropriate cellular machinery to properly fold, assemble, and posttranslationally modify complex human proteins, while bacteria boast high growth rates, cell densities, and product yields through more inexpensive cultivation techniques. Both systems are capable of producing recombinant proteins on a gram-per-liter scale, but both systems lack easy cost-effective scalability, or are limited by the classes of proteins they can produce.

Microalgae, on the other hand, offer scale and cost of production that can potentially rival that of agricultural production, once the platform is developed to produce recombinant proteins at the efficiencies of these other systems. Mammalian cell culture dominates the current therapeutic protein market, but the end products of this system are typically only available to those patients who can afford the

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extremely high price of these products. To make a comparison, costs of monoclonal antibody production are estimated to be approximately $150 per gram in mammalian cells, but only $0.05 per gram in plants (Dove, 2002). Furthermore, mammalian cell culture production facilities can cost several hundred million dollars in upfront construction and equipment costs (Dove, 2002). Microalgae are a promising system due to inexpensive cultivation costs where media costs are only $0.002 per liter, and the cost of algae production facilities can be a fraction of the cost of a mammalian cell culture facility. This is particularly significant for those recombinant proteins needed in massive, affordable quantities, such as animal feed, industrial enzymes, or vaccines for developing countries.

Besides being highly scalable and cheap, microalgae have several other advantageous attributes. The nuclear, chloroplast, and mitochondrial genomes are transformable, and the timeline from generating initial transformants to having characterized, scaled-up production cultures is relatively fast for eukaryotic cells at only a few weeks. In addition, algae cytosol and plastids both have the chaperones and protein disulfide isomerases that are required for assembling complex therapeutic proteins derived from higher organisms (Kim & Mayfield, 1997; Schroda, 2004). The chloroplast is a particularly attractive compartment for the production of certain proteins, because it is unique in its ability to accumulate proteins lacking glycosylation, which in the case of antibodies may avoid activating the complement system or antibody dependent cell cytotoxicity (Sawada-Hirai et al., 2004).

27.2 HIGH-VALUE RECOMBINANT PROTEINS PRODUCED IN MICROALGAE

Several highly valuable recombinant proteins have been produced in microalgae from heterologous genes transformed into either the nuclear or chloroplast genomes. Most of these have been produced in the chloroplast of *Chlamydomonas reinhardtii*, but there have been several recombinant proteins produced in other microalgae species. The first significant therapeutic protein produced in algae was a human single-chain antibody (Mayfield et al., 2003). More recently a full-length human monoclonal antibody was expressed in *C. reinhardtii* chloroplast and was shown to have antigen binding activity similar to the same antibody expressed in the traditional CHO system (Tran et al., 2009). A monoclonal antibody and its antigen were also expressed from the nucleus of *Phaeodactylum tricornutum*, and the antibody was reported to accumulate to 8% of total soluble protein (TSP) within the endoplasmic reticulum, levels sufficient to reach gram-per-liter amounts in some microalgae (Hempel et al., 2011). Although this antibody was glycosylated and thus potentially immunogenic, genetic engineering in the methylotrophic yeast *Pichia pastoris* demonstrated that human-like glycosylation pathways can be implemented in transgenic organisms (Choi et al., 2003; Hamilton et al., 2003), and presumably these same genetic modifications could be made in microalgae. Also from the nucleus, transgenic *Nannochloropsis oculata* expressing bovine lactoferricin (LFB) were able to prevent pathogen infection of the digestive tract when fed to medaka fish (Li & Tsai, 2009). Table 27.1 lists the recombinant proteins that have been produced from microalgae and assayed for bioactivity, to date.

Currently, the highest levels of recombinant protein accumulation have consistently been achieved in the chloroplast. For example, in *C. reinhardtii* the mammalian-gut mucin stimulant, mammary-associated serum amyloid protein (M-SAA) accumulated to 10% of TSP when grown heterotrophically (Manuell et al., 2007). Although there have been significantly more microalgae species with transformed nuclear genomes, relatively few regulatory elements, transformation vectors, and methods have been identified for this compartment which afford the levels of recombinant protein accumulation observed in chloroplasts.

