Chapter 11
Genetic Engineering for Microalgae Strain Improvement in Relation to Biocrude Production Systems

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Abstract An advanced understanding of the genetics of microalgae and the availability of molecular biology tools are both critical to the development of advanced strains, which offer efficiency advantages for primary production, and more specifically in the context of production for biocrude and renewable energy. Consequently, we outline the current state of the art in microalgal molecular biology including the available genome sequences, molecular techniques and toolkits, amenable strains for transformation of nuclear and plastid genomes, and the control of transgenes at both transcriptional and translational levels. We also examine some strategies for improvement of expression and regulation. We suggest the primary strategies in strain improvement that are most relevant to biocrude applications; briefly illustrate the process of photosynthesis to enable identification of targets for improvement of net photosynthetic conversion efficiency in mass cultivation; and further discuss how improvement of metabolic systems may also be achieved and benefit production models. Finally, we acknowledge the aspects of prudent risk assessment and consequent regulation that are developing and how our knowledge of natural algae in existing ecosystems, and GM work in conventional agriculture both contribute lessons to these discussions. We conclude that if properly managed, these developments provide significant potential for increasing global capacity for renewable fuel production from microalgae and that these developments could also have benefits for other applications.

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11.1 Introduction

The Need for Strain Improvement While microalgae are a proven and promising platform for the production of high-value products, their greatest potential arguably lies in their ability to capture solar energy and convert it to chemical energy in the form of high energy density fuel feedstocks with low net carbon emissions. The importance of this is highlighted by the fact that ~80 % of global energy demand is supplied in the form of fuels, while only ~20 % is utilised as electricity (BP 2014; Stephens et al. 2013b). Consequently, there is a great need for renewable fuel production systems that have an economic and energetically positive return on investment (ROI), and microalgae are one of the very few options for making this a reality at scale.

Thermochemical processing of whole biomass to biocrude is a promising area of research and current commercialisation. At first glance this processing strategy does appear to promise increased yields, since in addition to lipids, other organic molecules such as proteins, starch and cellulose can be converted. It may also address some of the conventional cost/energy bottlenecks, particularly as complete dewatering and cell disruption are not needed. But it must also be considered that, in contrast to the extraction of a relatively homogenous product such as TAGs and neutral lipids, the resultant output product from hydrothermal liquefaction (HTL) can vary in quality from a type I kerogen (a complex carbonaceous organic compound) (Speight 2006) to a higher grade biocrude, equivalent to the best petroleum crudes. The quality of the output depends upon the efficiency of the HTL process as well as the composition of the initial biomass. While the upgrading of kerogen to biocrude can be a much simpler process than lipid extraction from microalgae biomass, it remains an economic and energetic cost in the process. Thus, the technology can be streamlined partly by the development of microalgal production strains that have a more desirable composition for HTL processing and consequently improve the quality of biocrude output. The marketability of the biocrude product to fuel producers depends upon specific quality criteria including high carbon and hydrogen content and low oxygen, nitrogen and sulphur levels. Other qualitative considerations include acyl chain lengths and saturation, as well as finer points related to fuel standards. To achieve such standards and ultimately obtain a biocrude product that is comparable to conventional petroleum crude, HTL kerogens and oils can require fractionation to a higher grade product. This additional process step results in material losses and increased energy costs which offset some of the anticipated benefit of this production strategy. This poses a significant operational loss unless the residual fraction can be efficiently recycled back to production (e.g. through strategies such as anaerobic digestion or gasification) or otherwise contributes to cost recovery and energy balance.

Strain development through the use of molecular biology has greater flexibility than conventional breeding and strain development techniques. This may translate to increases in overall productivity (greater volumes to process) and greater carbon density (higher grade biocrude output) and so is of importance for advancing this

production strategy. Knowledge of algal genetics is not yet as sophisticated as other model systems. The ability to engineer algal biology is correspondingly limited at present, but is growing rapidly. Here, we discuss the ongoing development of molecular research for greater understanding of microalgae systems. In particular, we summarise the increasing set of available molecular techniques (Sect. 11.2), their application to microalgae technologies (Sect. 11.3) and the establishment of prudent regulatory systems to ensure these systems become environmentally responsible and socially accepted (Sect. 11.4).

11.2 Molecular Biology Capacity in Microalgae

11.2.1 Genomics and Molecular Biology of Microalgae

Microalgae-based biocrude production is an established technology, but compared to conventional fossil fuel extraction, it is energetically unattractive and the chemistry poorly understood. Improvements in process chemistry are necessary for microalgae biocrude to compete successfully with fossil fuels and non-algal biofuel technologies and to reach its full potential. While conventional strategies for strain development can yield significant improvements, genetic modification (GM) has the potential to improve aspects of biocrude production more rapidly and potentially to greater effect. As the primary aim of HTL is to generate more biocrude product per unit biomass with reduced energy costs, the manipulation of the initial biomass quality and yield, as well as aspects of the HTL chemistry (e.g. N & S content), may be amenable to GM strategies. The first step is to determine what traits would be helpful for HTL processing; the second is to identify how manipulating algal genetics can produce those traits.

Genetic Research in Microalgae To engineer beneficial traits into production strains, sufficient knowledge of algal biology is required to conduct targeted optimisation. This is embodied primarily in both the understanding of the most appropriate effects to target and of the methods to enable their engineering. Just as bacterial engineering rests upon a deep knowledge of bacterial biochemistry and genetics, algal GM biotechnology needs to rest upon a firm foundation of fundamental research into the way that algal genomes work. Despite the commonality of fundamental genetic mechanisms across the span of life on Earth, great variety is also present, and a consistent lesson is that ‘the devil is in the detail’ with respect to individual organisms. Consequently, the specifics matter greatly. Further, much biological variability will have accumulated from the ancient origins of algal phyla and their early divergence from plants and animals. Much specific knowledge of algal gene regulation will therefore be required before skilful, efficient and routine genetic manipulation will be possible. The recently expanded library of available algal genomes is a welcome advance but is of limited utility until these genomes are systematically mapped, curated, annotated and understood, a much more time-

consuming task than the actual sequencing. Systematic approaches such as the generation of knockout mutants of all *Chlamydomonas* genes at Stanford University (Zhang et al. 2014) and the transcriptomic (FANTOM) approaches pioneered at RIKEN in Japan (Forrest et al. 2014) are needed to provide the ability to quickly and with certainty assign biological functions to specific genes and curate algal genomes similarly to those of mammals. While microalgal genomes are undoubtedly simpler than the human genome, the resources allocated to studying them are miniscule by comparison, and the molecular toolkit is sparse, especially the lack of specific antibodies.

Advancements in Genomics Genome sequencing and sequence analysis is an important first step in deepening our understanding of microalgal systems and ultimately developing improved engineering processes. Only a very small number of genomes are available particularly when considered against the huge microalgal species diversity; however, the number of genome sequencing programs is steadily increasing (see Table 11.1). The National Centre for Biotechnology Information (NCBI) now contains 25 green algae genomes either in full, as scaffolds, or for which sequencing is currently underway (www.ncbi.nlm.nih.gov/genomes). Furthermore, there are novel bioinformatic tools (e.g. KEGG assignments accessible at www.genome.jp/kegg), and as BioModels databases accessible at www.ebi.ac.uk/biomodels-mainwww.ebi.ac.uk/biomodels-mainwww.ebi.ac.uk/biomodels-mainwww.ebi.ac.uk/biomodels-main) become available online, they will enable researchers to predict and characterise gene regulatory pathways, forecast outcomes of metabolic shifts and functionally annotate de novo genomes of diverse algal species.

Genetic Mechanisms The existence of functional microRNAs in *Chlamydomonas* (Molnar et al. 2007) demonstrates that much of the convoluted genomic biology being revealed in mammals can also be expected in these simple organisms. The general schema of molecular pattern receptors, signal transduction mechanisms, and complex transcription factor-mediated feedback control of nuclear genes is to be expected, and many of the protein motifs will be familiar (e.g. helix-loop-helix transcription factors). However, given the evolutionary distance between different algal clades and between algae and land plants, it is to be expected that apart from highly conserved central mechanisms (core metabolism, cell replication, and mitochondrial and photosynthetic machinery), many baroque variations remain to be discovered. Algal genetics lags far behind algal physiology, much of which is common to plants in specific detail as well as general principles. To fill this gap, high-throughput gene analysis and bioinformatics will be critical for rapid mapping of the overall territory, even if painstaking molecular analysis is still needed for final validation of proposed biochemical and information pathways.

The algal genes that have so far been studied in detail illustrate this need. Significant changes to cell status, such as nutrient limitation (sulphate, nitrogen, iron, copper), lead not to up-regulation of a few receptors or import proteins, but to coordinated changes of thousands of genes, which resemble those waves of altered

Table 11.1 Update on available algal genome sequences, ongoing and future genome sequencing projects

Class	Species	Strain	Project type	Genome size (Mb)	No. genes	References
Chlorophytes (green algae)	*Chlamydomonas reinhardtii*	CC-503	Genome	121	15,143	Merchant et al. (2007) and Proschold et al. (2005)
	Chlamydomonas incerta		EST	ND		http://tbestdb.bcm.umontreal.ca/searches/login.php
	Volvox carteri	UTEX2908	Genome	138	14,437	Prochnik et al. (2010)
	Dunaliella salina	CCAP19/18	Genome			Joint Genome Institute (JGI)
	Chlorella variabilis (former: *Chorella vulgaris)*	NC64A	Genome	46	9791	Blanc et al. (2010)
	Haematococcus pluvialis					Grossman (2007)
	Scenedesmus obliquus					Grossman (2007)
	Oedogonium cardiacum		Chloroplast genome			Grossman (2007)
	Pseudendoclonium akinetum		Chloroplast genome			Pombert et al. (2005)
	Coccomyxa subellipsoidea	C-169	Genome	49	9915	Blanc et al. (2012)
	Botryococcus braunii		Genome			Joint Genome Institute
	Mesostigma viride		EST			http://tbestdb.bcm.umontreal.ca/searches/login.php
	Nephroselmis olivacea		EST			http://tbestdb.bcm.umontreal.ca/searches/login.php
	Ulva linza	–	EST	–	6519	Zhang et al. (2012)
	Leptosira terrestris		Chloroplast genome			de Cambiaire et al. (2007)
	Pedinomonas minor		Plastid genomes			Grossman (2007), project to be
	Monoraphidium neglectum	SAG 48.87	Genome		16,761	Bogen et al. (2013)

(continued)

Table 11.1 (continued)

Class	Species	Strain	Project type	Genome size (Mb)	No. genes	References
Eustigmatophyta	*Nannochloropsis gaditana*		Genome	34	3558	Qingdao Inst. Bioe. Biop. Tech.
Prasinophytes	*Ostreococcus tauri*	OTH95	Genome	13	7892	Derelle et al. (2006)
	Ostreococcus lucimarinus	CCE9901	Genome	13	7651	Palenik et al. (2007)
	Ostreococcus sp.	RCC809	Genome	12		Joint Genome Institute
	Micromonas pusilla	CCMP1545	Genome	22	10,575	Worden et al. (2009)
	Micromonas sp.	RCC299	Genome	21	10,056	Worden et al. (2009)
	M. pusilla ?	RCC809	Genome	21		Worden et al. (2009)
	Bathycoccus prasinos	BBAN7	Genome	18		Joint Genome Institute
Rhodophytes	*Cyanidioschyzon merolae*	10D	Genome	17	6170	Matsuzaki et al. (2004)
	Galdieria sulphuraria		Genome	14	6723	Schonknecht et al. (2013)
	Porphyta yezoensis		EST	43	10,327	Kasuza DNA Research Institute
	Chondrus crispus		Genome	105	9843	Collen et al. (2013)
	Porphyridium purpureum		Genome	20	8355	Bhattacharya et al. (2013)
Glaucophytes	*Cyanophora paradoxa*		Genome	70	27,921	Price et al. (2012)
	Glaucocystis nostochinearum		EST			Uni Montreal

(continued)

Table 11.1 (continued)

Class	Species	Strain	Project type	Genome size (Mb)	No. genes	References
Stramenopiles (diatoms)	*Thalassiosira pseudonana*	CCMP1335	Genome	32	13,025	Armbrust et al. (2004)
	Thalassiosira oceanica		Genome	92	34,684	Lommer et al. (2012)
	Phaeodactylum tricornutum	CCP1055/1	Genome	27	10,398	Bowler et al. (2008)
	Fragilariopsis cylindrus	CCMP1102	Genome	81		Joint Genome Institute
	Pseudo-Nitzschia mutiseries	CLN-47	Genome			Joint Genome Institute
	Amphora sp.	CCMP2378	Genome			Raymond and Kim (2012)
	Attheya sp.	CCMP212	Genome			Raymond and Kim (2012)
	Fragilariopsis kerguelensis					T. Mock, U. East Anglia, USA
	Ectocarpus siliculosus	Ec32	Genome	214	16,256	Cock et al. (2010)
	Aureococcus anophagefferens	CCMP1984	Genome	57	11,522	Gobler et al. (2011)
Haptophytes	*Emiliania huxleyi*	CCMP1516	Genome	168	38,549	Read et al. (2013)
	E. huxleyi	RCC1217	Genome			The Genome Analysis Centre (TGAC), UK
	E. huxleyi	CCMP371	EST			University of Iowa, USA
	Phaeocystis antarctica					Joint Genome Institute
	Phaeocystis globosa					Joint Genome Institute
	Pavlova lutheri		EST			University Montreal
	Isochrysis galbana	CCMP1323	EST			University Montreal

(continued)

Table 11.1 (continued)

Class	Species	Strain	Project type	Genome size (Mb)	No. genes	References
Cryptophytes	*Guillardia theta*	CCMP2712	Genome	87	24,840	Curtis et al. (2012)
	Guillardia theta		Genome	350	302	Douglas et al. (2001)
	Hemiselmis andersenii		Nucleomorph genome	0.572		NCBI
	Gonomiomonas sp.	ATCC 50108	EST			University Montreal
	Gonomiomonas sp.		EST			University of Iowa, USA
	Chroomonas mesostigmatica	CCMP1168				Moore et al. (2012)
Chlorarachniophytes	*Bigelowiella natans*	CCMP2755		94.7		Joint Genome Institute
Alveolates (Dinoflagellates)						

gene expression seen in multicellular organisms. Only high-throughput mapping can provide the necessary background to support the efficient dissection of these biological responses. Apart from nutrient limitation, the kinds of coordinated responses which might be expected include photoacclimation, responses to predators and pathogens, differentiation-like developmental programs and adaptions to environmental niches. Fortunately, many of the tools developed for the study of other organisms can readily be adapted for algal biology. These include powerful genome-editing platforms either developed [zinc finger nucleases, TALENs (Gao et al. 2014; Sizova et al. 2013)] or under-development [CRISPR/Cas (Sander and Joung 2014)]. Although not yet routine, the ability to conduct precise genome engineering will greatly advance the speed and scope of algal GM production.

