Genetic and Metabolic Engineering of Microalgae

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Abstract Microalgae are promising producers of many valuable compounds serving the food, feed, healthcare, and pharmaceutical industries. Microalgae grow rapidly and generally tolerate a wide range of environments. They can serve as cell factories for economical and sustainable production of diverse products. Microalgae can be transformed through genetic and metabolic engineering methods to overproduce the desired chemicals. Advancements in the "omics" technologies are generating information to allow design and creation of super algal strains for producing biofuels and other products.

Keywords Microalgae · Genomics · Metabolomics · Genetic engineering · Metabolic engineering · Strain improvement · Biofuel

1 Introduction

The unicellular microalgae have been recognized as natural sources of valuable compounds such as carotenoids, long-chain fatty acids, hydrocarbons, and pharmaceuticals. Being eukaryotes and photoautotrophs, they may serve as attractive bioreactor systems that require no organic carbon source and allow cost-effective large-scale production of high value compounds including heterologous recombinant products. In addition, microalgae have high growth rates, are easy to culture, and are amenable to manipulation of their physiological, biochemical, and genetic processes. The feasibility of genetic modification and expression of heterologous

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genes in microalgae has widened the scope of products from traditional food and feed additives to new bioactive compounds for industrial and healthcare applications. Among the new products are human antibodies, hormones (Mayfield et al. 2003; Hempel et al. 2011), insecticidal proteins (Borovsky 2003), and vaccines (Geng et al. 2003). The high photosynthetic efficiency and broad environmental tolerance of microalgae, coupled with their high lipid productivity, have brought attention to them as biofuel feedstocks of great potential (Kleinováa et al. 2012; Blatti et al. 2013).

The domestication of microalgae, similar to other crops, is driven by the need to produce uniform, high-yielding organisms (Gressel et al. 2013). While nonrecombinant techniques such as breeding, sexual hybridization, and strain selection, together with biochemical engineering (BE), have less risk issues, developments in genetic engineering (GE), metabolic engineering (ME), and transcription factor engineering (TFE) with a focus on the regulation of metabolic pathways in the whole cell may prove to be more promising in the long term (Courchesne et al. 2009), as they provide good control and predictability of the system (Blatti et al. 2013). The success of the latter technologies is made possible by contributions from characterization of algal genomes (genomics) and other "omics" technologies (proteomics, metabolomics) allowing trait mining via high-throughput computational systems, for use with advanced genetic engineering tools (Yang and Cao 2012).

2 Microalgal Genomics

To date, more than 20 algal whole genomes have been sequenced, and together with nuclear, mitochondrial, and chloroplast genomes and expressed sequence tags (ESTs) provide the sequence information required for genetic and metabolic engineering of microalgae (Table 1). The National Centre for Biotechnology Information (NCBI) indicates that many more sequencing projects are on-going. The genomics databank provides the fundamental information on the key genes, metabolites, enzymes, proteins, and the biosynthetic pathways, for facilitating both genetic and metabolic manipulation.

The green microalga *Chlamydomonas reinhardtii* has been considered as the model for photosynthesis and chloroplast biogenesis for nearly 50 years (Rochaix 2002). The first set of expressed sequence data (EST) of *Chlamydomonas* was from *C. reinhardtii* growing under photoautotrophy, and was generated by the Kazusa Institute (Asamizu et al. 1999). In this study 3433 independent ESTs were generated of which 817 showed significant similarity to sequences registered at public protein databases, and 140 were matched with previously identified *C. reinhardtii* genes. The remaining ESTs were novel sequences. Over 60 % of ESTs had full-length protein coding regions. The subsequent launch of the *Chlamydomonas* Genome Project by Arthur Grossman was followed by generation of more than 40,000 cDNA data (Shrager et al. 2003). The cDNA was derived from cells

Table 1 List of selected microalgal genome sequences which have been completed

Phylum	Species	Strain	Description	GenBank	Length (nt)	References
Completed (*nuclear genomes sequenced*)						
Chlorophyta						
	Chlamydomonas reinhardtii	CC-503 cw92 mt+	Model species, freshwater	ABCN00000000	120,405,000	Merchant et al. (2007)
	Chlorella variabilis	NC64A	*Paramecium* symbiont, model for viral–algal interactions	ADIC01000000	46,200,000	Blanc et al. (2010)
	Micromonas pusilla	CCMP1545	Marine picoeukaryote	ACCP00000000	21,706,984	Worden et al. (2009)
	Ostreococcus lucimarinus	CCE9901	Marine picoeukaryote	CP000581-CP000601	13,200,000	Palenik et al. (2007)
	Ostreococcus tauri	OTH95	Marine picoeukaryote	CR954201-CR954220	12,578,000	Derelle et al. (2006)
	Volvox carteri f. nagariensis	Eve	Simple multicellular relative of *C. reinhardtii*	ACJH00000000	125,467,762	Prochnik et al. (2010)
Heterokontophyta						
	Nannochloropsis gaditana	CCMP526	Euryhaline eustigmatophyte, rich in PUFA		29,000,000	Radakovits et al. (2012)
	Nannochloropsis oceanica	CCMP1779	Marine eustigmatophyte, rich in PUFA		30,000,000	Pan et al. (2011)
Ochrophyta						
	Thalassiosira oceanica	CCMP1005	Marine diatom as model for tolerance to low-iron conditions		81,600,000	Lommer et al. (2012)

(continued)

Table 1 (continued)