27.3 GENETIC TRANSFORMATION OF MICROALGAE

Genetic transformation of photosynthetic microalgae was first achieved in the chlorophytes (green algae), but has since been demonstrated in rhodophytes (red algae), phaeophytes (brown algae), euglenoids, diatoms, and dinoflagellates as detailed below. Several barriers challenge exogenous DNA before integration into an algal genome. These can include a cell wall and several additional membranes depending on the target organelle and species being transformed. Once integrated, the transgene has to pass the scrutiny of the host cell expression machinery as well as potential repair mechanisms and regulatory checkpoints (Choquet et al., 1998). The following describes several methods of transgenesis along with strategies for achieving and maintaining the accumulation of recombinant proteins.

Many microalgae transformation techniques were first developed and refined in the green algae *C. reinhardtii* and then applied to other algae groups. Currently, particle bombardment and electroporation are the most frequently employed methods for introducing foreign DNA. Other methods include agitation in the presence of glass beads or silicon carbide whiskers, which require minimal equipment, but generally have lower transformation rates. However, a recently optimized glass bead-based technique in

Table 27.1. Bioassayed microalgae-derived recombinant proteins

Protein	Biotechnological application	Bioassay	Genetic source	Platform organism	Compartment	Reference
α-HBsAg full-length IgG1 mAb (CL4mAb)	Binds hepatitis B surface antigen	HBsAg binding ELISA	*Homo sapiens*	*Phaeodactylum tricornutum*	Cytosol	Hempel et al., 2011
Hepatitis B virus surface antigen (HBsAg)	Immunogen	α-HBsAg binding inhibition ELISA	Hepatitis B virus	*Phaeodactylum tricornutum*	Cytosol	Hempel et al., 2011
C-terminal domain from the apical major antigen AMA1 fused to a truncated granule-bound starch synthase (GBSS)	Immunogen	Red blood cell entry inhibition assay and lethal dose mouse survivability	*Plasmodium berghei*	*Chlamydomonas reinhardtii*	Nuclear encoded, chloroplast directed	Dauvillée et al., 2010
C-terminal domain from the Major Surface Protein (MSP1) fused to a truncated granule-bound starch synthase (GBSS)	Immunogen	Red blood cell entry inhibition assay and lethal dose mouse survivability	*Plasmodium falcipirum*	*Chlamydomonas reinhardtii*	Nuclear encoded, chloroplast directed	Dauvillée et al., 2010
D2 fibronectin-binding domain of *Staphylococcus aureus* fused with the cholera toxin B subunit (CTB-D2)	Immunogen	IgA and IgG ELISA, pathogen load qtPCR, lethal dose survivability in mice	*Staphylococcus aureus*, *Vibrio cholerae*	*Chlamydomonas reinhardtii*	Chloroplast	Dreesen et al., 2010
High mobility group protein B1 (HMGB1)	Inflammatory cytokine	Fibroblast chemotaxis	*Homo sapiens*	*Chlamydomonas reinhardtii*	Chloroplast	Rasala et al., 2010
Vascular endothelial growth factor (VEGF)	Therapeutic angiogenesis	VEGF receptor binding ELISA	*Homo sapiens*	*Chlamydomonas reinhardtii*	Chloroplast	Rasala et al., 2010
α-PA83 full-length IgG1 mAb (83K7C)	Binds anthrax protective antigen 83 (PA83); anthrax neutralization	PA83 binding ELISA	*Homo sapiens*	*Chlamydomonas reinhardtii*	Chloroplast	Tran et al., 2009

Bovine lactoferricin (LFB)	Digestive tract bacteriocidal	Fish-feeding survival assay	*Bos taurus*	*Nannochloropsis oculata*	Cytosol	Li & Tsai, 2009
Glutamic acid decarboxylase 65 (hGAD65)	Autoantigen	Sera immunoreactivity and spleen cell proliferation in NOD mice	*Homo sapiens*	*Chlamydomonas reinhardtii*	Chloroplast	Wang et al., 2008
Bovine mammary-associated serum amyloid (M-SAA)	Intestinal infection protectant for livestock	Mucin induction assay	*Bos taurus*	*Chlamydomonas reinhardtii*	Chloroplast	Manuell et al., 2007
Swine fever virus E2 viral protein (CSVF-E2)	Immunogen	Subcutaneous immunization in mice	Classical swine fever virus	*Chlamydomonas reinhardtii*	Chloroplast	He et al., 2007
Metalothionein-2 (hMT-2)	UV protectant	Cell survival after UV exposure	*Homo sapiens*	*Chlamydomonas reinhardtii*	Chloroplast	Zhang et al., 2006
A-glycoprotein D large single chain mAb (HSV8-lsc)	Binds herpes simplex virus glycoprotein D	HSV8 binding ELISA	*Homo sapiens*	*Chlamydomonas reinhardtii*	Chloroplast	Mayfield et al., 2003
Cholera toxin B subunit fused to FMD virus VP1 (CTBVP1)	Mucosal adjuvant fused to a livestock viral immunogen	GM1-ganglioside receptor binding ELISA	*Vibrio cholerae*, Foot-and-mouth disease virus	*Chlamydomonas reinhardtii*	Chloroplast	Sun et al., 2003
Flounder growth hormone (fGH)	Agricultural growth hormone	Dietary supplementation and growth promotion	*Panaeolus olivaceus*	*Chlorella ellipsoidea*, *Nannochloropsis oculata*	Cytosol	Kim et al., 2002; Chen et al., 2008