Case Studies A number of genetic responses in algae have been described, mainly in response to key physiological processes such as photosynthesis, nutrient limitation and circadian rhythm. These include the analysis of the transcriptional responses of the light-harvesting complex (LHC) genes to light and circadian signals, the carbon concentrating mechanism (CCM) in response to CO_2 limitation, and responses to iron, copper and sulphur limitation.

Few of the estimated 234 transcription factors and regulators initially identified bioinformatically in the *Chlamydomonas* genome (Riano-Pachon et al. 2008) have even tentative roles assigned to them. Although no promoters have been comprehensively analysed, several have been cloned and their behaviours studied and utilised for experimental systems. The best examples are the light-harvesting antenna genes which are regulated both by light and by circadian mechanisms. In addition to promoter regulation, post-transcriptional regulation has been demonstrated by an mRNA binding protein CHLAMY1, composed of two subunits (C1 and C3). In turn, an E-box-like promoter element has been shown to be involved in the regulation of the circadian rhythm protein C3 (Seitz et al. 2010) and some binding factors isolated. Regulatory factors controlling the CCM have been identified [CCMl (Fang et al. 2012; Fukuzawa et al. 2001); LCR1 (Ohnishi et al. 2010; Yoshioka et al. 2004)]. Iron-responsive elements have been identified in several genes [Fox1 (Allen et al. 2007; Fei et al. 2009), Atx1, Fbp1, Fld, Fea1], while the copper response regulator CRR1 has been shown to mediate copper and zinc responses (Malasarn et al. 2013; Sommer et al. 2010) and anaerobiosis [HydA1 (Lambertz et al. 2010; Pape et al. 2012) and Fdx5]; other nutrient uptake regulatory genes include those for sulphur SAC1 (Davies et al. 1996; Moseley et al. 2009) and phosphate PSR1 (Moseley et al. 2009; Wykoff et al. 1999). Although this represents a beginning, it pales in comparison with the extensive analyses of animal genomes, and when contrasted to the ~15,000 genes of *Chlamydomonas*, it is unlikely that this subset will provide an adequate basis for modelling promoter function in algae in general.

Some analysis has started in species other than *Chlamydomonas* including *Dunaliella* (Jia et al. 2012; Lu et al. 2011; Park et al. 2013), and some crossover is expected from plant gene analysis especially in *Arabidopsis.* A start has also been

made in understanding the role of mRNA regulation (Schulze et al. 2010; Wobbe et al. 2009) and chromatin remodelling in *Chlamydomonas* (Strenkert et al. 2011, 2013). While miRNA regulation has been demonstrated (Molnar et al. 2007; Yamasaki et al. 2013), little detail is available, nor is epigenetics well understood. In summary, the detail and breadth of examples typical of the regulation of mammalian promoters and their resultant mRNAs is sorely lacking for algal genomes. Consequently, close study of a set of promoter control mechanisms as models is badly needed and will greatly advance the level of understanding in this area, enabling much more sophisticated photosynthetic engineering, including the discovery of useful inducible/repressible promoters, and the ability to manipulate metabolic pathways and cellular strategies which are normally tightly regulated by photosynthesis. Lipid and starch accumulation, photoprotection and cellular replication, for example, are all cellular functions which are desirable to control for biotechnology applications. Abundant proteins including rubisco and LHC proteins represent substantial cellular resources. Some LHC adaptive functions are important to retain or enhance; others are potentially dispensable under bioreactor conditions or can even reduce biomass growth if allowed to operate naturally. Resource allocation within a cell is complex (Pahlow and Oschlies 2013) and only partly within our control as over- or under-production of specific metabolites can be detrimental to the fitness of the organism and feedback regulation in algae is incompletely understood. Therefore, opportunities exist for the development of the excretion of the end product (e.g. H_2 produced from water via the photosynthetic machinery; volatile metabolic intermediates (Melis 2013); specific secretion mechanisms for proteins and lipids).

The study of gene regulation has traditionally proceeded through intensive analysis of specific cases. As broad understanding evolves of the kinds of mechanisms that are present in biological systems, the emphasis has shifted to high-throughput analyses starting with microarrays and mass mutant libraries, and it is to be expected that this will quickly generate large amounts of data once algal genomics matures. Nonetheless, there are very few case studies of algal genetic mechanisms, and the study of particular cases will still be vital to anchor, interpret and calibrate the results of mass data acquisition.

11.2.2 Techniques for Genetic Modification of Algae

Characteristics such as high photon conversion efficiency, fast growth rate, high growth density, high oil/carbon content, ease of harvesting and high pathogen/predator resistance all represent aspects of importance for the development of high efficiency microalgal production strains. So far, however, there have not been any reports of a single species that is able to meet each of these criteria. The importance of microalgae bioprospecting and breeding, apart from establishing a solid basis for high efficiency strain development, lies in the identification of novel biological mechanisms and algal systems which can be exploited by genetic engineering.

Ideally, these will form libraries of traits, which in combination with tools to conduct species-specific engineering will enable strain customisation. In this context, it is of note that algae possess three genetic systems: the nuclear, the mitochondrial and the plastid genome, each of which may be genetically manipulated.

The green alga *Chlamydomonas reinhardtii* is arguably the most widely used model alga, at least in terms of fundamental biology; its physiology is well described, multiple mutants exist, all three of its genomes have been sequenced (Maul et al. 2002; Merchant et al. 2007; Popescu and Lee 2007), and a range of molecular tools have been developed to facilitate its genetic engineering. It contains one chloroplast with a 203 kb circular genome encoding about 100 genes (Maul et al. 2002). The chloroplast genome of *C. reinhardtii* is AT-rich and highly polyploid with a copy number of about 50–80 copies per chloroplast (Maul et al. 2002). With this high genome copy number, the chloroplast has been the choice of heterologous protein production since protein levels of over 40 % of total soluble protein (TSP) can be achieved (Surzycki et al. 2009). The nuclear genome of *C. reinhardtii* has a high GC content and frequent repeat regions. Protein accumulation is often lower compared to expression in the chloroplast, and transgenes can often be silenced. However, transgene expression from the nucleus offers several advantages such as inducible expression, post-translational modifications and heterologous protein-targeting to various compartments within the cell and secretion. The mitochondrial genome of *C. reinhardtii* is also polyploid with around 50–100 genome copies organised in about 20–30 nucleoids (Hiramatsu et al. 2006). It consists of a 15.8 kb linear DNA molecule which has been fully sequenced, and telomeres corresponding to inverted repeats of ~500 bp are located at each end, with 40-bp single-stranded extensions (Vahrenholz et al. 1993). Compared with the mitochondrial genome typically found in vascular plants, it is extremely compact with 14 genes encoding in total only eight proteins (Remacle et al. 2006) and three tRNAs. The low tRNA content suggests that cytosolic tRNAs are imported, a process known to take place in plant and human mitochondria, making it an interesting potential model system for more detailed process analysis (Remacle et al. 2012).

11.2.3 Molecular Toolkits—Genetic Engineering of the Different Compartments

Nuclear Transformation There are a variety of established transformation methods to integrate heterologous DNA into the nuclear genome including particle bombardment (Debuchy et al. 1989; Kindle et al. 1989; Mayfield and Kindle 1990), agitation of cell-wall-deficient strains with glass beads (Kindle 1990), electroporation (Shimogawara et al. 1998), agitation with silicon carbide whiskers (Dunahay 1993) and biologically mediated gene transfer by *Agrobacterium tumefaciens* (Kumar et al. 2004). In *C. reinhardtii*, transformation of the nuclear genome occurs

by random insertion through non-homologous end joining (Tam and Lefebvre 1993) or by using linear DNA that promotes the insertion of multiple copies in one locus (Cerutti et al. 1997b). Phenotypic and genetic screening of transformants can minimise undesirable non-target effects of the random insertion of a transgene that can lead to disrupted genes or regulatory elements. In return, this effect is used to study genes of unknown function using high-throughput insertional mutagenesis (Dent et al. 2005). In *C. reinhardtii,* targeted gene integration through homologous recombination (HR) using single-stranded transforming DNA (Zorin et al. 2005, 2009) is possible, but only at low frequencies to date. However, high rates of homologous recombination have been reported in another green algal species *Nannochloropsis* (Kilian et al. 2011), showing promise for reverse genetics and targeted gene knockouts.

Random and Insertional Mutagenesis The creation of mutants of an organism by the use of irradiation or chemical mutagenesis is the classic approach pioneered by Morgan in *Drosophila* and used for a century to study the effect of significant alteration in the behaviour of a gene, typically by partial or total deletion. These methods produce base pair changes leading to a range of disturbances including altered amino acid sequence, small deletions, truncations, frameshifts and splicing defects; the resultant mutants include temperature-sensitive and dominant mutants as well as functional knockouts sometimes giving rise to complex phenotypes. In the last 3 decades, the insertion of foreign transgenes (usually carrying a marker to allow selection and identification) has also been well established, as described above. Insertion mutants usually have knocked out or disabled genes, though insertion into promoters can lead to alterations in expression levels and splicing. Gene inactivation is largely on a random basis (Zhang et al. 2014) across the genome (at least where the chromatin structure is sufficiently open) which allows the unbiased identification of genes relevant to biological processes and has been very useful for research into biological mechanisms and physiology in *Chlamydomonas* and other algae. The difficulty is usually that a specific phenotypic screen is needed to identify relevant genes. Lethal mutations will not be identified, nor will mutants in genes which are redundant or which show no overt phenotype under the conditions of the screen. Of the estimated ~15,000 genes in the *Chlamydomonas* genome, only a few hundred have been reported in the literature, and many genes known to be important are not represented in collections of mutants. A good example is the multigene family of LHC genes. An insertion mutant in a single LHC gene will usually not produce any easily measurable phenotype, due to compensation by other LHCs, while the highly specific physiological or genetic tests needed to demonstrate the loss of a specific LHC gene are not suitable for screening assays. Finally, each transformation produces only a few hundred colonies, with very likely a biased set of genes being affected. This makes it a major project to uniquely identify mutants of each gene in an alga. Fortunately, this is being performed at Stanford University, and the resulting collection of mutants will be an invaluable research resource for the *Chlamydomonas* community (Zhang

et al. 2014). However, it is unlikely that this resource will be duplicated for every species of algae of research or commercial interest.

Homologous Recombination (HR) in the Nucleus Homologous recombination, the recombination between homologous DNA sequences, is essential for eukaryotes to repair DNA double-strand breaks and introduce genetic diversity during cell division, and two main pathways ('double-strand break repair' and 'synthesis-dependent strand annealing') have been proposed (Sung and Klein 2006). In plants and algae, nuclear located *RecA* homologues show high similarity to the prokaryotic *RecA* genes which suggests an endosymbiotic transfer from mitochondria and chloroplasts to the nucleus of ancestral eukaryotes (Lin et al. 2006). Although the introduction of foreign DNA into the nucleus occurs predominantly via random insertional mutagenesis, successful targeted homologous recombination has been reported in several algal species such as *C. reinhardtii* (Gumpel et al. 1994; Sodeinde and Kindle 1993; Zorin et al. 2009), *Nannochloropsis* sp. (Kilian et al. 2011) and *Cyanidioschyzon merolae 10D* (Minoda et al. 2004) with as little as 230-bp DNA sequence homology in the haploid cell (Gumpel et al. 1994). It has been demonstrated that the introduction of single-stranded repair DNA leads to a more than 100-fold reduction of non-homologous DNA integration in comparison with double-stranded DNA (Zorin et al. 2005). Attempts to increase the low frequency of homologous recombination in plants by the over-expression of well-characterised enzymes involved in homologous recombination such as the recA and ruvC proteins have been reported to increase homologous recombination and double-strand break repairs; however, these reports suggest that foreign gene targeting is not improved (Reiss et al. 1996, 2000; Shalev et al. 1999).