Phylum	Species	Strain	Description	GenBank	Length (nt)	References
Rhodophyta						
	Cyanidioschyzon merolae	10D	Thermo-acidophile	AP006483(DDBJ)	16,520,000	Matsuzaki et al. (2004)
	Galdieria sulphuraria		Thermo-acidophile		11,400,000	Muravenko et al. (2001), Barbier et al. (2005)
Haptophyta						
	Emiliania huxleyi	CCMP1516	Bloom-forming marine coccolithophore	AHAL00000000	167,700,000	Read et al. (2013)
Completed (mitochondrion genomes sequenced)						
Chlorophyta						
	Chlamydomonas reinhardtii	*CC-503 cw92 mt+*	–	U03843	15,758	Maul et al. (2002)
	Dunaliella salina	*CCAP19/18*	–	GQ250045	28,331	Smith et al. (2010)
Completed (chloroplast genomes sequenced)						
Bacillariophyta						
	Phaeodactylum tricornutum	*CCAP1055/1*	–	EF067920	117,369	Oudot-Le Secq et al. (2007)
Chlorophyta						
	Chlamydomonas reinhardtii	*CC3269*	–	BK000554	203,828	Maul et al. (2002)
	Chlorella vulgaris	*C-27*	–	AB001684	150,613	Wakasugi et al. (1997)
	Parachlorella kessleri	*SAG 211-11g*	–	FJ968741	123,994	Turmel et al. (2009)

(continued)

Table 1 (continued)

Phylum	Species	Strain	Description	GenBank	Length (nt)	References
Cryptophyta						
	Cryptomonas paramecium	*CCAP977/2a*	–	GQ358203	77,717	Donaher et al. (2009)
	Rhodomonas salina	*CCMP1319*	–	EF508371	135,854	Khan et al. (2007)
Haptophyta						
	Emiliania huxleyi	*1516*	–	AY741371	105,309	Sanchez Puerta et al. (2005)

exposed to various stress conditions such as nutrient deprivation. The other recombinant libraries were from other stress conditions such as anaerobiosis, oxidative stress, high light conditions, high-activity osmotic conditions, heavy-metal exposure, and iron and copper deprivation. These results allowed the identification of genes that are potentially activated under different stress conditions. In addition, it demonstrated the protocols for establishing high-quality unigene sets and microarray data (Eberhard et al. 2006; Jain et al. 2007) which can facilitate the investigation of gene function, structure, and regulation for *Chlamydomonas* and potentially other microalgae. The cDNA-based microarrays were used to study gene expression in various conditions such as understanding changing gene expression during the deprivation of sulfur (S) and phosphorus (P) (Zhang et al. 2004; Moseley et al. 2006). These studies not only focused on genes related to S and P metabolism but also on those involved in photosynthesis; carbon metabolism; respiration alternative electron transfer pathways, ATPases, transporter; oxidative stress, chaperones, proteolysis; and other metabolic and biosynthetic processes.

Phosphorus and sulfur are micronutrients that are essential for sustaining life. Studies have shown that the limitation of S and P will affect growth and reduce photosynthesis efficiency (Wykoff et al. 1998; Shimogawara et al. 1999; Zhang et al. 2002). Knowledge on the genes involved and level of expression are important for further application in genetic and metabolomic engineering where the expression of metabolites can be manipulated to produce desired bioproducts. Lohr et al. (2005) used comparative genome analyze to understand genes that are involved in the chlorophyll and carotenoid biosynthesis and to examine their phylogenetic relationships with the deduced sequence of the protein from vascular plants, other algae, and cyanobacteria. Results showed that there are additional conserved domains in the algal and plant proteins but not in cyanobacteria, which may directly influence protein activity, assembly, or regulation. This study was based on phylogenetic studies, theoretical evaluation of gene expression through analysis of expressed sequence tag data, and codon bias of each gene. It provided the hypotheses concerning the function and regulation of the individual genes, and proposed targets for future research. Quantitative polymerase chain reaction (PCR) was used to examine the effect of very low fluence light (VLFL) on the level of expression of key genes which are critical for chlorophyll and carotenoid biosynthesis. The expressions of some of the genes in chlorophyll synthesis such as Glu-1-semialdehyde aminotransferase (*GSA*) and ALA dehydratase (*ALAD*) were found to increase significantly by 15-fold and sevenfold, respectively, after 2 h of exposure to VLFL. Other genes that showed increased expressions were uroporphyrinogen III decarboxylase (*UROD1*), and protoporphyrin IX Mg-chelatase subunit H (*CHLH1* and *CHLH2*). Only the phytoene desaturase (*PDS)* gene was observed to be involved in carotenoid biosynthesis. These provided information on the specific photoreceptors in the biosynthesis of specific pigments.

RNA silencing or RNA interference (RNAi) has emerged as the tool for knocking down gene expression in eukaryotes since the 1990s (Hannon 2002). This technology has been used on several strains of *Chlamydomonas* in which 30

different genes have been reported to be down regulated by antisense or inverted approaches but the efficiency in silencing is often variable for different constructs (Schroda 2006). MicroRNAs (miRNAs) can also be used to down regulate the expression of endogenous genes through mRNA cleavage (Zhao et al. 2009). Zhao et al. (2009) developed two artificial miRNAs (amiRNAs) targeting the *MAA7* and *RBCS1/2* genes, respectively, in *Chlamydomonas*, and when overexpressed, they could cleave their respective targets precisely at the predicted sites, resulting in greatly decreased accumulation of *MAA7* and *RBCS1/2* transcripts and showed expected mutant phenotypes. This study showed that the miRNA technique gives much higher efficiency in silencing the same set of genes as compared to study by Rohr et al. (2004) which used the RNAi approach.