Dunaliella salina was shown to be more efficient than electroporation or particle bombardment (Feng et al., 2009). *Agrobacterium tumefaciens*-mediated transformation has also been demonstrated but has not been applied as extensively as the previously mentioned techniques, and thus less is known about the potential of this system to generate useful transgenic lines (Kumar et al., 2004; Kathiresan et al., 2009; Anila et al., 2011).

Particle bombardment is performed by introducing small metal particles, typically gold or tungsten that has been coated with recombinant DNA, into cells. The particles are delivered into cells by accelerating them with pressurized gas, allowing passage through the membranes surrounding the cell and target organelle. The microprojectiles can be accelerated using a helium-pressurized apparatus, commonly manufactured as a gun or vacuum chamber. This technique can be particularly useful in walled species of algae, as well as for transforming chloroplasts or mitochondria. Photosynthetic microalgae that have been transformed using this basic protocol include *C. reinhardtii* (Boynton et al., 1988), *Chlorella ellipsoidea* (Jarvis & Brown, 1991), *Volvox carteri* (Schiedlmeier et al., 1994), *Cyclotella cryptica* (Dunahay et al., 1995), *Navicula saprophila* (Dunahay et al., 1995), *P. tricornutum* (Apt et al., 1996), *Chlorella sorokiniana* (Dawson et al., 1997), *Chlorella kessleri* (El-Sheekh, 1999), *Thalassiosira weissflogii* (Falciatore et al., 1999), *Cylindrotheca fusiformis* (Fischer et al., 1999), *Euglena gracilis* (Doetsch et al., 2001), *Porphyridium* UTEX637 (Lapidot et al., 2002), *Haematococcus pluvialis* (Teng et al., 2002), *V. carteri* (Jakobiak et al., 2004), *D. salina* (Tan et al., 2005), *Gonium pectorale* (Lerche & Hallmann, 2009), and *Chaetoceros* sp. CCK09 (Miyagawa-Yamaguchi et al., 2011).

Electroporation uses an electric current to temporarily perforate the cell membrane in many strains of microalgae (Mayfield, 1991). Although it requires more equipment than glass beads, it can be more efficient when optimized, and transformation rates as high as 2×10^5 transformants per microgram of DNA have been reported in *C. reinhardtii* (Shimogawara et al., 1998). Several factors contribute to optimal transformation efficiency including osmolarity, temperature, concentration of the exogenous DNA, voltage, and capacitance. Optimal conditions vary among the transformed species, which include *Chlorella saccharophila* (Maruyama et al., 1994), *Chlorella vulgaris* (Chow & Tung, 1999), *C. ellipsoidea* (Chen et al., 2001), *Cyanidioschyzon merolae* 10D (Minoda et al., 2004), *D. salina* (Sun et al., 2005), *Dunaliella tertiolecta* (Walker et al., 2005), *Dunaliella viridis* (Sun et al., 2006), and *N. oculata* (Chen et al., 2008).