RNA Interference (RNAi) In the absence of tools for precise genome manipulation, RNAi-mediated knock-down of gene expression enables the creation of highly specific research mutants without the need for phenotypic screening or selection. RNAi techniques also enable the study of reduced levels of gene expression where total ablation would be lethal. The phenomenon of RNA interference is produced by the action of specific cellular machinery [Dicer and Argonaute (AGO) proteins (Casas-Mollano et al. 2008; Cerutti and Casas-Mollano 2006)] on mRNAs, guided by microRNAs which are naturally occurring small double-stranded RNAs (dsRNAs) in a process called mRNA cleavage (Bartel 2004). RNAi represents the use of this natural process for experimental ends by the artificial provision of small RNA molecules designed to interfere with the expression of a target gene. MicroRNAs (miRNAs) have also been discovered in *C. reinhardtii* (Molnar et al. 2009; Zhao et al. 2007) enabling a highly specific genetic tool (Moellering and Benning 2010; Molnar et al. 2009; Schmollinger et al. 2010; Zhao et al. 2007). Difficulties in achieving direct nuclear gene knockout via homologous recombination for *C. reinhardtii* (Nelson and Lefebvre 1995) led to RNAi becoming a widely used method to accomplish post-transcriptional gene silencing for gene function discovery (knock-down approaches via reverse genetics) and metabolic engineering (Fuhrmann et al. 2001; Molnar et al. 2009; Rohr et al. 2004; Schroda et al. 1999; Zhao et al. 2009). Artificial microRNAs have also been engineered to create

functional knock-downs of several nuclear genes in *Dunaliella salina* (Sun et al. 2008) and in the diatom *Phaeodactylum tricornutum* (De Riso et al. 2009). This method could potentially be used for algal genetic engineering for biofuel production (Cerutti et al. 2011; Grossman 2000; Wilson and Lefebvre 2004). The weakness of RNAi is the need to maintain the expression of the RNAi construct, typically requiring ongoing selective pressure, for example with antibiotics.

Genome Editing The ability to precisely edit the genome enables the specific deletion or mutation of genes and regulatory regions, with the resultant ability to create targeted mutations for study or industrial applications. As the resultant mutations are permanent, the stability problems inherent in RNAi knock-down constructs are eliminated. Genome editing also offers the prospect of precise mutants lacking foreign DNA, which consequently are technically non-GMO organisms. Several systems of genome editing have been developed in recent years, including zinc finger nucleases (ZFN), transcription activator-like effector nucleases (TALENs), meganuclease and the CRISPR/Cas system. Both ZFN (Sizova et al. 2013) and TALENS (Gao et al. 2014) have been used for genome editing in *Chlamydomonas*. The drawback of these systems is the substantial investment of time and effort required. In contrast, the CRISPR/Cas system promises a simpler, more facile approach. CRISPR (clustered regularly interspaced short palindromic repeats) sequences are found in prokaryotes and are short repetitive DNA sequences, corresponding to part of the DNA of bacteriophage genomes. In conjunction with a specific nuclease (Cas), they form the basis of a prokaryotic 'immune system' to recognise and eliminate viral genomes. The CRISPR RNA acts as a guide for Cas-mediated cleavage of the viral DNA or provirus. For genome editing, first described in 2013 (Cho et al. 2013; Mali et al. 2013), the addition of a targeted 'guide RNA' with co-expression of the Cas9 nuclease enables precise and specific genome editing and has been rapidly adopted in many organisms including both animals (Hsu et al. 2014) and plants (Feng et al. 2013). Although first attempts to use CRISPR/Cas in algae have encountered difficulties, it is anticipated that these will be overcome in the near future, enabling rapid and flexible genome editing in algae.

Chloroplast Transformation The chloroplast is the site of photosynthesis and storage of the resultant starch and is also important for the production of fatty acids and photosynthesis-related pigments especially carotenoids. Transformation of the chloroplast requires transfer of the transforming DNA to the interior of the chloroplast, and consequently, particle bombardment (biolistics) using DNA-coated gold or tungsten particles is the most commonly used method for chloroplast transformation. Some particles penetrate the cell wall, plasma and chloroplast membrane and deposit the transforming DNA in the plastid, which can then integrate into the local genome through homologous recombination (Boynton et al. 1988). Flanking endogenous sequences that are homologous to the targeted insertion site can make chloroplast transformation events highly targeted to any region in the genome (Rasala et al. 2013) which in *C. reinhardtii* is a great advantage over

nuclear transformation where homologous recombination occurs only at low efficiency (Sodeinde and Kindle 1993).

Homologous Recombination in the Chloroplast In the chloroplast, homologous recombination is mediated by an efficient *RecA*-type system which, due to its homology to the *Eschericia coli* RecA system, is suggested to be related to the cyanobacterial ancestors of chloroplasts (Cerutti et al. 1992, 1995; Inouye et al. 2008; Nakazato et al. 2003). In cyanobacteria, the RecA system is essential for cell viability especially under DNA damaging conditions (Jones 2014; Matsuoka et al. 2001). DNA repair is also believed to be the main function for homologous recombination in the chloroplast (Cerutti et al. 1995; Rowan et al. 2010). Several different models exist for the precise mechanism of homologous recombination and are not presented in detail here; however, the main steps of prokaryotic homologous recombination include initial strand breakage, formation of an enzyme complex to unwind the double-stranded DNA to form a single strand, followed by a mechanism called 'strand invasion' which searches for similar sequences on a homologous DNA fragment for pairing. This is then followed by DNA synthesis in accordance with the new template strand and resolution of the structure (Amundsen et al. 2007; Smith 2012).

Genome Stability in the Chloroplast With 50–100 genome copies, the chloroplast is highly polyploid, and apart from a few exceptions, each plastome shows a tetrapartite organisation containing two inverted repeat sequences that are mirror images of one another, separated by a large and a small single-copy unit. Though absent in present-day cyanobacteria and not essential for the general chloroplast genome function, the inverted repeat sequences show properties which suggest their involvement in gene maintenance and increased genome stability (Goulding et al. 1996). Chloroplast genomes can undergo homologous recombination between the inverted repeat sequences as several studies have shown two populations of plastomes in the same organism differing only in the single-copy sequence orientation (Aldrich et al. 1985; Palmer 1983; Stein et al. 1986). Furthermore, it has been observed that the inverted repeat regions accumulate nucleotide substitution mutations 2.3 times more slowly than the single-copy regions (Perry and Wolfe 2002; Ravi et al. 2008; Shaw et al. 2007). It was also demonstrated that DNA rearrangements occur more frequently when a large inverted repeat sequence is lost (Palmer and Thompson 1982). This suggests that homologous recombination between plastomes and inverted repeats contributes to this increased genome stability.

Manipulation of the chloroplast genome via homologous recombination offers the potential for exact gene deletions and insertions. Generally, chloroplast transformation vectors are *E. coli* plasmid derivatives carrying the foreign DNA flanked by DNA sequences (>400 bp each) which are homologous to the target region of the plastid DNA (Bock 2001; Hager and Bock 2000). Due to the high ploidy of the plastid genome and the fact that initially only a single plastome copy is transformed, the resulting phenotype may be weak in primary transformants and it is important to establish an efficient and suitable selection system or strategy to identify and enrich

cells containing transformed ptDNA copies, which may easily revert to wild type (Hager and Bock 2000; Rasala et al. 2013). Once the genome is homoplastomic and thus lacking template for further undesired homologous recombination events, the selectable marker can be removed (Day and Goldschmidt-Clermont 2011), though selective pressure against the transgene can still exist.

Mitochondrial Transformation Respiration and photosynthesis are coupled processes, and mitochondrial mutations are known to affect photosynthesis (Schönfeld et al. 2004) as well as many other aspects of cellular metabolism. Although in *Chlamydomonas* homologous recombination in mitochondria DNA is only detected after crosses between different mating types in mitotic zygotes (Remacle et al. 2012), it demonstrates that the cellular machinery is available for recombination-based foreign gene integration. Although the first transformation of the mitochondrion was published in 1993 (Randolph-Anderson et al. 1993) using biolistic bombardment, reports of mitochondrial transformation are rare and initially limited to the restoration of the wild-type mitochondria genome in mutant strains. In 2006, Remacle et al. reported the first and, to date, the only modification of the mitochondrial genome in vivo in a photosynthetically active organism. The work presented the introduction of a nucleotide substitution in the *cob* gene, conferring resistance to myxothiazol, and an internal deletion in *nd4* (Remacle et al. 2006). Homologous recombination was facilitated with as little as 28-bp homology between the introduced and endogenous DNA (Remacle et al. 2006).

Selection Techniques Following Transformation The identification of chloroplast and nuclear transformants can be achieved through the rescue of mutants impaired in endogenous cellular functions (non-photosynthetic, flagellar or auxotrophic mutants) or the introduction of antibiotic and herbicide resistances by point mutation of endogenous genes or the expression of heterologous resistance genes. A range of auxotrophic mutants have been developed (including mutations in arginine biosynthesis, nitrate reductase, nicotinamide biosynthesis and thiamine biosynthesis (Debuchy et al. 1989; Ferris 1995; Kindle et al. 1989; Rochaix and Vandillewijn 1982)) as well as mutants in photosynthetic capability or flagellar motility (Diener et al. 1990; Mayfield and Kindle 1990; Mitchell and Kang 1991; Smart and Selman 1993).

Classical antibiotic resistance markers include kanamycin [chloroplast: (Bateman and Purton 2000); nuclear: (Hasnain et al. 1985; Sizova et al. 2001)], spectinomycin/streptomycin [chloroplast: (Goldschmidt-Clermont 1991); nuclear: (Cerutti et al. 1997a)], neamine/kanamycin and erythromycin [chloroplast: (Harris et al. 1989)] paromomycin and neomycin [nuclear: (Sizova et al. 2001)], cryptopleurine and emetine [nuclear: (Nelson et al. 1994)], zeocin and phleomycin [nuclear: (Stevens et al. 1996)] and hygromycin B [nuclear: (Berthold et al. 2002)]. Screening for resistance to herbicides, such as atrazine, which inhibits the function of photosystem II [chloroplast: (Erickson et al. 1984)], or Basta, leading to disruption of the chloroplast structure [chloroplast: (Cui et al. 2014)], is also possible. The use of antibiotic resistance markers is not problematic for research purposes, but is less

desirable for industrial-scale production of vaccines or therapeutics, and for large-scale outdoor or agricultural production, antibiotic-free media is usually required for both sociopolitical and socio-economic reasons.

Recombinant Protein Expression Regardless of the energy return on investment (EROI) of microalgal biocrude, the low net value poses a significant problem for economic biocrude production from microalgae. One potential strategy to offset this is to exploit the fact that high-value products such as recombinant proteins are typically only a small fraction by weight of the biomass and could potentially be extracted prior to thermochemical processing of remaining biomass to biocrude. Consequently, an additional and lucrative revenue stream can arguably be obtained to subsidise the process without sacrificing overall biofuel productivity. The demand for recombinant proteins is growing with increasing population and biotechnological applications, and ultimately, the expression systems with the greatest cost-benefit are likely to dominate these markets. In this context, microalgal systems offer significant advantages over traditional microbial fermentation or mammalian cell culture systems. So-called molecular 'pharming' is the production of pharmaceutical proteins, therapy peptides, vaccine subunits, industrial enzymes and secondary metabolites or other compounds of interest in plants, and algae also offer commodity-scale opportunities. Proteins with biopharmaceutical or biotechnological relevance can be, e.g. monoclonal antibodies, vaccines, blood factors, hormones, growth factors and cytokines.

Nucleus Although the chloroplast is the expression system of choice for high protein expression levels, the nucleus as expression location has its advantages which have to be carefully considered in the context of each product. Despite the comparatively low expression rate of 1 % of TSP claimed by commercial vendors and the risk of gene silencing, nuclear expression tools enable the fusion of the desired product to a selection marker (to avoid silencing) with subsequent self-cleavage, as well as secretion of the expressed compound into the media (Lauersen et al. 2013; Rasala et al. 2012). So far, most proteins expressed from the nucleus have been reporter genes used to quantitatively measure protein expression levels as well as the localisation and accumulation (e.g. within the *C. reinhardtii* cell) (Rasala et al. 2013). Six fluorescent proteins (blue mTagBFP, cyan mCerulean, green CrGFP, yellow Venus, orange Tomato and red mCherry) were expressed from the nuclear genome to allow protein detection in whole cells by fluorescence microplate reader analysis, live-cell fluorescence microscopy and flow cytometry.