The publication of the *Chlamydomonas reinhardtii* nuclear genome (Merchant et al. 2007) followed and pushed forward microalgal metabolic engineering, including engineering of fatty acid biosynthesis for biofuel (Blatti et al. 2013). The smallest known eukaryotes of about 1 μm diameter which have been subjected for full genome sequencing are the *Ostreococcus*: *O. tauri* (Derelle et al. 2006) and *O. lucimarinus* (Palenik et al. 2007). The genome sequence of a multicellular green volvocine alga *Volvox carteri* with a genome size of 138 MB, approximately 17 % larger than the *Chlamydomonas*, was published in 2010 (Prochnik et al. 2010). The larger size is mainly due to larger repetitive DNA in *V. carteri*. Another commercially relevant species, *Chlorella variabilis* NC64A, of 46 Mb genome size was sequenced in 2010 (Blanc et al. 2010). The eustigmatophytes *Nannochloropsis gaditana* (Radakovits et al. 2012) and *N. oceanica* (Pan et al. 2011) with high productivity of polyunsaturated fatty acids provided the data necessary for enhancing lipid productivity in these oleaginous species. Of the cyanobacteria (or Cyanophyta, as referred to by phycologists), more than 30 species have been sequenced, including *Synechococcus elongatus* (2.7 Mb), *Synechocystis* sp. (3.6 Mb), and *Prochlorococcus marinus* (1.7–2.4 Mb) (Hallmann 2007). Much of the genome data are directed to understand gene structure, composition, arrangement, and evolutionary relationships. Efforts to quickly annotate and characterize genes and proteins, for example, those involved in fatty acid biosynthesis and lipid metabolism (Blatti et al. 2013), have to be made before genetic and metabolomic engineering approaches and can sustainably increase biomass and lipid productivity of oleaginous microalgae.

3 Genetic Engineering of Microalgae

The creation of new strain varieties with the incorporation of desired traits using recombinant DNA technology is the long-term objective of biotechnology. The development of genetic engineering and manipulation has provided opportunities to genetically imbue strains with beneficial properties, to produce heterologous recombinant proteins, and to create new applications of natural resources. The development of an efficient transformation system includes processes for

introducing transgenes into the desired host, identifying or selecting transformants, enhancing the expression of desired genes, and maintaining stable expression. The recent advances in the field of algal genomics may serve as a powerful catalyst for progressing genetic manipulation and engineering. The establishment of genome information for model microalgae such as *Chlamydomonas reinhardtii* (Shrager et al. 2003; Merchant et al. 2007), *Dunaliella salina* (Smith et al. 2010; Zhao et al. 2011), and *Phaeodactylum tricornutum* (Bowler et al. 2008) would facilitate significant progress in the field of algal genetic engineering. The availability of genome data has enhanced understanding of the metabolic pathways, enabled identification of possible DNA elements or endogenous genes that could be modified or utilized for genetic improvement which includes regulatory elements such as promoters to achieve efficient expression of genes.

3.1 Construction of Transformation and Expression Vectors

The design and construction of efficient expression vectors play a critical role in the development of genetically engineered microalgae. The vector design involves the choice of selectable marker for efficient detection or selection of transformed strains of the initial bacteria host and the final algal host; as well as the utilization of an efficient expression system such as the use of efficient endogenous regulatory elements and codon optimization.

Various antibiotic resistance genes have been applied as selectable markers in microalgae transformation. These include the gene for resistance to kanamycin (Bateman and Purton 2000), G418 (Dunahay et al. 1995; Hawkins and Nakamura 1999; Cheng et al. 2012), spectinomycin (Cerutti et al. 1997; Mayfield et al. 2003), phleomycin (Stevens et al. 1996; Kim et al. 2002), hygromycin (Berthold et al. 2002; Kathiresan et al. 2009), chloramphenicol (Toyomizu et al. 2001; Niu et al. 2011; Guo et al. 2013), and paromomycin (Jakobiak et al. 2004; Sizova et al. 2011). The use of a mutant gene encoding acetohydroxyacid synthase (AHAS) that confers resistance to the herbicide sulfometuron methyl was reported for the selection of transformed *Porphyridium* species (Lapidot et al. 2002). The application of antibiotic resistance genes as selectable markers is limited in microalgae transformation as most microalgae are resistant to antibiotics naturally. Moreover, the use of antibiotic resistance genes as selectable markers raises concerns about transfer of such resistance to other species (Manimaran et al. 2011). Other alternatives include the use of reporter genes such as β-galactosidase (lacZ), β-glucuronidase (GUS), green fluorescent protein (GFP), and luciferase (Luc) (Falciatore et al. 1999; Gan et al. 2003; Jiang et al. 2003; Tolonen et al. 2006; Kathiresan et al. 2009; Cheng et al. 2012; Guo et al. 2013). Synthetic versions of codon-optimized green fluorescent protein (GFP) and luciferase reporter genes have been designed for expression in the nucleus or chloroplast of *Chlamydomonas* (Fuhrmann et al. 1999,

2004; Franklin et al. 2002). The *C. reinhardtii* gene *ARG7* encoding the enzyme argininosuccinate lyase (ASL) is a popular selectable shuttle marker used for this alga. Its utilization resulted in the rescue of arginine-requiring arg7 mutants to prototrophy (Debuchy et al. 1989). Other *C. reinhardtii* genes used as transformation markers were the nitrate reductase gene (*NIT1*) (Fernández et al. 1989; Kindle et al. 1989) and the arylsulfatase gene (*ARS*) (de Hostos et al. 1989).

An efficient promoter plays a crucial role in obtaining high expression of the inserted gene. Various promoters have been utilized to drive transgene expression in microalgae, namely the CaMV35S promoter from plant virus (Kim et al. 2002; Tan et al. 2005), promoter of the maize ubiquitin-Ω (Chen et al. 2001; Geng et al. 2003), and endogenous promoters from specific microalgae. Successful application of the diatom fucoxanthin–chlorophyll a/c binding protein gene (*FCP*) promoter was reported in marine diatoms (Falciatore et al. 1999; Zaslavskaia et al. 2000). The ribulose bisphosphate carboxylase/oxygenase small subunit (RBCS2) promoter from *C. reinhardtii* showed higher efficiency than the CaMV 35 S promoter in transformed *Dunaliella salina* (Sun et al. 2005). Niu et al. (2011) used the promoter and terminator of the nitrate reductase gene from *Phaeodactylum tricornutum* for expression of the transgene in *Chlorella vulgaris.* Poulsen and Kroger (2005) observed transgene expression driven by the promoter (Pnr)/terminator (Tnr) cassette derived from the nitrate reductase gene, and would be switched on when cells were grown in the presence of nitrate. Expression of *ARS* gene driven by the NIT1 promoter (Ohresser et al. 1997) and β2-tubulin promoter (Davies et al. 1992) was also observed in transformed *C. reinhardtii*. In addition, fusion of the *Chlamydomonas* heat shock protein 70A (HSPp70A) promoter to the other promoters acted as a transcriptional enhancer of the existing *Chlamydomonas* promoters, namely, RBCS2, β2-tubulin, and HSP70B resulting in higher level of expression (Schroda et al. 2000). Stable nuclear transformation of *D. salina* was reported with the endogenous salt-inducible promoter of duplicated carbonic anhydrase 1 (DCY1) (Li et al. 2010a; Lu et al. 2011). It was observed that the salt-inducible expression of transgenes was regulated by highly repeated GT sequences of the promoter (Li et al. 2010a).