27.4 STRATEGIES FOR MAINTAINING ACCUMULATION OF RECOMBINANT PROTEINS

A significant contributor of a transgene's ability to be expressed and accumulate product seems to involve sharing a codon bias with the host microalgae. This is evident through a comparison of the genes successfully used to produce recombinant proteins with those that have not been successful. A useful resource for analyzing codon usage was published in 2000, and now contains over 3 000 000 complete protein coding sequences from almost 36 000 organisms (Nakamura et al., 2000). This database, the codon usage tabulated from Genbank (CUTG), is available at http://www.kazusa.or.jp/codon/

It is important to consider codon bias not only of the nuclear genome but for the organelles as well, which can be quite different from nuclear codon bias. For example, coding sequences from *C. reinhardtii* have a GC content of 62% in the nuclear genome, 48% in the mitochondrial genome, and 34% in the chloroplast genome. Many other chlorophytes also have a high GC content in nuclear DNA. Furthermore, in a majority of characterized microalgae, the wobble position for both the nucleus and the chloroplast tends to be the most restrictive. In diatoms such as *P. tricornutum* and *Thalassiosira pseudonana*, nuclear GC contents are more modest (53%, 50%) and less influential at the wobble position. Figure 27.1 illustrates the nucleoside preferences of codons by position of several microalgae species for both the nuclear and chloroplast genomes. The CUTG can be referenced to find actual codon frequencies for a given species and compartment, and should be considered when synthesizing transgenes.

Besides codon optimization, promoters and regulatory elements governing transgenes also significantly impact expression and accumulation of recombinant proteins. An optimization of chloroplast promoters and untranslated regions (UTRs) for expressing heterologous proteins in *C. reinhardtii* was recently accomplished (Rasala et al., 2011). In a photosynthetic-deficient genetic background, psbA elements were the most robust for the production of several recombinant proteins. A fusion of the 16s ribosomal promoter with the atpA 5′ UTR showed improved recombinant protein expression over atpA itself, and produced more protein than psbA elements when used in photosynthetic competent cells. In the nuclear genome fusing the hsp70 promoter to the rbcs2 promoter also increased transgene expression, suggesting that this strategy might work on other relevant genes (Schroda et al., 2000). Several other simple and chimeric elements have been described