Chloroplast The production of recombinant proteins including reporters (e.g. GUS, luciferase or GFP) (Franklin et al. 2002b; Mayfield and Schultz 2004; Minko et al. 1999; Sakamoto et al. 1993), protein therapeutics (antibodies, hormones, growth factors or vaccines) (Franklin and Mayfield 2005; Manuell et al. 2007; Mayfield and Franklin 2005; Mayfield et al. 2003; Rasala et al. 2010; Surzycki et al. 2009) and industrial enzymes has established the chloroplast as a suitable production platform for a broad range for protein candidates. The potential for high expression levels [over 40 % of TSP (Surzycki et al. 2009)] and the ability to form complex

proteins including disulphide bonds (Tran et al. 2009) allow for limited post-translational modification without interference caused by 'gene silencing' making the chloroplast the site of choice for protein expression. Furthermore, the prokaryotic characteristics of the chloroplast genome such as gene organisation into operon-like structures (Holloway and Herrin 1998) potentially allow the expression of multiple transgenes from a single operon (transgene stacking) and with this the introduction of complete biochemical pathways. The additional development of a Gateway-compatible transformation system (Oey et al. 2014) allows the rapid production of multiple transformants. Success rates and costs for successfully establishing expression of new proteins in *C. reinhardtii* are estimated to be similar to those of yeast and mammalian cells (Rasala et al. 2013), but with potentially much lower production and purification costs.

11.2.4 Transcriptional and Translational Control of Transgenes

Nucleus A major mechanism of nuclear gene regulation is transcriptional control. Strategies to enhance heterologous gene expression, which occurs at relatively low levels compared to expression in the chloroplast, are the search for more effective promoters (Rasala et al. 2013) and the enhancement of translation efficiency through codon optimisation (Fuhrmann et al. 1999, 2004; Heitzer and Zschoernig 2007; Mayfield and Kindle 1990). The glycosylation patterns of nuclear expressed and secreted proteins from *C. reinhardtii* remain to be resolved. Studies on the post-translational machinery of this alga would be helpful to exploit this as an option of algal protein production.

Chloroplast Ensuring high heterogeneous gene expression requires the identification of endogenous transcriptional and translational regulatory elements (Harris et al. 2009; Marin-Navarro et al. 2007; Purton 2007). Although chloroplast gene expression is usually regulated at the translational level (Barnes et al. 2005; Eberhard et al. 2002; Nickelsen 2003; Rasala et al. 2010, 2011; Zerges 2000), choice of promoter and 5′ UTRs sequences is of importance due to potential feedback regulations which can interfere with the heterologous protein expression and mRNA stability (Gimpel and Mayfield 2013; Hauser et al. 1996; Manuell et al. 2007). The 5′ UTRs are believed to regulate ribosome association, transcript stability and the rate of translation (Barnes et al. 2005; Marin-Navarro et al. 2007; Salvador et al. 1993; Zou et al. 2003) while 3′ UTRs influence mRNA stability (Herrin and Nickelsen 2004; Lee et al. 1996; Monde et al. 2000; Stern et al. 1991) or may interact with 5′ UTRs (Katz and Danon 2002).

Mitochondria In photosynthetic organisms, mitochondria are the organelle with the greatest diversity in size and structure, ranging from 15 kb linear DNA molecules in *Chlamydomonas* to 1.0 Mb in angiosperms. To date, *Chlamydomonas* is

the only photosynthetically active organisms for which mitochondrial DNA has been altered, which significantly limits insights into translational and transcriptional control. Attempts have been made with plant mitochondria to utilise in vitro DNA and RNA import, and electroporation of isolated mitochondria has been used to gain further information about transcription and post-transcriptional processing (Remacle et al. 2012).

11.2.5 Strategies to Improve Gene Expression Levels

Fusion Proteins Specific regions within the coding regions of chloroplast genes enhance efficient expression of foreign genes in plants and algae (Anthonisen et al. 2002; Gray et al. 2009; Kuroda and Maliga 2001b). Genetic fusion of endogenous regions to an exogenous protein of interest may represent another effective strategy for high-level transgene expression (Gray et al. 2011; Kasai et al. 2003). A disadvantage of the fusion protein approach may be reduced industrial or clinical values or increased cost of purifying the protein of interest from its fusion partner.

Codon Optimisation Codon optimisation has been shown to be an important factor of heterologous gene expression in algae (Heitzer et al. 2007). It has been demonstrated that protein expression levels can be improved in the chloroplast by up to ~80-fold (Franklin et al. 2002a) by adjusting the codon bias of the transgene to the AT-rich chloroplast codon bias. Despite this, the effects of codon bias on expression levels are largely heuristic and still not well understood at a theoretical level.

Replacement of Highly Expressed Photosynthesis Genes The highest protein expression levels in *Chlamydomonas* have so far been demonstrated in transformants carrying the *psbA* promoter and 5′ UTR for transcription and translation initiation in a *psbA* knockout background, which leads to a non-photosynthetic strain (Manuell et al. 2007; Surzycki et al. 2009). *PsbA* encodes for the D1 protein in the photosystem II reaction centre and is the most rapidly synthesised protein at high light intensity in higher plants and algal cells (Trebitsh et al. 2000). Although photosynthesis was restored by introducing the *psbA* gene at a different location, the presence of the psbA protein decreased the yield of foreign protein production (Manuell et al. 2007). This may reflect competition between the two genes, but could also be due to primary energetic or biosynthetic limitations.

Open Reading Frame Orientation In the chloroplast, 3′ UTR of the RNA often shows the potential for stem-loop formation, which serves for transcript stabilisation rather than transcription termination (Rott et al. 1998). Therefore, a degree of 'read through' from the upstream gene is possible (Oey et al. 2009), which in parallel can increase the amount of translatable RNA and thus potentially increase the amount of protein (Stern and Gruissem 1987).

Heterologous and Hybrid Regulatory Systems Rasala et al. (2011) have recently shown that an increase in protein production can be achieved by the fusion of the *16S* ribosomal promoter, which does not contain translation initiation signals such as Shine–Dalgarno sequences, to the endogenous *atpA* 5′ UTR containing the translation initiation signal. This, and the demonstration that heterologous regulatory elements can significantly induce the expression rate (Kuroda and Maliga 2001a; Oey et al. 2009; Ruhlman et al. 2010), suggests that designed regulatory elements could serve to improve expression.

Inducible Systems Environmental changes or developmental factors naturally influence the up- and down-regulation of genes. Understanding those regulatory mechanisms provides a valuable genetic tool, for example to switch protein expression automatically or under the control of specific circumstances. Examples of inducible algal promoters include light responsive genes (Falciatore et al. 1999), nitrogen starvation (Poulsen et al. 2006; Poulsen and Kroger 2005), a sulphur-regulated arylsulfatase gene and an ISG glycoprotein. Tightly controlled expression of toxic proteins (e.g. the growth factor DILP-2) is also often desirable (Surzycki et al. 2007). The expression of psbD (D2 component of PSII) is dependent on the Nac2 gene fused to the copper-sensitive cytochrome c6 promoter (cyc6) and induced in copper deficiency and repressed in the presence of copper.

Riboswitches Riboswitches that can be used to regulate protein expression at the translational level have been shown to be functional in *C. reinhardtii* and *Volvox carteri* (Croft et al. 2007) suggesting that it may be a useful technique for other algae species as well.

11.2.6 Genetic Engineering in Other Algae

Apart from *C. reinhardtii,* few algal species have been subjected to extensive genomic manipulation. As it seems unlikely that *C. reinhardtii* will be used for commercial biofuel applications, this needs to be remedied. Because of the phylogenetic and structural diversity of algae, methods established for *C. reinhardtii* cannot necessarily be easily transferred to other species and may require major adaptations. Therefore, recent efforts have been made to develop molecular toolkits to increase the range of other more suitable algal species for commercial production scenarios.

A number of algae species have been transformed successfully, and an overview is given in Table 11.2. For example, *Euglena gracilis* was transformed with an antibiotic resistance marker (Doetsch et al. 2001) and *Porphyridium* spp. using a herbicide resistance cassette (Lapidot et al. 2002) RNAi has also been used to engineer nuclear genes in the chlorophyte *Dunaliella salina* (Sun et al. 2008) and in the diatom *Phaeodactylum tricornutum* (De Riso et al. 2009). Applicable genetic modifications of green algae for industry are the transformation of *Haematococcus pluvialis* (Steinbrenner and Sandmann 2006; Teng et al. 2002), an important

Table 11.2 Amenable strains and transformation systems

Compartment	Phylum	Class	Species	Method	References
Nuclear	*Chlorophyta*	*Chlorophyceae*	*Chlamydomonas reinhardtii*	A, B, E, G, S	Debuchy et al. (1989)
					Dunahay (1993) and Kindle et al. (1989)
					Kindle (1990), Kumar et al. (2004) and Mayfield and Kindle (1990)
					Rochaix and Vandillewijn (1982), Shimogawara et al. (1998) and Fernandez et al. (1989)
					Molnar et al. (2009) and Stevens et al. (1996)
					Berthold et al. (2002) and Sizova et al. (2001)
					Cerutti et al. (1997a)
					Goldschmidt-Clermont (1991)
					Schroda et al. (2000)
			Dunaliella salina	B, E, G	Feng et al. (2009) and Geng et al. (2004)
					Sun et al. (2005) and Wang et al. (2007),
					Tan et al. (2005) and Li et al. (2007)
			Eudorina elegans	B	Lerche and Hallmann (2013)
			Gonium pectorale	B	Lerche and Hallmann (2009)
			Haematococcus pluvialis	A, B	Teng et al. (2002)
					Kathiresan et al. (2009) and Steinbrenner and Sandmann (2006)
			Volvox carteri		Hallmann and Rappel (1999) and Hallmann and Sumper (1994)
					Hallmann and Sumper (1996) and Jakobiak et al. (2004)
					(Schiedlmeier et al. 1994)
		Trebouxiophyceae	*Chlorella ellipsoidea*	PT-E	Jarvis and Brown (1991) and Kim et al. (2002)
					Bai et al. (2013) and Liu et al. (2013)
			Chlorella saccharophila	PT-E	Maruyama et al. (1994)
			Chlorella sorokiniana	B	Dawson et al. (1997) and Hawkins and Nakamura (1999)
			Chlorella vulgaris	B	Hawkins and Nakamura (1999)
	Dinoflagellate	*Dinophyceae*	*Amphidinium spp.*	S	ten Lohuis and Miller (1998)
			Symbiodinium microadriaticum	S	ten Lohuis and Miller (1998)
	Heterokontophyta	*Bacillariophyceae (diatoms)*	*Chaetoceros salsugineum*	*B*	Miyagawa-Yamaguchi et al. (2011)

(continued)

Table 11.2 (continued)

Compartment	Phylum	Class	Species	Method	References
			Chaetoceros debilis	B	Miyagawa-Yamaguchi et al. (2011)
			Chaetoceros setoensis	B	Miyagawa-Yamaguchi et al. (2011)
			Chaetoceros tenuissimus	B	Miyagawa-Yamaguchi et al. (2011)
			Cyclotella cryptica	B	Dunahay et al. (1995)
			Cylindrotheca fusiformis	B	Fischer et al. (1999) and Poulsen and Kroger (2005)
			Navicula saprophila	B	Dunahay et al. (1995)
			Phaeodactylum tricornutum	B	Apt et al. (1996), De Riso et al. (2009), Falciatore et al. (1999) and Zaslavskaia et al. (2000)
					Miyagawa et al. (2009), Sakaguchi et al. (2011) and Zaslavskaia et al. (2001)
			Thalassiosira weissflogii	B	Falciatore et al. (1999)
			Thalassiosira pseudonana	B	Poulsen et al. (2006)
		Eustigmatophyceae	*Nannochloropsis* sp.	A, E	Cha et al. (2011) and Kilian et al. (2011)
			Nannochloropsis gaditana	E	Li et al. (2014) and Radakovits et al. (2012)
			Nannochloropsis granulata	E	Li et al. (2014)
			Nannochloropsis oculata	PT-E	Chen et al. (2008), Li et al. (2014) and Li and Tsai (2009)
			Nannochloropsis oceanica	E	Vieler et al. (2012) and Li et al. (2014)
			Nannochloropsis salina	E	Li et al. (2014)
	Rhodophyta	*Cyanidiophyceae*	*Cyanidioschyzon merolae*	E, PEG	Fujiwara et al. (2013), Minoda et al. (2004) and Ohnuma et al. (2008), (2009)
Chloroplast	*Chlorophyta*	*Chlorophyceae*	*Chlamydomonas reinhardtii*	B, G	Boynton et al. (1988) and O'Neill et al. (2012)
			Haematococcus pluvialis	B	Gutierrez et al. (2012)
			Dunaliella sp	B	Purton et al. (2013)
			Scenedesmus sp	B	Purton et al. (2013)
		Prasinophyceae	*Platymonas subcordiformis*	B	Cui et al. (2014)
	Euglenophyta	*Euglenoidea*	*Euglena gracilis*	B	Doetsch et al. (2001)
	Porphyridiophyta	*Porphyridiophyceae*	*Porphyridium sp*	B	Lapidot et al. (2002)
Mitochondria	*Chlorophyta*	*Chlorophyceae*	*Chlamydomonas reinhardtii*	B	Randolph-Anderson et al. (1993), Remacle et al. (2006) and Yamasaki et al. (2005)

Methods A—*Agrobacterium*, B—biolistic bombardment, E—electroporation, G—glass bead agitation, S—silicon carbide whiskers, PT—protoplast transformation, and PEG—with polyethylene glycol

producer of astaxanthin, and *Dunaliella salina* (Feng et al. 2009; Geng et al. 2004; Sun et al. 2005; Tan et al. 2005) used for β-carotene production. Diatoms are also important commercial sources for aquaculture feedstock, specialty oils such as omega-3 fatty acids, and are used in nanotechnology due to their unique silica frustules. There has been one report of a nuclear transformation of dinoflagellates (ten Lohuis and Miller 1998). Red algae have been used for both chloroplast transformation (Lapidot et al. 2002) and nuclear transformation (Cheney et al. 2001; Minoda et al. 2004). A human growth hormone (hGH) has been successfully expressed in the nucleus of *Chlorella vulgaris* (Hawkins and Nakamura 1999) and a fish growth hormone (GH) in *Nannochloropsis oculata* (Chen et al. 2008). Transformation techniques using a cellulolytic enzyme to weaken the cell walls and make the cells more competent for the uptake of foreign DNA have been successfully applied to the green algae *Chlorella ellipsoidea* (Liu et al. 2013) and may be applicable for the transformation of other algal species with tough cell walls in future. A synthetic biology approach to engineer complex photosynthetic traits from diverse algae into a more controllable production strains has been shown using an ex vivo genome assembly to transfer genes for core photosystem subunits from *Scenedesmus* into multiple loci in the *Chlamydomonas* plastid genome (O'Neill et al. 2012).