In vector design, inclusion of species-specific 5′,3′-untranslated regions (UTRs) would enhance expression of transgenes. The 5′ region of chloroplast genes has been reported to play a role in RNAs stability (Salvador et al. 1993) and its *cis*-acting elements regulate translation (Nickelsen et al. 1999). Nickelsen (1999) has utilized the 5′ regions of the spinach psbB and the wheat psbA genes including their promoters to drive the expression of a reporter gene in the chloroplast of *Chlamydomonas*. He reported that the resulting transcripts were unstable although the plant promoters were still active indicating different molecular mechanisms governing the posttranscriptional regulation in plants and *Chlamydomonas*. Stevens et al. (1996) observed the expression of the phleomycin-resistance gene in *Chlamydomonas* when they fused the *ble* gene which has similar codon usage as *C. reinhardtii* to the 5′ and 3′ untranslated regions of *C. reinhardtii* rbcS2. It was also

reported that the use of endogenous intron enhanced the expression of the transgene in *Chlamydomonas* (Lumbreras et al. 1998). Although endogenous sequence elements were reported to be species-specific and would enhance the expression of foreign genes, it was also demonstrated that selenocysteine insertion sequence (SECIS) element from either human Sep15 or *C. reinhardtii* selenoprotein W1 could drive the expression of a heterogeneous protein, human selenoprotein (Sep15) in *C. reinhardtii* (Hou et al. 2013). Three types of human Sep15 gene fragments were constructed, namely, Sep15ORF-hSECIS, Sep15ORF-chSECIS, and wtSep15 and upon transformation to *C. reinhardtii*, these transgenes integrated into its genome. Expression of the human Sep15 was detected at both mRNA and protein levels.

3.2 Methods of Gene Introduction

Several methods of gene introduction have been successfully used to achieve expression of transgenes in either the nucleus or the plastid of microalgae, although, in most cases, transgenes were transiently expressed. These methods include trans-conjugation, agitation in the presence of glass beads or silicon carbide whiskers, electroporation, biolistic microparticle bombardment, and *Agrobacterium tumefaciens*-mediated transformation (Table 2). Stable transformation has been achieved for both nuclear and chloroplast transformations of microalgae (Dunahay et al. 1995; Kim et al. 2002; Lapidot et al. 2002). The first report on the stable transformation of *Chlorella* via microparticle bombardment resulted in the rescue of nitrate reductase-deficient *C. sorokiniana* mutants using a homologous selectable marker, the nitrate reductase gene from *C. vulgaris* (Dawson et al. 1997). The use of microparticle bombardment and this homologous marker has also enabled stable transformation of *Volvox carteri* (Schiedlmeier et al. 1994). In microparticle bombardment high velocity microprojectiles are used to deliver transgenes into the host cells (Klein et al. 1987). In electroporation, or electropermeabilization, a high-intensity electric pulse is used to create transient pores in the cell membrane to facilitate the entry of transgenes (Rae and Levis 2002). The removal of the external field results in the resealing of the membrane electropores. This method has been successfully used for the introduction of transgene into *Chlamydomonas* (Brown et al. 1991). The optimal parameters for efficient transformation by electroporation vary among strains. These parameters include electric field strength, temperature, osmolarity, and the concentration of transgene (Shimogawara et al. 1998).

Agrobacterium, a gram-negative plant pathogen, has been widely used in the genetic engineering of plants. *Agrobacterium*-mediated transformation is a low-cost method that offers a high transformation efficiency. The presence of acetosyringone was reported to enhance the transformation efficiency of *Agrobacterium*-mediated transformation by activating the *vir* genes of *Agrobacterium* (Stachel et al. 1985; Men et al. 2003; Kumar et al. 2004). *Agrobacterium*-mediated transformation has

Table 2 Gene introduction methods applied to genetically engineered microalgae

Methods	Transformed microalgae
Conjugation	*Prochlorococcus* (Tolonen et al. 2006) *Pseudanabaena* (Sode et al. 1992) *Synechococcus* (Sode et al. 1992; Brahamsha 1996) *Synechocystis* (Sode et al. 1992)
Agitation with glass beads	*Chlamydomonas reinhardtii* (Kindle 1990; Purton and Rochaix 1995; Ohresser et al. 1997; León et al. 2007) *Dunaliella salina* (Feng et al. 2009)
Agitation with silicon carbon whiskers	*Amphidinium* sp. (Lohuis et al. 1998) *C. reinhardtii* (Dunahay 1993) *Symbiodinium microadriaticum* (Lohuis et al. 1998)
Electroporation	*C. reinhardtii* (Brown et al. 1991) *Chlorella vulgaris* (Niu et al. 2011) *C. zofingiensis* (Liu et al. 2014) *Nannochloropsis* (Kilian et al. 2011) *Scenedesmus obliquus* (Guo et al. 2013) *Spirulina platensis* (Toyomizu et al. 2001) *Synechococcus* (Matsunaga et al. 1990)
Biolistic microparticle bombardment	*Chaetoceros* sp. (Miyagawa et al. 2011) *C. reinhardtii* (Bateman and Purton 2000; Mayfield et al. 2003; Sun et al. 2003) *C. zofingiensis* (Liu et al. 2014) *D. salina* (Tan et al. 2005) *Navicula saprophila* (Dunahay et al. 1995) *Phaeodactylum tricornutum* (Falciatore et al. 1999; Miyagawa et al. 2009) *Porphyridium* sp. (Lapidot et al. 2002) *Thalassiosira pseudonana* (Poulsen et al. 2006)
Protoplast transformation with polyethylene glycol and dimethyl sulfoxide	*Chlorella* (Hawkins and Nakamura 1999) *C. ellipsoidea* (Kim et al. 2002)
Agrobacterium tumefaciens-mediated transformation	*C. reinhardtii* (Kumar et al. 2004) *D. bardawil* (Anila et al. 2011) *Haematococcus pluvialis* (Kathiresan et al. 2009) *Nannochloropsis* sp. (Cha et al. 2011) *Schizochytrium* (Cheng et al. 2012)