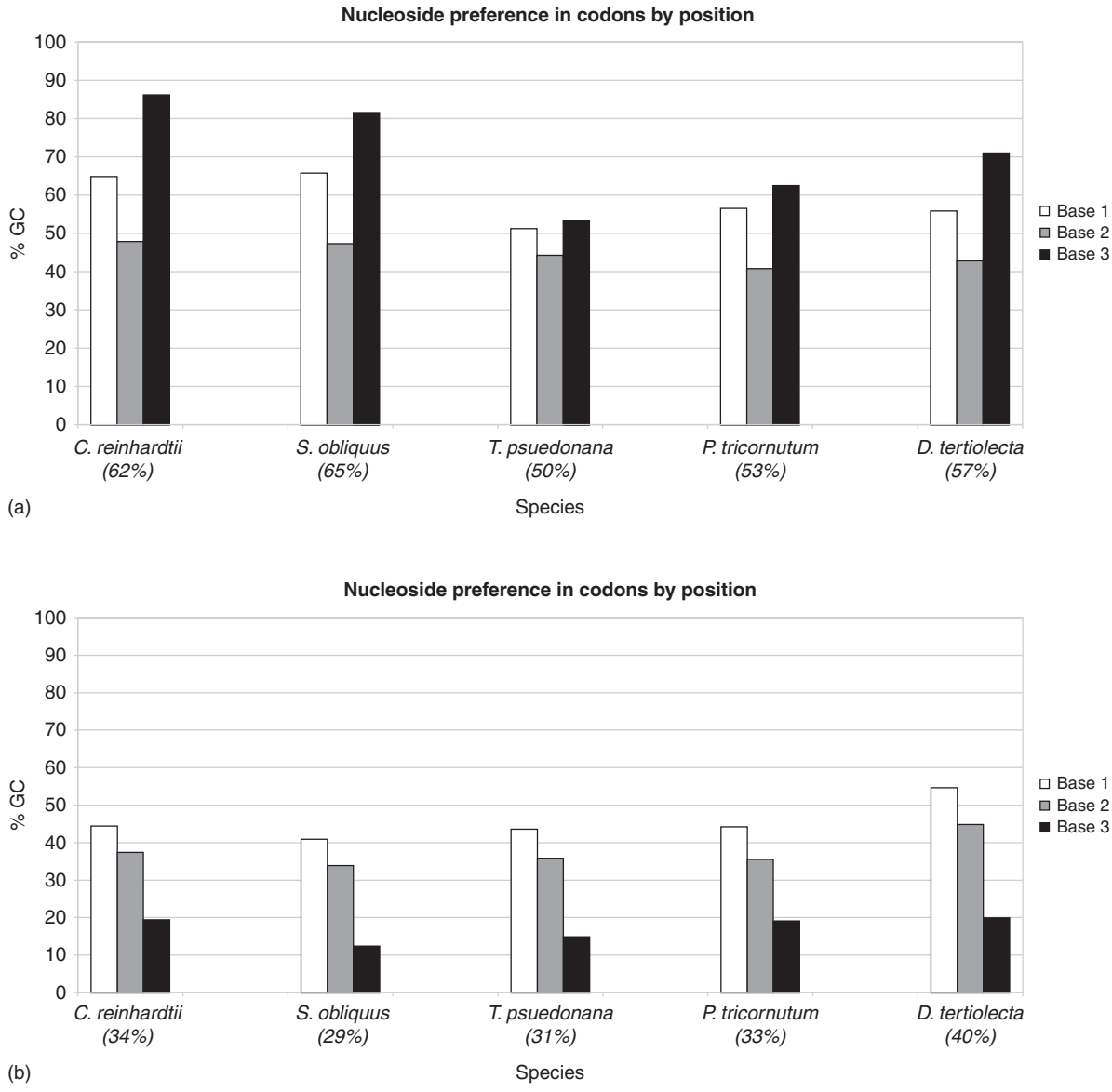

Figure 27.1. Nucleoside preference of codons by position. GC content of each position is presented based on all available coding sequences (CDSs) at the CUTG. The following photosynthetic microalgae (green algae, diatoms) are analyzed and include the average GC content in parenthesis for the (a) nucleus and (b) chloroplast: *Chlamydomonas reinhardtii*, *Scenedesmus obliquus*, *Thalassiosira pseudonana*, *Phaeodactylum tricornutum*, and *Dunaliella tertiolecta*.

for nuclear and chloroplast organelles and a list of these are presented in Table 27.2.

Table 27.3 describes several selectable markers available for transforming both the nuclear and chloroplast genomes of microalgae species. Historically, endogenous genes were the first selectable markers used to complement nutritional auxotrophic and photosynthetic-deficient mutants of haploid microalgae (Boynton et al., 1988). These prototrophic, recessive markers are valuable tools for generating strains without the use of heterologous DNA, something that may be essential for any strain that is to be used for outdoor growth. Antibiotic and herbicide resistance-conferring

Table 27.2. Nuclear and chloroplast promoters used to express transgenes in microalgae

Promoter	Source	Host	Description	Genome	Reference
vcp1	*Nannochloropsis oculata*	*Nannochloropsis oculata*	Violaxanthin/chlorophyll *a*-binding protein 1	Nuclear	Kilian et al., 2011
vcp2	*Nannochloropsis oculata*	*Nannochloropsis oculata*	Violaxanthin/chlorophyll *a*-binding protein 2; bidirectional	Nuclear	Kilian et al., 2011
psbD	*Chlamydomonas reinhardtii*	*Chlamydomonas reinhardtii*	Photosystem II protein D2	Chloroplast	Barnes et al., 2005
cyc6	*Chlamydomonas reinhardtii*	*Chlamydomonas reinhardtii*	Cytochrome c6	Nuclear	Quinn et al., 2003
psaD	*Chlamydomonas reinhardtii*	*Chlamydomonas reinhardtii*	Photosystem I complex protein	Nuclear	Fischer & Rochaix, 2001
hsp70A	*Chlamydomonas reinhardtii*	*Chlamydomonas reinhardtii*	Heat shock protein 70A	Nuclear	Schroda et al., 2000
psbA	*Chlamydomonas reinhardtii*	*Chlamydomonas reinhardtii*	Photosystem II protein D1	Chloroplast	Ishikura et al., 1999
atpA	*Chlamydomonas reinhardtii*	*Chlamydomonas reinhardtii*	ATPase alpha subunit	Chloroplast	Ishikura et al., 1999
rbcL	*Chlamydomonas reinhardtii*	*Chlamydomonas reinhardtii*	Ribulose bisphosphate carboxylase large subunit	Chloroplast	Ishikura et al., 1999
nia1	*Chlamydomonas reinhardtii*	*Chlamydomonas reinhardtii*	Nitrate reductase	Nuclear	Loppes et al., 1999
cop	*Chlamydomonas reinhardtii*	*Chlamydomonas reinhardtii*	Chlamyopsin	Nuclear	Fuhrmann et al., 1999
p1′2′	*Agrobacterium tumefaciens*	*Amphidinium* sp., *Symbiodinium microdriaticum*	Bidirectional promoter	Nuclear	Lohuis & Miller, 1998
rbcs2	*Chlamydomonas reinhardtii*	*Chlamydomonas reinhardtii*	Small subunit of ribulose bisphosphat carboxylase	Nuclear	Stevens et al., 1996
fcp	*Phaeodactylum tricornutum*	*Phaeodactylum tricornutum*	Fucoxanthin chlorophyll-*a* or -*c* binding protein	Nuclear	Apt et al., 1996
acc1	*Cyclotella cryptica*	*Cyclotella cryptica*, *Navicula saprophila*	Acetyl-CoA carboxylase	Nuclear	Dunahay et al., 1995
CaMV 35S	*Chlamydomonas reinhardtii*	*Chlamydomonas reinhardtii*	Cauliflower mosaic virus 35S	Nuclear	Dk et al., 1995
Nos	*Agrobacterium tumefaciens*	*Chlamydomonas*	Nopaline synthase	Nuclear	Hall et al., 1993
Nos	*Chlamydomonas reinhardtii*	*Chlamydomonas reinhardtii*	Nopaline synthase from *Agrobacterium tumefaciens*	Nuclear	Hall et al., 1993
cabII-1	*Chlamydomonas reinhardtii*	*Chlamydomonas reinhardtii*	Chlorophyll-*ab* binding protein	Chloroplast	Blankenship & Kindle, 1992
β-2-tub	*Chlamydomonas reinhardtii*	*Chlamydomonas reinhardtii*	*β*-2-tubulin	Nuclear	Davies et al., 1992