Given the recent expansion of interest in microalgae, a broader repertoire of genome sequences and analytical and molecular engineering tools are being reported and will provide the foundation for a broad range of biofuel applications, some of which are covered in the following section.

11.3 Application of Genetic Engineering for Practical Applications

11.3.1 Strategies for GM Microalgae Relevant to Biocrude Production

The potential benefits of GM in microalgae mass cultivation systems for the production of biocrude can be broadly divided into seven strategies:

1. To increase the net photosynthetic productivity of mass cultures
2. To increase nutrient assimilation capacity
3. To modify bulk energy and carbon flows (e.g. rerouting energy flows into lipids)
4. To enhance the alga's capacity to remain dominant in contaminated cultures (e.g. resistance to predators, pathogens)
5. To enhance the harvestability and processability of the algae biomass (biology of flocculation)
6. To improve economic viability through the manufacture of high-value products and services (HVP&S—e.g. recombinant vaccines or industrially useful properties such as the ability to digest cellulose)

7. To develop enabling technologies for biotechnology (e.g. export systems for proteins, lipids, or other products; internal signalling or reporter systems and switchable effectors, for example to stop and start growth, trigger programmed cell death or dissemble the cell wall upon demand).

11.3.2 Engineering Increases to the Net Photosynthetic Productivity of Mass Cultures

The two key requirements for any commercial renewable fuel system are a demonstrable positive energy balance and financial profitability. The optimisation of photon conversion efficiency (PCE) towards the desired end fuel product is central to this and requires optimisation of the following biological processes:

1. Solar energy capture
2. Storage of the captured energy as chemical energy
3. Minimising metabolic losses
4. Targeting the photosynthates into the chosen product stream

Solar Energy Capture While the theoretical PCE of microalgae may be as high as ~8–12 % of total incident solar energy (Melis 2009; Zhu et al. 2010), conventional commercial systems fall far short of this (up to ~2 % annual average for HRPs and ~5 % for PBRs). This, however, is already a significant advance on conventional agriculture and also shows the potential for further improvement. Well-operated high rate pond (HRP) systems cultivating suitable production strains in favourable climatic locations can achieve up to ~70 T ha^{-1} yr^{-1} which is equivalent to approximately 20 g m^{-2} d^{-1} though many current systems are achieving much less than this (Downes et al. 2013). The production of up to 70 T biomass dry weight yr^{-1} makes such microalgae systems ~5 times more productive than sugar cane [global average 71 T fresh weight ha^{-1} yr^{-1} (FAO—Food and Agriculture Organization of the United Nations 2014)], which as dry mass in comparison also generally has a lower calorific value than dried microalgal biomass, especially relative to oleaginous strains. At these levels (and assuming a nominal solar energy level of ~20 MJ m^{-2} d^{-1}), HRPs are producing microalgae biomass at ~2 % PCE (average value with variance between 1 and 4 %) in comparison with the global annual average of ~0.4 % for sugar cane yields when fallowing and rattooning are considered (Stephens et al. 2013a). Although microalgae can be produced at higher productivities, the capital cost and operating cost of these algae farms result in a higher relative cost of production. For example, more advanced PBR systems with improved designs and a larger surface area to volume ratio can achieve PCE rates of ~5 % in similar outdoor conditions (equivalent to ~175 T ha^{-1} yr^{-1}), but currently, the increase in capital cost is much higher than the returned benefit of the higher productivity. Higher PCE rates have been achieved in the laboratory, but this

is under artificial conditions. Consequently, biological methods of improving the PCE rate of microalgae production without incurring additional capital expenditure could play an important part in increasing the feasibility of a wide range of microalgal production systems. As the photosynthetic machinery comprises a number of potential targets for GM approaches, it is useful first to examine the molecular mechanisms of photosynthesis.

Photosynthesis and Electron transfer Photosynthesis drives the first step of all biofuel production and as such is a major target for genetic optimisation. In particular, the light reactions of photosynthesis capture solar energy and convert it into chemical energy in the form of ATP and NADPH. The ATP and NADPH generated is subsequently used to drive CO_2 fixation (dark reactions) and so ultimately the formation of biomass (*for biomethane and biomass-to-liquid (BTL)*), oils (*for biodiesel and aviation fuel*), sugars and starch (*for bio-ethanol*), and protons and electrons (*for bio-H_2*). Optimising the efficiency of light capture and its conversion to chemical energy is thus of critical importance for the development of all high efficiency/low-cost biofuel processes. Here, the complex processes of photosynthesis are briefly summarised to highlight key areas of potential genetic optimisation.

In higher plants and green algae, light is captured by the LHC proteins, which are commonly referred to as LHCI and LHCII based on their predominant interactions with photosystems I (PSI) or II (PSII). The LHC proteins belong to a large gene family, which in the green alga *C. reinhardtii* consists of over 20 members (Dittami et al. 2010). LHC proteins have a dual role:

1. To capture light and funnel the derived excitation energy to PSI and PSII.
2. To dissipate excess energy via the processes of non-photochemical quenching (NPQ) to reduce photodamage to PSII. Under high-light operational conditions such as in Australia, NPQ can result in energy losses of $\sim$90 % of the captured solar energy (Mitra and Melis 2008; Polle et al. 2003). To achieve the highest solar-to-chemical energy conversion efficiency, it is therefore imperative to minimise these losses.

The excitation energy transferred to PSII by LHCII drives the photosynthetic water splitting reaction, which converts H_2O into H^+, electrons and O_2. Under light-limited conditions, the photosynthetic electron transport chain is thought to be in the 'State 1 Transition' in which almost all of the captured photons are used to drive the transfer of electrons along the linear electron transport chain from the PSII–-LHCII supercomplex, via plastoquinone, cytochrome b_6f, the PSI–LHCI supercomplex and ferredoxin and on to NADPH.

Simultaneously, H^+ ions are released into the thylakoid lumen by PSII and the PQ/PQH_2 cycle. This generates a proton gradient across the thylakoid membrane, which drives ATP production via ATP synthase. The H^+ and electrons are recombined by ferredoxin–$NADP^+$ oxidoreductase to produce NADPH. NADPH and ATP are used in the Calvin–Benson cycle and other biochemical pathways to

produce the sugars, starch, oils and other biomolecules that are feedstocks for biofuel products (and which collectively form biomass). Alternatively, in some photosynthetic micro-organisms such as *C. reinhardtii*, the H^+ and electrons extracted from water (or starch) can be fed to the hydrogenase HydA, via the electron transport chain to drive the direct production of H_2 from H_2O. H_2 is potentially the most efficient form of biofuel production as, in contrast to the production of oils and carbohydrates, it requires no additional ATP or NADPH. Furthermore, far from only having a future use, H_2 is already used for synthetic fuel production and has significant industrial applications.

State Transitions The highest efficiency of ATP and NADPH production is achieved under low NPQ/linear electron transport conditions. This is because almost all of the captured energy is used to drive photochemistry and only two photons (*one to excite PSII and a second to excite PSI*) are required to transfer one electron from H_2O to NADPH. Consequently, microalgae have evolved the state transition process to maintain linear electron transport under changing light conditions. The state transition process balances the absorbed light energy between the two photosystems by relocating light-harvesting complex II proteins between the two PS reaction centres thus optimising the corresponding antenna. In State 1, CP29 and LHCII trimers are attached to PSII. Under high-light conditions, the PQ pool can become over-reduced (PQH_2) if the turnover rate of PSII exceeds that of PSI. To prevent photodamage, *C. reinhardtii* undergoes a transition from State 1 to State 2 in which CP29 and LHCII trimers are thought to detach from PSII, reducing its antenna size, and couple to PSI–LHCI to yield a PSI–LHCI–LHCII supercomplex which is to capture more light (Drop et al. 2011; Kargul et al. 2005). If the process of state transition is not sufficient and the PQ pool continues to be over-reduced, or the cell has a greater requirement for ATP, NADPH-dependent cyclic electron transport is induced.

Cyclic Electron Transport Cyclic electron transport transfers H^+ from the stroma and e^- from the stromal side of PSI to the luminal surface of the thylakoid membrane. H^+ is then released into the lumen, and the electrons cycled back to PSI via plastocyanin (PC). In this way, the over-reduced PQH_2 pool is re-oxidised to PQ. Converting PQH_2 to PQ helps to protect PSII from photoinhibition as the latter is available to accept electrons released by PSII. The H^+ transported to the lumen by cyclic electron transport is used for ATP synthesis via the ATP synthase. Although cyclic electron transport is not as efficient as linear electron transport, some of the energy is utilised for the production of ATP. Recently, Iwai et al. (2010) reported the isolation of a 1 MDa 'CEF–PSI supercomplex' and suggested it as the site of cyclic electron transport. This supercomplex is thought to consist of the PSI–LHCI and $Cytb_6f$ complexes, ferredoxin–NADPH oxido-reductase (FNR) and the integral membrane protein PGRL1. Independent studies revealed that PGRL1 is required for efficient cyclic electron transfer in *C. reinhardtii* (Petroutsos et al. 2009; Tolleter et al. 2011).

Theoretical Areas for the Genetic Optimisation of Photosynthesis The involvement of light-harvesting antenna proteins and the reaction centres in the dynamic function of state transitions, cyclic electron transport and specific biofuel production modes such as hydrogen remains an active area of structural biology (electron tomography, crystallography and high-resolution single particle analysis), protein biochemistry and genetic engineering research. Established and previously introduced methods such as RNA interference, random insertional mutagenesis as well as recent advantages in the development of gene-editing tools such as CRISPR/Cas (Sander and Joung 2014) or TALEN (Gao et al. 2014; Sizova et al. 2013) systems open up the ability to knock out/knock down specific genes or conduct precise gene editing.

Knockout/Knock-down of Specific Genes Deletion and knockout of specific genes such as LHC genes allow the establishment of stable mutants for structural and phenotypic characterisation. This in turn will help to develop pseudo-atomic resolution blueprints of the photosynthetic machinery to enable structure-guided design. Although quite unstable for commercial use, RNA interference to down-regulate particular proteins has been quite successful at increasing our understanding of optimal configurations for the photosynthetic apparatus and is therefore a useful tool to identify potential targets for further investigation and potential engineering applications.

Minimising Energy Losses At low-light levels, the rate of photosynthesis is limited by light, while under high-light levels, it is primarily limited by the rate of CO_2 fixation by rubisco. Increasing the atmospheric CO_2 concentration can increase the rate of CO_2 fixation. However, even under these conditions, wild-type microalgae are already supersaturated by irradiance levels in the $\sim$100–400 μE m^{-2} s^{-1} range, which is considerably lower than maximum incident irradiance levels (up to $\sim$2500 μE m^{-2} s^{-1}) as they generally possess large (dark green) chlorophyll-binding LHC antenna systems designed to capture a large proportion of the light incident upon them. Consequently, microalgae have evolved a range of photo-protective mechanisms to prevent oxidative damage under supersaturated illumination conditions. In the wild type with large antenna, algae cells at the illuminated surface of the bioreactor that are exposed to high-light levels capture the bulk of the light energy. The energy required to drive photochemistry is used, and the remainder of this captured energy is wasted via processes such as NPQ in which the excess is dissipated as heat (Mussgnug et al. 2007; Polle et al. 2003; Tolleter et al. 2011). As a result, light penetration into the culture is compromised and the cells located deeper in the culture are exposed to decreasing levels which impairs these cells in their photosynthetic efficiency. This in turn drastically reduces the efficiency of the overall culture. Although this problem can be overcome by reducing culture density or decreasing the light path, this reduces production levels or requires a larger surface area, increasing the cost of the bioreactors. Increasing the mixing rate is also of limited benefit as energy dissipation occurs on a shorter timescale ($<1 \times 10^{-6}$ s) than transferring cells from high- to low-light zones in the bioreactor ($\sim 1 \times 10^{-3}$ s) and requires a considerable energy input, significantly reducing the energy yield of

the process. Another mechanism of the cells to avoid photodamage is the ability to partially down-regulate their antenna systems yielding a light green phenotype due to their reduced chlorophyll content at the same cell density. Although this is an effective way of reducing light capture, a remarkable amount of energy is still wasted by NPQ. Reducing the amount of light captured per photosystem to the optimum light capture needed for each cell can markedly improve photobioreactor efficiencies (Beckmann et al. 2009; Mitra and Melis 2008; Polle et al. 2003).