also been successfully applied to microalgae. Kathiresan et al. (2009) reported successful *Agrobacterium*-mediated transformation of *Haematococcus pluvialis* cells without the presence of acetosyringone or induced injury. They have used continuous light which plays an important role in the *Agrobacterium*-mediated transformation process and enhances transformation frequency (Zambre et al. 2003). In addition, the cultures were maintained at 22 °C during co-cultivation which has been reported to be the optimal temperature for the *Agrobacterium*-mediated transformation of plants (Dillen et al. 1997).

3.3 Potential Applications of Genetic Transformation in Microalgae

The green microalga *Chlamydomonas* is widely used as a single-celled photosynthetic model organism for various biological studies. The establishments of various transformation methods for nuclear and plastid transformation of *C. reinhardtii* (Rochaix 1995; Kindle 1990) together with the characterization of genes from *C. reinhardtii,* namely, the *ARG7* (Debuchy et al. 1989; Purton and Rochaix 1995), *NITI* (Fernández et al. 1989), and *NIC7* (Ferris 1995), have enabled the genetic manipulation of this microalga. The achievement of random integration at nonhomologous sites through nuclear transformation of *C. reinhardtii* has been exploited to generate new mutants by tagging mutagenesis (Adam et al. 1993; Davies et al. 1994; Prieto et al. 1996) and to isolate genes of unknown products (Tam and Lefebvre 1993). Cagnon et al. (2013) screened transformants generated by random insertional mutagenesis and isolated oil mutants of *C. reinhardtii* with enhanced oil accumulation under the nitrogen-replete condition as well as mutants with altered oil content under nitrogen depletion. Adam and Loppes (1998) reported the cloning of a gene from *C. reinhardtii* which is required for derepressible neutral phosphatase activity, PHON24 using ARG7 as an insertional mutagen. Auchincloss et al. (1999) described the cDNA sequence of ARG7 and successfully generated vectors with smaller size and reduced repetitive DNA compared to the genomic DNA initially used for gene tagging.

Microalgae have been demonstrated as useful bioreactors for the expression of valuable proteins such as vaccines, antibodies, and hormones. In the first such report, a monoclonal antibody directed against the herpes simplex virus glycoprotein D was produced in the chloroplast of engineered *C. reinhardtii* (Mayfield et al. 2003). The fusion proteins of foot-and-mouth disease virus (VP1) and cholera toxin B subunit have also been expressed in the chloroplast of *C. reinhardtii* (Sun et al. 2003) and have shown potential application as a mucosal vaccine source. Transient expression of the hepatitis B surface antigen was reported in *Dunaliella salina* (Geng et al. 2003). A vector was also designed with an extracellular secretion signal sequence inserted between a promoter region and the human growth hormone (hGH) gene for the expression of this heterologous protein in *Chlorella* although no stable transformation was observed (Hawkins and Nakamura 1999). In addition, transgenic microalgae can be used to improve animal feed. Flounder fry fed with the transformed *Chlorella* expressing the flounder growth hormone gene showed enhanced growth (Kim et al. 2002). Similarly, a transgenic cyanobacterium expressing the *cryIVD* gene of *Bacillus thuringiensis* var. *israelensis* and a transgenic *Chlorella* expressing trypsin-modulating oostatic factor exhibited larvicidal activity for mosquito control (Stevens et al. 1994; Borovsky 2003). Microalgal strains were also engineered to be imbued with additional useful features. León et al. (2007) genetically engineered *Chlamydomonas reinhardtii* to express the β-carotene ketolase gene from *Haematococcus pluvialis* via the use of the *Chlamydomonas* constitutive promoter of the rubisco small subunit (*RbcS2*).

They utilized the *Chlamydomonas* transit peptide sequences of Rubisco small subunit (*RbcS2*) or ferredoxin (*Fd*) to direct the product to the chloroplast. Transgenic *Phaeodactylum tricornutum* that accumulated the high value omega-3 long-chain polyunsaturated fatty acid docosahexaenoic acid (DHA) was produced by expressing the Δ5-elongase gene from *Ostreococcus tauri* (Hamilton et al. 2014).

The instability of petroleum price and an uncertain long-term availability have focused attention on finding new sustainable biofuel resources. Globally, there are intensive efforts to enhance the accumulation of lipids, hydrocarbons, and other energy compounds in various organisms including microalgae through genetic and metabolic engineering. Nitrogen stress or starvation has been reported to decrease growth rate, protein content, photosynthetic activity, and cell size, but increases the lipid and carbohydrate content of algae (Li et al. 2012; Simionato et al. 2013; Pancha et al. 2014).