Table 27.3. Nuclear and chloroplast selectable markers used in microalgae

Marker	Selection	Microalgae transformed	Reference
Bsr	Blasticydin S resistance (deaminase)	*Nannochloropsis* sp. (strain W2J3B)	Kilian et al., 2011
ARG9	Arginine prototrophy (chloroplast encoded)	*Chlamydomonas reinhardtii*	Remacle et al., 2009
PDS	Norflurazon resistance (mutated endogenous phytoene desaturase)	*Chlorella zofingiensis*, *Haematococcus pluvialis*	Steinbrenner & Sandmann, 2006; Chen et al., 2008
aph7″	Hygromycin B resistance (aminoglycoside phosphotransferase)	*Chlamydomonas reinhardtii*	Berthold et al., 2002
ALS	Sulfometuron methyl resistance (mutated endogenous acetolactase gene)	*Chlamydomonas reinhardtii*	Kovar et al., 2002
aphVIII	paromomycin/kanamycin resistance (aminoglycoside phosphotransferase)	*Chlamydomonas reinhardtii*	Sizova et al., 2001
act-2	Cycloheximide resistance (mutated endogenous ribosomal L41)	*Chlamydomonas reinhardtii*	Stevens et al., 2001
aphA-6	Kanamycin/amikacin resistance (aminoglycoside phosphotransferase)	*Chlamydomonas reinhardtii*	Bateman & Purton, 2000
nat	Nourseothricin resistance (acetyltransferase)	*Phaeodactylum tricornutum*	Zaslavskaia et al., 2000
sat-1	Nourseothricin resistance (acetyltransferase)	*Phaeodactylum tricornutum*	Zaslavskaia et al., 2000
hup1	Nutritional marker or trophic conversion (hexose transporter)	*Phaeodactylum tricornutum*, *Cylindrotheca fusiformis*	Fischer et al., 1999; Zaslavskaia et al., 2001
PPX1	Porphyric herbicide resistance (mutated endogenous protoporphyrinogen oxidase)	*Chlamydomonas reinhardtii*	Randolph-Anderson et al., 1998
hpt	Hygromycin B resistance (phosphotransferase)	*Amphidinium*, *Symbiodinium*, *Nannochloropsis* sp. (strain W2J3B)	Ten Lohuis & Miller, 1998; Kilian et al., 2011
ble	Zeocin resistance (stoichiometrically neutralizes phleomycins)	*Chlamydomonas reinhardtii*, *Phaeodactylum tricornutum*, *Nannochloropsis* sp. (strain W2J3B)	Apt et al., 1996; Stevens et al., 1996; Lumbreras et al., 1998; Kilian et al., 2011
NIC7	Nicotinamide prototrophy	*Chlamydomonas reinhardtii*	Ferris, 1995
THI-10	Thiamine prototrophy	*Chlamydomonas reinhardtii*	Ferris, 1995
cat	Chloramphenicol resistance (acetyltransferase)	*Chlamydomonas reinhardtii*, *Phaeodactylum tricornutum*	Dk et al., 1995; Apt et al., 1996
CRY1-1	Cryptopleurine/emetine resistance (mutated endogenous ribosomal S14)	*Chlamydomonas reinhardtii*	Nelson et al., 1994
nptII	Neomycin resistance (phosphotransferase)	*Chlamydomonas reinhardtii*, *Symbiodinium* sp., *Phaeodactylum tricornutum*, *Amphidinium* sp., *Cyclotella cryptica*, *Navicula saprophila*	Hall et al., 1993; Dunahay et al., 1995; Ten Lohuis & Miller, 1998; Zaslavskaia et al., 2000
aadA	Spectinomycin/Streptomycin resistance (adenylyltransferase)	*Chlamydomonas reinhardtii*	Goldschmidt-Clermont, 1991; Cerutti et al., 1997
oee-1	Oxygen-evolving enhancer protein (restores photosynthesis)	*Chlamydomonas reinhardtii*	Mayfield & Kindle, 1990
ARG7	Arginine prototrophy	*Chlamydomonas reinhardtii*	Debuchy et al., 1989
NIT1 (NIA1)	Nitrate prototrophy	*Chlamydomonas reinhardtii*	Kindle et al., 1989