Engineered small antenna cell lines with reduced LHC levels offer the potential of improving the light penetration into the bioreactor and the ability to better match solar energy input with the energy requirements of each photosynthesising cell. Thus, 'small antenna' cells at the bioreactor surface absorb only the light that they need, largely eliminating the need to dissipate excess energy through NPQ and to switch into the cyclic electron transport mode. This in turn allows more light (i.e. the light dissipated in the WT) to penetrate into the bioreactor so that cells deeper in the culture have a near optimal exposure to light (Mussgnug et al. 2007; Oey et al. 2013). Although the maximum efficiency of individual cells remains unchanged under saturating light levels, the overall efficiency of the small antenna culture, and with this the bioreactor photosynthetic efficiency, increases.

Initially, studies were conducted to evaluate the effect of down-regulating LHC levels more generally than specifically. Insertional mutagenesis was utilised to generate *C. reinhardtii tla1* mutant (Polle et al. 2003) with <50% of total chlorophyll per cell and a Chl a/Chl b ratio of almost triple, relative to parental strain indicating a reduction in the chlorophyll a/b binding proteins of the LHCII antenna. *Tla1* was shown to have a similar light saturation curve to the *C. reinhardtii* cbs3 mutant (Polle et al. 2000, 2003). Crossing of the cell-wall-deficient *tla1* with a cell wall intact strain CC1068 (CW^+) led to strain *tla1*–CW^+ which had higher oxygen evolution and cell densities relative to wild type at 1500 μE m^{-2} s^{-1}. Berberoglu et al. (2008) reported comprehensively upon the radiation characteristics of these two strains as well as a new mutant *C. reinhardtii tlaX* which has significantly lower total chlorophyll and substantially lower chlorophyll b. Similar such examinations of strain-specific radiation characteristics (Berberoglu et al. 2008; Heng et al. 2014; Kandilian et al. 2013; Lee et al. 2013) provide a good framework for comparisons and further advancement.

Translational control was also studied when NAB1 was postulated to be an LHC mRNA-specific repressor protein that, when disrupted through insertional mutagenesis, led to the *C. reinhardtii* Stm3 mutant with high LHC and chlorophyll levels (Mussgnug et al. 2005). Thus, the potential up-regulation of NAB1 and similar regulatory proteins like it could assist in the art of engineering light capture. Alternatively, it was found that replacement of 1 or 2 of the NAB1 cysteine residues can perturb its deactivation mechanism, and this in vitro led to enhanced repression of LHC translation, and consequently smaller LHCII antennae (Wobbe et al. 2008). This was validated when a permanently active NAB1 variant was used to generate the mutant *C. reinhardtii* Stm6Glc4T7 (Beckmann et al. 2009) although LHC antennae reduction effect was not as pronounced as that achieved by other methods.

RNA interference was used to facilitate targeted down-regulation of particular proteins of interest. Using a common LHC protein target DNA sequence, Mussgnug et al. (2007) was able, in the *C. reinhardtii* Stm3LR3 strain, to down-regulate LHCII proteins by ~95 % and LHCI proteins by ~80 %. As the LHC proteins perform a complex set of roles, a more refined strategy was developed in which specific sequences were chosen to target only LHCBM1, 2 and 3 in *C. reinhardtii* Stm6Glc4L01 (Oey et al. 2013). Both of these strategies have been reported to be successful at improving the growth rates of high cell density cultures under high-light conditions. Perrine et al. (2012) also employed an RNAi strategy to down-regulate CAO in *C. reinhardtii* and compared CAO-RNAi (CR) cell lines to the previously developed *C. reinhardtii* cbs3. Collectively, these RNAi projects have shown that although peripheral antennae can indeed be minimised, there are advantages in having precisely engineered antennae systems (e.g. which enables state transitions to function normally) for high efficiency biotechnology applications. More recently, Synthetic Genomics and ExxonMobil have reported on their collaborative work identifying light acclimation regulator (LAR) genes Lar1 and Lar2 (Bailey 2013) and developed *Nannochloropsis* mutants that are locked in a high-light-acclimated state. In terms of future developments, in this area, the above RNAi data can be used to identify particular targets for more permanent editing, deletion and upregulation using the emerging CRISPR/Cas and TALEN approaches.

Expanding the Available Solar Spectrum The currently accepted range for photosynthetically active radiation (PAR) absorbed by chlorophylls a and b only comprises ~43 % of the total solar spectrum, however, other chlorophylls include c1, c2, d and f and utilising additional chlorophyll types could enable the capacity for microalgae to exploit a wider range of wavelengths (Blankenship and Chen 2013; Chen and Blankenship 2011). In this context, it is of note that chlorophyll d (Manning and Strain 1943; Miyashita et al. 1996) and chlorophyll f (Chen et al. 2010) are red shifted and reported to be in oxygenic organisms. This indicates that these chlorophylls may therefore be able to capture light in the infrared range which is usually excluded from conventional calculations on microalgal productivity for most oxygenic algae which more commonly incorporate chlorophylls a and b into the photosynthetic machinery.

The engineering of photosystems incorporating alternative chlorophyll molecules requires the introduction of biosynthetic pathways for these specific pigments, the accompanying mechanisms of their targeted incorporation into the photosystems and the engineering of PSI, PSII and the light-harvesting proteins themselves to enable the precise coordination of these new pigments. In this context, it is of note that the disruption of the chlorophyll a oxygenase (CAO) gene which is reported to be involved in the synthesis of chlorophyll b yielded the chlorophyll b-depleted strain *C. reinhardtii* cbs3 (Tanaka et al. 1998). Subsequently, Polle et al. (2000) showed that in the absence of chl b, some substitution by chl a occurred in *C. reinhardtii* cbs3. All apoproteins of the LHC were reported to be present, although trimeric LHCII did not appear to assemble and the total antennae size was

truncated. Furthermore, cbs3 had a higher chlorophyll per cell relative to wild type even though chl b is absent. Photon use efficiency was significantly decreased in cbs3, but P_{max} depended greatly on the carbon source. Also, there has been some evidence that reduction in carotenoid levels can also have an effect in reducing PSII antennae although results are less pronounced (Polle et al. 2001). While this suggests that it is possible to engineer cell lines with tailored chlorophyll compositions, much detailed work must be completed to achieve real increases in photon use efficiency. In addition to potentially enhancing natural photosynthesis, such genetic engineering studies coupled with advanced structural biology analysis could also yield valuable insights for the design of artificial photosynthetic systems.

Precise Gene Editing Precise engineering of genes and the creation of stable cell lines, e.g. via the replacement of individual codons for specific amino acids will enable the fine-tuning of the photosynthetic process for biotechnological applications, for example using structure-guided design and gene-editing tools to modify individual chlorophyll-binding sites to facilitate the binding of specific chlorophylls or alter cyclic and linear electron transport controls and state transitions.

Photosystem Reaction Centres Aside from work on the optimisation of the LHC antennae and chlorophyll density, there has recently been progress in the investigation of variants of the D1 protein subunit of photosystem II (Vinyard et al. 2014) in which it was reported that different naturally occurring isoforms are tuned to either high- or low-light conditions and that through introducing point mutations, photochemical design concepts could be elucidated to enable fine-tuning of photosystem reaction centres towards specific environmental conditions. This work is still at early stages but has the potential to complement the work on optimisation of antennae systems for synergistic effect.

Other Recent Developments Also, there are other more generalist strategies for improving photosynthesis that are not specifically centred around light harvesting. Zhu et al. (2010) and Evans (2013) have recently reviewed some of the most promising advancements including the improvement of rubisco [or substitution of rubisco from other biological sources, e.g. (Lin et al. 2014)], and enhanced carbon concentrating and metabolic enzymes (e.g. sedoheptulose biphosphatase) that are associated with photosynthesis. It is important however to remember the distinction between modifications that enhance the productivity of a single cell and modifications that actually compromise the cell in some way but enhance the productivity of the overall culture (this is further discussed in Sect. 11.4 in the context of government regulation).

Challenges for Effective Deployment of These Technologies in Commercial Systems While growth rates in the laboratory appear to be better for high-density cultures and high-light conditions, as yet there have been no successful demonstrations of pigment/antennae mutants proving this capacity in scale up systems. Huesemann et al. (2009) did perform the first scale up cultivation of pigment mutants of *Cyclotella* that had been developed for them by Jürgen Polle; however, the mutant culture showed no significant growth benefit over the control culture.

The complexity of the antenna systems and their dynamic interactions makes the precise improvement of light capture and utilisation without the introduction of detrimental effects challenging and underlines the consideration that random mutagenesis approaches may be most useful where subsequent screening can verify that no functional units other than the target genes have been affected. In the absence of these checks, growth could actually be compromised rather than improved. Precision strategies for genetic engineering of light harvesting address this concern (as discussed above), but either system is acceptable. Furthermore, despite strains that have been developed specifically for high-density mass cultivation and to have the highest photon to chemical energy conversion efficiency show great promise, the state of being 'locked in' to a certain growth condition can also present a challenge. For example, other strains that retain the photo-adaptive capacity and can modulate their antennae size, pigment density and appropriate reaction centres have the capacity to invade cultivations of industrial production strains and grow more competitively in the photo-limited zone. Although they may not grow fast, in long-term semi-continuous culture, they could over time alter the population distribution and affect overall culture productivity (Perrine et al. 2012).

11.3.3 Engineering Improved Metabolism Systems

The downstream metabolic pathways which utilise the ATP and NADPH generated by photosynthesis offer a second area of opportunity for genetic improvement. Essentially, biocrude is reduced carbon dioxide and photosynthesis is a process of reduction. Metabolic engineering based, for example, on flux analysis (Dal'Molin et al. 2011) offers the opportunity to increase the capture of the energy derived from solar irradiation into desired 'chemical energy-rich' product (e.g. triacylglycerides, secondary metabolites or recombinant proteins) in production models utilising extraction processes or for sale of raw biomass.

To achieve the maximum PCE for a particular product, it is important to achieve four key things:

1. The optimised channelling of the captured solar energy into the biochemical pathway producing the target product
2. The optimisation of the efficiency of the biochemical pathway producing the product
3. To minimise energetic losses through cell division and metabolism
4. To export the product to avoid inhibitory feedback loops

It could be argued that while the engineering of pathways for production models utilising extraction processes or for the sale of biomass is likely to benefit most from genetic optimisation, the benefits of metabolic engineering are attenuated when whole biomass processing strategies such as HTL to biocrude are intended. However, even in this scenario, the elemental composition of microalgal biomass is still relevant to the quality of biocrude output and biomass. For example, oil-rich

biomass can be considered to be more reduced than its more oxygenated carbohydrate-rich counterpart and as such tends to have a higher energy content or calorific value in terms of MJ kg^{-1}. Thus, for example, while TAG accumulation has traditionally been examined as a feedstock for biodiesel production, for which it is particularly suited, its accumulation can also be beneficial for the production of biocrude. Similarly, protein accumulation results in high nitrogen and sulphur levels which incurs additional nutrient requirements and poses a problem for biocrude quality. Generally, algae are considered to be ~50 % carbon; however, this is not an inflexible rule. The Redfield ratio purports that the stoichiometric ratio of main elements is expected to be 106C:16N:1P; however, this is a molar ratio, and when masses are calculated, the corresponding mass ratios would be approximately 41C:7N:1P. Thus, algae can vary significantly in their carbon content, and where algae can accumulate large amounts of lipid stores, then a reasonable carbon range could be anticipated from ~40–60 % carbon. In this respect, where carbon assimilation can be increased without compromising total productivity, metabolic engineering can play an important role in strain development for whole biomass processing.