Studies were conducted to elucidate the role of genes and their products in the regulation of algal lipid accumulation. Inhibiting cell cycle progression has been observed to result in the accumulation of lipids in microalgae (Guckert and Cooksey 1990). Transcriptomic and proteomic analysis showed that under nitrogen stress, mRNAs and proteins associated with fatty acid and lipid biosynthesis were up-regulated (Miller et al. 2010; Guarnieri et al. 2011). In addition, a number of potential gene targets which are involved in cell signaling, transcriptional regulation, lipid biosynthesis, and cell cycle control were identified. These discoveries would facilitate further studies such as knockdown or inducible repression of these genes as novel approaches to mimic nitrogen stress-induced cell cycle arrest and hence, enhance the accumulation of lipid. Reports strongly suggested that abolishment of the starch synthesis pathway would switch photosynthetic carbon flux toward triacylglyceride (TAG) synthesis as an alternative carbon reserve for cells under stress (Wang et al. 2009; Li et al. 2010b). Hence, this has become an effective strategy rather than direct manipulation of the lipid synthesis pathway to obtain high accumulation of TAG. Direct manipulation and over-expression of the acetyl-CoA carboxylase gene in the microalgae *Cyclotella cryptica* and *Navicula saprophila* did not successfully enhance the lipid content (Dunahay et al. 1996). In another attempt, over-expression of three type-2 acyl-CoA, diacylglycerol acyltransferase genes (DGATs) in *Chlamydomonas reinhardtii,* also did not increase TAG accumulation (Russa et al. 2012).

4 Metabolic Engineering

Metabolic engineering is defined as "directed improvement of product formation or cellular properties through the modification of specific biochemical reactions or the introduction of new genes with the use of recombinant DNA technology" (Stephanopoulos et al. 1998). Metabolic engineering is different from genetic engineering: the main focus of the latter is on enzymes and genes while the former is concerned with the modification of biochemical pathways or the integrated

metabolic pathways rather than the individual reaction (Stephanopoulos and Sinskey 1993). The metabolic engineering approach in combination with advances in high-throughput computing enables efficient investigation of cellular metabolism and physiology at the systems level, leading to enhancement of multiple traits like product concentrations, yield, productivity, and tolerance (Jang et al. 2012). This new approach is termed systems metabolic engineering.

Investigations into cellular functions have been made possible by efficient comparative genome sequence analysis to identify genes for system manipulation toward desired metabolic phenotypes through transcriptomics, proteomics, and metabolomics profiling. Transcriptome profiling uses DNA microarrays and allows for identifying target genes through differential expression profiling under various environmental conditions (Sindelar and Wendisch 2007; Jang et al. 2012). Proteomics provide protein profiling. In metabolomics, an array of advanced tools like mass spectrometry–chromatography and nuclear magnetic resonance identify metabolites including substrates, products, and intermediates associated with different metabolic states of the cell (Jang et al. 2012). All such information can then be incorporated into in silico metabolic models (Kim et al. 2008; Schellenberger et al. 2010) and algorithms which point the way toward metabolic engineering (Park et al. 2009, 2010; Choi et al. 2010).

4.1 Metabolic Engineering of Lipid Metabolism

Microalgae are a promising feedstock for biofuels such as biodiesel, biohydrogen, and bioethanol. Many microalgae achieve maximal lipid yields under stress conditions (Hu et al. 2008) that hinder growth and result in cell compositions which are not ideal for biofuel applications (Courchesne et al. 2009). Metabolic engineering through genetic manipulation presents a promising strategy for the over-production of algal oils. The available approaches may include random and targeted mutagenesis and gene transformation. In this review, we will focus on some of the examples on how metabolic engineering can be used to enhance algal biofuel production.

The lipid metabolism of microalgae is highly complex but an understanding of the biosynthetic pathways is essential before the creation of the best strain for biodiesel production can take place. Recent work on the introduction of the genes for enzymes related to lipid biosynthesis, such as acetyl-CoA carboxylase (ACC), KAS III, and ACL into higher plants like *Arabidopsis, Brassica napus*, and tobacco, has shown increased production of lipid (Courchesne et al. 2009). A similar approach can be applied for microalgae. ACC is the first enzyme in the lipid biosynthesis pathway of triacylglycerol and its over-expression may enhance lipid yield as shown in *Arabidopsis* (Roessler et al. 1997). The ACC gene from *Cyclotella cryptica* was introduced into two species of diatoms, *C. cryptica* and *Navicula saprophila*, but disappointingly there was no increase of oils in the cells (Dunahay et al. 1995; Sheehan et al. 1998). The lipid increase in plants, as

compared to rather low increase in microalgae, suggested there is a mechanism or regulatory control that needs further study. Recently, the plastidic acetyl-CoA carboxylase (ACCase) was shown to be the rate-limiting enzyme in the fatty acid synthesis in *Brassica napus* (Andre et al. 2012).

The cloning of single genes related to fatty acid synthesis did not increase the fatty acid contents, as shown above. In plants, a multi-gene approach has successfully enhanced lipid production (Courchesne et al. 2009). A similar approach of cloning multiple genes related to fatty acid synthesis in *Haematococcus pluvialis* under different stress conditions showed the expression of the key genes correlated with fatty acid synthesis (Lei et al. 2012). Lei et al. (2012) cloned seven key genes of fatty acid synthesis: 3-keto acyl-acyl carrier protein synthase gene (*KAS*), acyl-acyl carrier protein thioesterase (*FATA*), ω-3 fatty acid desaturase (*FAD*), acyl carrier protein (*ACP*), malonyl-CoA:ACP transacylase (*MCTK*), biotin carboxylase (*BC*), and stearoyl-ACP-desaturase (*SAD*) into *H. pluvialis*. The clones were grown in various stress conditions: nitrogen depletion, salinity, and high or low temperature. In general, the results showed that high temperature, high salinity, and nitrogen depletion favored fatty acid (FA) synthesis and the FA quality was not affected much. At the same time, the cells were also harvested for RNA in order to quantify the expressions of the seven key genes. The correlations between different fatty acid syntheses and gene expressions were different. ACP, KAS, and FATA shared close correlations with fatty acid synthesis, while the other enzymes did not. The ACP, important in both fatty acid and polyketide biosynthesis, had its gene expression up-regulated to 8.7 times with high temperature compared to only 2.6 times at low temperature. The expression of KAS (catalyzes the initial condensing reaction in FA biosynthesis) was increased by Fe + AC (acetate) supplementation approximately 3.5 times, while individual treatments with Fe and AC promoted its gene expression to 81 and 42 %, respectively, in comparison to the control. The FATA functions as a chain-length-determining enzyme in de novo biosynthesis of plant fatty acid synthesis, and the FATA mRNA levels were up-regulated significantly under all treatments in this study: 2.0 times under nitrogen depletion; 2.9 times with Fe + AC combined; 3 times under low temperature; 9.9 times with Fe; 13.8 times under high temperature; and 18.8 times with AC. The information derived from this research was significant in identifying the potential candidate genes for use in metabolic engineering to enhance the production of FA in terms of quantity and quality.