genes are dominant markers commonly employed across many microalgae species, and are particularly useful for transforming diploid species. However, heterologous drug resistance genes are undesirable in commercial production systems, and will likely need to be eliminated before the strains can be used to produce any commercial product. One strategy for marker removal or recycling has been developed for the chloroplast and utilizes homologous recombination to move the heterologous gene after integration and selection of a stable transgenic line (Fischer et al., 1996).

Reporter proteins are indispensible when developing a protein production line, and several are available with a range of applications. Luciferase genes from several different species have been codon optimized for *C. reinhardtii* for both chloroplast and nuclear expression, and have also been developed for the nucleus of *P. tricornutum* and *G. pectorale* (Falciatore et al., 1999; Minko et al., 1999; Fuhrmann et al., 2004; Mayfield & Schultz, 2004; Shao & Bock, 2008; Lerche & Hallmann, 2009). Besides luminescence, there are also colorimetric assays made possible through enzymatic reporters. Beta-glucuronidase (GUS) is a bacteria-derived reporter available in many microalgae species, and arylsulfatase (ARS) has been used as a reporter in *C. reinhardtii* (Davies et al., 1992; Klein et al., 1992).

Many variants of green fluorescent protein (GFP) have been optimized for the chloroplast and the nucleus of *C. reinhardtii*, and in *P. tricornutum* a humanized enhanced GFP (eGFP) with similar codon bias accumulated well (Fuhrmann et al., 1999; Zaslavskaia et al., 2000; Franklin et al., 2002). Fluorescent proteins provide excellent means of quickly quantitating transgene expression in populations and their individuals. For example, a 1 mL culture of *C. reinhardtii* can have 10^7 individuals, all of which can be analyzed for GFP expression (fluorescence) in minutes using flow cytometry, which can produce robust statistical data on transgene expression. Furthermore, interesting individuals can be selected from a population using fluorescence-activated cell sorting (FACS) (Montero et al., 2011).

27.5 CONCLUSIONS

Photosynthetic microalgae are a promising platform for meeting the increasing demand for high-value recombinant proteins. More importantly, because they can be grown inexpensively on a large scale, those high-demand recombinant proteins can potentially be produced at a cost low enough to positively impact significant global problems such as disease eradication or livestock nutrition, where cost is an important factor. Furthermore, the potential for oral delivery offers an ability to eliminate many processing steps, which could reduce production costs even further.

It is clear from examining the history of transgenic microalgae that a variety of fully functional recombinant proteins can be produced with a wide range of applications. In order to successfully express a given transgene, the coding sequence should be optimized and governing regulatory elements should be appropriately selected based on the target species and compartment. Several methods of genetic transformation have been developed for both the nucleus and the chloroplast in combination with selectable markers and reporter genes for developing production strains. These strategies seem likely to work in a variety of algal species, including those now being developed for large-scale industrial processes.

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