Balancing Carbon Storage in Microalgae (Case Studies) Studies have shown that the content and biochemical composition of microalgae can vary significantly as their metabolic pathways shift in response to environmental stimuli and changes in nutritional conditions—protein (6–52 %), carbohydrate (5–23 %) and lipid (7–23 %) (Brown et al. 1997; Chen et al. 2011; Guschina and Harwood 2006; Johnson and Alric 2013; Poerschmann et al. 2004; Roessler 1990). While some algae use lipids as energy storage, e.g. diatoms, for many species cultivated under nutrient replete normal growth conditions, carbon reserves are preferentially stored as starch (as in higher plants) which are readily catabolised to glucose for ATP (Johnson and Alric 2013). Though in nutrient deplete unfavourable growth conditions where temperature, light, salinity, pH, etc., may be suboptimal, the carbon can be stored in the form of triacylglycerols as a reserve for future use (Roessler 1990; Sheehan et al. 1998). By taking a nutritional and/or an environmental approach, the regulatory processes involved in these carbon storage schemes can be manipulated, although the effects may be species specific. While some of these approaches may not be practical in a commercial context, it is interesting to understand genetic activities related to such phenomena.

Nitrogen depletion was first demonstrated by Spoehr et al. in *Chlorella pyrenoidosa* and has since been applied to a number of other strains as the standard for lipid induction (Ben-Amotz et al. 1985; Spoehr and Milner 1949). Studies have shown significant increases in lipid content (from 1 to 85 % of cellular mass) in microalgal cultures at the expense of cellular growth (Rodolfi et al. 2009; Roessler 1990; Siaut et al. 2011). As the synthesis of proteins and nucleic acids rely on nitrogen, these pathways inevitably cease to function and the flow of carbon diverts to storage compounds (Berges et al. 1996; Roessler 1990). Similarly, silicon

depletion in diatoms such as *Cyclotella cryptica* has been observed to induce a multifold increase in ACCase activity leading to increased lipid accumulation, while a reported 50 % reduction in carbohydrate storage was noted. Pulse chase experiments suggested in the first 12 h after silicon depletion, ~55–68 % of the lipids produced via de novo synthesis (Roessler 1988a, b and 1990).

The effects of salinity on metabolism have been mainly studied in halotolerant species such as *Dunaliella* which is found to draw upon starch reserves to physiologically respond to osmotic stress (Craigie and McLachlan 1964). As salinity within the medium is increased, *Dunaliella* cells contract rapidly, and the cells then metabolise glucose and fructose into a glycerol pool within the cytoplasm in order for the cell to regain its volume (Baird and DeLorenzo 2010). On the other hand, when salinity in the medium is decreased, the available glycerol pool is metabolised back into starch reserves (Ben-Amotz and Avron 1973; Borowitzka and Brown 1974). Other growth factors such as temperature stress, pH variation and light intensity have also been reported to influence the lipid composition in a range of species (Guckert and Cooksey 1990; Roessler 1990).

Knowledge in how different species respond to different nutrients and environmental conditions will greatly aid researchers in developing a greater understanding of mass culture and biological response. The disadvantage of these simple strategies for increasing lipid content is that they are fundamental responses to stress, represent a loss of net productivity and can be subject to operational limitations. However, elucidating the genomic changes elicited by these responses can enable engineering strategies which offers the prospect of more rapid, direct and controllable ways to siphon off the biological gains of photosynthesis into a desirable form.

Engineering of Lipid Pathways While much is known about lipid metabolism in higher plants from research models such as *Arabidopsis* (Beisson et al. 2003), lipid metabolism in microalgae is substantially different relative to higher plants and also between microalgal genera. The neutral and polar lipids, and the enzymes and metabolic pathways involved in their biosynthesis and catabolism have been recently described with the current focus being upon gene identification to enable proper metabolic engineering (Dal'Molin et al. 2011; Guschina and Harwood 2013; Khozin-Goldberg and Cohen 2011; Liu and Benning 2013; Rismani-Yazdi et al. 2011). *C. reinhardtii* remains the most extensively studied model for microalgal lipid metabolism (Liu and Benning 2013; Merchant et al. 2012), but it does not appear to use phosphatidylcholine as a substrate in TAG synthesis or to accumulate TAG unless under stress conditions (unlike *Nannochloropsis* which can synthesise TAG under normal cultivation conditions) or in starch accumulation (*sta*) mutants (Li et al. 2010; Work et al. 2010; Zabawinski et al. 2001). Recently, the metabolic pathways of *Dunaliella tertiolecta* (Rismani-Yazdi et al. 2011) and *Monoraphidium neglectum* (Bogen et al. 2013) have been reported, and there is some knowledge of other microalgae (Guschina and Harwood 2013), but knowledge of specific enzymatic processes and the genes involved requires further advancement before

effective metabolic engineering strategies become commonplace and metabolic flux models will also assist with this (Dal'Molin et al. 2011).

In early work on acetyl-CoA carboxylase (ACCase) in which Dunahay et al. (1996) transformed the diatom *Cyclotella cryptica* with additional copies of the ACCase gene within the TCA cycle to increase the flux of carbon towards lipid biosynthesis, the resultant increase in enzyme activity did not increase lipid accumulation. Similarly, the recent engineering of increased expression DGAT strains in *C. reinhardtii* (La Russa et al. 2012) saw enhanced DGAT mRNA levels but failed to increase intracellular TAG accumulation. Thus, claims that this field of engineering lipid metabolism in microalgae is still in its infancy are valid (Merchant et al. 2012), and much more work needs to be completed before we are likely to see the potential gains in industrial production candidates that have been anticipated. Possible reasons for this are that microalgae have some form of feedback regulation and that they have multiple and divergent DGAT2 isoforms (Chen and Smith 2012), and while all algal species have at least one DGAT2 from the animal clade, it currently appears that only green algae have DGAT2s similar to higher plants. Thus, single gene engineering strategies may be of limited application when dealing with a gene network which we do not currently comprehend sufficiently. Chen and Smith (2012) call for further investigation of DGAT2 enzymatic characteristics as functionality and substrate preferences are currently not fully understood. PDAT is the other of the two enzymes involved in the final step of TAG production and it has recently been examined (Boyle et al. 2012; Yoon et al. 2012). Yoon et al. (2012) demonstrated that PDAT is indeed involved in TAG biosynthesis in *C. reinhardtii* through RNAi-induced PDAT knock-down mutants. Thus, both DGAT and PDAT represent valid targets for metabolic engineering, but more must be understood about the other metabolic processes acting in the TAG 'neighbourhood'. By simultaneously manipulating all of the critical genes that influence the metabolic flux, success is far more likely. RNAi and new CRISPR/Cas and TALEN technologies offer the potential to dissect these pathways and indeed optimise individual catalytic steps through genetic editing and the amino acid level.

Another promising genetic approach has focused upon the engineering of lipid catabolism rather than biosynthesis. Using this approach, Trentacoste et al. (2013) incorporated antisense and RNAi into the diatom *Thalassiosira pseudonana* targeting a newly identified gene Thaps3_264297, which was reported to be a multifunctional lipase–phospholipase–acyltransferase, which showed consistent decrease in microarray transcript abundance throughout the lipid accumulation phase of silicon withdrawal. Thaps3_264297 is homologous to human CGI-58 whose mutation in humans can lead to excessive accumulation of neutral lipid droplets in various tissues. Trentacoste et al. (2013) found that these knock-down mutants had increased accumulation of TAG droplets and total lipid production without negatively affecting cell division and biomass growth. Examples of other targetable enzymes may include malate dehydrogenase (*mme* gene), pyruvate formate-lyase (*pfl* gene) or the fatty acid synthase complex (FAS) to drive carbon

towards fatty acid synthesis (Perez-Garcia et al. 2011; Yu et al. 2011), and these targets will be further refined with the improvement of metabolic flux models.

Metabolic engineering is not just about increasing the flux towards fatty acid synthesis and TAG accumulation, but also qualitatively concerning the types of lipids that are produced. For example, metabolic engineering has been successful in altering the fatty acid profile of *Phaeodactylum tricornutum* (Radakovits et al. 2011) to yield shorter acyl chains. The capacity to manipulate both chain length and the degree of saturation has significant potential for adjusting fuel properties.

Engineering of Carbohydrate Pathways Similar to the metabolic models for fatty acid biosynthesis and catabolism, models for carbohydrate metabolism are also under-development, for example, in *Phaeodactylum* (Kroth et al. 2008). For bio-crude production, increasing the carbohydrate concentration in cells can also increase the total carbon content although oxygen content increases and this strategy seems secondary relative to strategies maximising lipid content. In the *C. reinhardtii Stm6* mutant, the deletion of the *Moc1* gene via random gene insertion resulted in modified respiration metabolism with the downstream effect of accumulating large starch reserves within the chloroplast (Schönfeld et al. 2004). There are also ambitions for microalgae, in particular cyanobacteria, to produce carbohydrates at the industrial scale (Ducat et al. 2012; Wijffels et al. 2013), and the company Algenol that utilises GM algae to produce and secrete ethanol is a good example of this.

Challenges for Effective Deployment of these Technologies in Commercial Systems Where microalgae are accumulating energy storage compounds, they become better candidates for production; however, within the ecology of mass cultivations, they also become better candidates for predation, increasing the energy return for micro-organisms grazing upon them.

11.3.4 Engineering Improvements to Process Streams and Economics of Algal Biotechnology

While ultimately the hard physical metrics for microalgal biocrude are essentially the energy returned on investment (EROI) and the economic viability, the maturing and scaling of the technology still require further development. During this early development phase, commercial viability requires a profitable path to technology deployment. Several approaches are possible for dealing with this problem.

High Value Products and Services (HVP&S) Algal GM is in its infancy compared to other systems. A challenge of generating GM strains for HVP&S production is to provide useful products and services that cannot be easily generated in more mature technologies. There is no rational point to replicating in algae a service that can be easily and economically performed by yeast or *E. coli* aside from reasons such as marketing appeal. The relative advantages of algae as GMO vehicles must therefore

be carefully considered on a case by case basis. Recombinant products such as peptides larger than those able to be chemically synthesised, but small enough to be extracted with relatively harsh techniques, may be particularly suitable. Many HVP&S GM strains will be designed to operate under heterotrophic conditions which simplify reactor design.

The difference between these approaches is that modification of bulk mass and energy flows is focussed on energy production and is strictly limited by the thermodynamics of light harvesting and carbon fixation, whereas HVP&S approaches are less thermodynamically constrained (and indeed, may not even utilise photoautotrophic systems) but are focussed mainly on economic gains.

Enabling and Supportive Technologies Given the constraints outlined above, it is clear that GM strains for HVP&S and those for biofuels applications will have little in common and it is unlikely that a single strain (or industrial facility) will serve both purposes, which argues against the 'biorefinery' concept if it is confined to a single strain or process. Nonetheless, the common biology underpinning all algal systems means that most of the enabling technologies invented in this space will apply similarly to a multitude of different algal biotechnology systems, yielding substantial cross-fertilisation. It is here that the biorefinery concept may be most profitable.

Many supportive technologies will therefore need to be developed before the industry matures, and GM can make major contributions to these. Protein and lipid export systems, for example, may reduce internal product inhibition while reducing harvest costs; modified photosynthetic systems may improve the efficiency of utilisation of incident light; and fluorescent signals may be generated to monitor internal biochemical processes. None of these technologies would intrinsically compromise the ability to convert light to fuel, but might greatly simplify or reduce costs for other biotechnological aspects. Clearly, there is a vast creative space for innovative GM approaches in this area. To the extent that such technologies reduce energy wastage during production, they can improve the EROI even without an alteration of the fundamental light-harvesting efficiency.

Advantages of Algae as Heterologous Expression Systems Algae as heterologous expression systems are comparable to plant systems primarily for their ability to produce proteins with post-translational modifications. They may not replace the established and commercialised bacterial and mammalian expression systems but offer the potential for biological products which are difficult to produce in an active form in prokaryotic systems and are expensive to make in eukaryotic systems (e.g. antibodies). They also offer advantages over conventional systems to be chosen for new products which cannot be produced in other systems [e.g. anti-cancer toxin (Tran et al. 2013)] and therefore provide a valuable opportunity for the industry.

One advantage that can make transgenic microalgae systems competitive in the field of pharmaceutical proteins is that many algae lack endotoxins or human pathogens (Mayfield and Franklin 2005; Walker et al. 2005) and are therefore 'Generally Recognized As Safe' (GRAS). This could allow for a reduction of

necessary purification steps during downstream processes as well as simplify quality control and therewith allay production costs. Another advantage of algae compared to higher plants is vegetative reproduction, leading to uniform clones with comparable production rates. This relates to product quality, e.g. demonstrated as certain beneficial post-translational modifications, product stability or biosafety. Microalgae systems display high growth rates and need only a short time from transformation to product formation so that scale up could be implemented within a few weeks within commercial processes. The cultivation can be inexpensive due to the relatively low costs of typical mineral media needed, therefore supplying a large-scale robust growing system which can yield cheaply extractable high-volume production. This provides possible cost savings during production processes, which could play a role in special fields, where large quantities of products are required at low costs such as recombinant antibodies or veterinary products.