Fatty acids derived from microalgae need to be of the correct chain length for use in production of biodiesel. Thioesterases (TE) are key enzymes in fatty acid biosynthesis that determine fatty acid carbon chain length in most plant tissues. TEs have been engineered into a variety of plant species to successfully alter the fatty acid profiles (Thelen and Ohlrogge 2002). This same approach has been applied to microalgae. The thioesterase PtTE was overexpressed in *Phaeodactylum tricornutum*, resulting in an increase of 72 % in the total fatty acids, although it did not change the fatty acid composition (Gong et al. 2011). In 2012, Blatti et al. (2012) manipulated the fatty acid biosynthesis of *C. reinhardtii* through interactions between the fatty acid acyl carrier protein (ACP) and thioesterase (TE) that regulate

fatty acid hydrolysis within the chloroplast of *C. reinhardtii.* The results showed that TE functionally interacts with CrACP to release fatty acids. In this case, increased levels of short-chain fatty acids in *C. reinhardtii* chloroplast were observed and the fatty acid profile remained unaltered. This shows that in order to engineer microalgae for the desired composition of fatty acids, the alteration of fatty acid biosynthesis can be done through protein–protein interactions.

In addition to the traditional genetic engineering (GE) approach of inserting single or multiple key genes relating to lipid production in the microalgae to increase the FA production, the TFE approach has been proposed as an alternative method. The transcription factors (TFs) may regulate or increase the activity of multiple enzymes controlling the production of microalgal lipids (Courchesne et al. 2009). This metabolic engineering approach has successfully increased the production of valuable metabolites in plants and animals (Segal et al. 2003; Broun et al. 2004; Reik et al. 2007). In order to use the TF strategy for improving lipid production, the TFs for microalgae have to be identified. For plants and animals, several TFs that are related to regulation of lipid biosynthesis have been identified. For instance, the sterol regulatory element-binding protein (SREBP) has been well known as a regulator of lipid homeostasis in mammals (Hitoshi 2005; Porstmann et al. 2005; Goldstein et al. 2006; Todd et al. 2006; Espenshade and Hughes 2007; Kotzka et al. 2010). In *Arabidopsis* a few transcription factors such as *LEC1*, *LEC2*, and *WRI1* have been found to regulate the seed oil content (Cernac and Benning 2004; Baud et al. 2007, 2009; Santos-Mendoza et al. 2008).

The manipulation of TFs can enhance the production of fatty acids (Mu et al. 2008; Tan et al. 2011). Wang et al. (2007) discovered 28 DNA-binding-with-one-finger (*Dof*) type transcription factors (*GmDof1*-21 and *β*-Tubilin) in soybean, an important oil crop. These TFs were found to affect the gene expressions of various organs. Among the 28 types of *Dof*, two genes, *GmDof*4 and *GmDof*11, were found to increase the total fatty acids and lipids contents in *GmDof*4 and *GmDof*11 transgenic *Arabidopsis* seeds. The *Dof* transcription factor family genes are found in various groups of organisms, including the green unicellular alga *C. reinhardtii* (Moreno-Risueno et al. 2007).

Riaño-Pachón et al. (2008) identified 234 genes encoding 147 TFs and 87 TRs (transcription regulators) in *C. reinhardtii*; however, there is not much information on their functions. The first study in making use of TFs to over express algal lipid production was carried out by Ibăñez-Salazar et al. (2014). Only one *Dof* gene in *C. reinhardtii* was found that was located in chromosome 12 at position 4426.865–4427.015 bp. This was discovered after a thorough blast analysis of the conserved domain in plants and comparison with the genome of *C. reinhardtii*. The Phytozome database (http://www.phytozome.net/cgi-bin/gbrowse/chlamy/) was used. Phylogenetic tree analysis further revealed the *Dof* sequence from *C. reinhardtii* to have a close relationship with *Volvox carteri*. Although the function of *Dof* in *C. reinhardtii* is unknown, the authors suggested it may have a possible role in increasing the fatty acid and lipid production. Based on the *Dof* sequences from *C. reinhardtii*, a synthetic *Dof*-type transcription factor gene was designed and plasmid constructed. The plasmid was transformed into the *C. reinhardtii* nucleus

using *Agrobacterium tumefaciens.* The transformation successfully yielded two transgenic lines (*Dof* 9 and *Dof* 11). In order to verify the possible function of the introduced *Dof* transcription factor, a transcription profile of 14 genes [eight genes involved in fatty acid biosynthesis: β-carboxyltransferase (*BCX1*), biotin carboxylase (*BCR1*), acyl carrier protein (*ACP1*), 3-ketoacyl-ACP synthase 2 (*KAS2*), 3-ketoacyl-ACP synthase 3 (*KAS3*), 3-ketoacyl-ACP reductase (*KAR1*), enoyl-ACP-reductase (*ENR1*), and acyl-ACP thiolase (*FAT1*); and six genes involved in glycerolipid biosynthesis: UDP-sulfoquinovose synthase (*SQD1*), the sulfolipid synthase (*SQD2*), monogalactosyldiacylglycerol synthase (*MGD1*), digalactosyldiacylglycerol synthase (*DGD1*), CDP-DAG-synthetase (*CDS1*), and phosphatidylglycerophosphate synthase (*PGP1*)] was examined. Among these enzymes, enoyl-ACP-reductase (ENR1) [fatty acid biosynthesis], phosphatidylglycerophosphate synthase (PGP1), monogalactosyldiacylglycerol synthase (MGD1), and sulfolipid synthase (SQD2) (glycerolipid biosynthesis) were over expressed in the two transgenic strains in comparison to the wild strain. The transgenic lines showed increased production in total lipids as well as fatty acids in comparison to the wild strain. The fatty acid composition of both transgenic lines and wild strain were similar, being dominated by palmitic acid (C16:0), γ-linolenic acid (C18:3, n3), and stearidonic acid (C18:4). This study showed the potential future application of TFs for increasing total lipid and fatty acid production in microalgae.