Microalgae have already been established as biotechnological production systems and approved by the US Food and Drug Administration for a number of secondary metabolites useful as food additives or cosmetics (Administration 2003, 2004, 2010a, b, 2011, 2012; Plaza et al. 2009) and for the production of carotene using *Dunaliella salina* (Hosseini Tafreshi and Shariati 2009) and lutein as an antioxidant and food colourant. Antiviral activities have been shown. Vaccination concepts for a large number of diseases prevalent in developing nations based on recombinant antigen expression in microalgae could result in inexpensive production and distribution as well as long-term storage at room temperature (Dreesen et al. 2010; Specht et al. 2010). Edible vaccines are a possible field of application for algal expression systems, combining biosafety issues with inexpensive production and storage and therefore opening up making products accessible for less developed countries (Gregory et al. 2013). In the context of regulatory aspects in the pharmaceutical sector, novel expression systems have to offer enormous advantages over conventional systems to be chosen for new products. The possibility to use a closed photobioreactor system contributes to reducing the risk of contamination and prevents transgenes dispersing into the environment.

11.4 Regulatory Considerations in the Risk Assessment of GM Microalgae

The responsible production of genetically modified (GM) microalgae and its appropriate regulation in many ways parallels the previous emergence of GM crops utilised in terrestrial crop-based systems. GM crops have been in field testing for approximately three decades now and with their global scale now approaching almost 200 million hectares, their benefits have been demonstrated, although they have been beset by much controversy, and there are also some cautionary lessons

learned. There are some important distinctions between the two forms of production (i.e. aquatic versus terrestrial), and microalgae systems are generally capable of much greater containment than conventional cropping systems. In order to preface this discussion, it is important to first examine the current issues with wild (non-GM) algae, both in the environment and in commercial production systems, and the current state of regulatory oversight.

Wild Algae in Aquatic Ecosystems 'Toxic algae blooms' are a regular headline in the mainstream media resulting in a public perception that algae are a menace. In water treatment industries, this fear of algal toxins is also relatively well established. In reality, the number of algae that produce any toxins is a tiny fraction of the existing biodiversity. Almost all of the known toxins attributed to algae are actually found in certain types of cyanobacteria and dinoflagellates, with a much smaller representation from some bacillariophytes (diatoms), haptophytes, pelagophytes and euglenoids. In some cases, there are groups who are cultivating specific species, e.g. dinoflagellates, to utilise toxic compounds for applications such as biomedical cytotoxins (generally under laboratory conditions), but this is the exception rather than the rule, and the overwhelming majority of the industry is focused upon avoiding toxic species. For example, the cultivation of the cyanobacteria *Arthrospira* (*Spirulina*) for human food consumption must be free from the cyanobacteria *Microcystis*.

Environmental algal blooms, while an ongoing concern, are usually the result of anthropogenic nutrient outflows or natural processes of nutrient cycling. They are not generally the result of well-managed microalgae farming practices. Such blooms can occur during periods of elevated nutrient levels due to either natural processes (e.g. weather effected nutrient run-off from land or oceanic currents and upwellings) or from anthropogenic nutrients (e.g. municipal, agricultural or industrial waste waters), with the latter being more closely correlated with the increase in the frequency and the intensity of environmental algal blooms.

Algal blooms can be broadly divided into classes as (1) blooms that are transient and innocuous (2) both transient and persistent blooms that are generally considered to be harmful, and (3) blooms that are clearly detrimental and disruptive to ecosystems. As the algae themselves are by and large ubiquitously present, the primary underlying issue is the management of nutrients and eutrophication processes. While innocuous algae blooms are generally rapidly consumed by organisms higher up the food web (e.g. plankton and filter feeders) and are therefore transient, harmful algal blooms (HABs) (Anderson 2009; Anderson et al. 2002; Van Dolah et al. 2001) and ecosystem disruptive algal blooms (EDABs) (Sunda et al. 2006) can be comprised of algal species that are generally unpalatable to aquatic herbivores or that contain toxins. This is important because it disrupts the food web and the concordant transition of nutrition and chemical energy to higher trophic levels which can result in a loss of ecosystem biodiversity (with ecosystem biodiversity being closely correlated with ecosystem resilience). Historically, these problems are largely caused by agricultural nutrient outflows, and there has been significant

analysis of how outflows of nutrients and chemicals from agricultural production can vary greatly in their 'pollution footprint', e.g. (Hill et al. 2006). The potential for reducing the pollution footprint is one of the strong benefits of microalgal production systems (Smith et al. 2010) in that they generally have no chemical outflows, and due to greater containment relative to fields of crops in soil, they can have much lower nutrient outflow, and in some cases a negative footprint where they utilise anthropogenic nutrients from other systems, e.g. wastewater integration and bioremediation systems. Nevertheless, forward thinking risk management strategies are needed to ensure that microalgae production systems at very large scale do not induce similar concerns to those experienced in traditional agriculture.

Proper Management of Microalgal Production Systems Proper management of microalgal systems is an important aspect of any commercial operation. This will be increasingly important as systems are scaled for large-scale production and the varieties of engineered strains used increase. The establishment of production models aiming to exploit the benefits of GM microalgae contributes additional complexity to prudent regulatory frameworks. There is a duality to the responsible management of GM microalgae production systems in that (1) from a product perspective, farmed microalgae cultivations must be maintained at adequate purity and free from contaminants that can compromise product quality (e.g. in the *Arthrospira* example given above), and (2) from an environmental perspective, the release of nutrients or microalgal biomass must be properly managed in order to mitigate any risk to local ecosystems. Given that for the production of biocrude, biomass will be subjected to thermochemical processing, it is the latter point which is central to this discussion.

Both the type of release (nutrients or biomass) and the scale of release are important parameters in a proper risk assessment. Gressel et al. (2013) have added to the discussion on mitigating spills and propose that spills from large-scale cultivations will be inevitable—however, there is an important consideration here regarding the terminology moving forward in this discussion. We expect that implementation of proper standards in prudent farm management should be able to mitigate the chances of large-scale spills into the environment; however, it is widely agreed that microalgae have a relatively high capacity for dispersion (e.g. microscopic size, and potential to form aerosols). Thus, if some aerosolised cells escape to the environment, it is certainly a release, but is this considered a spill? In terms of nutrients, the scale and/or persistence of release is generally the most important variable in terms of subsequent eutrophication potential and the corresponding risk assessment, but in terms of biomass, a single cell escaping as an aerosol particle has the potential to establish itself outside of the farm boundary even if there is no 'spill'. Thus, in this respect, species release is indeed inevitable, and it is in this context that any discussion of GM strains must be conducted. Hence, if small-scale release cannot ultimately be avoided, then the discussion is inexorably dependent upon the biological character of what is released.

GM Microalgae and Their Regulation Considering the inevitability of release, risk assessments of GM microalgae must be conducted on a case-by-case basis, with specific attention to the nature of the modification and whether it actually conveys a competitive advantage of some kind to the strain in question when it is relocated within a natural ecosystem or whether the modification can result in disruption to ecosystems in some other way. Henley et al. (2013) have recently published an excellent examination of GM algae risk assessment which should serve well as a foundation study for this evolving discussion. They rightly stipulate that for a GM-specific environmental risk assessment, primary considerations of potential ecological impact include the following:

1. The potential of GM microalgae to be more highly competitive in natural ecosystems.
2. The potential of GM microalgae to result in altered communities of aquatic herbivores in terms of composition, dominance or biodiversity.
3. The potential of GM microalgae to be involved in horizontal gene transfer (HGT) to other micro-organisms.

Given that it is anticipated that, for the most part, new algae producers will not be cultivating microalgal species that are invasive or toxic—the primary considerations will be the genetic modifications themselves rather than the host strains (indeed popular host strains such as *C. reinhardtii* are quite easily outcompeted by many wild-type species); however, it has previously been seen that some potential production candidates have already been involved in bloom events that have resulted in their classification as EDABs (Sunda et al. 2006). Thus, we encourage a careful and iterative investigation of all aspects of microalgae production, but emphasise that in this discussion it is the specific genetic modifications relevant to high-density microalgae cultivations that is in need of far greater discussion in the literature. Consequently, we discuss here the implications of the engineering applications highlighted in section two, with respect to associated risk of species establishment, dominance and ecosystem disruption. Much can be gleaned from the parallels with GM crop species, especially pollen dispersal; however, there are distinct differences between terrestrial crops and communities of aquatic micro-organisms.

For microalgal strains engineered to have varied light-harvesting and photosynthetic efficiency, the general desire is to increase net biomass productivity. As discussed above, this can be achieved through different methods. The down-regulation of LHC proteins or pigmentation can provide an overall net benefit to high-density cultures in high-light conditions (i.e. the artificial farming environment), e.g. (Oey et al. 2013); however, this generally makes individual cells less competitive in natural ecosystems where competing wild-type cells retain the capacity to modulate their antennae size and pigmentation levels. In theory though, genetic modifications that unilaterally increase total productivity (e.g. a higher efficiency rubisco enzyme or strains that can utilise a wider range of the spectrum) could potentially convey a competitive advantage irrespective of the growth environment.

Where strains are photosynthetically superior irrespective of environment, they could potentially affect ecosystem dominance and diversity, and while microalgae composition might not be significantly changed, the increased availability of these microalgae could result in additional effects like changes in plankton composition. In contrast, LHC/pigment-reduced cells could lead to some immediate compositional changes when consumed, but this would be insubstantial at the community level, and as these strains are outcompeted by wild-type organisms, there would be no net change to dominance or biodiversity. Again, the real concern for HGT would be that microalgae with superior generic photosynthetic efficiency would be capable of transferring this trait to other phototrophic organisms enabling them to also have greater competitiveness in the natural ecosystem. The transfer of disabled antennae/pigment modulation would not convey an advantage to other species.

GM strains that have a greater capacity for nutrient scavenging may have an increased competitiveness if released, but there is already a diverse range of strategies for nutrient uptake and usage among naturally occurring algae (Henley et al. 2013). Thus, while the predicted risk for these modifications is considered to be low, there has not yet been sufficient data from field trials to properly draw a conclusion.

Metabolic engineering is intended to alter the composition of microalgal biomass. While for biocrude producing systems, this will ideally result in strains that have higher overall carbon content, and it is not producing strains outside of the range of what occurs in nature. Nevertheless, if the available proportion of the population containing high carbon (i.e. abundance of GM microalgae relative to wild-type microalgae) is shifted, there is potential for an effect even if the consequences are low. If the nutritional value of the microalgae is altered, then this could also lead to changes in the nutritional value of plankton and filter feeders and subsequently lead up the food web to higher trophic levels. By the ecological risk assessment proposed by Henley et al. (2013), this risk is considered to be very low; however, this should be monitored in the longer term to obtain confirmation. In general, the accumulation of energy storage products in the form of reduced carbon molecules does not convey a competitive advantage to GM microalgae and it is likely that they will also be outcompeted by wild types within natural ecosystems.

GM traits that enhance the capacity of a microalga to remain dominant in the presence of predators, pathogens and competitors are varied in their approach and range from resistance to chemicals (e.g. herbicides and pesticides) to the use of allelopathy and toxins to maintain dominance. The use of chemicals is unlikely to become widespread for low-value commodity products such as biocrude due to the economic pressure it places upon business models; however, the engineering of endogenous chemicals into GM microalgae that prevent contamination is a potential risk that must be properly examined. Henley et al. (2013) propose that the risk of this approach is low to moderate depending upon whether the allelopathic chemical is naturally occurring or novel; however, we suggest that the range of potential risk assessment outcomes can be as variable as the potential allelopathic chemicals that can be engineered and that even for relatively low-level allelopathy, at the very large scales of production proposed for addressing fuel demand, even

mild allelopathy could have ecosystem disruptive effects. Thus, we advise a strict examination of these strategies; though to the best of our knowledge, these strategies have not yet been employed. We do agree though that where traits are selected for from large populations and then elucidated and reproduced through engineering (rather than engineering of novel chemicals), the risk will be attenuated.

Other GM strategies to increase the harvestability and processability of microalgae are unlikely to affect their dominance in natural systems, and the risk for these traits is considered very low. Similarly, where protein expression is used to create a primary revenue stream from a high-value product before HTL of residual biomass, these strains are unlikely to compete in natural systems due to diversion of much of their energy flow towards a product that is not useful to the microalga.

The theoretical risk assessment discussed here and that presented by Henley et al. (2013) can be quite informative, relying on an analysis of whether similar traits are already part of the ecosystem. However, a physical risk assessment strategy will be more convincing where laboratory-scale simulated ecosystems are developed from natural water bodies and the long-term survivability of GMO algae in mixed culture can be evaluated, e.g. by PCR.

11.5 Conclusion

The commercially profitable production of algal biocrude, at scale, will represent the culmination of a long and parallel development of algal agronomy, biology, GM, bioreactor engineering, harvesting and chemical conversion processes and the development of suitable sensors and control systems, along with their associated modelling and control software. No one innovation will suffice to overcome the formidable challenges faced by this nascent industry, and no actor will have ownership of all the important intellectual property. Since the most significant competitive challenges are between algal technologies and other fuel systems and secondary markets, the field of algal biotechnology stands to benefit greatly from relative openness of sharing data, technology and experience. This suggests that the modified algal strains used for biocrude production in the future will be heavily modified fuel factories equipped with streamlined metabolism, externally controllable cellular programs, and both sensors and reporting systems for monitoring the state of the system. Biocrude production appears, at this stage, to offer one of the most promising production pathways for algal biofuel production, and genetic manipulation offers a powerful tool for fine-tuning microalgal biofuel production all the way along the development pathway.

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