There have been several successes in manipulation of the prokaryotic cyanobacteria (or Cyanophyta) that make them good alternative biofactories for biofuels. Liu et al. (2010) successfully inserted the genes related to lipid biosynthesis from plants and *Escherichia coli* into mutant strains of *Synechocystis* sp. PCC 6803. They constructed five strains of PCC 6803 in which the inserted genes replaced the genes imparting properties that either competed with or inhibited the production of free fatty acids. The genetically modified strains successfully overproduced fatty acids (C10–C18) and secreted them into the medium at levels of up to 133 ± 12 mg/L of culture per day at a cell density of 1.5×10^8 cells/mL (0.23 g of dry weight/L). According to the authors, the genetically constructed strains could theoretically produce 6500 gallons of biodiesel per acre per year in a cost-effective system that eliminated the extraction costs. Subsequently, a sixth generation was constructed by Liu et al. (2011) which was genetically enabled to grow in high light and a maximum fatty acid secretion level of 197 ± 14 mg/L of culture at a cell density of 1.0×10^9 cells/mL was obtained.

4.2 Metabolic Engineering of Biohydrogen Production

Photosynthetic microalgae have the ability to produce hydrogen, another potential biofuel. Under the normal aerobic growing conditions, microalgae will not produce hydrogen. However, if anaerobiosis can be induced with low oxygen levels at night, a hydrogenase enzyme is expressed in the chloroplast for light-mediated generation of hydrogen (Melis et al. 2007). The presence of oxygen inhibits the transcription

and activity of hydrogenase(s), but production may be continued with the addition of the herbicide DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea; a PSII electron chain uncoupler) (Esquivel et al. 2011). Several approaches have been used to increase hydrogen production of microalgae that grow photosynthetically but under anoxic conditions. Sulfur depletion can enhance hydrogen production in *C. reinhardtii* through induction of anoxia and consequent expression of hydrogenase. The Leghemoglobin (*LBS*) genes from the soybean root nodules that have high affinity to O_2 were genetically engineered into *C. reinhardtii* and successfully increased the hydrogen production by 22 % (Wu et al. 2011). Scoma et al. (2012) introduced the high hydrogen producer D1 protein mutant strain of L159I-N230Y of *C. reinhardtii* where leucine residue L159 was replaced by isoleucine, and the N230 asparagine was replaced by tyrosine to improve hydrogen production. The mutant strain had higher carbohydrate and hydrogen production capacity compared to the control.

5 Limitations and Risks in Genetic and Metabolic Engineering of Microalgae

Microalgal biotechnology has entered an exciting era in which advances in analytical and computational tools allow redirecting metabolic processes toward desired outcomes. The intense search for the cost-effective lipid producers and production system has pushed emerging technologies like the "omics," genetic and metabolic engineering to new limits. Integration of information derived from genomics, proteomics, and metabolomics continues to improve and fundamental eco-physiological responses of algae to a changing environment are becoming better understood.

While genetic engineering appears to be a powerful tool in algal biotechnology, there are hurdles to overcome. Availability of cost-effective and efficient tools for gene delivery and detection of expression is one limitation (Qin et al. 2012). Potential ecological impact of the engineered strains is another concern. Biosafety entails both elimination of harm to humans and the natural ecosystem (Qin et al. 2012). At present, the most pressing issue is that of the efficiency of the transformation systems. For example, a low success rate in gene expression and poor stability of the transformants are persistent issues. Transgenic algal clones exhibit suppression of exogenous genes when cultured under nonselective conditions (Leon-Banares et al. 2004). Better understanding of regulation of gene expression including transgene silencing, as well as cellular responses to the vector or gene construct, is required (Wu-Scharf et al. 2000; Leon-Banares et al. 2004; Hallmann 2007). Anila et al. (2011) showed stable transgene integration in *Dunaliella bardawil* after 18 months of continuous culture in the absence of selection pressure. The transformant had been produced via *Agrobacterium*-mediated transformation.

Risks from transgenic algae relate to human health and the environment (Hallmann 2007). Transgenics may introduce toxic compounds and allergens

causing dietary problems, while transfer of novel genes to non-target species may occur through the use of transgenics. Escaped transgenics may outcompete indigenous species and become dominant, resulting in major ecological upsets (Henley et al. 2013). Use of enclosed photobioreactors offers some protection against escape but additional mechanisms are needed to prevent survival of escaped cells in nature. Use of "completely algae-derived vectors" may have some benefits (Qin et al. 2012). Henley et al. (2013) recommended that multiple biocontainment strategies could be implemented through simultaneous introduction of traits or mutations into the transgenics to reduce risks. These include reduced growth fitness especially in relation to the wild type (Gressel et al. 2014) and conditioned lethality in the wild, as well as impaired reproduction, both asexual and sexual (Henley et al. 2013). Finally, risk assessments based on actual experimental data (Gressel et al. 2013) would allow the development of regulatory guidelines for monitoring and management of algal transgenics.

6 Concluding Remarks

Advances in the "omics" technologies have accelerated the development of more refined genetic and metabolic engineering tools to transform algal cells into bio-factories for producing valuable chemicals. The earlier and perhaps the most successful approaches of physiologically stressing the cells to produce desired compounds had the limitation of reducing the product yields. Genetic and metabolic engineering are more promising in the long run and potentially allow better controlled and predictable bioprocesses enhanced with the use of regulation of multiple enzymes to control metabolism (Courchesne et al. 2009). A thorough analysis of potential risks of using transgenics is required and robust methods of managing such risks need to be developed.

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